



Bisacurone inhibits adhesion of inflammatory monocytes or cancer cells to endothelial cells through down-regulation of VCAM-1 expression

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Vascular cell adhesion molecule-1

Abstract

Bisacurone, one of the active compounds of the traditionally used indigenous herb *Curcuma longa* Linne (Zingiberaceae), has anti-oxidant, anti-inflammatory, and anti-metastatic activities. We studied how the level of vascular cell adhesion molecule-1 (VCAM-1), one of the key molecules in the development of atherosclerosis as well as carcinogenesis and metastasis, might be affected by bisacurone in tumor necrosis factor-alpha (TNF- α)-activated human umbilical vein endothelial cells (HUVECs). Bisacurone dose-dependently inhibited TNF- α -mediated expression of VCAM-1. It showed significant suppressive effect on ROS generation in response to TNF- α stimulation and it blocked nuclear factor-kappa B (NF- κ B) p65 translocation into the nucleus and phosphorylation of inhibitory factor κ B α (I κ B α). It also inhibited phosphorylation of Akt and PKC, which are upstream in the regulation of VCAM-1 by TNF- α . Furthermore, bisacurone decreased U937 monocyte and human oral cancer cell (Hep-2, QLL-1, SCC-15) adhesion to HUVECs stimulated by TNF- α , suggesting that it may inhibit the binding of these cells by regulating the expression of critical adhesion molecules by TNF- α . Thus, bisacurone may be beneficial in the treatment of inflammatory diseases, such as atherosclerosis, where inflammatory monocytes are involved in their pathology, and, moreover, in the development of tumors.

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Abbreviations: ANOVA, analysis of variance; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; ECAM, endothelial cell adhesion molecule; ECGS, endothelial cell growth supplements; ECL, enhanced chemoluminescence; ERK, extracellular regulated kinase; FBS, fetal bovine serum; HUVEC, human umbilical vein endothelial cell; ICAM, intercellular adhesion molecule; NF- κ B, nuclear factor κ B; SDS, sodium dodecyl sulfate; PKC, protein kinase C; PVDF, polyvinylidene difluoride; ROS, reactive oxygen species; TBS-T, Tris-buffered saline/Tween 20; TNF, tumor necrosis factor; VCAM, vascular cell adhesion molecule.

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1. Introduction

Adhesion molecules play an important role in inflammatory response and in the development of metastasis with respect to tumorigenesis through mediating migration of leucocytes or cancer cells to the blood vessel wall [1,2]. Vascular cell adhesion molecule-1 (VCAM-1) is an inducible cell surface glycoprotein that belongs to the immunoglobulin supergene family. It was first identified as an adhesion molecule induced on endothelial cells by the inflammatory cytokine interleukin-1 (IL-1) and tumor necrosis factor (TNF) or lipopolysaccharide (LPS) [3,4]. Previously, it was reported that some adhesion molecules are constitutively expressed on human oral cancer cells and that the expression of these molecule is enhanced after stimulation with TNF-alpha, IL-2, or interferon gamma [5]. Elevated expression of adhesion molecules and integrins in oral carcinoma cells may lead to a growth advantage and increased invasive potential of the primary oral tumor [6–8]. Additionally, a recent study showed that some highly metastatic human cancer cells have high affinity conformations at the cell surface and adhered to and migrated on VCAM-1, rather than on other adhesion molecules [9]. Therefore, discovering therapeutic agents that have specific suppression effects on adhesion molecules, such as VCAM-1, has become even more important.

Traditionally, *Curcuma longa* Linne (Zingiberaceae) has been used for centuries in indigenous medicine for the treatment of a variety of medical conditions [10]. Curcumin is one of the well-known bioactive phytochemicals that is derived from *C. longa* Linne. It possesses anti-carcinogenic activity in vivo and in vitro [11,12]. This plant is abundant with bioactive compounds called curcuminoids, which have similar chemical structures to curcumin. They also exhibit remarkable anti-inflammatory and anti-oxidant effects as well as anti-cancer properties [13,14]. Recently, bisacurone, an active compound, was isolated from *C. longa* and identified in comparison of spectral data with those of previous report [15]. There is considerable interest in the various health-promoting benefits of this drug, but its effects on EC function have not been elucidated.

Reactive oxygen species (ROS) generated by the disturbance of the oxidation/reduction state of the cell have been implicated in the pathogenesis of various inflammatory diseases and cancer [16,17]. ROS may be a common intracellular messenger for a variety of redox-sensitive transcription pathways that lead to the expression of cell adhesion molecules in vascular endothelial cells [18–20]. Recently, it was discovered that PI3K and PKC are involved in TNF- α -induced oxidant generation in ECs and that they act through activation of the NF- κ B transcription factor [21]. Interestingly, in our previous study, we found that inhibitors of PI3K/Akt (LY204002) and PKC (GF109203) specifically down regulated the expression of VCAM-1 mediated by TNF- α [22]. Meanwhile, an inhibitor of ERK1/2 (PD98059) failed to suppress VCAM-1 induction [22]. Thus, in this study, we aimed to investigate whether bisacurone inhibits VCAM-1 expression and if so, the molecular mechanisms by which bisacurone inhibit VCAM-1 expression.

2. Materials and methods

2.1. Extraction and isolation

Bisacurone (Fig. 1) was isolated from *C. longa* Linne (Zingiberaceae). Briefly, dry and powdered radix of *C. longa* (10 kg) was extracted

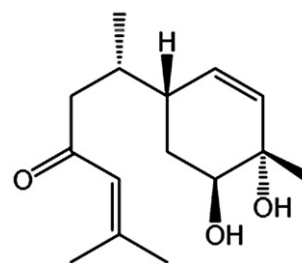


Figure 1 Chemical structure of bisacurone.

with methanol at room temperature and the methanol extract (750 g) was partitioned in CHCl_3 - H_2O mixture. The concentrated organic layer was subjected to repeated silica gel column chromatography eluted by a step gradient of hexane-ethylacetate to afford six fractions A1–A6. Of these, A5 (31 g) was chromatographed on a silica column using a step gradient of CHCl_3 -methanol to obtain 8 fractions. Bisacurone (220 mg) was purified from the fraction 4 (2 g) by HPLC (YMC Pack Pro C18, 250 \times 20 mm, a gradient of acetonitrile from 10% to 100% in water) as a viscous oil; ESI-MS m/z : 251.2 ($\text{M}-\text{H}$) $^-$. NMR data were coincided with those reported [17] and its purity was more than 95% in HPLC analysis.

2.2. Materials

Tissue culture medium 199, fetal bovine serum (FBS), antibiotics (penicillin/streptomycin), glutamine, and collagenase were supplied by Gibco-BRL (Rockville, MD). Anti-VCAM-1, anti-phospho-ERK1/2, and anti-NF- κ B (p65) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA); anti-p-Akt, anti-p-PKC, and anti-p-I κ B α antibodies were obtained from Cell Signaling Technology (Beverly, MA). Enhanced chemiluminescence (ECL) Western blotting detection reagent was from Amersham (Buckinghamshire, UK). All other chemicals, including endothelial cell growth supplements (ECGS) and heparin, were supplied by Sigma-Aldrich (St. Louis, MO).

2.3. Cell culture

HUVECs were isolated from umbilical cord veins by collagenase treatment [23] and growth in medium 199 supplemented with 20% fetal bovine serum (FBS), 2 mM L-glutamine, 5 U/ml heparin, 100 IU/ml penicillin, 10 μ g/ml streptomycin, and 50 μ g/ml ECGS. Cells were cultured in 100 mm dishes and grown in a humidified 5% CO_2 incubator. HUVECs were plated at a density of 1×10^7 cells per 100 mm dish. Cells were used between passage numbers 3 and 6. Hep-2 and SCC-15 were obtained from American Type Culture Collection (ATCC, Rockville, MD), and U937 human monocytes and QLL-1 cells were obtained from the Korea Cell Line Bank (KCLB, Seoul, Korea). Cells were grown in RPMI 1640 supplemented with 10% FBS, 2 mM L-glutamine, 25 mM HEPES, 25 mM NaHCO_3 , 100 IU/ml penicillin, and 10 μ g/ml streptomycin.

2.4. Western blot analysis

To separate nuclear and cytoplasmic protein fractions, cells were washed with ice-cold phosphate-buffered saline (PBS; pH 7.4), lysed in buffer A (10 mM HEPES pH 7.9, 1.5 mM MgCl_2 , 0.5 mM dithiothreitol [DTT], 5 μ M leupeptin, 2 μ M pepstatin A, 1 μ M aprotinin, and 20 μ M phenylmethylsulfonyl fluoride) by repeated freezing and thawing, and separated by centrifugation at 1000 g. The supernatant (cytoplasmic extract) was obtained by further centrifugation at 10,000 g for 15 min. The pellets were washed once with buffer A and resuspended in buffer B (10 mM Tris-Cl pH 7.5, 0.5% deoxycholate, 1% Nonidet P-40, 5 mM EDTA, 0.5 mM DTT, 5 μ M leupeptin, 2 μ M pepstatin A, 1 μ M aprotinin, and 20 μ M phenylmethylsulfonyl fluoride). The suspension was agitated for 30 min at

4 °C and centrifuged at 10,000 g for 20 min. The supernatant fraction containing nuclear proteins was collected. For isolation of total cell extracts, cells were lysed in PRO-PREP protein extract solution. The sample was centrifuged at 13,000 rpm for 20 min at 4 °C. Protein concentration was determined by the Bradford method. An equal volume of 2×SDS sample buffer (0.1 M Tris-HCl, 20% glycerol, 4% SDS and 0.01% bromophenol blue) was added to an aliquot of the supernatant fraction from the lysates, and the samples were boiled for 5 min. Thirty µg of protein were subjected to 10% SDS-polyacrylamide gel electrophoresis for 1 h 30 min at 110 V. The separated proteins were transferred to PVDF membrane for 2 h at 20 mA with SD Semi-dry Transfer Cell® (Bio-Rad). The membranes were blocked with 5% nonfat milk in Tris-buffered saline (TBS) containing 0.05% Tween 20 (TBS-T) for 2 h at room temperature. Next, the membranes were incubated with anti-VCAM-1, anti-p-ERK1/2, anti-p-Akt, anti-p-PKC, anti-NF-κB (p65), and anti-p-IκBα

antibodies at a 1:500 concentration (4 µg/ml) in 5% skim milk in TBS-T overnight at 4 °C, and the bound antibody was detected by horseradish peroxidase-conjugated anti-rabbit IgG. The membranes were washed and then developed using a Western blotting Luminol Reagent system (Amersham).

2.5. Plasmid construction

For construction of the pVCAM1-258-luc plasmid, the human VCAM-1-promoter region (-258/+42) was cloned into the *KpnI/HindIII* site of the pGL3-basic vector (Promega, Madison, WI) [24]. Polymerase chain reaction was used to amplify the fragment using primers as follows: forward primer with *KpnI* site (5'-CAAGTACCTTTATCTTTCCAGTAAAGATAGCC-3') and reverse primer with a *HindIII* site (5'-GATAGCTTAGCTCCTGAAGCCAGTGAG-3'). pVCAM1-258-luc was double digested with *KpnI/HindIII*, and the

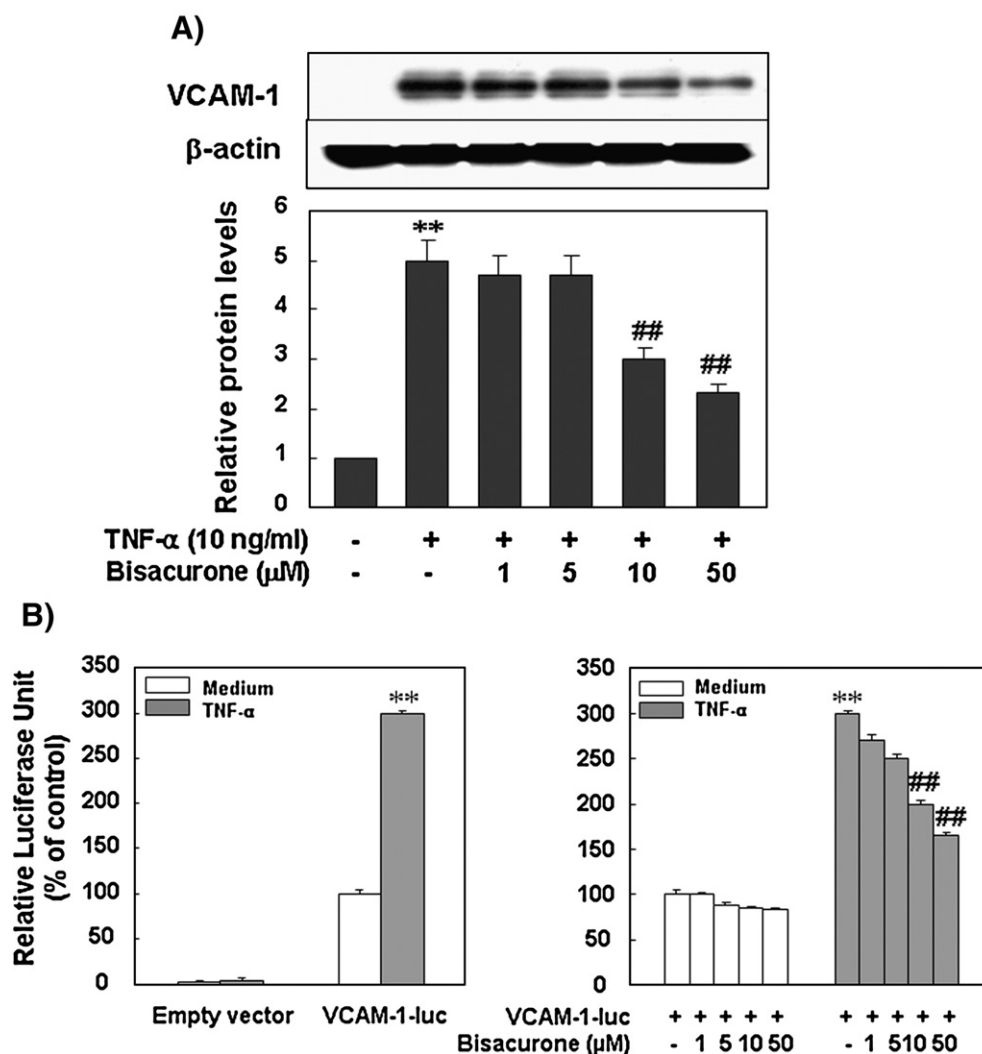


Figure 2 Inhibitory effect of bisacurone on VCAM-1 protein expression and VCAM-1-luciferase activity in TNF-α-stimulated HUVECs. (A) Cells were pretreated with bisacurone (1, 5, 10, and 50 µM) for 24 h and then stimulated with TNF-α (10 ng/ml) for 6 h. After treatment, protein was extracted from the cells and VCAM-1 protein level was determined by Western blot analysis as described in Materials and methods. The band intensities were assessed by scanning densitometry. (B) Cells were transfected with 1 µg of VCAM-1-luciferase, allowed to recover for 24 h, and then treated with 10 ng/ml TNF-α with or without bisacurone (1, 5, 10, and 50 µM). Cells were harvested 6 h after treatment. Luciferase activities are presented as the fold activation relative to that of the untreated cells. Data are presented as means±SD of three independent experiments. Significance compared to control, ***P*<0.01; significance compared with TNF-α, ##*P*<0.01.

VCAM-258 fragment was inserted into the corresponding enzyme sites of the pUC19 plasmid to construct the pUC19-VCAM1-258 plasmid. NF- κ B-luciferase constructs (the consensus NF- κ B binding sequence was cloned into the pGL3-basic luciferase expression vector) were kindly provided by Dr G. Koretzky (University of Pennsylvania).

2.6. Transfection

NF- κ B-luciferase and VCAM-1-luciferase transient transfections were performed using Lipofectin (Gibco-BRL) according to the manufacturer's protocol as described previously [23]. Briefly, 5×10^5 cells were plated in a 60 mm dish plate the day before transfection and grown to about 70% confluence. Cells were transfected with empty vector (pGL3 and/or pcDNA3), 1 μ g of NF- κ B-luciferase, or VCAM-1-luciferase+0.5 μ g of pRL-TK-luciferase. Transfections were allowed to proceed for 12 h. The transfected cells were washed with 4 ml of PBS and then stimulated with 10 ng/ml TNF- α . The cells were continually cultured in serum-free Medium-199 until they were harvested. Luciferase activity was normalized using pRL-TK-luciferase activity (*Renilla* luciferase activity) in each sample.

2.7. Luciferase assay

After experimental treatments, the cells were washed twice with cold PBS, lysed in a passive lysis buffer provided in the Dual Luciferase kit (Promega, Madison, WI), and assayed for luciferase activity using a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA) according to the manufacturer's protocol. All transfections were done in triplicate. Data were presented as a ratio between Firefly and *Renilla* luciferase activities.

2.8. Prooxidant production

Production of intracellular peroxides was monitored spectrofluorometrically using dichlorofluorescein diacetate (DCFH-DA) as a fluorescent dye [25]. Cells were suspended 12 h after serum deprivation and then DCFH-DA dissolved in ethanol was added at a final concentration of 10 μ M in the medium. Oxidation of DCFH by peroxides yielded dichlorofluorescein (DCF). Fluorescence was monitored at the excitation and emission wavelengths of 485 and 530 nm, respectively, using a fluorescence plate reader (50 cycles per 20 s at 37 $^{\circ}$ C) (Tecan[®], Tecan US Inc.). Data were expressed as changes relative to the initial fluorescence.

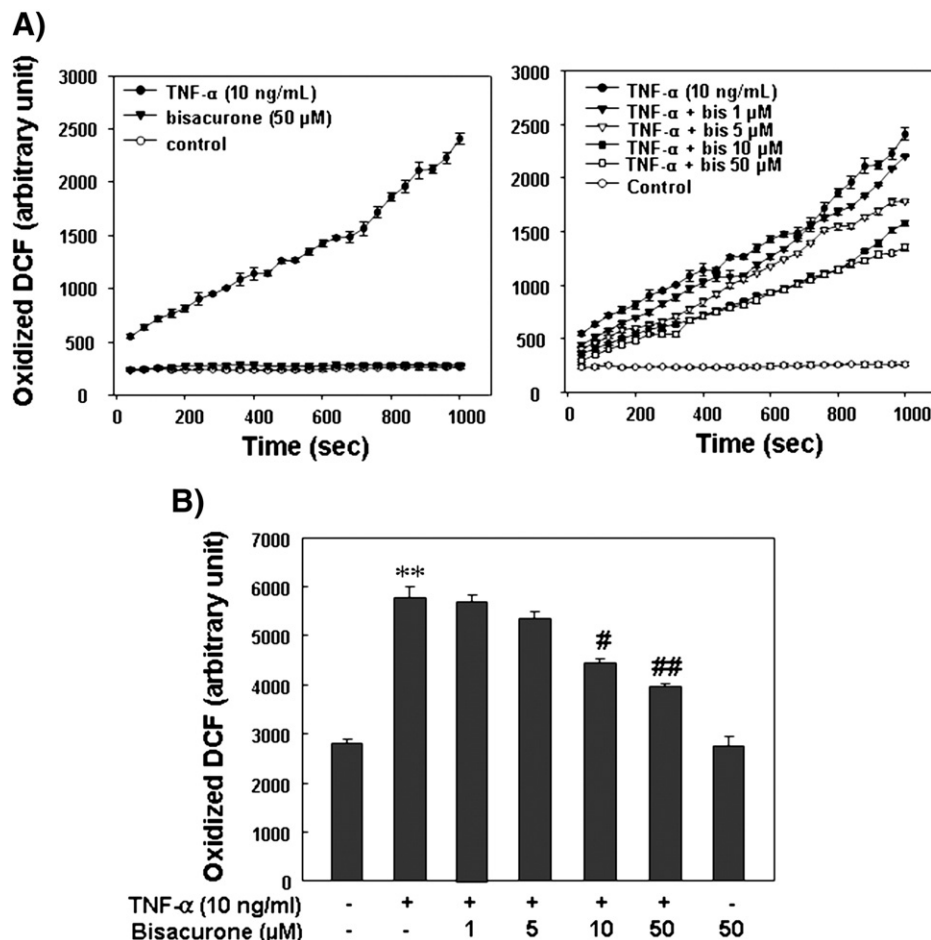


Figure 3 The effect of bisacurone on pro-oxidant production and accumulation in TNF- α -stimulated HUVECs. (A) The cells were preincubated without serum for 12 h and then loaded with 10 μ M DCFH-DA. Dye-loaded cells were subjected to control, TNF- α (10 ng/ml) alone, bisacurone (50 μ M) alone, or TNF- α with bisacurone (1, 5, 10, and 50 μ M). (B) The cells were pretreated with bisacurone (1, 5, 10, and 50 μ M) and then co-treated with TNF- α (10 ng/ml) for 4 h. Next, the cells were loaded with 10 μ M DCFH-DA. Fluorescence of DCF was monitored at the excitation wavelength of 485 nm and the emission wavelength of 530 nm. Data are presented as means \pm SD of three independent experiments. Significance compared to control, ** P <0.01; significance compared with TNF- α , # P <0.05 and ## P <0.01.

2.9. Adhesion assay

HUVECs were seeded in 2 well chamber slides for 48 h before experiments. Fresh medium was provided before stimulation with TNF- α . Monocyte U937 cells, Hep-2, SCC-15, or QLL-1 cells (3×10^7) were incubated in RPMI 1640 medium containing 2% FBS and 10 $\mu\text{g}/\text{ml}$ of the fluorescent dye BCECF/AM (Boehringer, Mannheim, Germany) at 37 $^\circ\text{C}$ for 30 min. Fluorescence labeled cells were pelleted and resuspended ($7.5 \times 10^5/\text{ml}$) in medium 199 with 10 mM HEPES buffer (M199H). HUVECs were washed three times with M199H before the addition of loaded cells and incubated at 37 $^\circ\text{C}$. After 30 min, cell suspensions were withdrawn and HUVECs were gently washed with M199H. Fluorescent images were selected using a high-resolution video camera (DXC-960MD; Sony) mounted on a BH-2 Olympus microscope (Melville, NY, USA). We then picked images 0.2 mm in width within the first selected areas, and the immunoreactivity of these was measured using SigmaGel 1.0 (Jandel Scientific, Germany). Analyses were repeated three times over the same region, and the results are the means of three independent experiments.

2.10. Statistical evaluations

Data are expressed as the mean \pm SD of results obtained from the number (*n*) of independent experiments. Differences between data sets were assessed by one-way analysis of variance (ANOVA) followed by Newman-Keuls tests. $P < 0.05$ or $p < 0.01$ was accepted as statistically significant.

3. Results

3.1. Inhibitory effect of bisacurone on the VCAM-1 expression by TNF- α in HUVECs

The effect of bisacurone on the expression of VCAM-1 by TNF- α was determined in HUVECs. The cells were pretreated with various concentrations of bisacurone (1, 5, 10, and 50 μM) for 24 h and then co-treated with TNF- α (10 ng/ml) for 6 h. The VCAM-1 level induced by TNF- α was significantly reduced as the concentration of bisacurone increased (Fig. 2A). To probe whether bisacurone acts transcriptionally to inhibit TNF- α -induced VCAM-1 gene expression, we conducted a VCAM-1 promoter reporter assay. Cells were transfected with VCAM-1 promoter driven luciferase and then stimulated with TNF- α with and without bisacurone in a dose-dependent manner. As shown in Fig. 2B, bisacurone significantly inhibited VCAM-1-luc promoter activity dose-dependently.

3.2. Effect of bisacurone on the pro-oxidant production and accumulation by TNF- α

Numerous studies have shown that oxidative stress (e.g., reactive oxygen species) was highly associated with development of inflammatory disorders and that it plays an important role for cancer cells in inducing angiogenesis and tumor growth [17,26]. Thus, we tested the effect of bisacurone on pro-oxidant production by TNF- α . Here, we determined fluorescence of oxidized DCF in dye-loaded HUVECs cultured in medium alone or in the presence of TNF- α (10 ng/ml) or bisacurone (50 μM) (Fig. 3A). Our results show that TNF- α markedly increased the rate of DCF oxidation in a time-dependent manner in HUVECs, whereas bisacurone alone at a high concentration did not affect pro-oxidant production (Fig. 3A). Next, we investigated the dose dependence of the effect of bisacurone (1, 5, 10, and 50 μM) in the presence of TNF- α (Fig. 3A). Our result showed that bisacurone was active in inhibiting pro-oxidant production in a

concentration-dependent manner in TNF- α -stimulated HUVECs (Fig. 3A). Additionally, the effect of bisacurone on TNF- α -mediated pro-oxidant accumulation was tested. HUVECs were treated with bisacurone in a dose-dependent manner for 24 h and then co-treated with TNF- α for 4 h. TNF- α significantly increased DCF oxidation, which was inhibited by bisacurone in a concentration-dependent manner (Fig. 3B).

3.3. Differential effects of bisacurone on the phosphorylation of ERK1/2, PKC and Akt in TNF- α -stimulated HUVECs

Previously, we reported that the PKC and PI3K/Akt signaling pathways but not ERK1/2 are involved in VCAM-1 expression in TNF- α -stimulated HUVECs [22]. Therefore, we investigated here the effect of bisacurone on the phosphorylation of these kinases by TNF- α . HUVECs were stimulated with TNF- α for 10 min for the detection of ERK1/2 and for 30 min for the detection of PKC and Akt in the presence of bisacurone (1, 5, 10, and 50 μM). As shown in Fig. 4, TNF- α alone markedly activated phosphorylation of ERK1/2, PKC, and Akt. Bisacurone dose-dependently suppressed PKC and Akt phosphorylation but failed to inhibit ERK1/2 phosphorylation. These findings suggest that TNF- α -induced PKC and Akt activation are involved in VCAM-1 expression, which can be regulated by bisacurone. Interestingly, bisacurone showed stronger inhibition of the p-PKC level than the p-Akt level activated by TNF- α . Bisacurone inhibited PKC phosphorylation even at a low dose of 1 μM and completely suppressed it at 50 μM , while it inhibited Akt phosphorylation starting at 10 μM and significantly decreased it at 50 μM (Fig. 4).

3.4. Inhibitory effects of bisacurone on the activation of NF- κB and phosphorylation of I $\kappa\text{B}\alpha$ by TNF- α

NF- κB is activated by various inflammatory stimuli, including TNF- α , and VCAM-1 contains binding sites for NF- κB . Therefore,

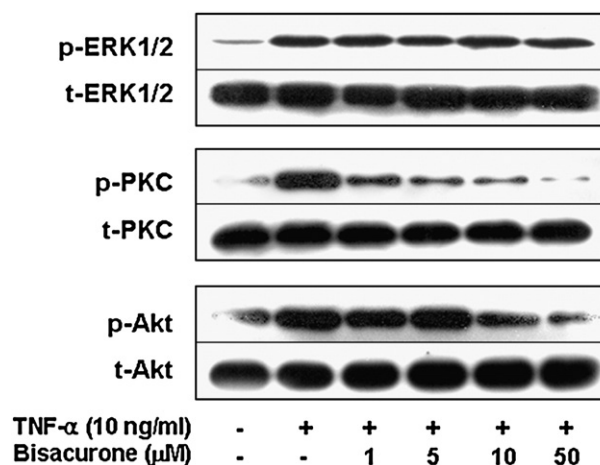


Figure 4 The effect of bisacurone on the phosphorylation of ERK1/2, Akt, and PKC by TNF- α in HUVECs. Cells were pretreated with bisacurone in a concentration-dependent manner (1, 5, 10, and 50 μM) for 24 h and then treated with TNF- α for 10 min for the detection of phospho-ERK1/2 or for 30 min for the detection of phospho-Akt and PKC. Cells were extracted, and protein level was detected by Western blot analysis. Data were confirmed by two independent experiments.

to determine whether NF- κ B activated by TNF- α was inhibited by bisacurone, we performed NF- κ B translocation experiments and a luciferase reporter assay. As shown in Fig. 5A, TNF- α caused NF- κ B (p65) to translocate from the cytosol into the nucleus, which was inhibited by pretreatment with bisacurone in a concentration-dependent manner. Phosphorylation of I κ B α , an inhibitor of NF- κ B activity, by kinases results in the degradation of I κ B α , which then releases NF- κ B, allowing it to translocate to the nucleus where it is active in the regulation of gene transcription. Thus, we determined if bisacurone could prevent this phosphorylation of I κ B α by TNF- α . As shown in Fig. 5B, bisacurone dose-dependently prevented the phosphorylation of I κ B α . Moreover, TNF- α increased NF- κ B-luciferase activity by about 2.5 fold compared with untreated control, which was efficiently inhibited by bisacurone in a concentration-dependent

manner (Fig. 5C). These results suggest that bisacurone inhibits NF- κ B activation by TNF- α , resulting in the inhibition of VCAM-1 expression.

3.5. Inhibition of TNF- α stimulated adhesion of monocytes or oral cancer cells to ECs by bisacurone

The monocyte/EC interaction is in part regulated by the expression of specific adhesion molecules. Among these, VCAM-1 expression plays an important role in the adhesion of monocyte (U937) cells to ECs [22,27]. Moreover, VCAM-1 plays an important role in the adhesion of cancer cells to EC [9]. Our results showed that incubation of HUVECs with bisacurone significantly attenuated the level of VCAM-1. Therefore, we investigated the effect of bisacurone on adhesion of monocytes

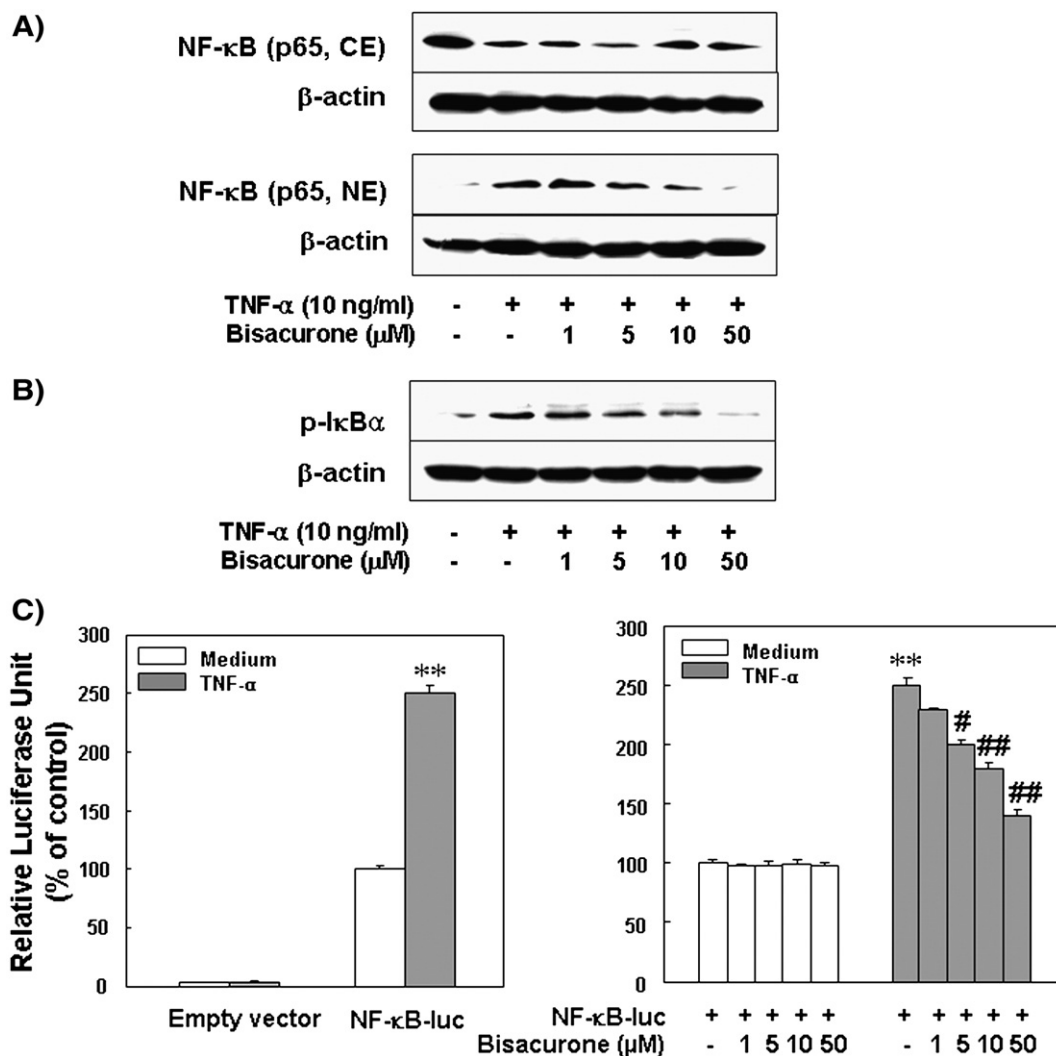


Figure 5 Inhibitory effect of bisacurone on TNF- α -induced NF- κ B activation in HUVECs. (A) Cells were pretreated with bisacurone (1, 5, 10, and 50 μ M) for 24 h and then treated with TNF- α (10 ng/ml) for 1 h. After treatment, nuclear and cytoplasmic fractions were analyzed for the detection of NF- κ B translocation from the cytosol into the nucleus (A), or total protein was analyzed for the detection of the p-I κ B α protein level (B) by Western blot analysis as described in the Methods section. Data were confirmed by two independent experiments. (C) Cells were transfected with 1 μ g of NF- κ B-luciferase and were allowed to recover for 24 h before being treated with 10 ng/ml of TNF- α with or without bisacurone (1, 5, 10, and 50 μ M). Cells were harvested 6 h after treatment. Luciferase activities are presented as the fold activation relative to that of the untreated cells. Data are presented as means \pm SD of three independent experiments. Significance compared to control, ** P <0.05; significance compared with TNF- α , # P <0.05 and ## P <0.01.

to HUVECs after stimulation with TNF- α . Adhesion of human monocyte U937 cells to HUVECs increased by 4-fold after stimulation with TNF- α at 10 ng/ml for 6 h (Fig. 6A), whereas very few U937 cells adhered to inactivated HUVECs (Fig. 6A). In contrast, when HUVECs were pretreated with bisacurone for 24 h, the results showed significant reduction of adherent cells to ECs from 4-fold to 2-fold (Fig. 6A). Furthermore, treatment with 1 to 50 μ M bisacurone for 24 h before TNF- α stimulation showed significant reduction of three human oral neoplastic cell lines (Hep-2, QLL-1 and SCC 15) adhering to ECs (Fig. 6B, C and D); adhesion of cancer cells to HUVECs increased about six times after TNF- α stimulation but decreased by about 2.5 fold with 50 μ M bisacurone.

4. Discussion

In this study, we clearly showed that bisacurone inhibited TNF- α -induced VCAM-1 expression in HUVECs and that it effectively suppressed the production of ROS in TNF- α -stimulated HUVECs. It was reported that ROS are produced rapidly in HUVECs in response to TNF- α [28] and that 10 ng/ml TNF- α stimulates ROS generation and subsequently upregulates VCAM-1 activation in HUVECs [29]. Thus, we may speculate that bisacurone might downregulate VCAM-1 expression through inhibition of ROS production. In addition, we found that bisacurone inhibited TNF- α -caused transcriptional activation of NF- κ B, which was due to blocking the

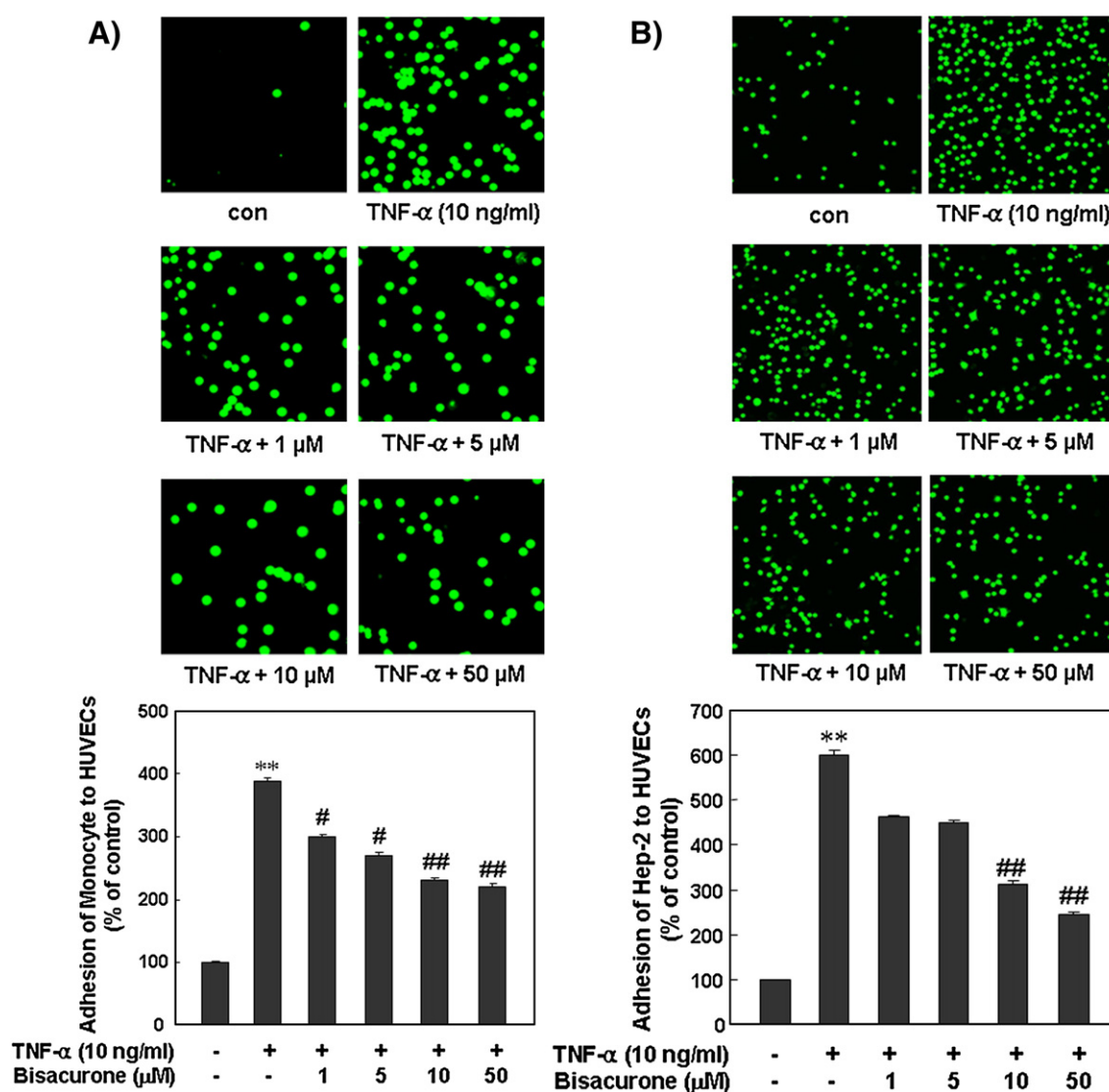


Figure 6 Inhibition of TNF- α -stimulated adhesion of monocyte or oral cancer cells to endothelial cells by bisacurone. HUVECs were pretreated with or without bisacurone (1, 5, 10, and 50 μ M) for 24 h and then stimulated with TNF- α for 6 h. After co-incubation of fluorescence labeled U937 monocytes (A) with HUVECs for 30 min at 37 $^{\circ}$ C, monocyte adhesion was determined in the images and is presented as a percentage of U937 cells bound to endothelial cells. Another group of HUVECs were co-incubated with fluorescence labeled human oral cancer Hep-2 (B), QLL-1 (C), and SCC-15 (D) cells for 30 min at 37 $^{\circ}$ C. The cancer cell adhesion was determined in the images and is presented as a percentage of cancer cells bound to the endothelial cells. Data represent the mean \pm SD of the three separate experiments performed in triplicate. Significance compared to control, ** P <0.01; significance compared with TNF- α , # P <0.05; ## P <0.01).

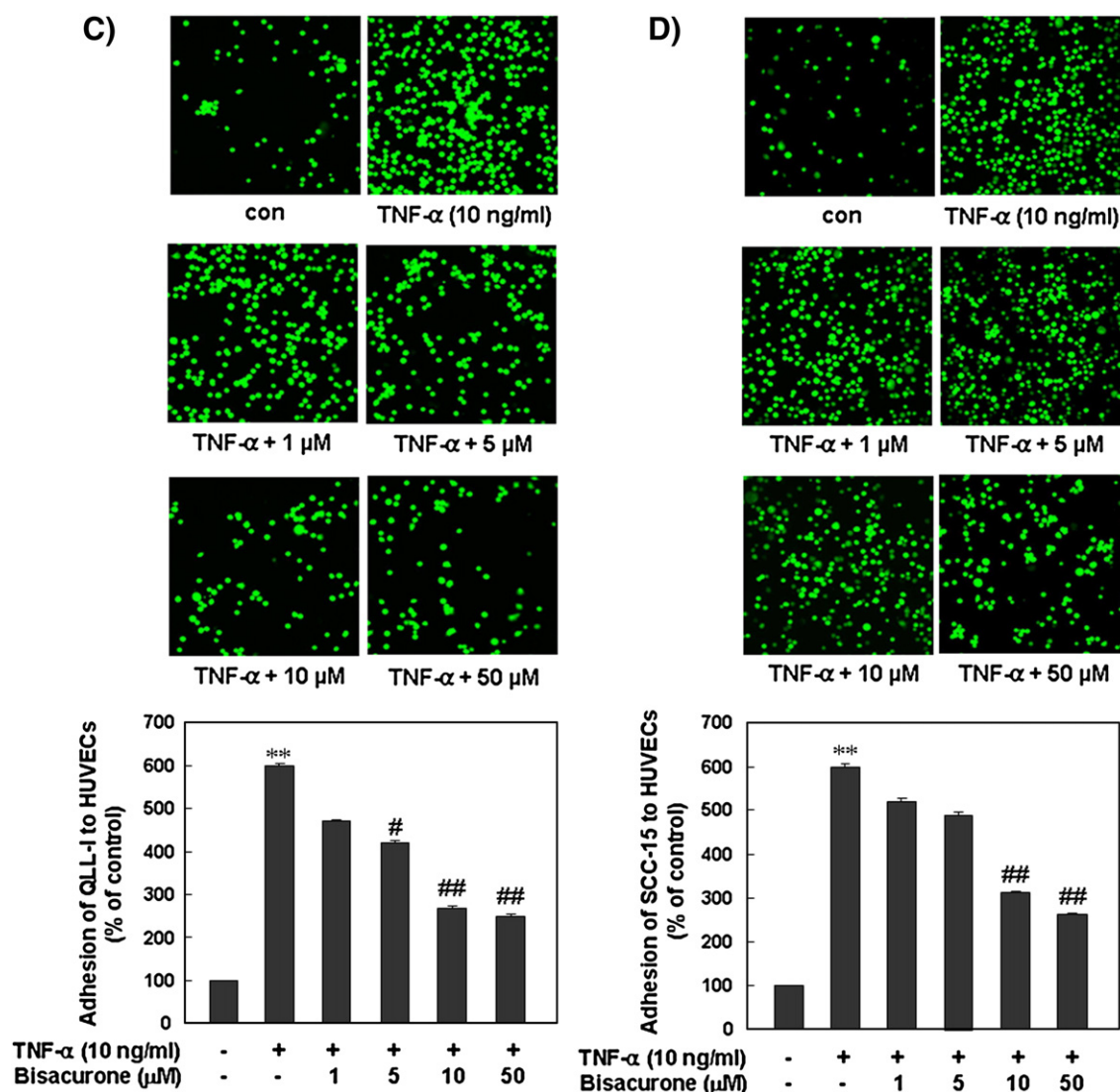


Figure 6 (continued).

phosphorylation of I κ B α . Moreover, bisacurone markedly suppressed TNF- α -mediated phosphorylation of Akt and PKC but not of ERK1/2 activation. The role of PI3K/Akt and PKC in the regulation of VCAM-1 activation was shown previously [22], whereas ERK1/2 is involved in ICAM-1 expression [23]. Of course, NF- κ B is a transcription factor involved in the induction of ICAM-1 as well as VCAM-1. It has been reported, however, that the ICAM-1 promoter does not contain the octamer binding protein, IRF, and GATA binding motifs, which are present in the VCAM-1 gene promoter region [30–33]. Therefore, we suggest that bisacurone suppresses VCAM-1 expression through regulation of the ROS, PI3K/Akt, PKC and NF- κ B pathways, where the NF- κ B pathway is partially involved.

The leukocyte adhesion cascade is documented to have receptor-ligand functional overlap, as VCAM-1 has been shown to support tethering and rolling of lymphocytes [34]. Compounds that suppress VCAM-1 expression may be effective in blocking leukocyte adhesion in a variety of inflammation settings. In contrast, reducing the expression of all ECAMs may cause detrimental side effects, and such a broad spectrum may not be necessary to achieve a ther-

apeutic effect. For example, VCAM-1 is present on aortic endothelium that covers early foam cell lesions [35]. Bisacurone showed an inhibitory effect on the adhesion of monocytic U937 cells to TNF- α -treated HUVECs. This inhibitory effect on the adhesion of monocytes to vascular endothelium seems likely to contribute to the anti-atherosclerotic activity of bisacurone. Importantly, the ability of tumor cells to form metastatic foci correlates with their ability to interact with and migrate through endothelial cell layers. This process involves multiple adhesive interactions between tumor cells and the endothelium. Recently, several melanoma cell lines with different metastatic properties were compared, and it was shown that, as the metastatic capacity of cells increases, the cells express more α 4 β 1 integrin (VCAM-1 receptor) rather than integrin for ICAM-1 [9]. Consequently, blocking VCAM-1 on endothelial cells may be a very valuable approach to interfere with tumor metastasis. Here, we demonstrated that bisacurone dose-dependently inhibited the adhesion of three different human oral cancer cell lines (Hep-2, QLL-1, SCC-15) to ECs. Therefore, these results suggest that bisacurone may be developed as a useful therapeutic for

the treatment of pathological processes, in particular diseases involving VCAM-1 (e.g. atherosclerosis and metastasis).

As a whole, our results suggest that the suppression of TNF- α -induced VCAM-1 expression by bisacurone might be mediated through blocking phosphorylations of PI3K/Akt and PKC, and at least in part by down regulation of NF- κ B activation. To our knowledge, this is the first demonstration of the capacity of bisacurone which is derived from the radix of *C. longa*, to modulate adhesion molecules, in particular VCAM-1.

Acknowledgements

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Appendix A. Supplementary Data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.intimp.2008.05.006](https://doi.org/10.1016/j.intimp.2008.05.006).

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