Arch. Pharm. Res. (2016) 39:83–93
DOI 10.1007/s12272-015-0687-3

Archives of Pharmacal Research
www.springer.com/12272

RESEARCH ARTICLE

Inhibition of VCAM-1 expression on mouse vascular smooth muscle cells by lobastin via downregulation of p38, ERK 1/2 and NF-κB signaling pathways

Kyoungran Lee[1] · Joung-Han Yim[2] · Hong-Kum Lee[2] · Suhkneung Pyo[1]

Received: 8 August 2015 / Accepted: 18 November 2015 / Published online: 23 November 2015
© The Pharmaceutical Society of Korea 2015

Abstract Atherosclerosis is a chronic inflammatory disease, the progression of which is associated with the increased expression of cell adhesion molecules on vascular smooth muscle cells (VSMCs). Lobastin is a new pseudodepsidone isolated from *Stereocaulon alpinum*, Antarctic lichen, which is known to have antioxidant and antibacterial activities. However, the nature of the biological effects of lobastin still remains unclear. In the present study, we examine the effect of lobastin on the expression of vascular cell adhesion molecules (VCAM-1) induced by TNF-α in the cultured mouse VSMC cell line, MOVAS-1. Pretreatment of VSMCs for 2 h with lobastin (0.1–10 μg/ml) concentration-dependently inhibited TNF-α-induced protein expression of VCAM-1. Lobastin also inhibited TNF-α-induced production of intracellular reactive oxygen species (ROS). Lobastin abrogated TNF-α-induced phosphorylation of p38 and ERK 1/2, but not JNK, and also inhibited TNF-α-induced NK-κB activation. In addition, lobastin suppressed TNF-α-induced IκB kinase activation, subsequent degradation of IκBα and nuclear translocation of p65 NF-κB. Our results indicate that lobastin downregulates the TNF-α-mediated induction of VCAM-1 in VSMC by inhibiting the p38, ERK 1/2 and NF-κB signaling pathways and intracellular ROS generation. Thus, lobastin may be an important regulator of inflammation in the atherosclerotic lesion and a novel therapeutic drug for the treatment of atherosclerosis.

✉ Suhkneung Pyo
snpyo@skku.edu

[1] School of Pharmacy, Sungkyunkwan University, Suwon City, Gyeonggi-do 440-746, South Korea

[2] Polar BioCenter, Korea Polar Research Institute, KORDI, Incheon, Republic of Korea

Keywords Lobastin · Vascular cell adhesion molecule-1 · Atherosclerosis · NF-κB · MAPK

Introduction

Atherosclerosis is a progressive, chronic inflammatory disease characterized by the recruitment of leukocytes, engulfment of lipid and accumulation of fibrous factors in the large ateries, which is the fundamental cause of cardiovascular disease (Lusis 2000). Several lines of evidence indicate that vascular cell adhesion molecule-1 (VCAM-1) expression is detected on vascular smooth muscle cells (VSMCs) and is important in the progression of atherosclerosis (Braun et al. 1999; Cybulsky et al. 2001; Huo and Ley 2001). In addition, it has been known that adhesion molecule expression on intimal VSMCs facilitates the migration of leukocytes such as monocytes and inflammatory cytokines including TNF-α which can contribute to the induction of VCAM-1 in VSMCs (Braun et al. 1999; Cybulsky et al. 2001; Huo and Ley 2001; Vazquez et al. 2012). Moreover, direct inhibition of VCAM-1 expression could be a therapeutic avenue for atherosclerosis compared with long-term intervention with ICAM-1, because VCAM-1 is an inducible gene requiring de novo protein synthesis and its expression is limited primarily to specific inflammation sites (Saxena and Medford 2000). Thus, direct inhibition of VCAM-1 expression, leading to suppression of the interaction of leukocytes to the VSMCs, may provide a potential therapeutic strategy for the treatment of chronic inflammatory diseases like atherosclerosis.

Many lichens have been used in folk medicine across the world. It has been reported that some lichens have been used in treatment of various inflammatory disorders such as

bronchitis (Saklani and Upreti 1992). Nowadays, the research and demand for lichens in human health applications are growing rapidly. The use of lichens as a medicine is often related to the secondary metabolites. A variety of bioactive metabolites has been isolated from lichen species of Antarctic origin (Seo et al. 2009). Accumulated data show that many biologically active lichen metabolites have antibiotic, antimycobacterial, antiviral, analgesic, antioxidant, and antipyretic properties (Kumar and Muller 1999). In addition, recent studies have demonstrated that longisiminone isolated from *Vsnea longissima* and usnic acid isolated from *Roccella montagnei* have anti-inflammatory activities in vitro and in vivo, respectively (Choudhary and Jalil 2005; Vijayakumar et al. 2000). Furthermore, our previous study shows that stereocalpin A and ramalin obtained from the Antarctic lichen *Ramalina terebrate* inhibited TNF-α-induced adhesion molecule (Byeon et al. 2012; Park et al. 2014). Lobastin (Fig. 1), a structurally new pseudodepsidone-type metabolite, was found from the new collection batch of *S.alpinum* from Antarctica, which is known to have antibacterial and antioxidant activities (Bhattarai et al. 2013). However, other biological activities of lobastin have not yet been fully clarified.

In the present study, we examine the effect of lobastin on the expression of VCAM-1 as well as the mechanism involved in the anti-atherosclerotic effect of lobastin in cultured vascular smooth muscle cells. The data from the present study suggest that lobastin suppresses TNF-α-stimulated VCAM-1 expression through the inhibition of ERK 1/2, p38, and NF-kB signaling pathways and intracellular ROS production.

Materials and methods

Plant material

Stereocaulon alpinum 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 (Øvstedal and Smith 2001). A voucher specimen was deposited in the Polar Lichen Herbarium, Korea Polar Research Institute, KOPRI, Incheon, Republic of Korea.

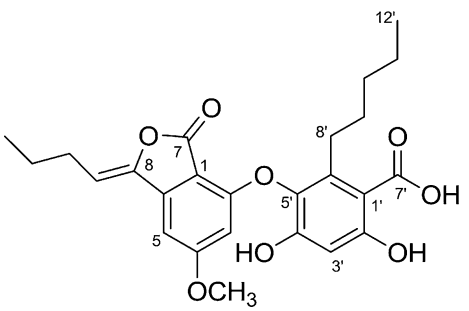

Fig. 1 Chemical structure of lobastin

Extraction and isolation

The extraction of lobastin was performed using a modification of the technique described previously (Bhattarai et al. 2013). Briefly, a dried sample of *Stereocaulon alpinum* (100 g) was extracted with MeOH (1 l × 2) for 24 h. The resulting crude MeOH extract (10.2 g) was subjected to C18 functionalized silica gel (5 × 30 cm) mild pressure liquid chromatography (MPLC), eluting with a stepwise gradient of MeOH in H_2O. The gradient program was as follows: 50 % methanol for 5 min, 75 % methanol in 5 min for 30 min, 85 % methanol in 5 min for 30 min, 95 % methanol in 5 min for 10 min. The flow rate was fixed at 45 ml/min. The collected broad peak (t_R = 43 min to 60 min) yielded lobastin. The analysis indicated that the lobastin was present in the crude extract as 16.3 % (w/w).

Reagents

Unless otherwise indicated, all chemicals used in this study were purchased from Sigma Chemical Co. (St Louis, MO). Anti-VCAM-1 antibody was purchased from R & D Systems, USA. DMEM medium and fetal bovine serum were purchased from Life Technologies, Inc. (Carlsbad, CA). The reporter plasmid pGL3-NF-κB used in the luciferase assay system was obtained from Promega (Madison, WI), and pCMV-β-gal was obtained from Lonza (Walkersville, MD). 3-Amino-1,2,4-triazole was purchased from Calbiochem (La Jolla, CA). Antibodies against IκBα, p65, JNK, phospho-JNK (p-JNK), ERK, phospho-ERK (p-ERK), phospho-38, p38, lamin A, TNF-α receptor 1 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 grown in DMEM medium supplemented with 0.2 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 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 the first to sixth passage. 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.

Assessment of cell viability

The cell viability was determined by a MTT assay as previously described (Nguyen et al. 2015). MOVAS-1 cells were seeded at a concentration of 2×10^4 cells/well in 96-well tissue culture plates and pretreated with various concentrations of lobastin (0.1, 1, 5, and 10 μg/ml) for 24 h. Cell viability was measured using a quantitative colorimetric assay with MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], indicating 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). The blank control only contained cell culture medium and the absorbance of untreated cultures was set at 100 %. Cell viability was expressed as a percentage of the untreated control. At least three independent experiments were performed.

Measurement of cell surface VCAM-1 expression

The cell surface expression of VCAM-1 on muscle cell monolayers was determined by an ELISA assay as previously described (Ju et al. 2002) with slight modifications. VSMCs (MOVAS-1) were seeded at a concentration of 2×10^4 cells/well in 96-well gelatin-coated plates, cultured to confluence and pretreated with lobastin (0.1–10 μg/ml) for 2 h at 37 °C. The cells were then incubated with TNF-α (10 ng/ml) for 8 h. 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. Cells were blocked with 1 % bovine serum albumin in PBS for 1 h, and subsequently incubated with monoclonal antibodies against VCAM-1 or isotype matched control antibody (0.25 g/ml, diluted in blocking buffer) overnight at 4 °C. After washing with PBS, cells were incubated with alkaline phosphatase-conjugated goat anti-mouse secondary antibody (1 μg/ml, diluted in PBS) for 1 h. After repeating the wash steps, fresh solution of peroxidase substrate (1 mg/ml *p*-nitrophenyl phosphate in 0.1 M glycine buffer, pH 10.4, containing 1 mM $MgCl_2$ and 1 mM $ZnCl_2$) was added, and the absorbance was monitored 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.

Measurement of mRNA levels by quantitative real-time polymerase chain reaction (qRT-PCR)

Following exposure to lobastin or TNF-α, total RNA was isolated using TRIzol (Life Technologies, Pioneer Valley, WI) according to the manufacturer's instructions. The yield and purity of the RNA were confirmed by measuring the ratio of the absorbance at 260 and 280 nm. RNA was reverse transcribed to cDNA using 0.2 μg/ml random primers, 10 mM dNTP-mix and reverse transcriptase (Promega, Madison, W1). Nested PCR was performed in a 20 μl volume comprising 3 μl cDNA, 2 μl each primer, and 2× QuantiTect SYBR Green PCR Master Mix and the fluorescence was monitored at each cycle. The mouse VCAM-1 RT-PCR primers were 5′-CCCAAGGATCCA GAGATTCA-3′ (sense) and 5′-TAAGGTGAGGGTGG CATTTC-3′ (antisense). The PCR primers for the mouse GAPDH control were 5′-GGTCCTCAGTGTAGCCCA AG-3′ (sense) and 5′-AATGTGTCCGTCGTGGATCT-3′ (antisense).

Western blotting analysis

Western blotting analysis was performed as previously described (Cho et al. 2003). The cells were pretreated with lobastin (0.1–10 μg/ml) for 2 h and then incubated with TNF-α (10 ng/ml) for 30 min or 8 h. Following treatment, the cells were lysed in 70 μl ice-cold lysis buffer A [10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 0.5 % Nonidet P-40 and protease inhibitor cocktail], followed by vortexing for 10 s. Cytosolic cell extracts were obtained after centrifugation at 1500×*g* for 10 min at 4 °C. The collected nuclei were resuspended in 50 μl 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. Whole cell lysates and cytosolic and nuclear extracts (20 μg) were electrophoretically resolved on a 7.5 % sodium dodecyl sulfate (SDS)-polyacrylamide gel, followed by transfer onto an immobilon polyvinylidene difluoride membrane (Amersham, Arlington Heights, IL), and probed with the appropriate antibodies. After extensive washing with TTBS (20 mM Tris pH7.5, 150 mM NaCL, and 0.2 % Tween 20), a Horseradish Peroxidase (HRP)-conjugated secondary antibody was applied. 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.

Transfection and reporter assays

VSMCs (MOVAS-1) were chosen for its high transfection efficiency. 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, pCMV-β-gal and pcDNA3.1 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 NF-κB luciferase, the cells were transfected with 0.5 μg NF-κB luciferase reporter using Metafectene PRO. After 4 h, the cells were pretreated with lobastin for 2 h followed by the addition of TNF-α (10 ng/ml) 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. Luciferase activity in the cells was determined. The values shown represent an average of three independent transfections and each transfection was carried out in triplicate.

ROS production assay

ROS production was determined as previously described (Kim et al. 2012) with a minor modification. CMH_2-DCFDA (5,6-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate; acetyl ester, Molecular Probes, Eugene, OR), a redox-sensitive fluorescent dye, was used to analyze the intracellular ROS level by flow cytometry. VSMCs (2×10^6 cells/ml) were pretreated with a indicated concentration of lobastin for 2 h, followed by addition of TNF-α (10 ng/ml) for 4 h. The cells were then stained for 30 min at 37 °C with 10 μM CMH_2-DCFDA. The cells were kept on ice in the dark and at least 5000 cells for each sample were analyzed using a Becton–Dickinson FACSCalibur (BD Biosciences, San Jose, CA), and data were analyzed by FCS Express 5 Flow Cytometry software (De Novo Software, CA, USA).

Statistical analysis

All experiments were performed at least three times (unless otherwise indicated) and each result is reported as the mean ± SEM. For comparisons between two groups, the Student's *t* test was used. Multi-group comparisons of mean values were analyzed by a one-way ANOVA. The significant values are represented by an asterisk ($*p < 0.05$).

Results

Effect of lobastin on VSMC viability

To examine the toxicity of lobastin on VSMCs in culture, MOVAS-1 cells were cultured in the presence of various concentrations (0–10 μg/ml) of lobastin for 24 h and cell viability was subsequently determined using an MTT assay. Cell viability was not influenced by lobastin up to a concentration of 10 μg/ml (Fig. 2a). Similarly, combined TNF-α and lobastin treatment did not affect cell viability at used concentration (Fig. 2b). Thus, in subsequent experiments, cells were treated with up to 10 μg/ml lobastin.

Effect of lobastin on the expression of VCAM-1 in VSMCs

We investigated the effect of lobastin on the expression of TNF-α-induced VCAM-1 in VSMCs. MOVAS-1 cells were pretreated with or without lobastin at the indicated concentrations for 2 h and then stimulated with TNF-α (10 ng/ml) for 8 h. As shown in Fig. 3a, VCAM-1 surface protein expression in VSMCs induced by TNF-α was significantly inhibited by lobastin at 1 and 10 μg/ml. Additionally, the expression of total cellular adhesion molecules on VSMCs was suppressed by lobastin in a concentration-dependent manner (Fig. 3b). Our data also shows that lobastin blocked TNF-α stimulated VCAM-1 expression in another smooth muscle cell line, HASMCs (Fig. 3d).

To further clarify the inhibitory effect of lobastin on TNF-α-stimulated VCAM-1 expression at the transcriptional level, the level of VCAM-1 mRNA expression was determined by qRT-PCR. As shown in Fig. 3c, the expression of VCAM-1 was significantly inhibited by lobastin in VSMCs stimulated with TNF-α for 4 h. Taken together, these data suggest that lobastin treatment results in the inhibition of VCAM-1 protein expression and causes post-transcriptional modulation of VCAM-1 expression.

Inhibitory effect of lobastin on TNF-α-induced activation of NF-kB

It is well known that the VCAM-1 gene contains two adjacent NF-kB binding sites in the upstream promoter region and these sites are key factors in TNF-α-induced VCAM-1 expression (Iademarco et al. 1992; Shu et al. 1993). We therefore examined the effect of lobastin on TNF-α-induced activation of NF-kB. The cells were pre-incubated with various concentrations of lobastin for 2 h prior to stimulation with TNF-α for 4 h. We measured transcriptional activity to determine whether lobastin regulates NF-kB. As shown in Fig. 4a, stimulation of the cells

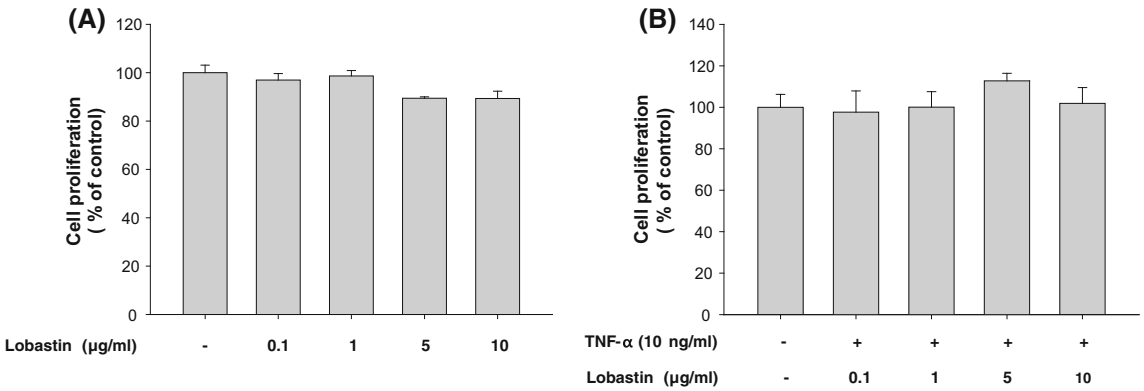

Fig. 2 Effects of lobastin on the proliferation of MOVAS-1 cells. **a** MOVAS-1 cells were pre-incubated with the indicated concentrations of lobastin (0.1, 1, 5 and 10 μg/ml) for 24 h and cell proliferation was determined by an MTT assay. **b** MOVAS-1 cells were cultured in the medium containing indicated concentrations of lobastin (0.1, 1, 5 and 10 μg/ml) and TNF-α for 24 h and cell proliferation was determined by an MTT assay. Cell viability was expressed as a percentage of the absorbance value obtained without lobastin treatment. Data were analyzed using one way ANOVA with an appropriate post-test. *$p < 0.05$, significantly different from the untreated control

with TNF-α increased the luciferase activity and this increased activity was significantly reduced by lobastin in a concentration-dependent manner. By Western blotting analysis, we also investigated the effects of lobastin on TNF-α-induced nuclear translocation of NF-kB in MOVAS-1 cells and HASMCs. As expected, lobastin inhibited the expression of nuclear p65 protein which is the subunit of NF-kB that regulates the transcription of genes associated with inflammation in both cells (Fig. 4b, d). These results suggest that lobastin reduced the TNF-α-induced activation of NF-kB.

Next, to further explore the mechanism of NF-kB inhibition by lobastin, we investigated the effect of lobastin on the degradation of IκBα. Western blotting showed that there was significant degradation of IκBα after 30 min of stimulation with TNF-α, whereas treatment of the cells with lobastin resulted in the interruption of the TNF-α-induced IκBα degradation (Fig. 4c). These results further demonstrate that lobastin inhibits TNF-α-stimulated NF-kB activation in VSMCs.

Effect of lobastin on MAP kinase and TNFR1 in TNF-α-stimulated smooth muscle cells

Since it has been reported that the expression of VCAM-1 is regulated through the mitogen activated protein kinase (MAPK) pathway in various cell types (Lee et al. 2006; Luo et al. 2010), we examined whether the inhibitory effect of lobastin on VCAM-1 expression was mediated through blocking the activation of the MAPK pathway. As shown in Fig. 5a, lobastin significantly inhibited the phosphorylation of p38 and ERK 1/2, but not JNK, in TNF-α-stimulated smooth muscle cells. Further support for this conclusion comes from observation that pretreatment of MAPK inhibitors (p38 MAPK inhibitor, SB203580 and ERK1/2 inhibitor, PD98059) enhanced the inhibitory effect of lobastin on VCAM-1 expression (Fig. 5b). These results suggest that lobastin inhibits TNF-α-induced VCAM-1 expression via a decrease in the p38 and ERK 1/2 signaling pathways.

Next, we investigated the effect of lobastin on the TNF-α receptor 1 (TNFR1) which is the main receptor through which TNF-α activates the NF-κB signaling pathway and adhesion molecules (Kitagaki et al. 2012; Sawa et al. 2007; Zhang et al. 2007). Our results show that TNF-α treatment resulted in an increase in TNFR1 expression and lobastin did not affect the total cellular protein expression of TNFR1 (Fig. 5c), suggesting that the inhibitory effects of lobastin are not mediated through direct inhibition of TNFR1 surface expression in muscle cells.

Effect of lobastin on the production of ROS in TNF-α-stimulated smooth muscle cells

It has been known that oxidative milieu induced by cytokines (IL-1β and TNF-α) leads to an enhanced expression of adhesion molecules via activation of various transcription factors including NF-κB (Kunsch and Medford 1999). Additionally, it was reported that lobastin shows antioxidant activity (Bhattarai et al. 2013). Thus, we examined the effect of lobastin on the production of TNF-α-induced ROS. MOVAS-1 cells and HASMCs were pretreated with lobastin for 2 h and then stimulated with TNF-α for 4 h. Lobastin significantly attenuated the level of ROS production in TNF-α-stimulated VSMCs (Fig. 6). This suggests that lobastin has an antioxidant activity which is involved in the inhibition of TNF-α-induced VCAM-1.

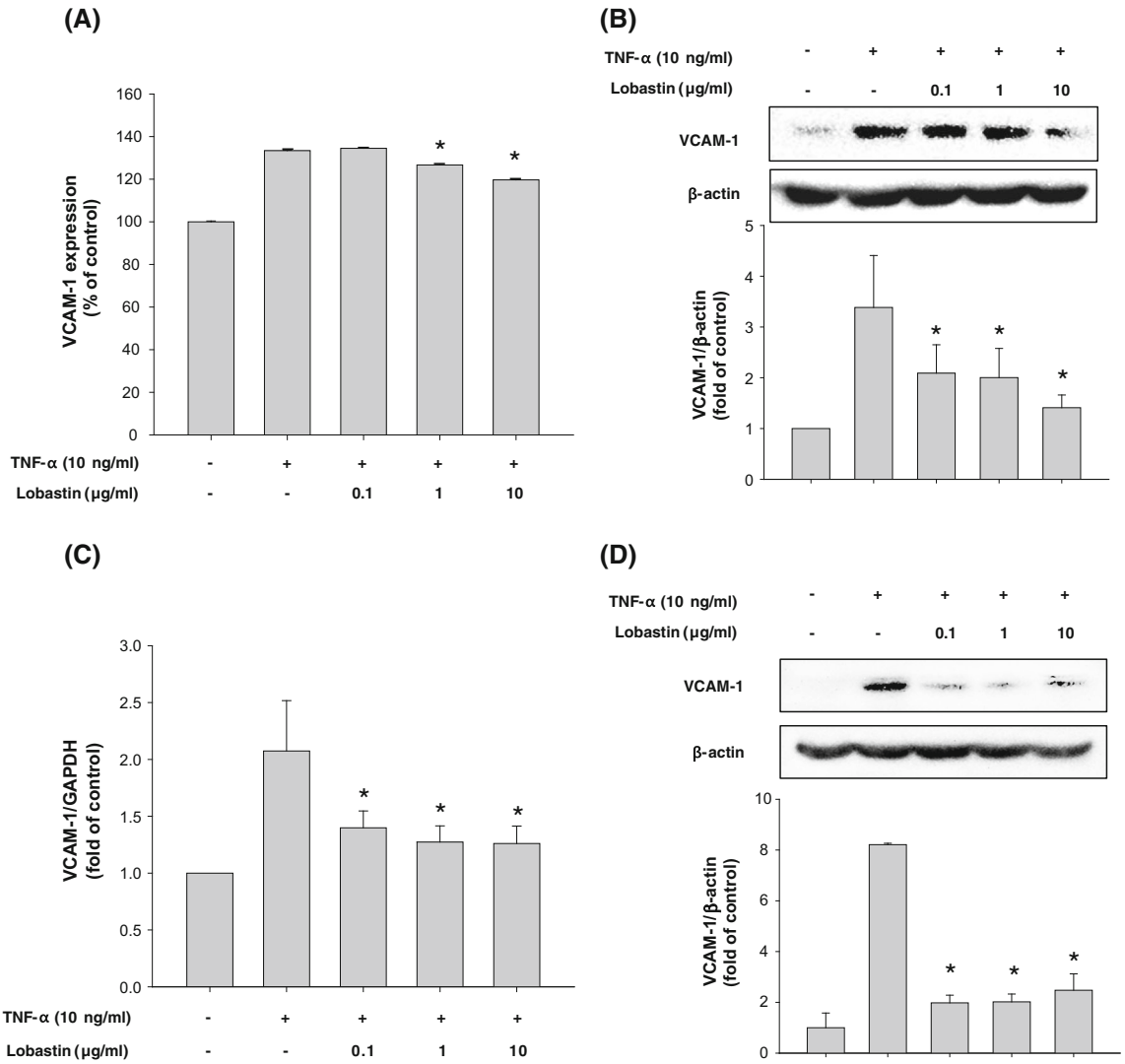

Fig. 3 Effect of lobastin on TNF-α-induced VCAM-1 protein and mRNA expression in VSMCs. **a** MOVAS-1 cells were pretreated with the indicated concentrations of lobastin for 2 h and stimulated with TNF-α (10 ng/ml) for 8 h. VCAM-1 surface protein expression was measured by ELISA. The data are expressed as a percentage of TNF-α-induced VCAM-1 expression. **b** The total cellular VCAM-1 protein levels were determined in MOVAS-1 cells by Western blotting. The β-actin protein level was considered as an internal control. The results illustrated are 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 the respective amount of β-actin protein. **c** MOVAS-1 cells were pre-incubated with the indicated concentrations of lobastin for 2 h and stimulated with TNF-α (10 ng/ml) for 4 h. The mRNA level of VCAM-1 was determined by qRT-PCR. GAPDH served as the internal control. One of the three separate experiments is shown. The levels of VCAM-1 mRNA expression are in arbitrary units, and data are normalized to the respective amount of GAPDH mRNA. Data are expressed using one way ANOVA with an appropriate post-test. *Significantly different from TNF-α-stimulated cells not treated with lobastin ($p < 0.05$)

Discussion

Adhesion molecules participate in inflammatory processes such as atherosclerosis and VCAM-1 expression is induced by inflammatory cytokines such as TNF-α in vascular smooth muscle cells at the base of atherosclerotic lesions. Modulation of VCAM-1 expression may be a promising strategy to prevent and treat chronic inflammatory diseases like atherosclerosis. Here, we addressed the question of pharmacological modulation of VCAM-1 expression upon treatment of VSMCs with TNF-α.

TNF-α is one of the pro-inflammatory cytokines that induce the proliferation of smooth muscle cells and contribute to the development of atheroma via the induction of pro-inflammatory mediators such as VCAM-1 (Dzau et al. 2002; Lee et al. 2006; Warner and Libby 1989). Additionally, VCAM-1 expression is strongly implicated in the development of atherosclerosis. In the present study, our

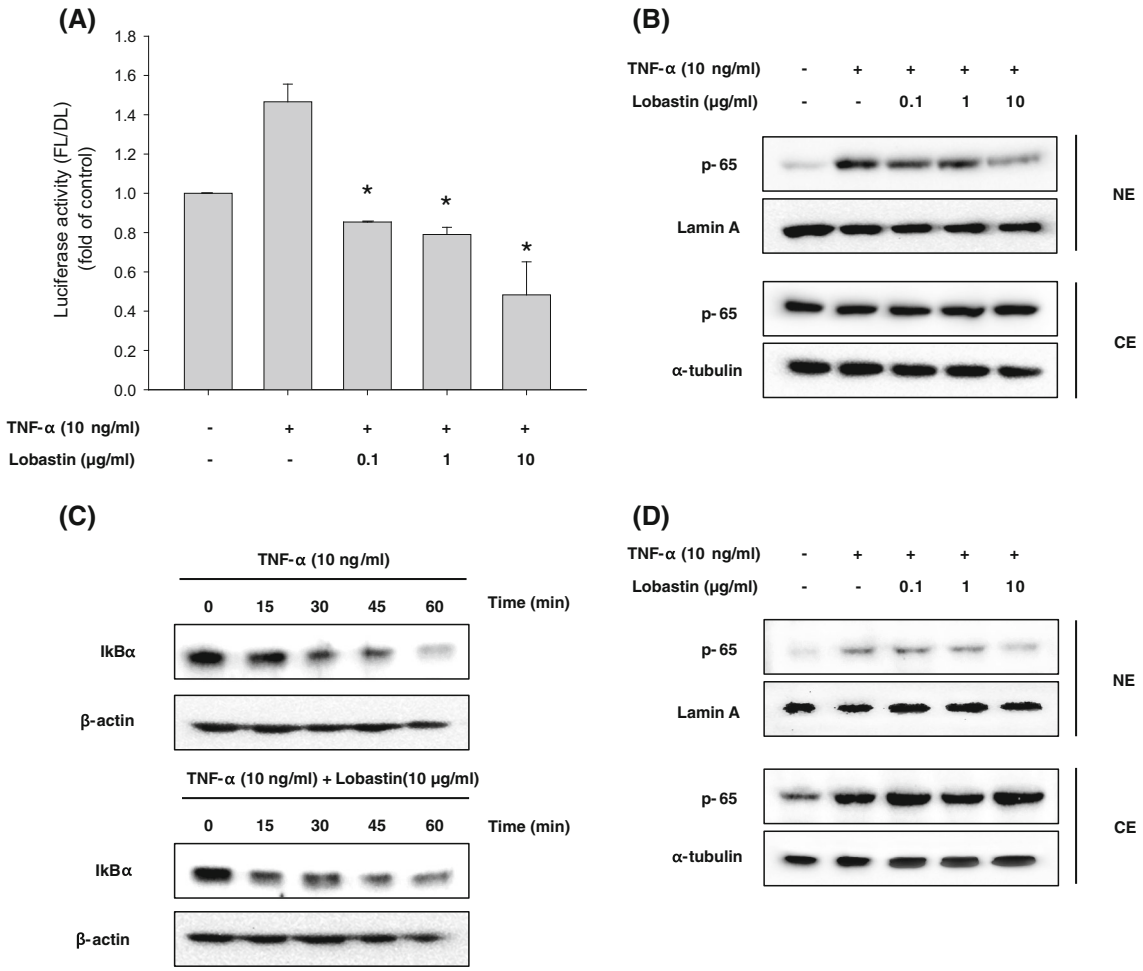

Fig. 4 Influence of lobastin on NF-κB activation and IκBα degradation in TNF-α-stimulated VSMCs. *NE* nuclear extracts, *CE* cytoplasmic extracts. **a** MOVAS-1 cells were transfected with a pGL3-NFκB-Luc reporter plasmid and pCMV-β-gal, pretreated with various concentrations of lobastin for 2 h, and then induced with TNF-α (10 ng/ml) for 4 h. The results are the mean ± SEM (one-way ANOVA followed by post test; $p < 0.05$; $n = 3$). **b**, **d** VSMCs were preincubated with or without various concentrations of lobastin for 2 h and then treated with TNF-α (10 ng/ml) for 4 h in MOVAS-1 (**b**) and HASMCs (**d**). Cytoplasmic and nuclear levels of NF-κB p65 were detected by Western blotting to analyze the translocation of NF-κB. α-Tubulin and Lamin A were used as loading controls for cytosolic and nuclear protein fractions, respectively. **c** MOVAS-1 cells were pre-incubated with or without lobastin (10 μg/ml) for 2 h and then stimulated with TNF-α (10 ng/ml) for the indicated times. The whole cell lysates were analyzed by Western blotting with an anti-IκBα antibody. The β-actin protein level was considered as an internal control. The results illustrated are representative of three separate experiments

results reveal that lobastin has the ability to modulate VCAM-1 expression in TNF-α-stimulated VSMCs. Treatment with lobastin is associated with the inhibition of the NF-κB and ERK/p38 pathways, resulting in a decrease in VCAM-1 promoter activity, the level of mRNA and protein expression. This could lead to reduced monocyte adhesion to VSMCs and may be the mechanism by which lobastin regulates monocyte recruitment during atherosclerotic lesion formation.

It is well known that TNF-α stimulates the activation of MAPKs which correspond to a family of serine/threonine kinases that is divided into p38 MAPK, ERK 1/2 and JNK which relay, amplify and integrate signals from various stimuli and elicit an appropriate physiological response including inflammatory responses (Ho et al. 2008; Manzoor and Koh 2012). Furthermore, several lines of evidence including us have suggested that the expression of VCAM-1 is mainly regulated by MAPKs in various cell types (Goetze et al. 1999; Kim et al. 2012; Luo et al. 2010). Cells may use diverse MAPK signaling pathways for TNF-α-induced expression of adhesion molecules and the MAPK signaling pathways are differently involved in response to anti-inflammatory compounds (Ho et al. 2008; Hoshiya and Awazu 1998; Law et al. 1996; Rao et al. 1995). Additionally, inhibition of MAPK pathway has been successfully used in treating inflammation. Therefore, in this study, we evaluated the effect of lobastin on the p38 MAPK, ERK 1/2 and JNK pathways in TNF-α-stimulated VSMCs. The

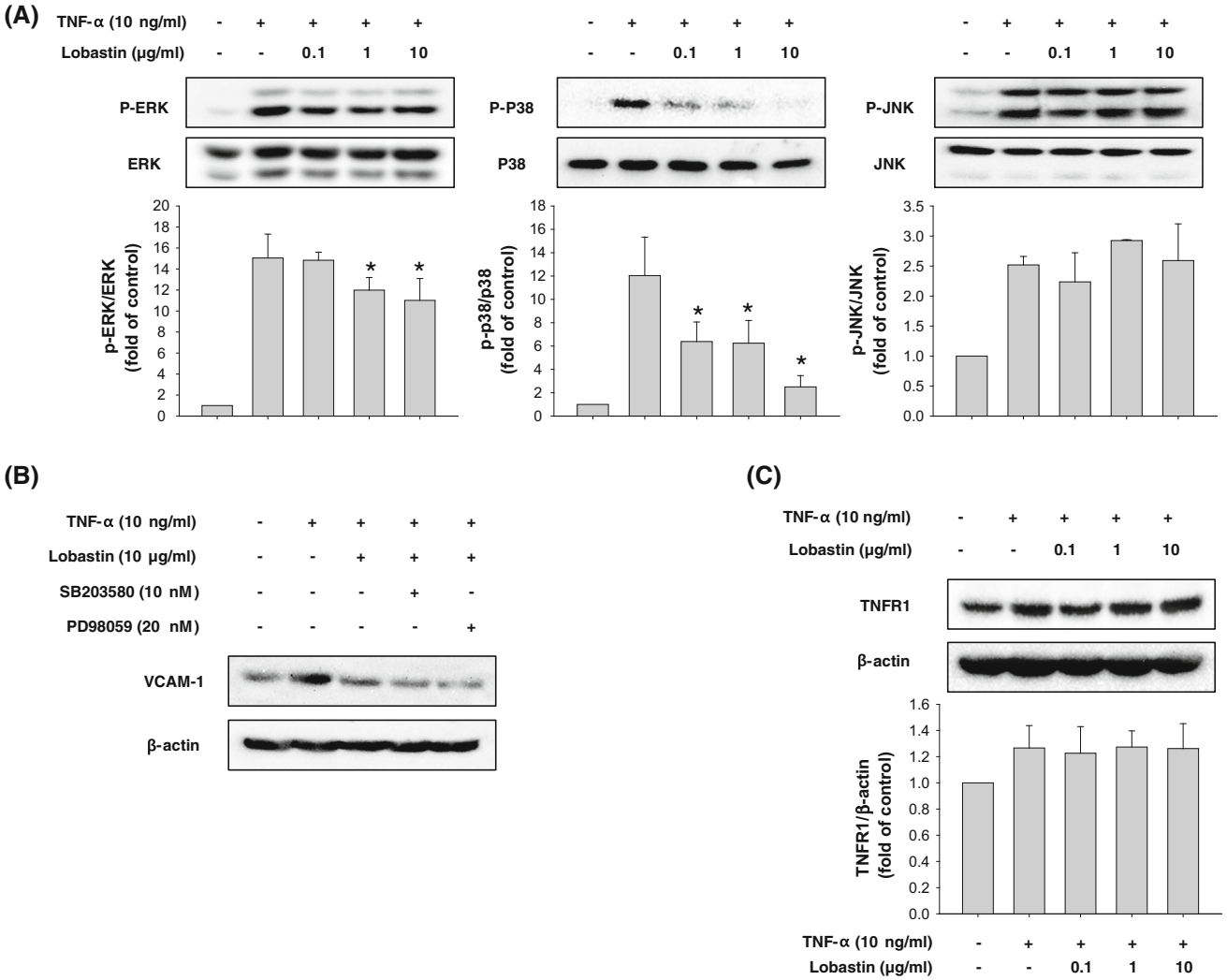

Fig. 5 Effect of lobastin on the activation of MAP kinases and TNFR1 expression in TNF-α-stimulated VSMCs. **a** MOVAS-1 cells were pretreated with the indicated concentrations of lobastin for 2 h and then incubated with TNF-α (10 ng/ml) for 15 min. The whole cell lysates were analyzed by Western blotting. The relative intensities were expressed as the ratio of phospho-MAPK to total MAPK. A typical result from three independent experiments is shown. **b** MOVAS-1 cells were pretreated with MAPK inhibitors (p38 inhibitor, SB203580, ERK1/2 inhibitor and PD98059) for 2 h and then incubated in the presence of lobastin (10 μg/ml) for 2 h followed by the stimulation of TNF-α (10 ng/ml) for 8 h. Cell lysates were harvested and subjected to Western blotting analysis **c** MOVAS-1 cells were pretreated with or without the indicated concentrations of lobastin for 2 h and then treated with TNF-α (10 ng/ml) for 4 h. The expression of TNFR1 was detected by Western blotting with an anti-TNFR1 antibody. The β-actin protein level was considered as an internal control. A typical result from three independent experiments is shown. The intensity of the bands was quantitated by densitometry. The levels of TNFR1 expression are in arbitrary units, and data are normalized to the respective amount of β-actin protein. The data are analyzed as mean ± SEM (one-way ANOVA followed by post test; $p < 0.05$; $n = 3$). Results are representative of at least three independent experiments

present data show that lobastin significantly reduced phosphorylation of p38 MAPK and ERK 1/2, but not JNK, Moreover, the inhibitory effect of lobastin on VCAM-1 expression was enhanced by MAPK inhibitors. These results suggest that lobastin treatment results in a decrease in TNF-α-induced VCAM-1 expression via the inhibition of the p38 MAPK and ERK 1/2 pathways. Additionally, we examined the effect of lobastin on the expression of TNFR1 in VSMCs due to the fact that TNFR1 is involved in adhesion molecule expression as well as VSMC proliferation (Sawa et al. 2007; Zhang et al. 2007) and the numerous biological effects of TNF-α are signaled via TNFR1, the major signaling receptor in most cells (McCoy and Tansey 2008). Interestingly, TNFR1 expression in cells treated with lobastin was not altered compared with TNF-α-stimulated cells, suggesting that the inhibitory effect of lobastin on VCAM-1 expression is not related to TNF-α-induced TNFR1 surface expression in muscle cells.

Many studies have shown that MAPKs activate many transcription factors that are important molecular targets

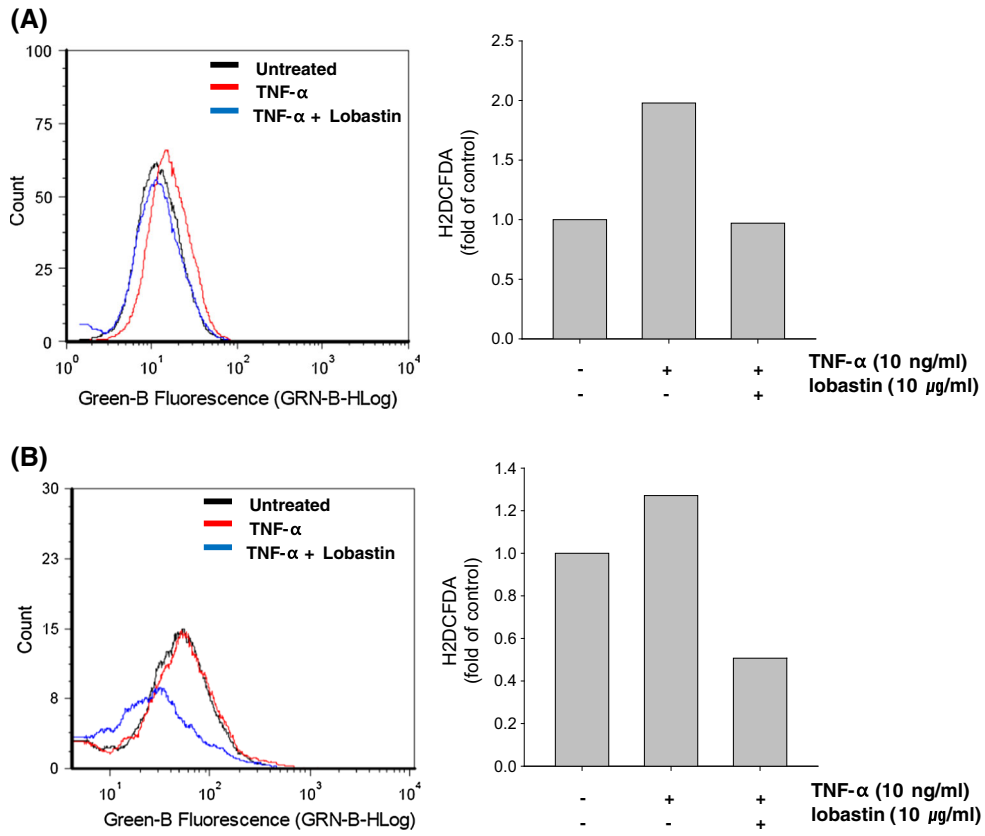

Fig. 6 Effect of lobastin on the production of ROS in TNF-α-stimulated VSMCs. VSMCs were pretreated with lobastin (10 μg/ml) for 2 h and then induced with TNF-α (10 ng/ml) for 4 h in MOVAS-1 cells (**a**) and HASMCs (**b**). The level of ROS generation was measured as described in "Materials and methods"

for therapeutic intervention, because they are central to the control of many genes involved in the regulation of inflammation and immunity. It is widely accepted that NF-κB plays a dominant role in the transcription of cell adhesion molecule genes in TNF-α-treated cells (Beg et al. 1993; Collins et al. 1995) and the 5′-regulatory region of the VCAM-1 gene contains the NF-κB binding site (Iademarco et al. 1992; Shu et al. 1993). In addition, it has been suggested that inducible NF-κB activation requires the phosphorylation and degradation of IκBα and the nuclear translocation of p65 (Tak and Firestein 2001). Therefore, we examined the effect of lobastin on NF-κB activation. In the present study, the activation of NF-κB was concentration-dependently inhibited by lobastin through the repression of IκBα degradation and subsequent p65 nuclear translocation in VSMCs stimulated with TNF-α. Thus, this inhibitory mechanism appears to be involved in the suppressive effect of lobastin on VCAM-1 expression. We cannot formally rule out the possibility that VCAM-1 expression induced by TNF-α is mediated via other transcription factors such as Nrf2, IRF-1and SP-1 which are known to be associated with the gene expression of adhesion molecules (Chen et al. 2009; Neish et al. 1995a, b). Nevertheless, the simplest interpretation of our data is that the MAPK and NF-κB pathways represent a novel insight into the mechanism of lobastin effects on VCAM-1 expression in VSMCs.

Oxidative stress plays an important in the regulation of VCAM-1 expression. Inflammatory cytokines including TNF-α and IL-1β induce the cellular synthesis of reactive oxygen species, which results in atherosclerotic plaque development via endothelial VCAM-1 expression and subsequence accumulation of monocyte (Cybulsky and Gimbrone 1991; Li et al. 1993; Meier et al. 1989). Our results reveal that lobastin blocked the production of TNF-α-stimulated ROS, suggesting that lobastin has an antioxidant activity in VSMCs. It has been known that the regulation of vascular gene expression by ROS is mediated through several transcriptional signaling pathways such as MAPK and NF-κB activation (Kunsch and Medford 1999). Furthermore, previous studies demonstrated that antioxidant, NAC, significantly inhibits TNF-α-induced VCAM-1 expression (Kim et al. 2012; Park et al. 2014). Based on these findings, it is possible that the inhibitory effect of lobastin on the

TNF-α-induced expression of VCAM-1 and activation of MAPK and NF-κB may be due to its antioxidant properties.

In conclusion, the data in the present study indicate that lobastin suppresses the expression of VCAM-1 through the inhibition of the ROS production, p38 MAPK, ERK 1/2 and NF-κB signaling pathways in TNF-α treated VSMCs. Thus, the inhibitory activity of lobastin on VCAM-1 in vascular smooth muscle cells implies the possibility of pharmacological intervention specifically directed towards inflammatory diseases including atherosclerosis. Further investigation including in vivo studies using animal models, will contribute to the elucidation of the clinical use of lobastin for the treatment of atherosclerosis.

Acknowledgments This research was supported by the grant of the Ministry of Oceans and Fisheries' R&D project (PM13030) and the Korea Polar Research Institute (KOPRI) project (PE13040).

Compliance with ethical standards

Conflict of interest The authors have no conflicts of interest to declare.

References

Beg AA, Finco T, Nantermet PV, Baldwin A (1993) Tumor necrosis factor and interleukin-1 lead to phosphorylation and loss of I kappa B alpha: a mechanism for NF-kappa B activation. Mol Cell Biol 13:3301–3310

Bhattarai HD, Kim T, Oh H, Yim JH (2013) A new pseudodepsidone from the Antarctic lichen Stereocaulon alpinum and its antioxidant, antibacterial activity. J Antibiot 66:559–561

Braun M, Pietsch P, Schrör K, Baumann G, Felix SB (1999) Cellular adhesion molecules on vascular smooth muscle cells. Cardiovasc Res 41:395–401

Byeon H-E, Park B-K, Yim JH, Lee HK, Moon E-Y, Rhee D-K, Pyo S (2012) Stereocalpin A inhibits the expression of adhesion molecules in activated vascular smooth muscle cells. Int Immunopharmacol 12:315–325

Chen X-L, Dodd G, Kunsch C (2009) Sulforaphane inhibits TNF-α-induced activation of p38 MAP kinase and VCAM-1 and MCP-1 expression in endothelial cells. Inflamm Res 58:513–521

Cho S-J, Kang N-S, Park S-Y, Kim B-O, Rhee D-K, Pyo S (2003) Induction of apoptosis and expression of apoptosis related genes in human epithelial carcinoma cells by *Helicobacter pylori* VacA toxin. Toxicon 42:601–611

Choudhary MI, Jalil S (2005) Bioactive phenolic compounds from a medicinal lichen, *Usnea longissima*. Phytochemistry 66: 2346–2350

Collins T, Read M, Neish A, Whitley M, Thanos D, Maniatis T (1995) Transcriptional regulation of endothelial cell adhesion molecules: NF-kappa B and cytokine-inducible enhancers. FASEB J 9:899–909

Cybulsky MI, Gimbrone M (1991) Endothelial expression of a mononuclear leukocyte adhesion molecule during atherogenesis. Science 251:788–791

Cybulsky MI, Iiyama K, Li H, Zhu S, Chen M, Iiyama M, Davis V, Gutierrez-Ramos J-C, Connelly PW, Milstone DS (2001) A major role for VCAM-1, but not ICAM-1, in early atherosclerosis. J Clin Investig 107:1255–1262

Dzau VJ, Braun-Dullaeus RC, Sedding DG (2002) Vascular proliferation and atherosclerosis: new perspectives and therapeutic strategies. Nat Med 8:1249–1256

Goetze S, Xi X-P, Kawano Y, Kawano H, Fleck E, Hsueh WA, Law RE (1999) TNF-α-induced migration of vascular smooth muscle cells is MAPK dependent. Hypertension 33:183–189

Ho AWY, Wong CK, Lam CWK (2008) Tumor necrosis factor-α up-regulates the expression of CCL2 and adhesion molecules of human proximal tubular epithelial cells through MAPK signaling pathways. Immunobiology 213:533–544

Hoshiya M, Awazu M (1998) Trapidil inhibits platelet-derived growth factor-stimulated mitogen-activated protein kinase cascade. Hypertension 31:665–671

Huo Y, Ley K (2001) Adhesion molecules and atherogenesis. Acta Physiol Scand 173:35–43

Iademarco M, McQuillan JJ, Rosen G, Dean D (1992) Characterization of the promoter for vascular cell adhesion molecule-1 (VCAM-1). J Biol Chem 267:16323–16329

Ju JW, Kim SJ, Jun CD, Chun JS (2002) p38 kinase and c-Jun N-terminal kinase oppositely regulates tumor necrosis factor α-induced vascular cell adhesion molecule-1 expression and cell adhesion in chondrosarcoma cells. IUBMB Life 54:293–299

Kim J-Y, Park H-J, Um SH, Sohn E-H, Kim B-O, Moon E-Y, Rhee D-K, Pyo S (2012) Sulforaphane suppresses vascular adhesion molecule-1 expression in TNF-α-stimulated mouse vascular smooth muscle cells: involvement of the MAPK, NF-κB and AP-1 signaling pathways. Vascul Pharmacol 56:131–141

Kitagaki M, Isoda K, Kamada H, Kobayashi T, Tsunoda S, Tsutsumi Y, Niida T, Kujiraoka T, Ishigami N, Ishihara M (2012) Novel TNF-α receptor 1 antagonist treatment attenuates arterial inflammation and intimal hyperplasia in mice. J Atheroscler Thromb 19:36–46

Kumar KC, Muller K (1999) Lichen metabolites. 1. Inhibitory action against leukotriene B4 biosynthesis by a non-redox mechanism. J Nat Prod 62:817–820

Kunsch C, Medford RM (1999) Oxidative stress as a regulator of gene expression in the vasculature. Circ Res 85:753–766

Law RE, Meehan WP, Xi X-P, Graf K, Wuthrich DA, Coats W, Faxon D, Hsueh WA (1996) Troglitazone inhibits vascular smooth muscle cell growth and intimal hyperplasia. J Clin Investig 98:1897

Lee CW, Lin WN, Lin CC, Luo SF, Wang JS, Pouyssegur J, Yang CM (2006) Transcriptional regulation of VCAM-1 expression by tumor necrosis factor-alpha in human tracheal smooth muscle cells: involvement of MAPKs, NF-kappaB, p300, and histone acetylation. J Cell Physiol 207:174–186

Li H, Cybulsky MI, Gimbrone M, Libby P (1993) An atherogenic diet rapidly induces VCAM-1, a cytokine-regulatable mononuclear leukocyte adhesion molecule, in rabbit aortic endothelium. Arterioscler Thromb Vasc Biol 13:197–204

Luo SF, Fang RY, Hsieh HL, Chi PL, Lin CC, Hsiao LD, Wu CC, Wang JS, Yang CM (2010) Involvement of MAPKs and NF-κB in tumor necrosis factor α-induced vascular cell adhesion molecule 1 expression in human rheumatoid arthritis synovial fibroblasts. Arthritis Rheum 62:105–116

Lusis AJ (2000) Atherosclerosis. Nature 407:233–241

Manzoor Z, Koh Y-S (2012) Mitogen-activated protein kinases in inflammation. J Bacteriol Virol 42:189–195

McCoy MK, Tansey MG (2008) TNF signaling inhibition in the CNS: implications for normal brain function and neurodegenerative disease. J Neuroinflamm 5:45

Meier B, Radeke H, Selle S, Younes M, Sies H, Resch K, Habermehl G (1989) Human fibroblasts release reactive oxygen species in response to interleukin-1 or tumour necrosis factor-alpha. Biochem J 263:539–545

Neish AS, Khachigian LM, Park A, Baichwal VR, Collins T (1995a) Sp1 is a component of the cytokine-inducible enhancer in the promoter of vascular cell adhesion molecule-1. J Biol Chem 270:28903–28909

Neish AS, Read MA, Thanos D, Pine R, Maniatis T, Collins T (1995b) Endothelial interferon regulatory factor 1 cooperates with NF-kappa B as a transcriptional activator of vascular cell adhesion molecule 1. Mol Cell Biol 15:2558–2569

Nguyen CT, Luong TT, Kim G-L, Pyo S, Rhee D-K (2015) Korean Red Ginseng inhibits apoptosis in neuroblastoma cells via estrogen receptor β-mediated phosphatidylinositol-3 kinase/Akt signaling. J Ginseng Res 39:69–75

Øvstedal DO, Smith RL (2001) Lichens of Antarctica and South Georgia: a guide to their identification and ecology. Cambridge University Press, Cambridge

Park B, Yim J-H, Lee H-K, Kim B-O, Pyo S (2014) 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. Biosci Biotechnol Biochem 79:1–14

Rao GN, Delafontaine P, Runge MS (1995) Thrombin stimulates phosphorylation of insulin-like growth factor-1 receptor, insulin receptor substrate-1, and phospholipase C-γ1 in rat aortic smooth muscle cells. J Biol Chem 270:27871–27875

Saklani A, Upreti D (1992) Folk uses of some lichens in Sikkim. J Ethnopharmacol 37:229–233

Sawa Y, Sugimoto Y, Ueki T, Ishikawa H, Sato A, Nagato T, Yoshida S (2007) Effects of TNF-alpha on leukocyte adhesion molecule expressions in cultured human lymphatic endothelium. J Histochem Cytochem 55:721–733

Saxena U, Medford R (2000) Vascular adhesion molecule-1 (VCAM-1), an inflammatory gene target for new therapeutics. Curr Opin Cardiovasc Pulm Renal Invest Drugs 2:258–262

Seo C, Sohn JH, Ahn JS, Yim JH, Lee HK, Oh H (2009) Protein tyrosine phosphatase 1B inhibitory effects of depsidone and pseudodepsidone metabolites from the Antarctic lichen Stereocaulon alpinum. Bioorg Med Chem Lett 19:2801–2803

Shu HB, Agranoff A, Nabel E, Leung K, Duckett C, Neish A, Collins T, Nabel G (1993) Differential regulation of vascular cell adhesion molecule 1 gene expression by specific NF-kappa B subunits in endothelial and epithelial cells. Mol Cell Biol 13:6283–6289

Tak PP, Firestein GS (2001) NF-κB: a key role in inflammatory diseases. J Clin Investig 107:7–11

Vazquez G, Smedlund K, Tano J-YK, Lee R (2012) Molecular and cellular aspects of atherosclerosis: emerging roles of TRPC channels. Coron Artery Dis 51:91–102

Vijayakumar C, Viswanathan S, Reddy MK, Parvathavarthini S, Kundu A, Sukumar E (2000) Anti-inflammatory activity of (+)-usnic acid. Fitoterapia 71:564–566

Warner S, Libby P (1989) Human vascular smooth muscle cells. Target for and source of tumor necrosis factor. J Immunol 142:100–109

Zhang L, Peppel K, Sivashanmugam P, Orman ES, Brian L, Exum ST, Freedman NJ (2007) Expression of tumor necrosis factor receptor-1 in arterial wall cells promotes atherosclerosis. Arterioscler Thromb Vasc Biol 27:1087–1094