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ORIGINAL ARTICLE

Etlingera pavieana extract attenuates TNF-α induced vascular adhesion molecule expression in human endothelial cells through NF-κB and Akt/JNK pathways

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Abstract

The aim of this study was to determine whether ethanol extracts of *Etlingera pavieana* rhizomes (EPE) can inhibit the expression of ICAM-1 and VCAM-1 in TNF-α-stimulated human vascular endothelial cells. EPE significantly reduced ICAM-1 and VCAM-1 expression in a concentration-dependent manner. EPE also suppressed phospho-IκB level and nuclear translocation of NF-κB. EPE significantly inhibited phosphorylation of JNK and c-Jun, a major component of AP-1, but had no effects on ERK and p38 MAPK pathways. Akt phosphorylation was increased in the presence of EPE, and wortmannin and SP600125 reversed the inhibitory effects of EPE on ICAM-1 and VCAM-1 expression. Furthermore, the active EPE constituents 4-methoxycinnamyl *p*-coumarate and *trans*-4-methoxycinnamaldehyde attenuated TNF-α-induced expression of ICAM-1 and VCAM-1. Taken together, our data indicate that EPE protects against vascular inflammation in endothelial cells, in part via NF-κB and Akt/JNK signalings. In future studies, *E. pavieana* may be developed as a therapeutic agent or dietary supplement for treating and preventing inflammatory diseases.

Keywords *Etlingera pavieana* · Inflammation · Cell adhesion molecules · Endothelial cell

Abbreviations

Akt	Protein kinase B
AP-1	Activator protein-1
CVDs	Cardiovascular diseases
ERK	Extracellular signal-regulated kinase
H2DCF-DA	2,7-Dichlorodihydrofluorescein diacetate
ICAM-1	Intercellular adhesion molecule-1
IκBα	Inhibitor of κBα
JNK	C-Jun NH_2-terminal kinase
MAPKs	Mitogen-activated protein kinases
MCC	4-methoxycinnamyl *p*-coumarate
MCD	*Trans*-4-methoxycinnamaldehyde
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NF-κB	Nuclear factor-kappa B
PI3K	Phosphatidylinositol 3′-kinase
ROS	Reactive oxygen species
TNF-α	Tumor necrosis factor-α
VCAM-1	Vascular cell adhesion molecule-1

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Introduction

Chronic vascular inflammation plays an important role in the initiation and development of atherosclerosis, which is the major cause of most cardiovascular diseases (CVDs) (Stoner et al. 2013; Khan et al. 2015). The endothelial adhesion molecules intercellular adhesion molecule (ICAM-1) and vascular cell adhesion molecule (VCAM-1) mediate leukocyte adhesion to vascular endothelia, and subsequent transendothelial migration into the arterial intima promotes the development of atherosclerotic lesions (Fotis et al. 2012; Khan et al. 2015).

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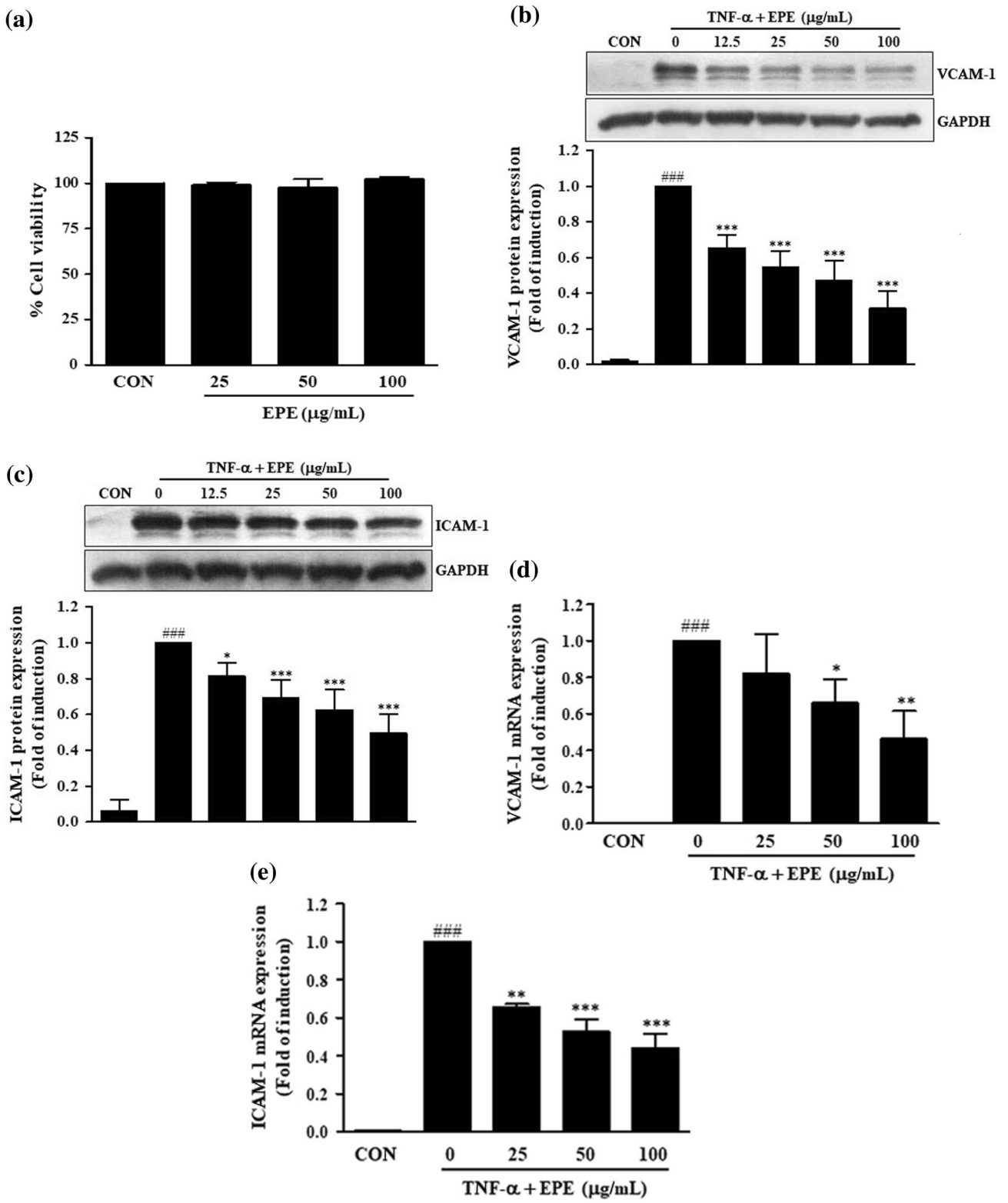


Fig. 1 Effects of EPE on cell viability (**a**) and TNF-α-induced ICAM-1 and VCAM-1 protein (**b**, **c**) and mRNA (**d**, **e**) expression levels. EA.hy926 cells were pretreated with EPE and were then stimulated with TNF-α for 6 or 3 h. ICAM-1 and VCAM-1 protein and mRNA expressions levels were determined by Western blotting and real time reverse transcriptase-polymerase chain reaction (real time RT-PCR) analyses, respectively; $^{###}p < 0.001$ compared with unstimulated control cells; $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared with TNF-α-treated cells

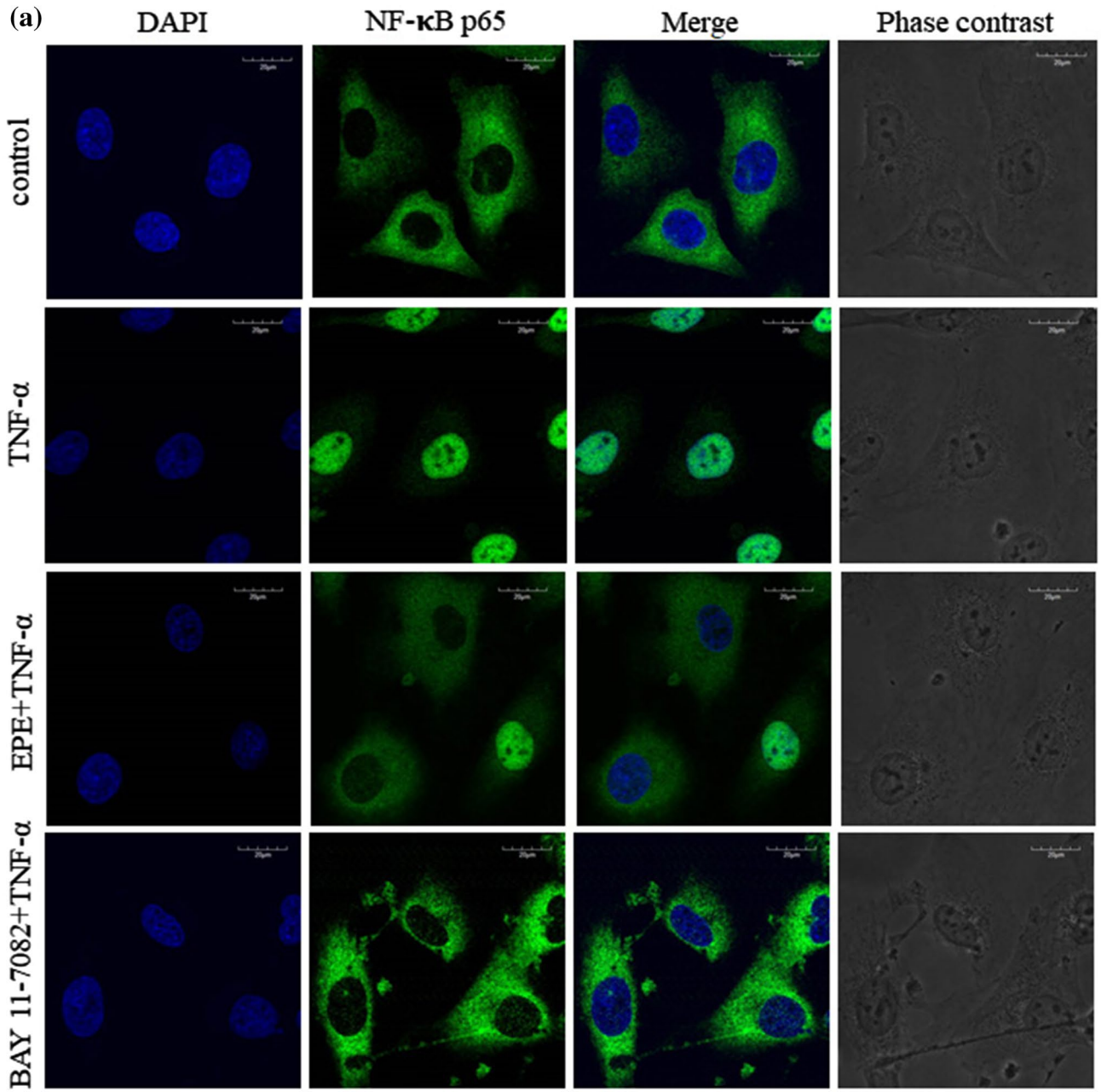


Fig. 2 Inhibitory effects of EPE on NF-κB activation; cells were pretreated with EPE for 1 h prior to stimulation with TNF-α for 30 min. Localization of the NF-κB p65 subunit was investigated using immunofluorescence analyses (**a**) and phospho-IκB levels were examined using Western blot analyses (**b**). Cells were pretreated with EPE and BAY11-7082 before stimulation with TNF-α for 6 h. ICAM-1 and VCAM-1 expression levels were determined using Western blot analyses (**c**, **d**); $^{\#\#\#}p < 0.001$ compared with unstimulated control cells; $^{***}p < 0.001$ compared with TNF-α-treated cells

Hence, ICAM-1 and VCAM-1 play critical roles in the early stages of atheroma formation and inflammation. Interventions that reduce the expression of these molecules may therefore be promising therapeutic strategies for preventing the development of atherosclerosis and inflammation-related diseases.

ICAM-1 and VCAM-1 expression levels are up-regulated in response to pro-inflammatory cytokines such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) (Moon et al. 2009; Stoner et al. 2013). TNF-α is produced by activated macrophages and is commonly expressed in atherosclerotic lesions (Cook-Mills et al. 2011). Several studies show that TNF-α induces ICAM-1 and VCAM-1 via the major signaling molecules nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinases (MAPKs) (Ledebur and Parks 1995;

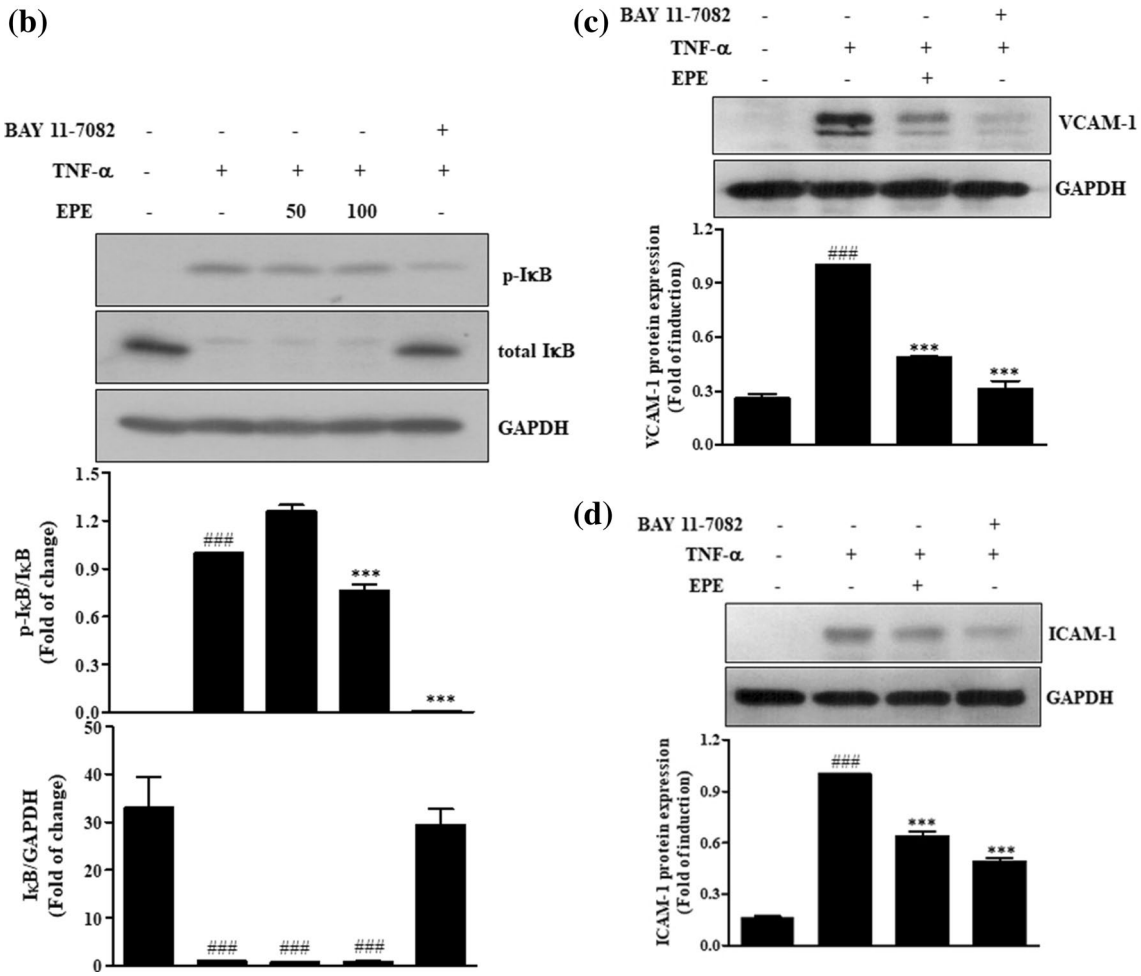


Fig. 2 (continued)

Chen et al. 2001; Ho et al. 2008). Moreover, phosphatidylinositol 3′-kinase/Akt (PI3K/Akt) cascades have been shown to be involved in TNF-α-induced endothelial adhesion molecule expression (Choi et al. 2010, 2012).

Etlingera pavieana (Pierre ex Gagnep.) R.M.Sm. is a plant of the Zingiberaceae family and is widely distributed in Southeast Asia countries, including Thailand, Cambodia, and Loas. *E. pavieana* rhizomes are consumed as a spice, culinary vegetable, and medicine (Poulsen and Phonsena 2017). Previous studies show that *E. pavieana* rhizomes has various pharmacological activities. In 2017, we reported potent anti-inflammatory effects of *E. pavieana* rhizome extracts in LPS-stimulated RAW 264.7 macrophages (Srisook et al. 2017). Among compounds from *E. pavieana* rhizomes, 4-methoxycinnamyl *p*-coumarate (MCC) and *trans*-4-methoxycinnamaldehyde (MCD) suppressed the production of inducible nitric oxide synthase (iNOS)-catalyzed nitric oxide (NO) and cyclooxygenase-2 (COX-2)-catalyzed prostaglandin E_2 (PGE_2), leading to anti-inflammatory effects (Mankhong et al. 2019; Srisook et al. 2019). We also showed that ethanol extracts from *E. pavieana* rhizomes ameliorated oxidative stress in TNF-α-stimulated human endothelial cells (Srisook et al. 2018). Moreover, *E. pavieana* rhizomes and their constituent MCD exhibited antitumor effects against breast, cervix, liver, and colon cancer cell lines (Iawsipo et al. 2018). However, the effects of *E. pavieana* rhizomes on vascular inflammation have not been demonstrated. Thus, we investigated the anti-inflammatory effects of *E. pavieana* rhizomes and characterized the related mechanisms in TNF-α-induced human endothelial cells. Our data provide evidence for the efficacy of *E. pavieana* rhizome extracts as neutraceuticals and medicine for preventing and treating inflammatory diseases.

Materials and methods

Materials

Phosphatase inhibitor cocktail and protease inhibitor cocktail were purchased from Thermo Scientific

(Massachusetts, USA). 2 × iTaq™ Universal SYBR® Green Supermix and iScript Reverse Transcription Super mix were purchased from Bio-Rad (California, USA). Antibodies against ICAM-1, VCAM-1, GAPDH, p–c-Jun (Ser63), p-SAPK/JNK (Thr183/Tyr185), p-p38 MAPK (Thr180/Tyr182), p-ERK1/2 (Thr202/Tyr204), SAPK/JNK, Akt, and p-Akt (Ser473) were purchased from Cell Signaling Technology (Massachusetts, USA). Antibodies against total ERK1/2 and total p38α were purchased from Santa Cruz Biotechnology (California, USA). We purchased 2,7-dichlorodihydofluorescein diacetate (H_2DCF-DA) from Sigma-Aldrich (St. Louis, MO, USA).

Plant extraction

E. pavieana (Pierre ex Gagnep.) R.M.Sm. rhizomes were collected from Chantaburi Province, Thailand. Plants were identified by Dr. B. Chewprecha, Department of Biology, Faculty of Science, Burapha University. A voucher specimen (KS-SCBUU-0012-1) was preserved at the Faculty of Science, Burapha University. Ethanol extracts of *E. pavieana* rhizomes were prepared as described by Srisook et al. (2017).

Cell culture

Human umbilical vein endothelial cells (EA.hy 926 cells) were purchased from ATCC. Cells were cultured in DMEM supplemented with 10% heat-inactivated fetal bovine serum and were incubated at 37 °C in 5% CO_2.

Cell viability assays

Cell viability was estimated according to the conversion of the tetrazolium dye MTT into purple colored formazan crystals by mitochondrial dehydrogenase in viable cells. Briefly, cells were subcultured in 24-well plates (5×10^4 cells/well) and were treated with extracts for 24 h. Cells were then washed in PBS and incubated in MTT solution (5 mg/mL) for 3 h. The resulting formazan crystals were dissolved in DMSO and absorbance was measured at 550 nm.

Western blot analysis

EA.hy 926 cells were pretreated with EPE for 1 h before exposure to 10-ng/mL TNF-α for indicated times. Whole cell protein lysates were then extracted in RIPA buffer containing 1 × protease inhibitor cocktail and 1 × phosphatase inhibitor cocktail. After centrifugation at 13,700*g* for 5 min, supernatants were collected and protein concentrations were determined using quick start Bradford dye reagent. Equal amounts of protein were separated using 10% SDS-PAGE. Fractionated proteins were then transferred onto PVDF membranes and were incubated with appropriate primary antibodies followed by corresponding secondary antibodies conjugated with HRP. Protein bands were visualized using ECL substrate. Relative band intensities were quantified densitometrically using Image Studio Lite-Free Western Blot Analysis Software.

RNA isolation and real time reverse transcription-polymerase chain reaction (real time RT-PCR)

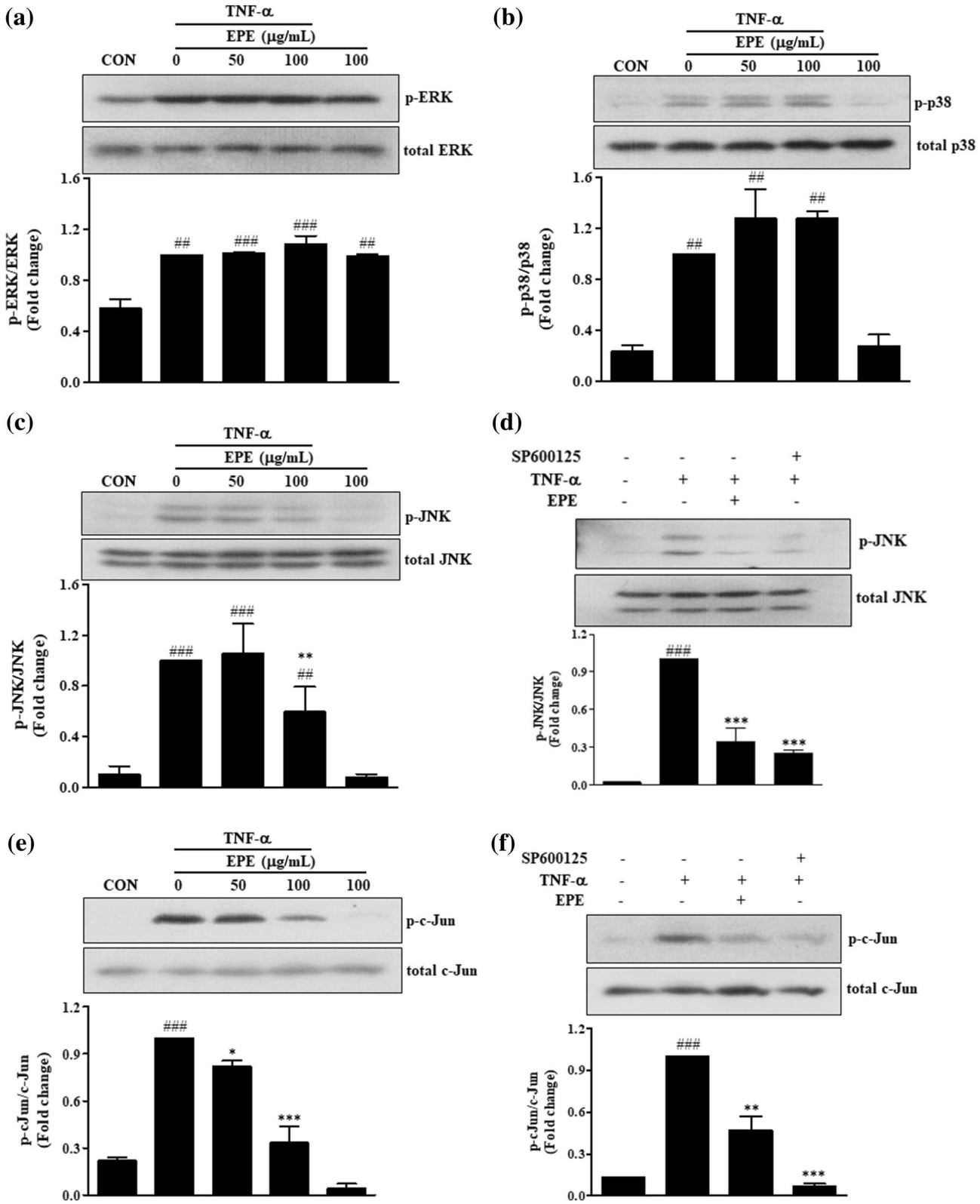
Cells were incubated with EPE for 1 h and were then treated with 10-ng/mL TNF-α for 3 h. Cells were harvested and total RNA was isolated using Nucleospin RNA (Macherey–Nagel, Germany) according to the manufacturer's instructions. Subsequently, cDNA was synthesized from 2-µg aliquots of total RNA using iScript Reverse Transcription Super mix. Real time PCR was conducted on a CFX96 TouchTM Real time PCR (Bio-Rad, USA.) instrument in 20-µL reaction mixtures containing 2 µL of cDNA, 0.25 µM of forward and reverse primers, and iTaq SYBR Green Supermix. The forward (F) and reverse (R) primer sequences for ICAM-1, VCAM-1, and GAPDH were as follows: ICAM-1, 5′-CCTGATGGG CAGTCAACAGCTA-3′ (F) and 5′-ACAGCTGGCTCCCGT TTC A-3′ (R); VCAM-1, 5′-GGGACCACATCTACGCTG ACAA-3′ (F) and 5′-GGCCACTCAAATGAATCTCTGGA-3′ (R); GAPDH, 5′-GTCAAGGCTGAGAACGGG AA-3′ (F) and 5′-AAATGAGCCCC AGCCTTCTC-3′ (R).

Immunofluorescence analyses

EA.hy 926 cells were pretreated with EPE for 1 h and were then incubated with TNF-α for 30 min. NF-κB p65 locations were investigated using immunofluorescence staining and confocal laser scanning microscopy as described by Srisook et al. (2019).

HPLC profile analysis

HPLC analyses were performed on a HPLC Agilent 1260 infinity II (Santa Clara, CA, USA) instrument with a diode array detector at 190–400 nm. Samples were eluted through a Phenomenex Luna C18 column (5 µ × 250 mm × 4.6 mm, 100A) at 35 °C. The mobile phase of methanol:water (70:30; v/v) was applied in isocratic mode with an injection volume of 10 µL, a flow rate of 1 mL/min, and UV detection at 320 nm.


(a)
TNF-α
EPE (μg/mL)
CON 0 50 100 100
p-ERK
total ERK
p-ERK/ERK (Fold change)
(b)
TNF-α
EPE (μg/mL)
CON 0 50 100 100
p-p38
total p38
p-p38/p38 (Fold change)
(c)
TNF-α
EPE (μg/mL)
CON 0 50 100 100
p-JNK
total JNK
p-JNK/JNK (Fold change)
(d)
SP600125 - - - +
TNF-α - + + +
EPE - - + -
p-JNK
total JNK
p-JNK/JNK (Fold change)
(e)
TNF-α
EPE (μg/mL)
CON 0 50 100 100
p-c-Jun
total c-Jun
p-cJun/c-Jun (Fold change)
(f)
SP600125 - - - +
TNF-α - + + +
EPE - - + -
p-c-Jun
total c-Jun
p-cJun/c-Jun (Fold change)

◂**Fig. 3** Effects of EPE on TNF-α-induced activation of MAP kinase signaling in endothelial cells. Cells were pre-incubated with EPE at various concentrations for 1 h and were then stimulated with TNF-α for 30 min. The phosphorylation levels of ERK1/2 (**a**), p38 MAPK (**b**), and JNK (**c**) were determined in Western blot analyses. Cells were pretreated with EPE and SP600125 for 1 h prior to treatment with TNF-α for 30 min. The levels of p-JNK (**d**) and p–c-Jun (**e**, **f**) were examined. Data are expressed as fold changes relative to cells treated with TNF-α only; $^{\#\#}p<0.01$ and $^{\#\#\#}p<0.001$ compared with unstimulated control cells; $^{*}p<0.05$, $^{**}p<0.01$ and $^{***}p<0.001$ compared with TNF-α-treated cells

Statistical analysis

All data are expressed as means ± standard deviations. Differences were identified using ANOVA followed by Tukey's multiple comparison tests, and were considered significant when $p<0.05$.

Results

Inhibitory effects of EPE on ICAM-1 and VCAM-1 expression in TNF-α-induced human endothelial cells

At 25–100 μg/mL, EPE did not significantly affect cell viability in MTT assays, compared with the viability of untreated control cells (Fig. 1a). Thus, EPE was used at non-cytotoxic doses in further experiments. In Western

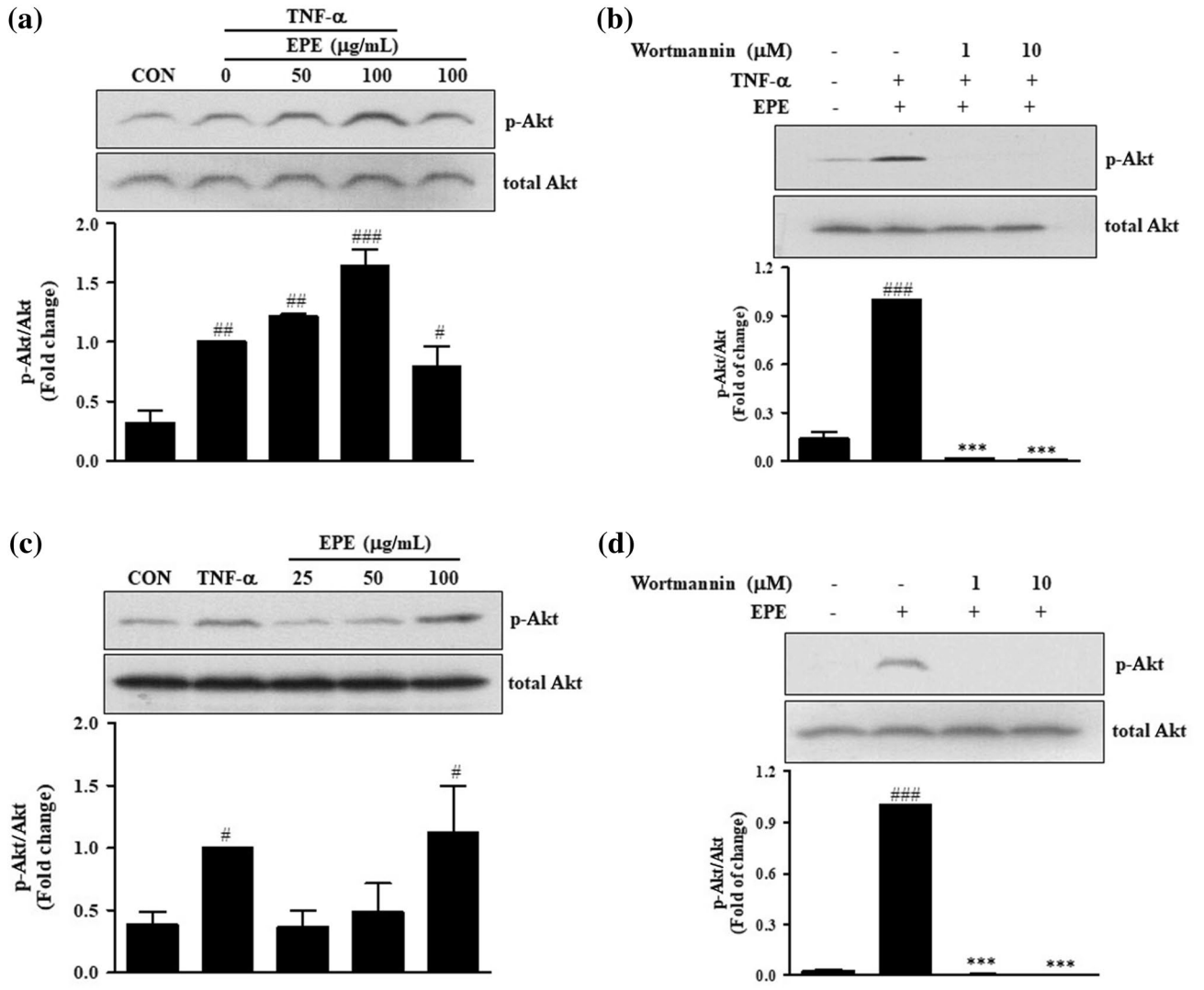
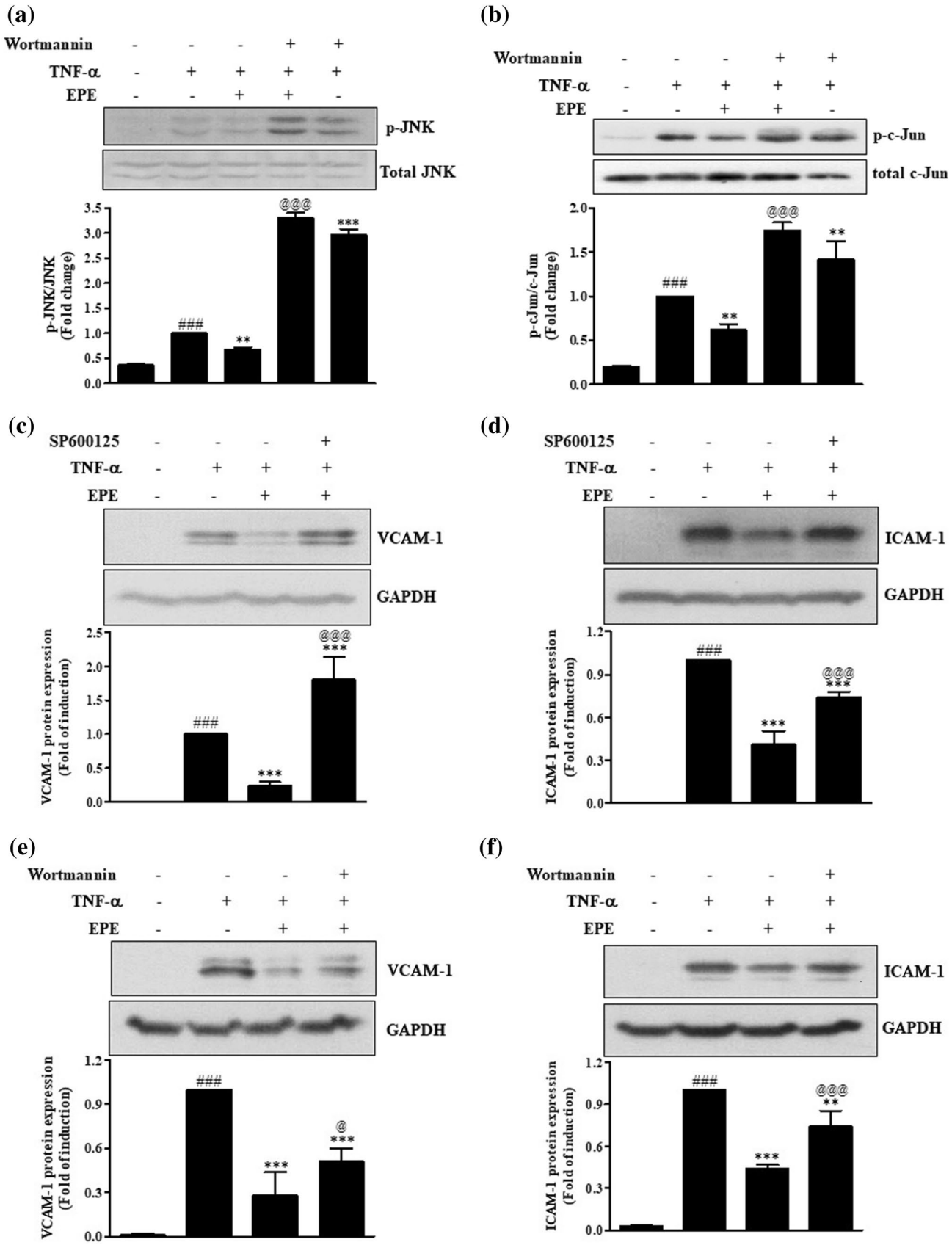


Fig. 4 Effects of EPE on TNF-α-induced phosphorylation of Akt (**a**) in human endothelial cells; cells were pretreated with EPE at various concentrations for 1 h and were then stimulated with 10-ng/mL TNF-α for 30 min; Akt phosphorylation after treatment with EPE alone (**c**) or with EPE and wortmannin (**b, d**); $^{\#}p<0.05$, $^{\#\#}p<0.01$, and $^{\#\#\#}p<0.001$ compared with control cells; $^{***}p<0.001$ compared with TNF-α-treated cells


(a)
Wortmannin
TNF-α
EPE
p-JNK
Total JNK
p-JNK/JNK (Fold change)
(b)
Wortmannin
TNF-α
EPE
p-c-Jun
total c-Jun
p-cJun/c-Jun (Fold change)
(c)
SP600125
TNF-α
EPE
VCAM-1
GAPDH
VCAM-1 protein expression (Fold of induction)
(d)
SP600125
TNF-α
EPE
ICAM-1
GAPDH
ICAM-1 protein expression (Fold of induction)
(e)
Wortmannin
TNF-α
EPE
VCAM-1
GAPDH
VCAM-1 protein expression (Fold of induction)
(f)
Wortmannin
TNF-α
EPE
ICAM-1
GAPDH
ICAM-1 protein expression (Fold of induction)

◂**Fig. 5** The effects of wortmannin and SP600125 on phosphorylation of JNK (**a**) and c-Jun (**b**) and on consequent expression levels of VCAM-1 (**c**, **e**) and ICAM-1 (**d**, **f**); cells were pretreated with EPE, wortmannin, or SP600125 for 1 h and were then exposed to TNF-α for 30 min or 6 h; $^{\#\#\#}p < 0.001$ compared with unstimulated control cells; $^{**}p < 0.01$ and $^{***}p < 0.001$ compared with TNF-α-treated cells; $^{@}p < 0.05$ and $^{@@@}p < 0.001$ compared with TNF-α and EPE treated cells

blot analyses of VCAM-1 and ICAM-1, TNF-α treatments resulted in a substantial increase in VCAM-1 and ICAM-1 protein levels (Fig. 1b, c). Pretreatments with EPE at non-cytotoxic doses, however, significantly decreased the expression of VCAM-1 and ICAM-1 proteins in a concentration-dependent manner. VCAM-1 expression was inhibited by EPE to a greater extent than ICAM-1 expression.

Real time RT-PCR analyses showed corresponding upregulation of VCAM-1 and ICAM-1 mRNA expression levels after TNF-α stimulation, and concentration-dependent downregulation of these mRNAs following EPE treatments (Fig. 1d and 1e).

NF-κB inactivation is involved in the anti-inflammatory effects of EPE in TNF-α-induced human endothelial cells

To elucidate the mechanisms underlying the anti-inflammatory effects of EPE in TNF-α endothelial cells, we determined the effects of EPE on NF-κB signaling. NF-κB is a well-known transcription factor that regulates the expression of numerous inflammatory genes, including those encoding VCAM-1 and ICAM-1 (Ledebur and Parks, 1995; Chen et al. 2001). Initially, we compared nuclear translocation of the NF-κB p65 subunit using immunofluorescence analyses. Incubation of cells with TNF-α induced nuclear translocation of NF-κB p65, whereas EPE treatment significantly suppressed this process (Fig. 2a).

Because NF-κB is activated by phosphorylation of the inhibitor of κBα (IκBα) by IκB-kinase (IKK), leading to degradation of IκB, we determined phosphorylated IκB levels in endothelial cells. Under these conditions, TNF-α treatments substantially increased the phosphorylation of IκB, whereas EPE treatments at 100 μg/mL decreased IκB phosphorylation. However, EPE did not affect total IκB levels as compared with TNF-α treated cells (Fig. 2b). In positive control experiments, the NF-κB inhibitor BAY11-7082 (5 μM) inhibited TNF-α induced nuclear translocation of NF-κB p65 and increased IkB phosphorylation as well as total IκB levels (Fig. 2a and 2b).

To confirm the roles of NF-κB signaling in the inhibitory effects of EPE on VCAM-1 and ICAM-1 expression, cells were pretreated with BAY11-7082 and were then incubated with TNF-α. As shown in Fig. 2c, d, inhibition of NF-κB reduced VCAM-1 and ICAM-1 expression levels.

Inhibitory effects of EPE on MAPK phosphorylation in TNF-α-induced human endothelial cells

In addition to NF-κB signaling, the MAPK signaling pathway has been implicated in TNF-α-induced expression of VCAM-1 and ICAM-1 (Ho et al. 2008). In analyses of the effects of EPE on TNF-α-induced MAPK signaling, extracellular signal-regulated kinase (ERK), c-Jun NH_2-terminal kinase (JNK), and p38 MAPK phosphorylation levels were increased following TNF-α treatments (Fig. 3a–c). Moreover, JNK phosphorylation was reduced in the presence of EPE (Fig. 3c), but at the tested concentrations, EPE did not suppress p-ERK or p-p38 MAPK protein expression levels (Fig. 3a, b). In positive control experiments, the JNK inhibitor SP600125 (10 μM) significantly inhibited phosphorylation of JNK (Fig. 3d).

Because c-Jun is a component of the activator protein-1 (AP-1) transcription factor complex and is a major target of JNK (Raivich et al. 2004), we investigated c- Jun phosphorylation using Western blot analysis. TNF-α induced phosphor-c-Jun levels in these endothelial cells, whereas EPE markedly decreased the phosphorylation of c-Jun in a concentration-dependent manner (Fig. 3e). To confirm the roles of JNK signaling in the phosphorylation of c-Jun in EA.hy926 endothelial cells, we pretreated cells with the JNK inhibitor SP600125 and then subjected cells to TNF-α for 30 min. As shown in Fig. 3f, SP600125 treatments reduced the phosphorylation of c-Jun in EA.hy926 cells.

Effects of EPE on Akt phosphorylation in human endothelial cells

The PI3K/Akt signaling pathway reportedly plays negative roles in the regulation of JNK activity (Fey et al. 2012; Zhao et al. 2015). Thus, we examined the effects of EPE on TNF-α induced phosphorylation of Akt. Exposure of endothelial cells to TNF-α led to increased Akt phosphorylation at Ser473 (Fig. 4a). EPE treatments, however, strongly increased phopho-Akt levels in TNF-α endothelial cells. Phospho-Akt levels were also notably higher in cells treated with EPE and TNF-α than in those treated with TNF-α alone. In experiments with the PI3K/Akt pathway inhibitor wortmannin, phospho-Akt levels were dramatically decreased, even after treatments with EPE and TNF-α (Fig. 4b). In subsequent experiments (Fig. 4c), EPE at 100 μg/mL strongly induced the phosphorylation of Akt. This observation was further confirmed by treatments with wortmannin, which abolished EPE-induced phosphorylation of Akt (Fig. 4d).

The inhibitory effects of EPE on VCAM-1 and ICAM-1 expression levels are mediated through Akt/JNK signaling

To confirm the findings described above, we determined whether the inhibitory effects of EPE on VCAM-1 and ICAM-1 expression are mediated through the Akt pathway. As shown in Fig. 5a, b, EPE inhibited p-JNK and p–c-Jun expression. Wortmannin attenuated these suppressive effects of EPE, and VCAM-1 and ICAM-1 levels were significantly decreased by treatments with both TNF-α and EPE. Interference with PI3K/Akt and JNK pathways by wortmannin and SP6000125, respectively, diminished the inhibitory effects of EPE on VCAM-1 and ICAM-1 expression (Fig. 5c–f).

Phytoconstituents of EPE inhibit VCAM-1 and ICAM-1 expression

In our previous study four phenolic compounds were identified in EPE using bioassay-guided isolation. Among these, 4-methoxycinnamyl *p*-coumarate (MCC) and *trans*-4-methoxycinnamaldehyde (MCD) had potent inhibitory effects on NO production in LPS-treated macrophages (Srisook et al. 2017). Therefore, we generated HPLC profiles of EPE and analyze its components. As shown in Fig. 6, MCC and MCD were identified as polyphenols in EPE. In subsequent experiments, MCC and MCD (6.25–25 μM) significantly suppressed TNF-α-induced VCAM-1 and ICAM-1 expression in EA.hy926 cells (Fig. 7). These data suggest that MCC and MCD might contribute to the anti-inflammatory effects of EPE in TNF-α-stimulated endothelial cells.

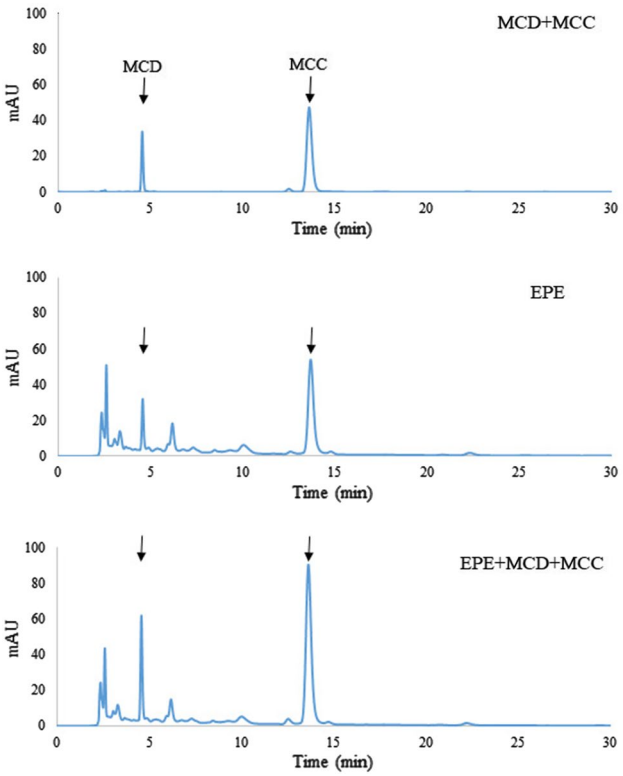


Fig. 6 HPLC chromatograms of EPE; 4-methoxycinnamyl *p*-coumarate (MCC) and *trans*-4-methoxycinnamaldehyde (MCD) contents were monitored at 320 nm

Discussion

It is widely accepted that vascular inflammation contributes to the pathogenesis of atherosclerosis, which is a major cause of CVDs (Stoner et al. 2013; Khan et al. 2015). After stimulating endothelial cells with inflammatory risk factors, the adhesion molecules ICAM-1 and VCAM-1 are induced and transmigration of monocytes is increased. Following migration into the sub-endothelial matrix, monocytes differentiate into macrophages and mediate the early vascular inflammatory changes that lead to atherosclerosis (Stoner et al. 2013; Khan et al. 2015). In addition to vascular inflammation, oxidative stress has been associated with all stages of atherosclerosis (Channon 2006; Vanhoutte et al. 2009). Reactive oxygen species (ROS) are the major mediators of oxidative stress. Over recent years, increasing numbers of studies have identified natural plant extracts that suppress vascular inflammation and have potential as dietary supplements that prevent chronic inflammatory diseases. As reported in our studies, extracts and isolated compounds from *E. pavieana* rhizomes had anti-inflammatory effects in LPS-treated RAW 264.7 macrophages (Srisook et al. 2017). In the present study, we demonstrated anti-atherosclerotic activities of EPE, as indicated by concentration-dependent decreases in TNF-α-induced ICAM-1 and VCAM-1 expression in endothelial cells. Furthermore, we previously showed that EPE treatments reduce ROS levels in TNF-α-stimulated endothelial cells (Srisook et al. 2018). Taken together, these data suggest that EPE might suppress leukocyte adhesion to endothelial tissues, resulting in decreased leukocyte infiltration, atherogenesis, and inflammation.

NF-κB plays a major role in signaling pathways that are implicated in the regulation of VCAM-1 and ICAM-1 by TNF-α (Ledebur and Parks 1995; Chen et al. 2001). Moreover, numerous studies suggest that NF-κB is involved in the pathogenesis of atherosclerosis (Brand et al. 1996; Zapolska-Downar et al. 2012). We therefore designed experiments to assess the effects of EPE on NF-κB activation. Under normal conditions, NF-κB is sequestered into the cytosol after binding IκBα. Yet, after stimulation with pro-inflammatory cytokines such as TNF-α, NF-κB is activated by the IKK complex, which phosphorylates IκB, resulting in its degradation (Won et al. 2016). Subsequently, NF-κB dimers

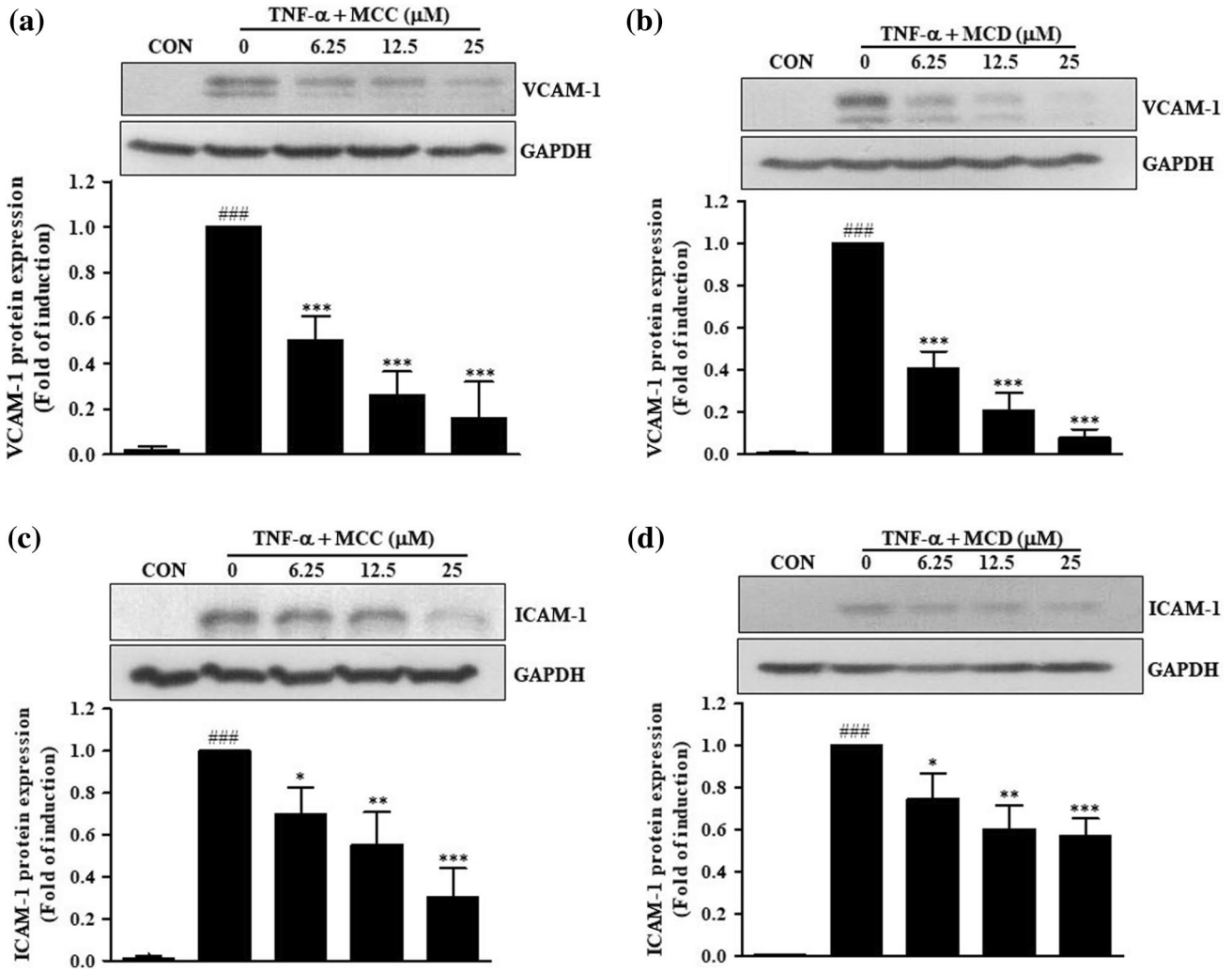


Fig. 7 Inhibitory effects of MCC and MCD on VCAM-1 (**a**, **b**) and ICAM-1 (**c**, **d**) expression in TNF-α-induced; endothelial cells were pre-incubated with MCC and MCD for 1 h before 6-h TNF-α treatments. ICAM-1 and VCAM-1 protein expression levels were determined using Western blot analysis; $^{###}p < 0.001$ compared with control cells; $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared with TNF-α-treated cells

translocate into the nucleus and regulate target genes. Thus, we determine whether EPE inhibits VCAM-1 and ICAM-1 expression via NF-κB signaling in endothelial cells. Our data show that EPE inhibits IκB phosphorylation and nuclear translocation of the NF-κB 65 subunit. Blocking of NF-κB activity by BAY 11-7082 also reduced nuclear translocation of NF-κB 65 and p-IκB expression levels. Moreover, BAY 11-7082 inhibited TNF-α-induced VCAM-1 and ICAM-1 expression. These observations suggest that EPE suppresses the expression of VCAM-1 and ICAM-1 by blocking NF-κB signaling.

MAPKs are serine/threonine protein kinases that participate in various physiological and pathological processes, including inflammation (Kim and Choi 2010). Three major MAPKs include ERK, JNK, and p38. MAPK signaling pathways have also been associated with the expression of inflammatory genes (Kim and Choi 2010). As transcription factor target of these MAPK signaling pathways, AP-1 (Whitmarsh 2007; Kim and Choi 2010), in addition to NF-κB, is regulated by TNF-α and induces the expression of VCAM-1 and ICAM-1 (Chao et al. 2011; Yan et al. 2015). Thus, to investigate whether MAPK pathways are involved in the inhibition of VCAM-1 and ICAM-1 expression by EPE, we analyzed MAPK phosphorylation levels. These experiments demonstrated that EPE inhibits TNF-α-induced JNK phosphorylation but not that of ERK or p38. Surprisingly, EPE alone caused significant phosphorylation of ERK. The phosphorylated level was the same extent as TNF-α treated cells (Fig. 3a). Besides, EPE plus TNF-α treatment did not have an additive effect on phosphorylated ERK level. Although the mechanism underlying the activation of ERK by EPE has not been fully described, the obtained data from

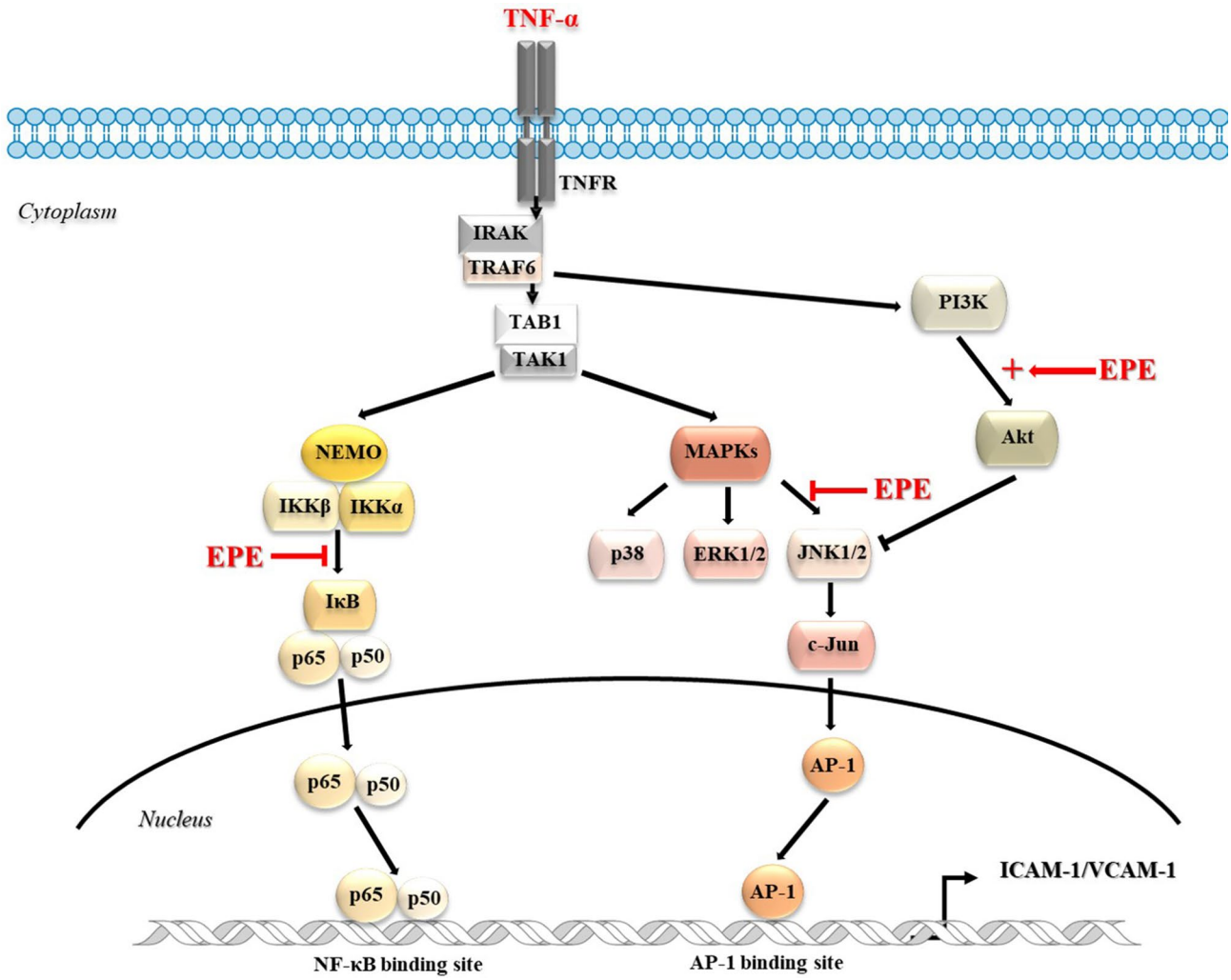


Fig. 8 The proposed mechanisms of EPE action on inflammation in TNF-α-treated endothelial cells

the present study suggest that anti-inflammatory effect of EPE was independent of ERK. JNK efficiently phosphorylates the AP-1 family member c-Jun at Ser63, leading to increased AP-1 activity (Whitmarsh 2007; Zhao et al. 2015). As shown in the present study, EPE suppressed phosphor-c-Jun levels in TNF-α-induced endothelial cells. Moreover, blockade of JNK by SP600125 attenuated c-Jun phosphorylation, suggesting that EPE inhibits c-Jun phosphorylation in part by suppressing JNK phosphorylation.

Apart from NF-κB and MAPK signaling pathways, the PI3K/Akt pathway has been abundantly implicated in the regulation of ICAM-1 and VCAM-1 expression (Choi et al. 2010, 2012). Moreover, crosstalk has been demonstrated between MAPK and Akt pathways (Aksamitiene et al. 2012; Fey et al. 2012; Zhao et al. 2015), Akt activation was also shown to contribute to JNK activity in previous studies (Fey et al. 2012; Zhao et al. 2015). In support of the involvement of Akt and JNK signaling pathways in the inhibition of TNF-α-induced ICAM-1 and VCAM-1 expression by EPE, our experiments (Fig. 4) show that significant increases in Akt phosphorylation due to EPE treatments are abolished by the PI3K/Akt inhibitor wortmannin. Similarly, inhibition of PI3K/Akt signaling by wortmannin attenuated the effects of EPE on JNK and c-Jun phosphorylation, and in turn, expression levels of VCAM-1 and ICAM-1. Based on these data, the inhibitory effects of EPE on VCAM-1 and ICAM-1 expression are likely mediated by Akt, which limits JNK and c-Jun phosphorylation. The findings of this study are similar to a previous report of Akt pathway activation by resveratrol, which led to decreased production of pro-inflammatory mediators and cytokines in LPS-induced RAW 264.7 macrophages (Zong et al. 2012). In contrast, many compounds are assoicated with positive regulation of inflammation by PI3K/Akt signaling (Choi et al. 2010, 2012; Mankhong et al. 2019). After isolating MCC from *E. pavieana* rhizomes, we showed that it inhibits Akt phosphorylation and

LPS-induced iNOS and COX-2 expression in macrophages (Mankhong et al. 2019). Accordingly, diosgenin and extracts from aerial bulbils of *Dioscorea batatas* suppressed TNF-α-induced cell adhesion molecules in vascular smooth muscle cells by downregulating Akt signaling (Choi et al. 2010, 2012). Yet, conflicting observations of PI3K/Akt mediated inflammatory responses have been generated in various cell types and with various agonists.

Based on our previous reports showing potent anti-inflammatory activities of MCC and MCD in RAW 264.7 macrophages, we examined the effects of these compounds on inflammatory responses in endothelial cells. In separate treatments with these phytochemicals, both compounds downregulated the expression of VCAM-1 and ICAM-1. Hence, MCC and MCD may be responsible for the anti-inflammatory effects of EPE in TNF-α-stimulated endothelial EA.hy926 cells. Moreover, MCC at 25–150 mg/kg (Srisook et al. Unpublished data) and MCD at 75–300 mg/kg (Srisook et al. 2019) exerted the inhibitory effect in ethyl phenylpropiolate-induced ear edema as well as carrageenan-induced hind paw edema in rats. However, further bioactive compounds that inhibit vascular inflammation need to be isolated and identified from *E. pavieana* rhizomes.

Conclusions

Collectively, our results suggest that ethanol extracts from *E. pavieana* rhizomes inhibit vascular inflammation by suppressing ICAM-1 and VCAM-1 expression in human endothelial cells. These effects were partially mediated by NF-κB and Akt/JNK signaling pathways as shown in Fig. 8. Our findings warrant further developments of *E. pavieana* extract based neutraceuticals and therapeutic agent that prevent and treat atherosclerosis and CVDs.

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Compliance with ethical standards

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

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