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PII: S0891-5849(11)00414-X
DOI: doi: 10.1016/j.freeradbiomed.2011.06.023
Reference: FRB 10718

To appear in: *Free Radical Biology and Medicine*

Received date: 3 January 2011
Revised date: 10 June 2011
Accepted date: 18 June 2011

Please cite this article as: Chan-Jung Liang, Shu-Huei Wang, Yung-Hsiang Chen, Shih-Sheng Chang, Tong-Long Hwang, Yann-Lii Leu, Ying-Chih Tseng, Chi-Yuan Li, Yuh-Lien Chen, Viscolin reduces VCAM-1 expression in TNF-$\alpha$-treated endothelial cells via the JNK/NF-$\kappa$B and ROS pathway, *Free Radical Biology and Medicine* (2011), doi: 10.1016/j.freeradbiomed.2011.06.023

# Viscolin reduces VCAM-1 expression in TNF-α–treated endothelial cells via the JNK/NF-κB and ROS pathway

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## Abstract

Viscolin, a major active component in a chloroform extract of *Viscum coloratum*, has antioxidative and anti-inflammatory properties. We focused on its effects on the expression of vascular cell adhesion molecule-1 (VCAM-1) in tumor necrosis factor-α (TNF-α)-treated human umbilical vein endothelial cells (HUVECs). The TNF-α-induced expression of VCAM-1 was significantly reduced by, respectively, 38±7%% or 34±16% when HUVECs were pretreated with 10 or 30 μM viscolin, as shown by Western blotting, and was also significantly reduced by pretreatment with the antioxidants N-acetylcysteine, diphenylene iodonium chloride, and apocynin. Viscolin also reduced TNF-α-induced VCAM-1 mRNA expression and promoter activity, decreased reactive oxygen species (ROS) production, nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity, and significantly reduced the binding of monocytes to TNF-α-stimulated HUVECs. The attenuation of TNF-α-induced VCAM-1 expression and cell adhesion was partly mediated by a decrease in JNK phosphorylation. Furthermore, viscolin reduced VCAM-1 expression in the aorta of TNF-α-treated mice in vivo. Taken together, these data show that viscolin inhibits TNF-α-induced JNK phosphorylation, nuclear translocation of NF-κB p65, and ROS generation and thereby suppresses VCAM-1 expression, resulting in reduced adhesion of leukocytes. These results also suggest that viscolin may prevent the development of atherosclerosis and inflammatory responses.

*Keyword*: viscolin, adhesion molecules, reactive oxygen species, inflammation, mitogen-activated protein kinases.

## Introduction

Vascular inflammation is a critical risk factor in the initiation and development of cardiovascular disorders, such as atherosclerosis [1]. Activation of the endothelium at the inflammatory site results in leukocyte adhesion to the endothelium and subsequent transmigration of leukocytes into the subendothelial space, an early event in atherosclerosis [2]. The leukocyte adhesion is primarily mediated by adhesion molecules expressed on the surface of the endothelium. Previous studies have shown that vascular cell adhesion molecule 1 (VCAM-1), but not intercellular cell adhesion molecule-1 (ICAM-1), mediates leukocyte recruitment to early lesions of atherosclerosis and appears to be the dominant adhesion molecule on the endothelial surface of the vascular wall in the initiation of atherosclerosis [3, 4]. In addition, levels of VCAM-1 expression have been suggested to be closely associated with the generation of reactive oxygen species (ROS) [5]. Thus, inhibition of VCAM-1 expression and ROS generation might be a useful therapeutic strategy for treating vascular inflammation and cardiovascular diseases [6].

Herbal medicines have been widely used in Asian countries for many centuries and the active components of herbs and their structural backbones might provide a useful platform for the development of effective pharmacological agents. *Viscum coloratum* Nakai, a traditional Chinese herbal medicine, has been used for a long time

to treat inflammatory diseases, such as rheumatism and atherosclerosis. However, its active components and pharmacological effects have not been extensively studied. *V. coloratum* inhibits superoxide anion generation by human neutrophils [7] and viscolin (4′,4″-dihydroxy-2′,3′,6′,3″-tetramethoxy-1,3-diphenylpropane), a new chalcone from *V. coloratum*, inhibits human neutrophil superoxide anion and elastase release [8]. Although previous studies have shown that viscolin has antioxidative and anti-inflammatory effects on human neutrophils [7-9], it is unclear whether it has anti-inflammatory and antioxidative effects on human vascular endothelial cells. Furthermore, the regulation of adhesion molecule expression involves a complex array of intracellular signaling pathways including mitogen activated protein kinases (MAPKs), transcriptional factors, and ROS [10-13]. Although these multiple signaling molecules have received considerable attention [10-13], little is known about the effects of viscolin on adhesion molecule expression and the mechanisms of these effects, and a better understanding of this might provide important insights into the prevention of atherogenesis and inflammation. We therefore tested the ability of viscolin to modulate the expression of adhesion molecules, MAPKs, and transcriptional factors in tumor necrosis factor-α (TNF-α)-treated human umbilical vein endothelial cells (HUVECs). In addition, we examined its effects on VCAM-1 expression in TNF-α-treated mice. Our results showed that viscolin reduced VCAM-1

expression both in vitro and in vivo and that this effect is partly mediated by inhibition of JNK phosphorylation, NF-κB activation, and ROS generation. Viscolin also significantly inhibited the adhesion of the human monocytic cell line U937 to TNF-α-treated HUVECs.

## Materials and Methods

*Materials*

Polyclonal rabbit IgG against human VCAM-1, GAPDH, phospho-p38, or histone (H1) and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG or anti-rabbit IgG antibodies were purchased from Santa Cruz (Santa Cruz, CA). Polyclonal rabbit IgG against human phospho-ERK 1/2 or phospho-JNK was purchased from Cell Signaling (Beverly, MA). Monoclonal rabbit against human p65 and phospho-p65 antibodies were purchased from GenText (Irvine, CA). Diphenylene iodonium chloride (DPI), PD98058, SP600125, and SB203580 were purchased from Biomol (Plymouth meeting, PA). Apocynin (APO) was purchased from ChromaDex (Irvine, CA). The amplex red hydrogen peroxide/peroxidase assay kit and Trizol reagent were purchased from Invitrogen (Carlsbad, CA). Recombinant human TNF-α was purchased from Peprotech (Rocky Hill, USA). N-acetylcysteine (NAC) was purchased from Sigma-Aldrich (St. Louis, MO). PVDF membranes were purchased

from Millipore (Billerica, MA).

*Extraction and purification of viscolin*

Viscolin was purified as described previously [7, 8]. In brief, dried stems of V. coloratum Nakai (family Loranthaceae) (471 g) were extracted six times with 1 L of methanol and the combined extracts evaporated and partitioned to yield chloroform and aqueous extracts. The chloroform extract (10.5 g) was subjected to column chromatography on a silica gel column and eluted with chloroform and methanol step gradients to obtain the active extract, PPE-SVC ($CHCl_3$:MeOH=9:1) (4.7 g). PPE-SVC was rechromatographed on a silica gel column and eluted with a gradient of *n*-hexane and acetone to give a chalcone derivative, viscolin (Figure 1, 53.6 mg).

*Cell culture*

Primary cultures of HUVECs were prepared as previously described [14]. The cells were grown in medium 199 containing penicillin-streptomycin (1%), endothelial cell growth supplement (30 μg/mL), and fetal bovine serum (FBS, 10%) at 37°C in a humidified atmosphere of 95% air, 5% $CO_2$ and were used between passages 2 and 5.

*RNA extraction and reverse transcriptase-polymerase chain reaction (PCR)*

Total RNA was extracted using Trizol reagent (Invitrogen; Carlsbad, CA) according to the manufacturer's protocol. The reverse-transcriptase reaction was carried out using M-MLV reverse transcriptase (Invitrogen; Carlsbad, CA).

Complementary DNA was generated by addition of 1 μg of total RNA to a reaction mixture containing 0.5 μg/μL of oligo-deoxythymidine, 20 mM dNTP, 0.1 M dithiothreitol, 250 mM Tris-HCl pH 8.3, 375 mM KCl, and 15 mM $MgCl_2$ and reaction at 37$^{o}$C for 90 min. The oligonucleotide primers used were 5'-GGAACCTTGCAGCTTACAGTGACA-3' (forward) and 5'-CAAGTCTACATATCACCCAAG-3' (reverse) for VCAM-1 and 5'-GTAACCCGTTGAACCCCATT-3' (forward) and 5'-CCATCCAATCGGTAGTAGCG-3' (reverse) for 18S subunit ribosomal RNA. The amplification profile was 1 cycle of initial denaturation at 94$^{o}$C for 5 min and 30 cycles of denaturation at 94$^{o}$C for 1 min, primer annealing at 62 $^{o}$C for 1 min, and extension at 72 $^{o}$C for 5 min. PCR products were analyzed on ethidium bromide-stained 2% agarose gels.

*Preparation of cell lysates and Western blot analysis*

To prepare cell lysates, the cells were lysed for 1 h at 4ºC in 20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 % Triton X-100, 1 mM phenylmethylsulfonyl fluoride, pH 7.4, then the lysate was centrifuged at 4000 *g* for 30 min at 4ºC and the supernatant retained. Western blot analyses were performed as described previously [15]. Briefly, samples of cell lysate (20 μg of protein) were subjected to 10% SDS-PAGE and transferred to PVDF membranes, which were then

treated with 3% nonfat milk in 0.1 M phosphate buffer for 1 h at room temperature (RT) to block nonspecific binding of antibody. The membranes were then incubated overnight at 4ºC with rabbit antibodies against human phospho-JNK, human phospho-ERK1/2, or human phospho-p38, all 1:1000 in PBS, then for 1 h at RT with HRP-conjugated goat anti-rabbit IgG antibodies (1:2000 in PBS, Santa Cruz), bound antibodies being detected using Chemiluminescence Reagent Plus (NEN, MA, USA). The intensity of each band was quantified using a densitometer. Antibodies against GAPDH (1:5000, Santa Cruz) were used as loading controls.

*VCAM-1 luciferase activity assay*

The VCAM-1-luc plasmid was constructed by cloning the human VCAM-1 promoter (a region spanning bp -1716 to -119) into the pGL3-basic vector (Promega, Madison, WI) and was used to transfect mouse embryonic fibroblast cells (NIH 3T3 cells) using Lipofectamine 2000 reagent (Invitrogen; Carlsbad, CA). To measure promoter activity, the cells were disrupted by sonication in lysis buffer (Promega), then, after centrifugation at 13000 g at 4ºC for 10 min, aliquots of the supernatants were tested for luciferase activity using the luciferase assay system (Promega). The luciferase activity was then normalized to the amount of cellular protein.

*Immunocytochemical localization of NF-κB p65*

To localize NF-κB expression in situ, confluent HUVECs (controls or cells treated

for 24 h with different drugs) on slides were incubated in the absence or presence of 10 ng/mL of TNF-α for 30 min, fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS), pH 7.4, for 15 min at 4 °C, then reacted for 1 h at RT with rabbit anti-human NF-κB p65 antibodies (1: 100 dilution in PBS; Genetex, Inc). After washes, the slides were incubated for 1 h at 37 °C with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG antibodies (Sigma) and viewed on a fluorescence microscope.

*Eelectrophoretic mobility shift assay (EMSA)*

The preparation of nuclear protein extracts and the EMSA conditions have been described previously [15]. Nuclear proteins were extracted using NE-PER reagent (Pierce, Rockford, IL) according to the manufacturer's protocol. The AP-1 and NF-κB binding activity of equal amounts (10 μg) of nuclear protein was analyzed using a LightShift Chemiluminescent EMSA Kit (Pierce, Rockford, IL). The synthetic double-stranded oligonucleotides used as the probes in the gel shift assay were 5'-AGT TGA GGG GAC TTT CCC AGG C-3' and 3'-TCA ACT CCC CTG AAA GGG TCC G-5' for NF-κB and 5'-CGC TTG ATG AGT CAG CCG GAA-3' and 3'-GCG AAC TAC TCA GTC GGC CTT-5' for AP-1.

*Detection of ROS ($O_2^{\bullet-}$ and $H_2O_2$) production*

The effect of viscolin on $O_2^{\bullet-}$ and $H_2O_2$ production by HUVECs was determined by a fluorometric assay using dihydroethidium (DHE) and amplex red as the probe,

respectively [16]. Confluent HUVECs were incubated with or without 30 μM viscolin for 24 h or 10 μM DPI for 2 h. HUVECs were incubated with 20 μM DHE for 20 min or with 50 μM amplex red/HRP for 10 min at 37℃, and then 10 ng/mL of TNF-α was added to the well for the indicated time. The fluorescence density (relative fluorescence units) was detected at 588/630 nm and 544/590 nm for excitation/emission for ethidium corresponding to $O_2^-$ and resorufin to $H_2O_2$, respectively, using a multi-detection reader (SpectraMax M5, Molecular Device).

*Measurement of superoxide anion ($O_2^{\bullet-}$) production*

The assay for the production of $O_2^{\bullet-}$ was based on the superoxide dismutase-inhibited reduction of ferricytochrome *c* and performed as described previously [17]. $O_2^{\bullet-}$ generation was measured after addition of 160 μM NADPH to 800 μL of relaxation buffer containing $4 \times 10^6$ cell equivalents of membrane extract, $1.2 \times 10^7$ cell equivalents of cytosol, 2 μM GTP-γ-S, 0.5 mg/mL of ferricytochrome *c*, and 100 μM sodium dodecylsulfate (SDS). To facilitate the assembly of NADPH oxidase components, all constituents (excluding NADPH) were incubated at RT for 3 min, then any test drug was added and the mixture incubated for 1 min at RT, then NADPH was added and the mixture incubated for 10 min at 37 ℃. Changes in absorbance at 550 nm due to reduction of ferricytochrome *c* were monitored.

*Plasma membrane preparation, NADPH oxidase activity assay, and Western blot*

*analysis of p47$^{phox}$*

The cytosolic and plasma membrane fractions were prepared as described previously, with modification [18]. Briefly, HUVECs were lysed in lysis buffer A (20 mM Tris-HCl, 10 mM EGTA, 2 mM EDTA, 2 mM DTT, 1 mM PMSF, 25 μg/mL aprotinin, and 10 μg/mL leupeptin). Cell lysates were centrifuged at 16,000 *g* for 20 min at 4°C. The supernatant was collected and designated the cytosolic fraction. The pellets were resuspended in lysis buffer B (0.5% sodium dodecyl sulfate, 1% NP-40, 1 mM $Na_3VO_4$, 1mM NaF, 1 mM PMSF, 25 μg/mL aprotinin, and 10 μg/mL leupeptin). Western blot analysis for p47$^{phox}$ was performed on the plasma membrane fractions as described above using a monoclonal mouse antibody against p47$^{phox}$ (BD, Biosciences Pharmingen). For NADPH oxidase assay, HUVECs were lysed in lysis buffer containing 20 mM monobasic potassium phosphate (pH 7.0), 1 mM EGTA, 10 μg/mL aprotinin, 0.5 μg/mL leupeptin, 0.5mM PMSF. Plasma membrane fractions were measured in a lucigenin chemiluminescence assay using 1 mM lucigenin (Sigma) and 5 mM NADPH (Sigma) as described previously [18]. Chemiluminescence as relative light units was measured in a microtiter luminometer (SpectraMax M5, Molecular Device) as an indicator of enzyme activity.

*Endothelial cell-leukocyte adhesion assay*

U937 cells, originally derived from a human histiocytic lymphoma and obtained

from the American Type Culture Collection (Rockville, MD) and grown in RPMI 1640 medium (M.A. Bioproducts, MD), were labeled for 1 h at 37ºC with BCECF/AM (10 mM, Boehringer-Mannheim). Labeled U937 cells ($10^6$) were added to HUVECs ($10^6$) in a 48-well plate and incubation continued for 1 h, then non-adherent cells were removed by two gentle washes with PBS and the number of bound U937 cells evaluated by fluorescence microscopy.

*Mouse model and immunohistochemical staining*

Male 8-week-old C57BL6 mice (n=24), weighing between 25 and 35 g, were purchased from the National Taiwan University (Taipei, Taiwan). All procedures involving experimental animals were performed in accordance with the guidelines for animal care of the National Taiwan University and complied with the ‘‘Guide for the Care and Use of Laboratory Animals’’ NIH publication No. 86-23, revised 1985. The mice were randomly divided into four groups, which were to be treated with DMSO, TNF-α, TNF-α plus viscolin, or viscolin. The mice were injected intraperitoneally (i.p.) with viscolin (10 mg/Kg/day in 50 μL of DMSO) or DMSO (50 μL) for 5 days, then were left untreated or were injected i.p. with TNF-α (10 μg/Kg/day) for the next 3 days. They were then anesthetized by i.p. injection of 30-40 mg/kg of pentobarbital, sacrificed, and the thoracic aorta dissected out, immersion-fixed in 4% buffered paraformaldehyde, paraffin-embedded, and cross-sectioned for immunohistochemistry.

To determine the level of expression of VCAM-1 in aortic walls and whether it was associated with endothelial cells, two serial sections were examined by immunostaining for, respectively, von Willebrand factor (vWF; marker for endothelial cells) or VCAM-1. The first section was incubated sequentially for 1 h at 37ºC with mouse monoclonal anti-human vWF antibody (1:50 dilution, Neomarkers, CA, USA) and 1 h at RT with HRP-conjugated goat anti-mouse IgG antibodies (1:200 dilution in PBS, Sigma) and bound antibody visualized using 3, 3' diaminobenzidine (Sigma-Aldrich, St. Louis, MO). The second section was incubated with rabbit antibodies against human VCAM-1 (1:100; Santa Cruz, CA) at 4℃ for 1 h, washed with PBS and then incubated with HRP-conjugated second antibody, then with the same chromogen as above.

*Statistical analysis of data*

All values are presented as the mean ± SEM and were analyzed using Student's t test. Statistical significance was determined as $P<0.05$.

## Results

*Viscolin reduces VCAM-1 mRNA and protein expression in TNF-α-treated HUVECs*

When the cytoxicity of TNF-α or viscolin for HUVECs was assessed by MTT assay after 24 h of incubation, cell viability was not affected by the presence of 10 ng/mL of TNF-α or 1-30 μM viscolin (data not shown).

TNF-α (10 ng/mL) induced significant VCAM-1 protein expression in HUVECs,

which peaked at 6 h (Figure 2A). As shown in Figure 2B, when HUVECs were pretreated for 24 h with 1, 3, 10, or 30 μM viscolin before incubation with 10 ng/mL of TNF-α for 6 h, TNF-α-induced VCAM-1 expression was reduced, respectively, to 91±14%, 96±16%, 38±7%, or 34±16% of control levels, the reductions caused by the two highest concentrations being significant. In all subsequent experiments, unless otherwise specified, 10 ng/mL of TNF-α and 30 μM viscolin were used.

To determine whether the effects of TNF-α alone or together with viscolin on VCAM-1 expression were exerted at the transcriptional level, VCAM-1 mRNA levels were measured by RT-PCR. As shown in Figure 2C, unstimulated HUVECs produced low amounts of VCAM-1 mRNA, and 6 h treatment with TNF-α resulted in a marked increase in levels. This increase was markedly inhibited by 24 h preincubation with 10 μM viscolin (36± 2 % inhibition).

The effect of viscolin on VCAM-1 gene transcription was confirmed using the luciferase gene activity assay. The VCAM-1 luciferase reporter gene was transfected into NIH 3T3 cells, which have a high transfection efficiency, rather than the very hard to transfect primary HUVECs [19], which were then stimulated with TNF-α for 6 h. Figure 2D shows that TNF-α treatment stimulated VCAM-1 luciferase activity and that preincubation of the cells for 24 h with viscolin significantly reduced the effect of TNF-α by 35 ± 4%. These results suggest that viscolin significantly inhibited

TNF-α-induced VCAM-1 expression at the transcriptional level.

*The viscolin-induced reduction in TNF-α-induced VCAM-1 expression is partly dependent on inhibition of JNK phosphorylation*

Since TNF-α-induced inflammation involves the secretion of inflammatory cytokines via the MAPK pathways [20], we next investigated whether TNF-α-induced VCAM-1 expression was mediated by activation of MAPKs. As shown in Figure 3A-3C, TNF-α induced transient phosphorylation of ERK1/2, JNK, and p38 in HUVECs, with the maximal response being seen within 15 min, followed by a decline to the basal level within 60 min. In addition, pretreatment for 1 h with the indicated concentrations of PD98059 (an ERK1/2 inhibitor), SP600125 (a JNK inhibitor), or SB203580 (a p38 inhibitor) inhibited the TNF-α-induced VCAM-1 expression seen at 6 h of TNF-α treatment (Figure 3D-3F). These results suggest that TNF-α-induced VCAM-1 expression is mediated by activation of MAPKs.

To determine the potential targets that were negatively regulated by viscolin, the cells were preincubated with viscolin for 24 h, then incubated with TNF-α for 15 min. As shown in Figure 3G-3I, pretreatment with viscolin significantly inhibited TNF-α-induced JNK phosphorylation by 36 ± 4% (Figure 3H), but had no significant effect on ERK1/2 and p38 phosphorylation. These results suggest that viscolin inhibits TNF-α-induced VCAM-1 expression partly by inhibiting TNF-α-induced

JNK phosphorylation.

*Viscolin decreases NF-κB activation and NF-κB p65 nuclear translocation in TNF-α-treated HUVECs*

Since the VCAM-1 gene promoter contains consensus binding sites for AP-1 and NF-κB [21, 22], we investigated whether viscolin inhibited TNF-α-induced VCAM-1 expression via an effect on these transcription factors. Gel-shift assays were performed to determine the effect of viscolin on AP-1 and NF-κB activation in TNF-α-treated HUVECs. As shown in Figs. 4A and 4B, low basal levels of AP-1 and NF-κB binding activity were detected in untreated control cells and binding was significantly increased by 30 min treatment with TNF-α. Pretreatment with viscolin for 24 h had no effect on TNF-α-induced AP-1 activation, but blocked the increase in NF-κB binding activity. To determine whether NF-κB activation was involved in the pretranslational effects of viscolin on VCAM-1 expression, we examined NF-κB p65 protein levels in the nuclei of TNF-α-treated HUVECs by Western blot and immunofluorescent staining. Western blot (Figure 4C) showed that higher levels of p65 and phospho-p65 were found in the nuclei of TNF-α-stimulated HUVECs compared to control HUVECs, and that viscolin pretreatment significantly reduced the expression of p65 and p-p65. Consistent with the Western blot findings, HUVECs stimulated with TNF-α for 30 min showed marked NF-κB p65 staining in the nuclei (T on Figure 4D) by immunofluorescent staining, while

viscolin-pretreated cells (24 h;T+Vis) showed weaker nuclear NF-κB expression, but stronger staining in the cytoplasm. The NF-κB p65 translocation induced by TNF-α was also significantly inhibited by pretreatment for 24 h with NAC (10 mM), DPI (10 μM), or APO (100 μM) (Figures 4C, 4D). Furthermore, the stimulatory effect of TNF-α on VCAM-1 levels was blocked by co-incubation with 0-10 μM parthenolide, an NF-κB inhibitor (Fig. 4E). These results suggest that viscolin inhibits the TNF-α-induced VCAM-1 expression by inhibiting NF-κB activation.

*Viscolin inhibits TNF-α-induced ROS production, NADPH oxidase activity and p47$^{phox}$ translocation in HUVECs*

Since previous studies have shown that viscolin inhibits superoxide anion production in human neutrophils [7, 8], we investigated whether it had an antioxidant effect on TNF-α-treated HUVECs. First, we examined its effect on TNF-α-induced $O_2^{\bullet-}$ and $H_2O_2$ production using DHE and amplex red as the probe, respectively. As shown in Figure 5A and 5B, TNF-α induced $O_2^{\bullet-}$ and $H_2O_2$ production in a time-dependent manner. Twenty-four hours pretreatment with viscolin for 24 h dramatically decreased TNF-α-induced $O_2^{\bullet-}$ and $H_2O_2$ production by 45 ± 1% and 22 ± 2%, respectively, as did 2 h preincubation with the NADPH oxidase inhibitor, DPI (Figure 5C and 5D). These results suggest that viscolin has a potent antioxidant activity on TNF-α-induced ROS production and that this may be mediated through

inhibition of NADPH oxidase activity. Since NADPH oxidase contains membrane-bound components (NOX and $p22^{phox}$) and cytosolic components ($p40^{phox}$, $p47^{phox}$, $p67^{phox}$, and Rac) [23], the membrane and cytosolic subunits of NADPH oxidase isolated from unstimulated HUVECs were assembled using SDS and incubated with viscolin or APO in the presence of NADPH. As shown in Figure 5E, NADPH treatment induced marked superoxide anion production, which was significantly reduced by addition of viscolin or APO for 2 min before assay. Furthermore, HUVECs were treated with 10 ng/mL TNF-α for 20 min, and then the membrane fraction was assayed for NADPH oxidase activity. As shown in Figure 5 F, TNF-α addition resulted in a significant increase in NADPH oxidase activity which was inhibited by viscolin treatment. We then determined whether this effect of visvolin was associated with translocation of p47, as this translocation mechanism was been reported to play an important role in the activation of NADPH oxidase [23]. Stimulation of HUVECs with 10 ng/mL of TNF-α for 20 min increased membrane $p47^{phox}$ expression compared with the untreated cells., whereas 24h pretreatment with viscolin resulted in a decrease in the membrane $p47^{phox}$ content in TNF-α–treated HUVECs (Figure 5G), suggesting an effect on the translocation of $p47^{phox}$ from the cytoplam to the membrane.

Since several lines of evidence have indicated that ROS production is the

mediator inducing VCAM-1 expression [24, 25], the role of ROS production in TNF-α-induced VCAM-1 expression was investigated. As shown in Figure 5H-5J, 2 h pretreatment with the antioxidant NAC or the NADPH oxidase inhibitors DPI or APO significantly attenuated TNF-α-induced VCAM-1 expression in a concentration-dependent manner. In addition, as shown in Figure 5K, 2 h pretreatment with antioxidants (NAC, DPI, or APO) partly inhibited TNF-α-induced JNK phosphorylation, the effects being similar to that of viscolin. These results suggest that NADPH oxidase-derived ROS production plays a critical role in TNF-α-induced VCAM-1 expression.

*Viscolin reduces the adhesion of monocytes to TNF-α-treated HUVECs*

To explore the effects of viscolin on the endothelial cell-leukocyte interaction, we examined the adhesion of U937 cells to TNF-α-activated HUVECs. As shown in Fig. 6, control confluent HUVECs (panel C) incubated with U937 cells for 1 h showed minimal binding, but adhesion was substantially increased when the HUVECs were pretreated with TNF-α for 6 h (panel T). Pretreatment of HUVECs with viscolin for 24 h (panel T+Vis) reduced the number of U937 cells adherent to TNF-α-treated HUVECs by 46±4% compared to TNF-α alone. The involvement of VCAM-1 in the adhesion of U937 cells to TNF-α-treated HUVECs was examined by pretreatment of the cells with anti-VCAM-1 antibody. When HUVECs were pretreated with 1 μg/mL (panel VCAM-1

Ab-1) or 2 μg/mL (panel VCAM-1 Ab-2) of anti-VCAM-1 antibody for 1 h, then incubated with TNF-α, the binding of U937 cells to HUVECs was significantly lower than that to non-antibody-treated TNF-α-stimulated cells, showing that VCAM-1 plays a major role in the adhesion of U937 cells to TNF-α-treated HUVECs. The adherence of TNF-α-treated U937 cells to HUVECs was also inhibited by 1 h pretreatment with 10 μM PD98059 (panel T+PD), SP600125 (panel T+SP), SB203580 (panel T+SB), or parthenolide (panel T+Par). Similarly, the adherence of U937 cells to TNF-α-treated HUVECs was also inhibited by 2 h pretreatment with antioxidants (NAC, DPI, or APO).

*Viscolin reduces VCAM-1 protein expression in the thoracic aorta in TNF-α-injected mice*

To determine the effect of viscolin on VCAM-1 expression *in vivo*, mice were injected with viscolin for 5 days prior to injection with TNF-α for 3 days, then immunohistochemical staining was performed to detect the expression of VCAM-1 on serial section of thoracic aorta, using vWF as an endothelial cell marker. As shown in Figure 7, in the control (panel C) and viscolin-treated (Vis) groups, no VCAM-1 staining was seen on the vascular wall, while, in the TNF-α-treated group (panel TNF-α), strong VCAM-1 staining was seen on the luminal surface. In contrast, pre-administration of viscolin resulted in weak VCAM-1 staining in the TNF-α-treated animals (panel TNF-α+Vis).

## Discussion

In the present study, we demonstrated that viscolin treatment reduced VCAM-1 expression both *in vitro* in TNF-α-stimulated HUVECs and *in vivo* in the thoracic aorta of TNF-α-treated mice. Viscolin also inhibited the binding of the human monocytic cell line U937 to TNF-α-stimulated HUVECs. These effects were inhibited by SP600125, a JNK inhibitor, or parthenolide, a NF-κB inhibitor, showing that they were partly mediated through inhibition of JNK phosphorylation and NF-κB activation. In addition, viscolin attenuated the increase in VCAM-1 mRNA expression and VCAM-1 promoter activity induced by TNF-α. Furthermore, viscolin had a scavenging effect on the generation of ROS as well as on the decreased NADPH oxidase activity.

Viscolin, isolated from *Viscum coloratum*, was chosen for testing, as *Viscum coloratum* has long been used in traditional Chinese medicine to treat inflammatory diseases. Antioxidative and anti-inflammatory actions are two of the pharmacological properties proposed to underlie its beneficial effects [7-9]. A partially purified fraction from the chloroform extract of *V. coloratum* (PPE-SVC) has been shown to inhibit the generation of superoxide anions by formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP)-activated human neutrophils, and purified viscolin, a major active component of PPE-SVC, inhibits the generation of superoxide anion and the release of elastase in

fMLP-activated human neutrophils [7]. Viscolin suppresses ROS and nitric oxide (NO) generation in leukocytes and microglial cells, and, in addition, attenuates pro-inflammatory cytokine production [9]. The present study is the first to report that viscolin strongly reduces levels of VCAM-1 mRNA and protein in TNF-α-treated HUVECs. The present results also showed that viscolin reduced TNF-α-induced VCAM-1 promoter activity.

Our results demonstrated that TNF-α induced time-dependent phosphorylation of MAPKs (ERK1/2, JNK, and p38) and that the increases in VCAM-1 expression and U937 cell adhesion induced by TNF-α were inhibited by PD98059, SP600125, or SB203580. These results show that activation of MAPKs is necessary for TNF-α-induced VCAM-1 expression in HUVECs. Consistent with these findings, TNF-α-induced VCAM-1 expression in human tracheal smooth muscle cells requires activation of MAPKs [26]. Furthermore, our results demonstrated that viscolin inhibited the TNF-α-induced phosphorylation of JNK, but not that of ERK1/2 or p38 (Figure 3), suggesting that the inhibitory effect of viscolin on VCAM-1 expression is mediated, in part, by JNK inhibition. Since a previous study showed that ROS regulate both protein kinases and protein phosphatases [27], one of our future aims is to determine the protein phosphatases involved in the dephosphorylation of JNK that are regulated by viscolin. In addition, our results also showed that viscolin inhibited

the TNF-α-induced increase in VCAM-1 mRNA levels. Although we cannot rule out the possibility that viscolin may affect the stability of VCAM-1 mRNA, viscolin was found to inhibit the TNF-α-induced promoter activity of VCAM-1 (Figure 2D). These results suggest that viscolin attenuates VCAM-1 expression induced by TNF-α, at least in part, through a transcriptional mechanism.

Several lines of evidence indicate that TNF-α induces ROS production in endothelial cells [10, 24, 25, 28]. Consistent with these previous results, our study showed that it rapidly induced ROS production and that this was inhibited by the NADPH oxidase inhibitors DPI and APO. These results suggest that TNF-α induces ROS production via activation of NADPH oxidase. ROS appears to be a second messenger in the TNF-α-induced signal transduction pathway that regulates VCAM-1 expression [10, 24, 25, 28]. In our study, antioxidants (NAC, DPI, or APO) inhibited the TNF-α-induced increase in VCAM-1 expression (Figure 5H-J) and U937 cell adhesion (Figure 6A), showing that ROS mediated the effects of TNF-α on VCAM-1 expression. In addition, preincubation with viscolin effectively attenuated the ROS production induced by TNF-α in HUVECs. Moreover, pretreatment with antioxidants (NAC, DPI, APO) inhibited TNF-α–induced JNK phosphorylation to a similar extent as viscolin. These results suggest that viscolin inhibits TNF-α–induced VCAM-1 expression via its antioxidative properties. Furthermore, we demonstrate that viscolin

inhibited NADPH oxidase activity and p47expression in the membrane fraction of TNF-α–treated HUVECs. Because of their chemical structure, a benzene ring with adjacent methoxy-hydroxyl groups, flavonoids are potent inhibitors of NADPH oxidase activity [29]. As the chemical structure of viscolin (Figure 1) is similar to that of flavonoids, it may have a similar inhibitory effect on NADPH oxidase activity. Future studies are necessary to clarify the role of viscolin on NOX activity as DPI and apocyanin have been reported to inhibit NOX activity as well as affect other reactive species and enzymes.

The VCAM-1 gene promoter contains consensus binding sites for AP-1 and NF-κB [21, 22]. Our results showed that the binding activity of NF-κB and AP-1 was activated by TNF-α and that pretreatment with viscolin significantly inhibited the TNF-α-induced increase in binding activity of NF-κB, but not that of AP-1. In addition, several reports have shown that natural products with antioxidant activity inhibit the TNF-α–induced activation of redox-sensitive NF-κB [10, 24, 25, 28]. Pretreatment with an NF-κB inhibitor suppressed the TNF-α-induced increase in VCAM-1 expression and U937 cell adhesion, suggesting that viscolin attenuates VCAM-1 expression via a reduction in NF-κB binding activity. Our results showed that viscolin and the antioxidants NAC, DPI, and APO significantly attenuated NF-κB binding activity and NF-κB p65 translocation, and that these effects may be due to its

antioxidative activity. Viscolin has anti-inflammatory and antioxidative properties based on the above findings. Because atherosclerosis is a chronic inflammatory disease [1,2], viscolin may be beneficial for the prevention of inflammation and atherosclerosis.

In conclusion, our study demonstrated that viscolin reduces VCAM-1 expression under inflammatory conditions both *in vitro* and *in vivo*. Our results show that the inhibitory effect on VCAM-1 expression is partly mediated by inhibition of JNK phosphorylation, NF-κB activation, and ROS production. Our results demonstrated the anti-inflammatory and antioxidative effects of viscolin, an active component of *V. coloratum*, on endothelial cells and suggested that this compound may provide a chemical backbone for the development of therapeutic agents.

## Acknowledgements

This work was supported by research grants from the National Science Council (NSC-99-2320-B-002-0220MY3) and the Cooperative Research Program of the NTU and CMUCM (99F0080-303), Taiwan, ROC.

**Conflict of Interest:** None

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## Figure legends

**Fig. 1.** Chemical structure of viscolin and its putative functional groups ( ).

**Fig. 2.** Viscolin inhibits the TNF-α-induced increase in VCAM-1 mRNA and protein levels in HUVECs. (A) HUVECs were treated with TNF-α (10 ng/mL) for the indicated time, then protein levels in cell lysates were measured on Western blots. (B) HUVECs were incubated with the indicated concentrations of viscolin for 24 h, then with 10 ng/mL of TNF-α for 6 h in the continued presence of the same concentration of viscolin, and VCAM-1 protein in cell lysates was measured by Western blot. GAPDH was used as the loading control. (C) Analysis of VCAM-1 mRNA levels in untreated HUVECs or HUVECs preincubated with or without 10 μM viscolin for 24 h, then incubated with 10 ng/mL of TNF-α for 6 h. Total RNA was analyzed by RT-PCR after normalization to 18S levels. (D) NIH 3T3 cells were transfected with a luciferase plasmid containing the VCAM-1 promoter for 24 h, then were incubated with or without 10 μM viscolin for 24 h prior to the addition of TNF-α (10 ng/mL) for a further 6 h. In A-D, the data are expressed as a fold value compared to the control value and are the mean±SEM for three separate experiments. *$P$<0.05 compared to the untreated cells. $^{\dagger}P$<0.05 compared to the TNF-α-treated cells.

**Fig. 3.** The viscolin-mediated reduction in TNF-α-induced VCAM-1 expression is partly dependent on inhibition of JNK phosphorylation. (A-C) HUVECs were treated with 10

ng/mL TNF-α for the indicated time, then the cell lysate was analyzed for MAPK phosphorylation by Western blot using antibodies against p-ERK1/2 (A), p-JNK (B), or p-p38(C). (D-F) The cells were preincubated for 1h with the indicated concentration of PD98059 (ERK1/2 inhibitor) (D), SP600125 (JNK inhibitor) (E), or SB203580 (p38 inhibitor) (F), then were treated with TNF-α for 6 h and the cell lysate analyzed for VCAM-1 expression by Western blot. (G-I) Western blot analysis showing the effect of viscolin treatment on the phosphorylation of p-ERK1/2 (G), p-JNK (H), or p-p38 (I) in TNF-α-treated HUVECs. HUVECs were incubated for 24 h with or without 30 μM viscolin, then the cells were incubated with 10 ng/mL of TNF-α for 15 min and aliquots of cell lysate containing equal amounts of protein subjected to immunoblotting with the indicated antibodies. The data are expressed as a fold of the control value and are the mean±SEM for 3 separate experiments. GAPDH was used as the loading control. *$P$<0.05 compared to the untreated cells. $^{\dagger}P$<0.05 compared to the TNF-α-treated cells.

**Fig. 4.** The viscolin-induced downregulation of VCAM-1 expression in TNF-α-stimulated HUVECs is mediated by inhibition of NF-κB activation and NF-κB p65 nuclear translocation. (A-B) Nuclear extracts prepared from untreated cells or from cells with or without 24 h pretreatment with 30 μM viscolin, then incubated with 10 ng/mL of TNFα for 30 min were tested for AP-1 (A) or NF-κB (B) DNA binding activity by EMSA. (C, D) Western blot and immunofluorescent staining for NF-κB p65.

HUVECs were preincubated for 24 h with 30 μM viscolin or for 2 h with 10 mM NAC, 10 μM DPI, or 100 μM APO, then were treated with 10 ng/mL of TNF-α for 30 min. A representative result from three separate experiments is shown. Bar=100 μM. (E) Cells were coincubated for 24 h with 0-10 μM parthenolide (Par, NF-κB inhibitor) and 10 ng/mL of TNF-α, then cell lysates were prepared and assayed for VCAM-1 by Western blot. The data are expressed as a fold of the control value and are the mean±SEM for 3 separate experiments. GAPDH was used as the loading control. *$P$<0.05 compared to the untreated cells. $^{\dagger}P$<0.05 compared to the TNF-α-treated cells.

**Fig. 5.** Viscolin reduces TNF-α-induced ROS production, NADPH oxidase activity and $p47^{phox}$ translocation in HUVECs. (A, B) HUVECs were incubated with DHE and amplex red/HRP, and then 10 ng/mL of TNF-α was added to the well for the indicated time, and ethidium and resorufin fluorescence were measured for the generation of $O_2^{\bullet-}$ and $H_2O_2$, respectively. (C, D) The cells were pretreated with 30 μM viscolin for 24 h or with 10 μM DPI for 2 h prior to addition of 10 ng/mL TNF-α for 15 min. The methods for the measurement of $O_2^{\bullet-}$ and $H_2O_2$ was described in the Materials and Methods. (E) The cytosolic and membrane fractions from unstimulated HUVECs were incubated with the reaction mixture for NADPH oxidase assembly as described in the Materials and Methods, then were left untreated or were incubated for 2 min with 30 μM viscolin or100 μM APO, then ferricytochrome c reduction was measured

at 550 nm. (F) Control cells or cells pretreated with 30 μM viscolin for 24 h or 10 μM DPI for 2 h were incubated with 10 ng/mL of TNF-α for 20 min, then the plasma membrane protein was assayed with a superoxide-dependent lucigenin chemiluminescent assay. (G) Western blot of $p47^{phox}$ levels in the membrane fractions of HUVECs pretreated with viscolin for 24 h or with 10 μM DPI for 2 h, then stimulated with of 10 ng/mL of TNF-α for 20 min. (H-J) The cells were incubated for 2 h with the indicated concentration of 0-10 mM NAC (H), 0-10 μM DPI (I), or 0-200 μM APO (J), then were treated with TNF-α for 6 h and the cell lysate analyzed for VCAM-1 expression by Western blot. (K) The cells were preincubated for 2 h with 10 mM NAC, 10 μM DPI, or 100 μM APO, then were treated with TNF-α for 15 min and the cell lysate analyzed for JNK phosphorylation by Western blotting. Values were presented as the mean±SEM. *$P$<0.05 compared to the untreated cells. $^{\dagger}P$<0.05 compared to the TNF-α-treated cells.

**Fig. 6.** Viscolin reduces the adhesion of U937 cells to TNF-α-stimulated HUVECs. Cells were left untreated or were pretreated for 24 h with 30 μM viscolin, or for 1h with 1 μg/mL or with 2μg/mL of anti-VCAM-1 antibodies, or for 1h with 10 μM PD98059, SP600125, SB203580 or parthenolide, or for 2 h with 10 mM NAC, 10 μM DPI, or100 μM APO, then were incubated with 10 ng/mL of TNF-α for 6 h in the continued presence of the inhibitor. (A) Representative fluorescent photomicrographs showing the effect on

TNF-α-induced adhesion of fluorescein-labeled U937 cells to HUVECs. C is untreated cells. Bar=100 μm. (B) The number of U937 cells bound per high power field was counted. The data are expressed as the mean±SEM for three separate experiments. *$P$<0.05 compared to the untreated cells. $^{\dagger}P$<0.05 compared to the TNF-α-treated cells.

**Fig. 7.** Immunohistochemical staining for vWF or VCAM-1 expression in serial sections of thoracic aortas from mice. Mice were treated with DMSO (C), TNF-α, TNF-α+viscolin, or viscolin alone as described in the Materials and Methods, then serial sections were stained for vWF (endothelial cell marker) or VCAM-1. The lumen is uppermost in all sections. The reaction product and the internal elastic membrane are indicated by an arrowhead and an arrow, respectively. Bar=50 μM.

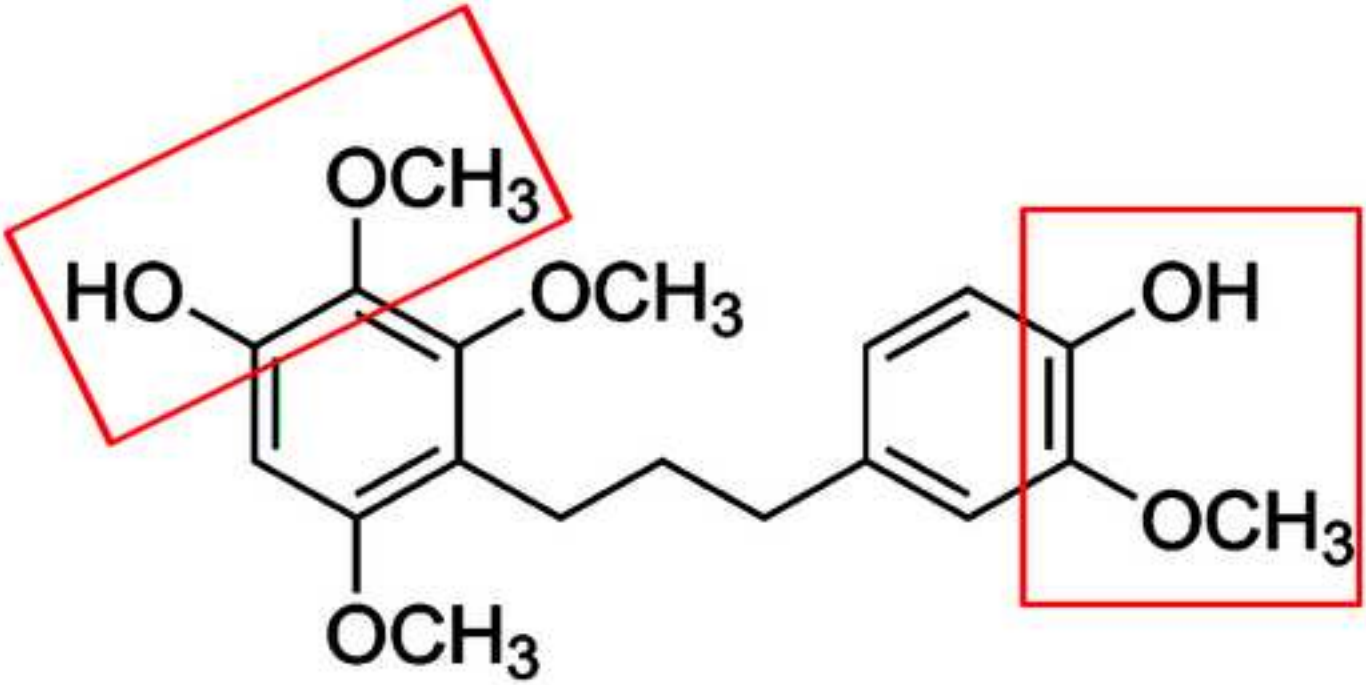


Fig. 1

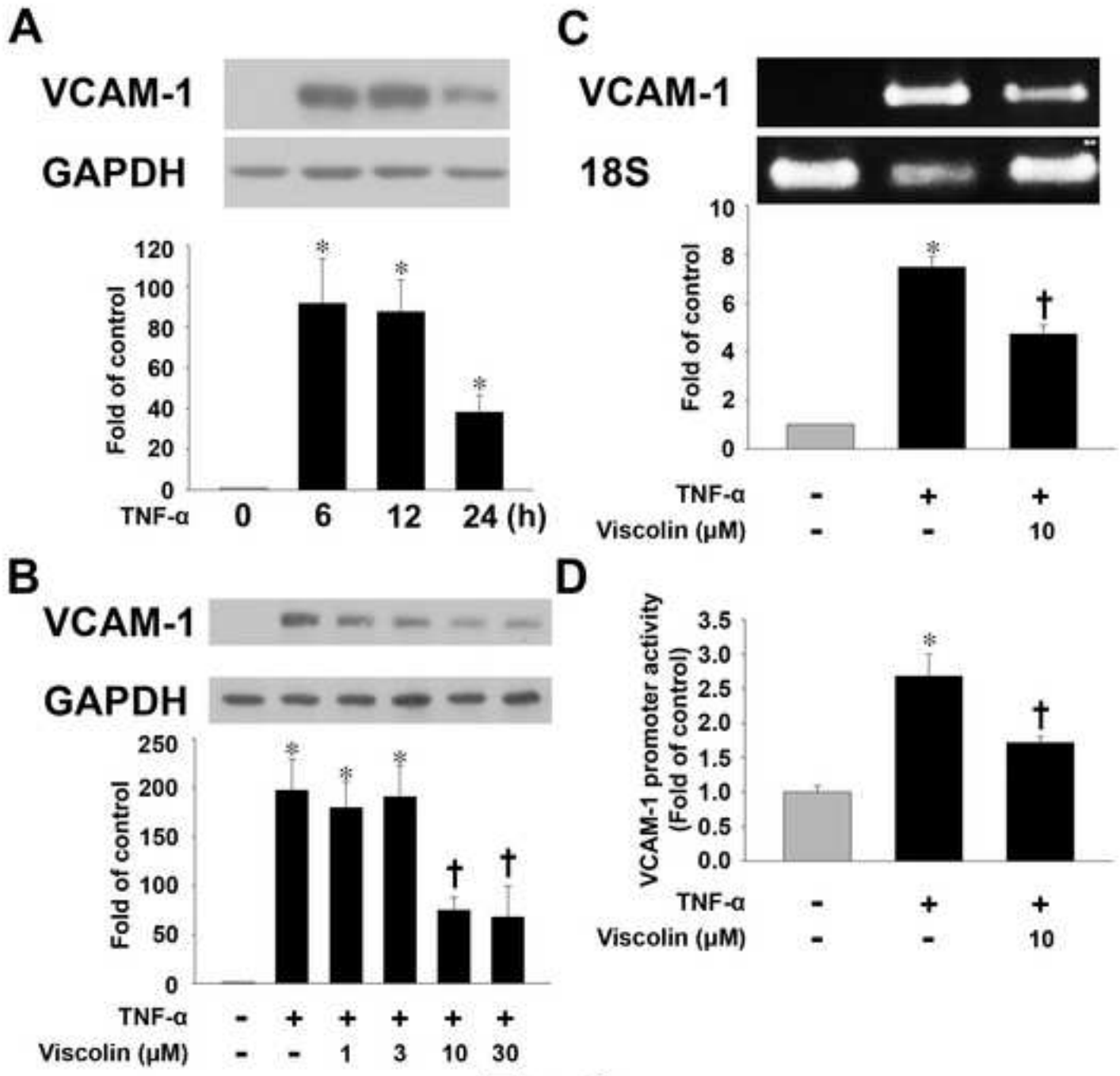


Fig. 2

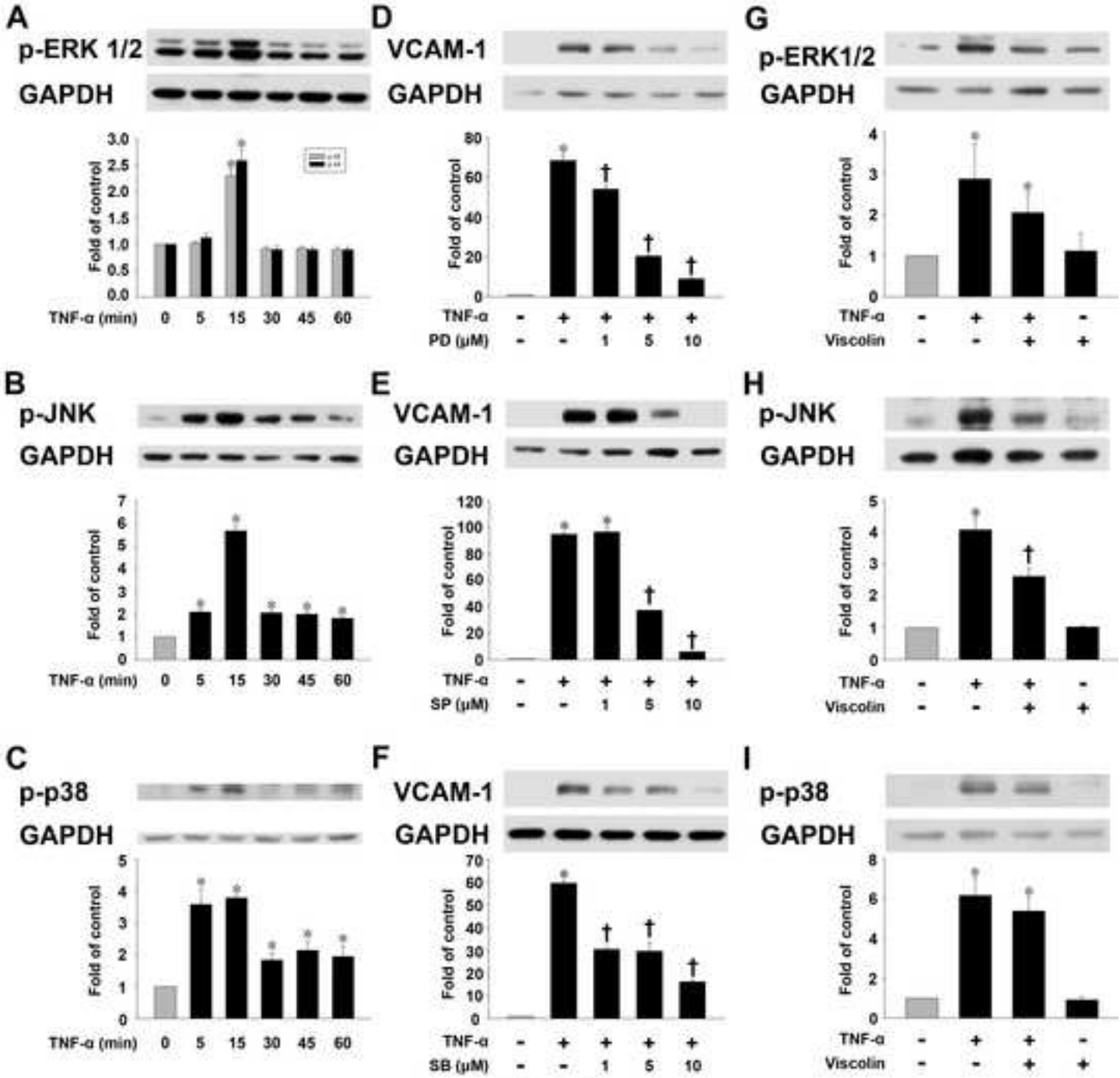


Fig. 3

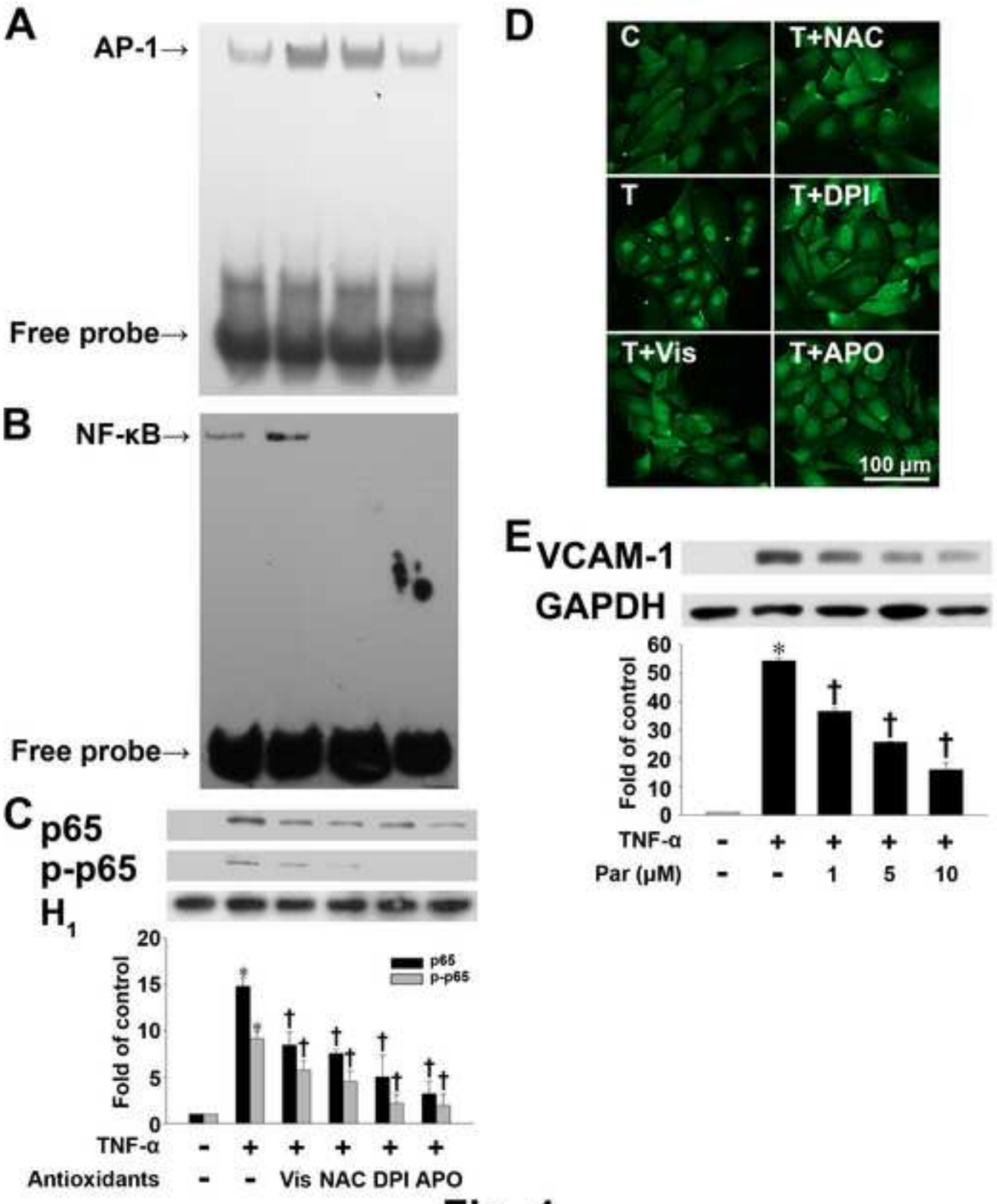


Fig. 4

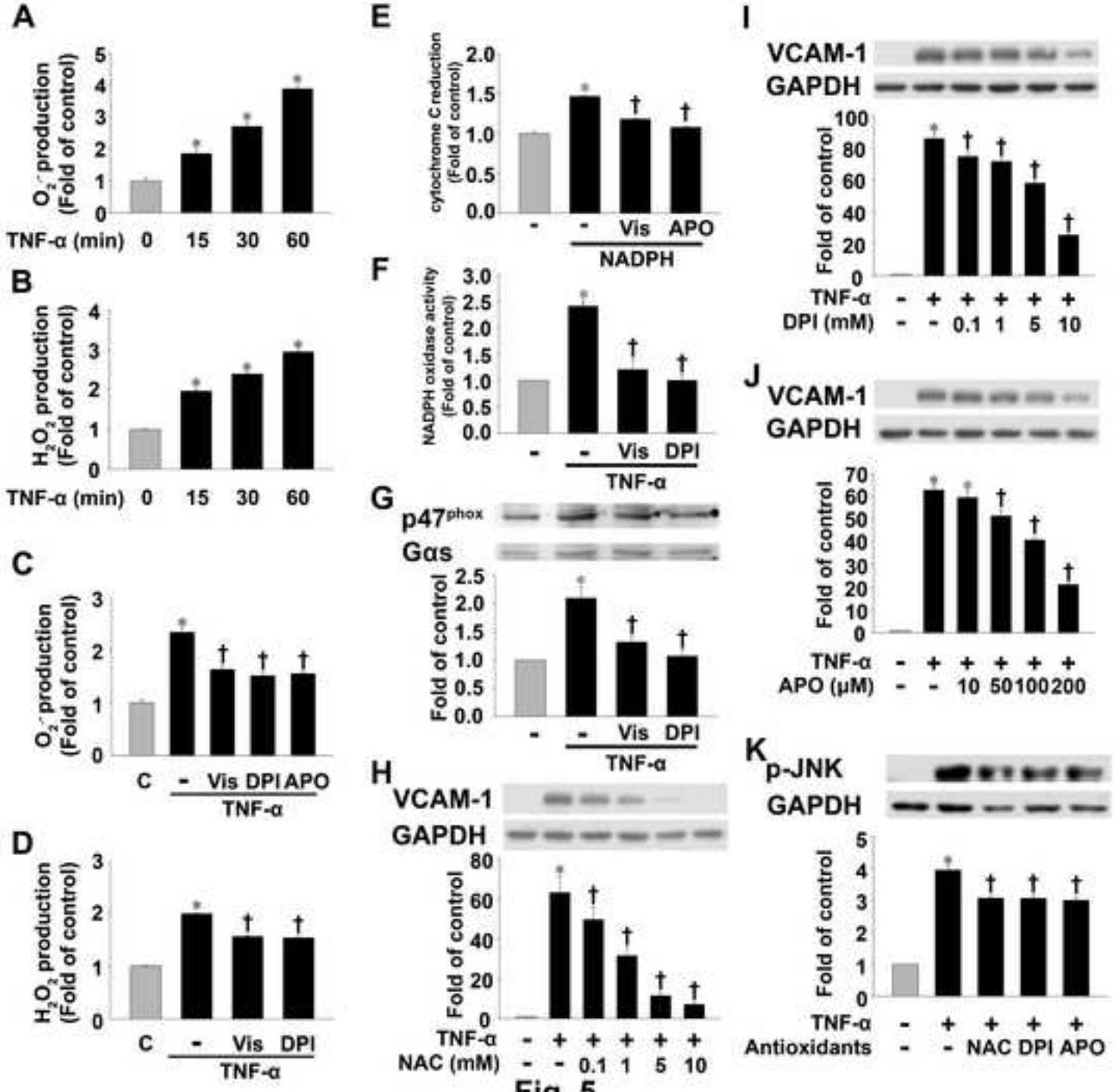


Fig. 5

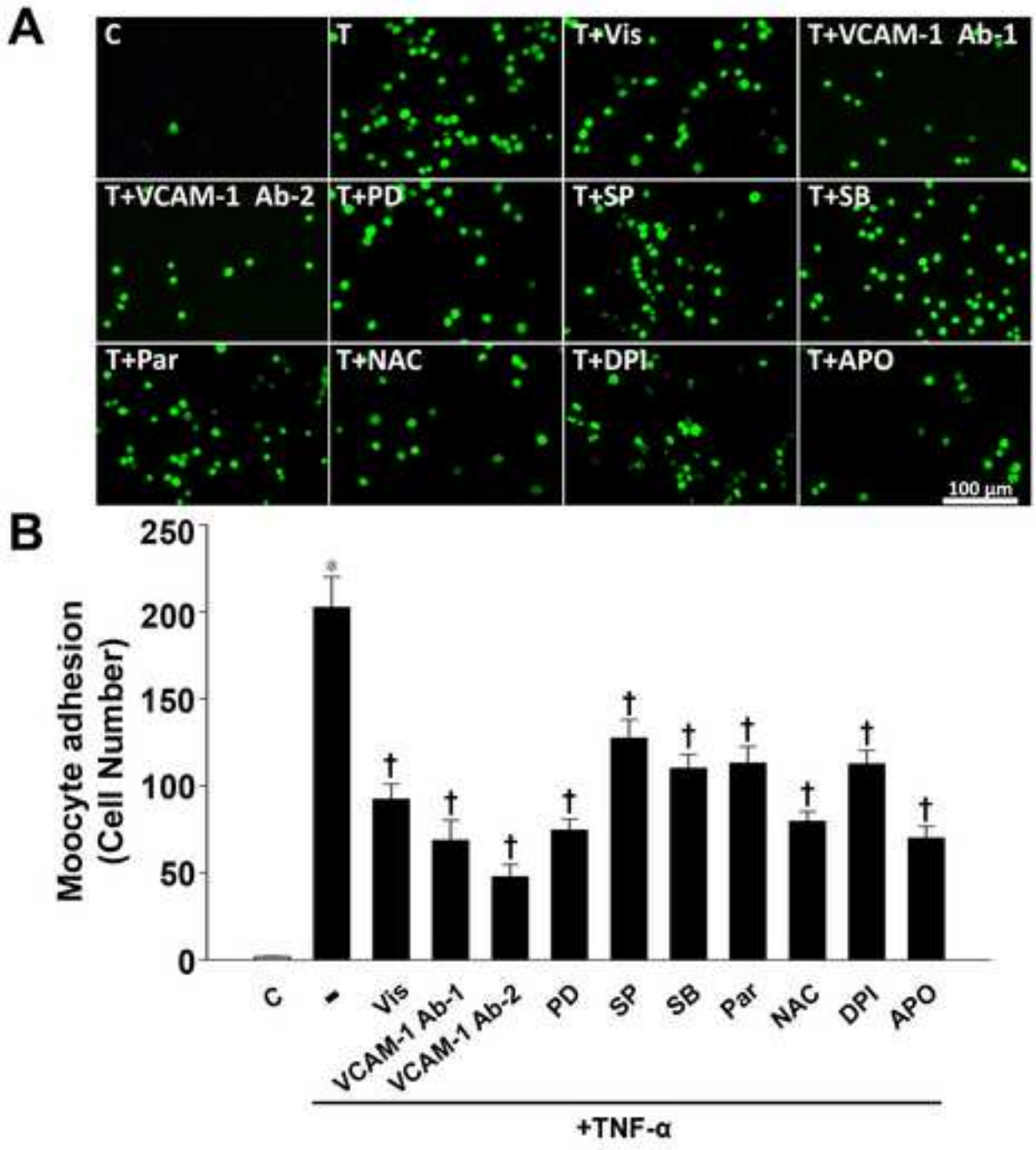


Fig. 6

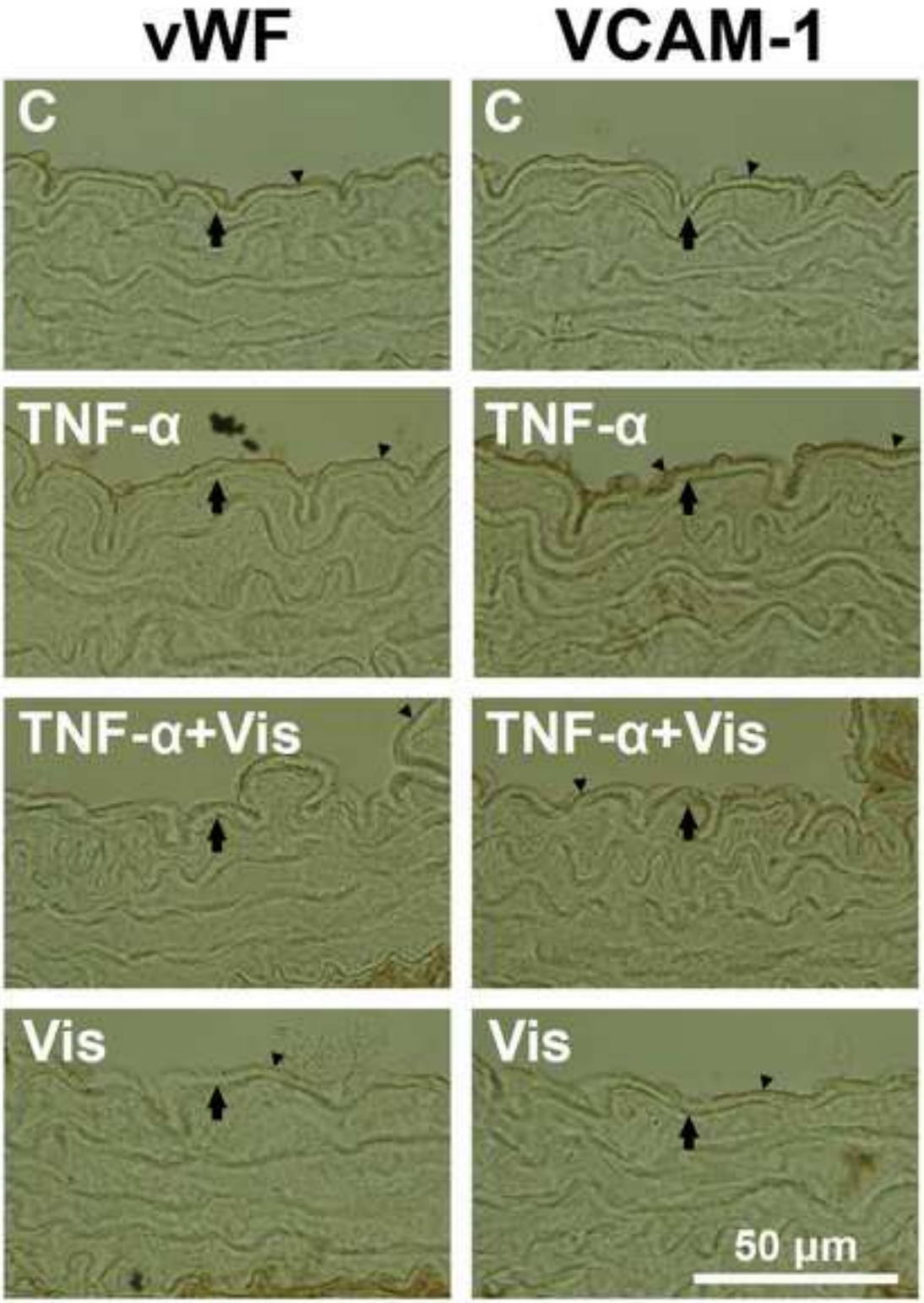


Fig. 7