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Original Article

Lavender Essential Oil and Its Main Constituents Inhibit the Expression of TNF-α-induced Cell Adhesion Molecules in Endothelial Cells

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Lavender essential oil (Lvn) has anti-inflammatory effects in an ovalbumin-sensitized murine model of asthma, and inhibits inflammatory cell infiltration into the lungs. The anti-inflammatory effects of Lvn on cell adhesion molecules are not clear. Here we evaluated the effects of Lvn and its main constituents, linalyl acetate (LA) and linalool (LO), on the expression of tumor necrosis factor-alpha (TNF-α)-induced cell adhesion molecules in murine brain endothelial bEnd.3 cells and human umbilical vein endothelial cells (HUVECs). The bEnd.3 cells were treated with Lvn, LA, or LO and subsequently stimulated with TNF-α. The mRNA expression levels of cell adhesion molecules were detected using RT-PCR. E-selectin and P-selectin protein and phosphorylated-NF-κB p65 were detected by western blotting. The effects of Lvn on HUVECs were measured by RT-PCR. In bEnd.3 cells, Lvn and LA suppressed TNF-α-induced E-selectin, P-selectin, vascular cell adhesion molecule-1, intercellular adhesion molecule-1, and phosphorylated-NF-κB p65 in the nucleus; LO did not suppress P-selectin or phosphorylated-NF-κB p65. Lvn inhibited TNF-α-induced E-selectin mRNA in HUVECs. These results indicate that Lvn and LA inhibit TNF-α-induced cell adhesion molecules in endothelial cells through the suppression of NF-κB activation. Consequently, Lvn or other essential oils including LA may be useful as alternative anti-inflammatory medicines.

Key words: lavender essential oil, linalyl acetate, inflammation, cell adhesion molecule, NF-κB

Essential oils are volatile organic compounds extracted from plants. The essential oil from lavender, *i.e.*, *Lavandula angustifolia* (Lvn) is one of the most common essential oils. As some clinical trials report, Lvn is useful for improving health and alleviating anxiety [1], insomnia [2], depression [3], and pain [4]. In animal experiments, Lvn exhibited anti-bacterial and anti-inflammatory effects [5,6]. We reported that Lvn suppressed the allergic inflammations in an ovalbumin (OVA)-sensitized murine model of asthma [7]. Lvn treatment reduced the infiltration of inflammatory cells such as macrophages, eosinophils, and lymphocytes into bronchoalveolar lavage fluid, peribronchial tissues, and perivascular tissues in the lungs in a murine model of asthma [7].

The essential oils include dozens of constituents at different concentrations, and generally two or three main constituents characterize the essential oils' biological properties [8]. The main constituents of Lvn are

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monoterpenes, such as linalyl acetate (LA) and linalool (LO) [9]. LA and LO showed anti-inflammatory effects in carrageenan-induced edema in rats [10]. LA and LO also inhibited nuclear factor-kappa B (NF-κB) activation in the human cervical adenocarcinoma cell line Hela and the murine macrophage cell line RAW264.7 cells, respectively [11, 12]. NF-κB is a transcription factor that induces the production of cell adhesion molecules such as E-selectin, P-selectin, vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1) [13, 14]. These cell adhesion molecules are involved in the infiltration of leukocytes and the pathogenesis of asthma [15-18]. In a murine model of asthma, asthmatic symptoms were suppressed by the inhibition of the NF-κB signaling pathway [19, 20]. In our murine model of asthma, Lvn treatment reduced the infiltration of inflammatory cells into the lungs [7].

These observations led us to hypothesize that the expression of cell adhesion molecules could be suppressed by Lvn treatment in vascular endothelial cells through the inhibition of NF-κB activation. We speculated that the main constituents of Lvn, such as LA and LO, might be involved in those effects. In the present study we used murine brain endothelial cell line bEnd.3 cells, which have frequently been used to study cell adhesion molecules in inflammation [13]. To extrapolate the anti-inflammatory effects of Lvn on the human body, we also used human umbilical vein endothelial cells (HUVECs). We examined the effects of Lvn, LA, and LO on the variations of tumor necrosis factor-alpha (TNF-α)-induced cell adhesion molecules in endothelial cells.

Materials and Methods

Reagents. The essential oil from *Lavandula angustifolia* (Lvn) was obtained from ROHTO Pharmaceutical (Osaka, Japan). The reagents' certificate of gas chromatography mass spectrometry analysis was supplied by ROHTO. The main constituents of the Lvn essential oil were as follows: linalyl acetate (LA) 34.65%, linalool (LO) 26.99%, β-caryophyllene 4.86%, trans-β-ocimene 4.63%, lavandulyl acetate 4.36%, cis-β-ocimene 2.89%, and terpinene-4-ol 2.89%; other constituents were < 2%. The specific gravity was 0.883 and the refractive index was 1.461.

LA was obtained from Wako Pure Chemical Industries (Osaka, Japan). LO was obtained from Tokyo Chemical Industry (Tokyo, Japan). The murine TNF-α and human TNF-α were purchased from Roche Applied Science (Penzberg, Bavaria, Germany).

Cell culture. The bEnd.3 cells, a murine brain endothelioma cell line, were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). The bEnd.3 cells were grown in Dulbecco's modified Eagle medium (DMEM) (Sigma Chemicals, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Life Technologies, Carlsbad, CA, USA) and 1% penicillin-streptomycin (Life Technologies) at 37°C in an atmosphere of 5% CO_2.

HUVECs were obtained from PromoCell (Heidelberg, Germany) and grown in Endothelial Cell Growth Medium 2 (PromoCell) at 37°C in an atmosphere of 5% CO_2. Sub-confluent, proliferating HUVECs at passages 2-5 were used.

Treatment of bEnd.3 cells and HUVECs with Lvn, LA, and LO, followed by TNF-α stimulation. The bEnd.3 cells were incubated for 2 h in the absence or presence of Lvn at one of two concentrations (0.01% or 0.005%). We confirmed that these concentrations of Lvn did not exhibit cytotoxicity by conducting an MTT assay (data not shown). The incubation time was determined by preliminary experiments with reference to Spelman *et al.* [21]. Lvn was diluted in dimethyl sulfoxide (DMSO) (Sigma Chemicals). To obtain consistent final concentrations of DMSO, all of the media were 0.09% DMSO. Subsequently, the cells were incubated without or with murine TNF-α (250 U/mL). The cultures intended for total RNA extraction were incubated for 3 h, and those intended for protein extraction were incubated for 5 h. These incubation times were determined by preliminary experiments with reference to previous reports [13, 22].

To assay the effects of LA and LO on the expression of cell adhesion molecules, we repeated this Lvn workflow, substituting LA or LO for Lvn. HUVECs were incubated in the absence or presence of one of two Lvn concentrations (0.01% or 0.005%) for 2 h. The cells were incubated without or with human TNF-α (100 U/mL) for 3 h to extract total RNA. Equal volumes of phosphate buffered-saline (PBS) were added to non-stimulated cells.

Reverse transcription-polymerase chain reaction (RT-PCR) for cell adhesion molecules. After the culture medium was removed, the cells were washed with

ice-cold PBS. We then extracted the total RNA using TRIzol® (Life Technologies) according to the manufacturer's protocol. Complementary DNA (cDNA) was synthesized from 1 μg RNA with random hexamer primers from a Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Mannheim, Germany). PCR amplifications were performed using a fixed amount of cDNA (1 μL), a pair of gene-specific primers, and TAKARA Taq (Takara Bio, Shiga, Japan) by a Veriti® Thermal Cycler (Applied Biosystems, Foster City, CA, USA). The mRNA expression levels of mouse E-selectin (*Sele*), mouse P-selectin (*Selp*), mouse VCAM-1 (*Vcam1*), mouse ICAM-1 (*Icam1*), mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH; *Gapdh*), human E-selectin (*SELE*), human VCAM-1 (*VCAM1*), human ICAM-1 (*ICAM1*), and human GAPDH (*GAPDH*) were measured. The amplified products were electrophoresed in a 1.5% agarose gel stained with ethidium bromide. The gel was imaged using a ChemiDoc™ Imaging System (Bio-Rad Laboratories, Hercules, CA, USA), and the band intensities were analyzed by Image Lab software (Bio-Rad Laboratories). We normalized these measurements to those of the constitutively expressed GAPDH gene in order to obtain the cell adhesion molecule mRNA levels. The primer sequences and PCR conditions are shown in Table 1.

Western blotting for E- and P-selectin. After the culture medium was removed, the cells were washed with ice-cold PBS and resuspended in 200 μL/well cold Mammalian Protein Extraction Buffer (GE Healthcare Biosciences, Buckinghamshire, UK). This buffer contained 5 mM Na_3VO_4 (MP Biomedicals, Santa Ana, CA, USA) and ×1 Protease Inhibitor Cocktail (Sigma Chemicals). The lysates were incubated on ice for 20 min and then allowed to cool for 15 min at −80°C. They were then centrifuged at 141 000*g* for 30 min at 4°C to remove cellular debris. Protein extracts were

Table 1 The primer sequences and PCR conditions used in this study

		Primer sequence	Annealing (°C) Cycles
Mouse			
Sele	Forward	5'-GATCCAACGCCAGAACAACA-3'	65°C
	Reverse	5'-CCCTTCCACACAGTCAAACG-3'	26 cycles
Selp	Forward	5'-CTATACCTGCTCCTGCTACCCAGGC-3'	60°C
	Reverse	5'-TTCACTCCACTGACCAGAGCCAGTG-3'	26 cycles
Vcam1	Forward	5'-CAAGGGTGACCAGCTCATGA-3'	55°C
	Reverse	5'-TGTGCAGCCACCTGAGATCC-3'	25 cycles
Icam1	Forward	5'-CAACTGGAAGCTGTTTGAGCTG-3'	55°C
	Reverse	5'-TAGCTGGAAGATCGAAAGTCCG-3'	29 cycles
Gapdh	Forward	5'-TTGGCATTGTGGAAGGGCTCAT-3'	55°C
	Reverse	5'-GATGACCTTGCCCACAGCCTT-3'	26 cycles
Human			
SELE	Forward	5'-CACTCAAGGGCAGTGGACACA-3'	60°C
	Reverse	5'-CAGCTGGACCCATAACGGAAAC-3'	20 cycles
VCAM1	Forward	5'-CATGACCTGTTCCAGCGAGG-3'	57°C
	Reverse	5'-CATTCACGAGGCCACCACTC-3'	20 cycles
ICAM1	Forward	5'-GGCTGGAGCTGTTTGAGAAC-3'	57°C
	Reverse	5'-CCTCTGGCTTCGTCAGAATC-3'	24 cycles
GAPDH	Forward	5'-GAAGGTGAAGGTCGGAGTC-3'	57°C
	Reverse	5'-GAAGATGGTGATGGGATTTC-3'	26 cycles

The primers were selected from the literature. The sequences of *Selp* primers are from Yao *et al*. [13]. The sequences of *Vcam1* and *Icam1* primers are from Lindsey *et al*. [42]. The sequences of *Gapdh* primers are from Nacher *et al*. [43]. The sequences of *SELE* and *VCAM1* primers are from Ito *et al*. and Xu *et al*., respectively [44,45]. The sequences of *ICAM1* and *GAPDH* primers are from Zhang *et al*. [46]. The number of amplification cycles and annealing temperature for each set was determined experimentally.

quantitated using a Bio-Rad Protein Assay (Bio-Rad Laboratories). Equal amounts of total protein were mixed with Laemmli sample buffer (Bio-Rad Laboratories), boiled for 3 min at 100°C, and cooled on ice. Protein samples were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 7.5% acrylamide gels for 2 h at 100 V. The proteins were transferred to polyvinylidene difluoride (PVDF) membranes using a Trans-Blot Turbo™ Blotting System (Bio-Rad Laboratories). The membranes were washed three times with Tween/Tris-buffered salt solution (T-TBS) (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween20; Bio-Rad Laboratories) for 5 min each time, then blocked with T-TBS containing 5% skim milk (BD Biosciences, Lexington, KY, USA) for 1 h at room temperature.

Afterward blocking, the membranes were washed three times with T-TBS for 5 min each time, and incubated with one of two primary antibodies overnight at 4°C: E-selectin, *i.e.*, rat monoclonal anti mouse E-selectin/CD62E antibody (R&D Systems, Minneapolis, MN, USA) (1 : 2500) or P-selectin, *i.e.*, goat polyclonal anti mouse P-selectin/CD62P antibody (R&D Systems) (1 : 1000). After the overnight bath, the membranes were washed and incubated with secondary antibodies for 1 h at room temperature: E-selectin, *i.e.*, peroxidase-labeled goat antibody to rat IgG (H + L) (Kirkegaard & Perry Laboratories, Gaithersburg, MD, USA) (1 : 5000), or P-selectin, *i.e.*, peroxidase-labeled rabbit antibody to goat IgG (H + L) (Kirkegaard & Perry Laboratories) (1 : 5000). The membranes were washed three times with T-TBS for 5 min each time. Protein detection was performed using an Amersham ECL Prime Western Blotting Detection Kit (GE Healthcare Biosciences).

Blots were imaged using the ChemiDoc system, and proteins were quantitated using the Image Lab software. Blots were stripped using Restore™ PLUS Western Blot Stripping Buffer (Thermo Fisher Scientific, Waltham, MA, USA) for 15 min at 37°C and redetected with rabbit anti-actin antibody (Sigma Chemical) (1 : 1000), followed by peroxidase-labeled goat anti rabbit IgG (H + L) (Kirkegaard & Perry Laboratories) (1 : 10000) as an internal control. E- and P-selectin band intensities were normalized to actin band intensities.

Preparation of cytoplasm and nucleus, and NF-κB detection. The bEnd.3 cells were incubated in the absence or presence of 0.01% Lvn, 125 μM LA, or 250 μM LO for 2 h. Next, the cells were stimulated by murine TNF-α (250 U/mL) for up to 10, 30, or 60 min. Corresponding volumes of PBS were added to control cultures. After the TNF-α stimulation step, viable cell suspensions were separated into nuclear protein lysates and cytoplasmic lysates using NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific), according to the manufacturer's protocol. As a protease inhibitor, 0.5 mM of phenylmethylsulfonyl fluoride (PMSF) (Sigma Chemical) was added. Nuclear and cytoplasmic protein extracts were quantitated using the Bio-Rad Protein Assay.

To determine whether Lvn, LA, and LO affected the activation of NF-κB, we performed western blotting for phosphorylated-NF-κB p65 in the nucleus and NF-κB p65 in the cytoplasm. Constant amounts of nuclear or cytoplasmic protein extracts were electrophoresed using SDS-PAGE, and western blotting was performed. As the primary antibody, rabbit anti-NF-κB p65 antibody (Cell Signaling Technology, Danvers, MA, USA) (1 : 1000) was used in cytoplasmic blots and rabbit anti-phospho-NF-κB p65 antibody (Cell Signaling Technology) (1 : 1000) was used in nuclear blots. As the secondary antibody, peroxidase-labeled goat anti rabbit IgG (H + L) (Kirkegaard & Perry Laboratories) (1 : 5000) was used. Actin was used as an internal control in the cytoplasm extracts.

Statistical analysis. The statistical analysis was performed with GraphPad Prism ver. 6.01 (GraphPad Software, La Jolla, CA, USA). The data are expressed as the mean ± SEM of at least three independent experiments. We used an analysis of variance (ANOVA) with Tukey's *post-hoc* test for multiple comparisons to assess statistical significance. Probability values <0.05 were considered significant.

Results

Lvn inhibited the TNF-α-induced E-selectin, P-selectin, VCAM-1, and ICAM-1 expression in bEnd.3 cells. We used RT-PCR to measure the expression levels of *Sele*, *Selp*, *Vcam1*, and *Icam1* in bEnd.3 cells (Fig. 1A-D). The mRNA expression levels of the cell adhesion molecules showed significant increases after TNF-α stimulation (lane 2). In the 0.01% Lvn-treated cells (lane 5), the TNF-α-induced expression levels of *Sele*, *Vcam1*, and *Icam1* were significantly lower than those of the TNF-α-stimulated cells ($p<0.05$, $p<0.01$,

and $p < 0.05$, respectively). The TNF-α-induced *Selp* expression was not significantly inhibited. In the 0.005% Lvn-treated cells (lane 6), the TNF-α-induced expressions of *Sele*, *Selp*, *Vcam1*, and *Icam1* were not inhibited.

The expression levels of E- and P-selectin in bEnd.3 cells were measured by western blotting. As shown in Fig. 1E, F, the expression levels of E- and P-selectin were significantly elevated in the TNF-α-stimulated cells (lane 2). The TNF-α-induced E-selectin (lane 5, $p < 0.001$; lane 6, $p < 0.05$) and P-selectin (lanes 5 and 6, $p < 0.05$ for both comparisons) expression levels were significantly lower in the Lvn-treated cells compared to those in TNF-α-stimulated cells.

Lvn inhibited TNF-α-induced NF-κB activation in bEnd.3 cells. To determine whether Lvn inhibits NF-κB activation, we performed western blotting for phosphorylated-NF-κB p65 in the nuclear extract and NF-κB p65 in the cytoplasmic extract (Fig. 2). The phosphorylated-NF-κB p65 in the nucleus was significantly increased in TNF-α-stimulated cells at 10, 30, and 60 min (lane 2, $p < 0.05$; lane 3, $p < 0.001$; lane 4, $p < 0.05$). The highest activation of NF-κB p65 was observed at 30 min after TNF-α stimulation.

Lvn significantly suppressed phosphorylated-NF-κB p65 in the nucleus at 30 min (lane 6, $p < 0.05$) and 60 min (lane 7, $p < 0.05$) after TNF-α stimulation. No significant differences in cytoplasmic NF-κB p65 were observed in any lane.

LA and LO inhibited the TNF-α-induced mRNA expression of cell adhesion molecules in bEnd.3 cells. At 0.01%, Lvn inhibited the expression levels of TNF-α-induced cell adhesion molecules which contained approx. 136 μM of LA and 142 μM of LO. We con-

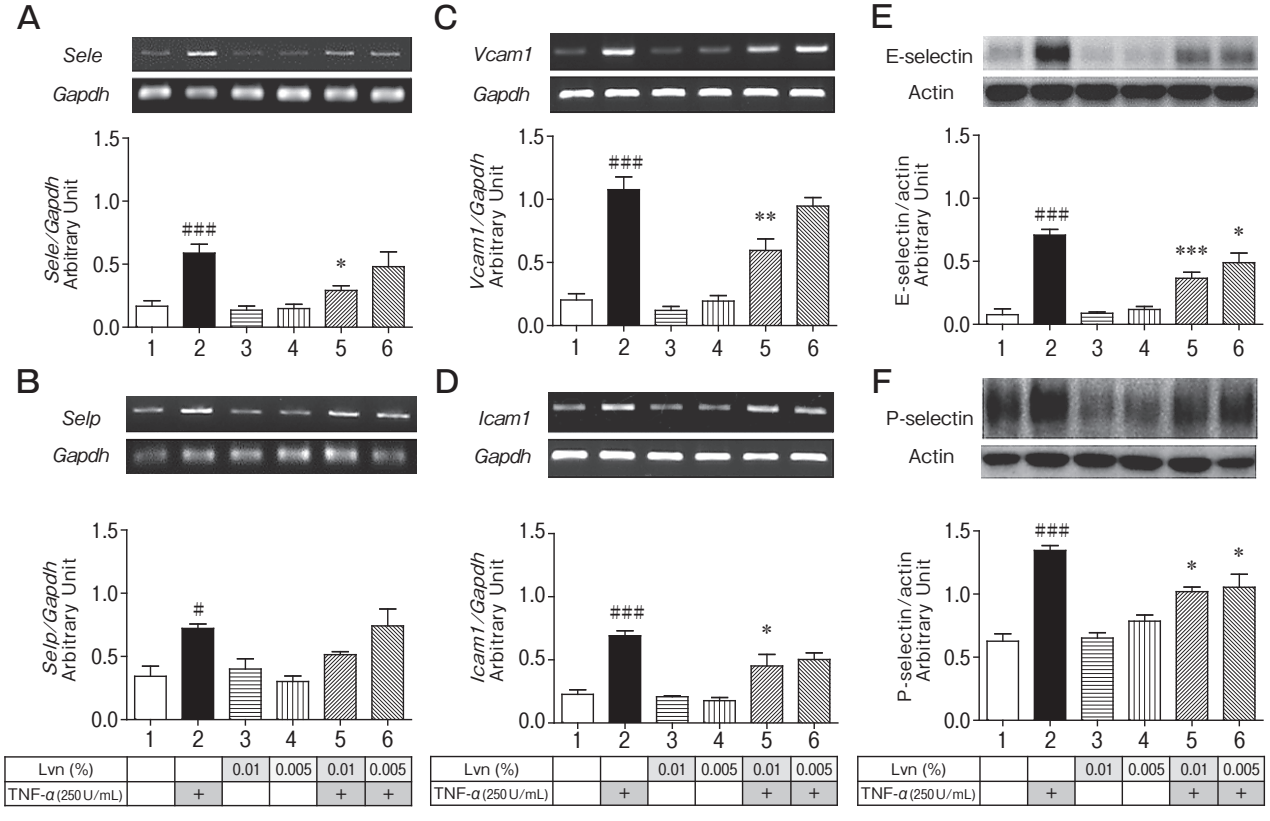


Fig. 1 Effects of Lvn on TNF-α-induced E-selectin, P-selectin, VCAM-1, and ICAM-1 expression in bEnd.3 cells. The expression levels of *Sele* (**A**), *Selp* (**B**), *Vcam1* (**C**), and *Icam1* (**D**) were quantified by RT-PCR. *Gapdh* was used as an internal control. The E-selectin (**E**) and P-selectin (**F**) were measured by western blotting. Actin was used as an internal control. The cells were pretreated with or without 0.01% or 0.005% Lvn for 2 h. The total RNA (A–D) and the cell lysates (E, F) were prepared from bEnd.3 cells incubated in the presence or absence of 250 U/mL murine TNF-α for 3 or 5 h. $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$ vs. non-stimulated cells; $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ vs. TNF-α-stimulated cells.

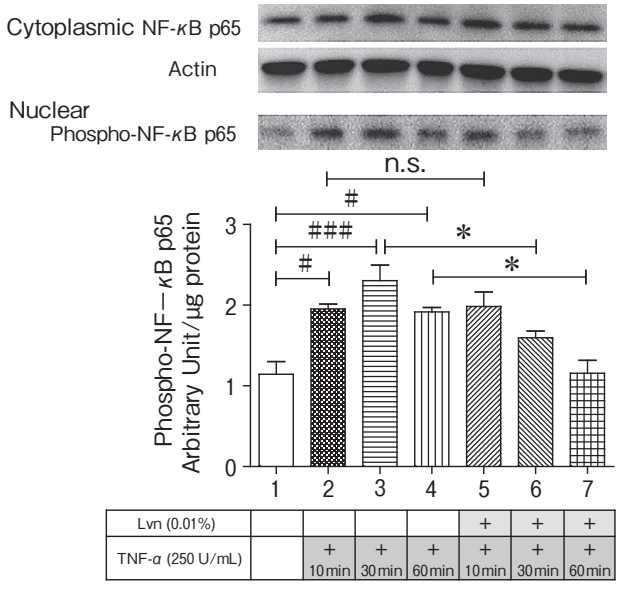


Fig. 2 Effects of Lvn on NF-κB activation in bEnd.3 cells stimulated with TNF-α. The nuclear phosphorylated-NF-κB p65 and the cytoplasmic NF-κB p65 were measured by western blotting. Actin was used as an internal control. The cells were stimulated by 250 U/mL murine TNF-α after treatment with 0.01% Lvn for 2 h. $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$ vs. non-stimulated cells; $^{*}p < 0.05$ vs. TNF-α-stimulated cells; n.s., not significant.

firmed that these concentrations of LA and LO did not exhibit cytotoxicity to bEnd.3 cells by MTT assay (data not shown). To determine the effects of LA on the TNF-α-induced *Sele*, *Selp*, *Vcam1*, and *Icam1* expression levels, we incubated bEnd.3 cells in the absence or presence of each of two LA concentrations (125 or 62.5 μM). As shown in Fig. 3A-D, the mRNA expression levels of the cell adhesion molecules were significantly increased by the TNF-α stimulation (lane 2). LA inhibited the TNF-α-induced expression levels of *Sele* ($p < 0.01$), *Selp* ($p < 0.01$), and *Vcam1* ($p < 0.05$) at 125 and 62.5 μM (lanes 5 and 6) relative to TNF-α-stimulated cells. LA also inhibited the TNF-α-induced *Icam1* expression at 125 μM (lane 5, $p < 0.01$) relative to the TNF-α-stimulated cells.

We also analyzed the effects of LO on the TNF-α-induced mRNA expression levels of cell adhesion molecules. The bEnd.3 cells were incubated in the absence or presence of each of two LO concentrations (250 or 125 μM). As shown in Fig. 3E-H, in the presence of 250 μM LO, the TNF-α-induced mRNA expression levels were inhibited for *Sele*, *Vcam1*, and *Icam1* (lane 5, $p < 0.05$, $p < 0.001$, and $p < 0.01$, respectively) relative to the TNF-α-stimulated cells (lane 2). In the presence of 125 μM LO, the TNF-α-induced mRNA expression levels were inhibited for *Sele* and *Vcam1* (lane 6, $p < 0.05$ and $p < 0.01$, respectively) relative to the TNF-α-stimulated cells. However, LO did not inhibit the TNF-α-induced *Selp* expression (Fig. 3F).

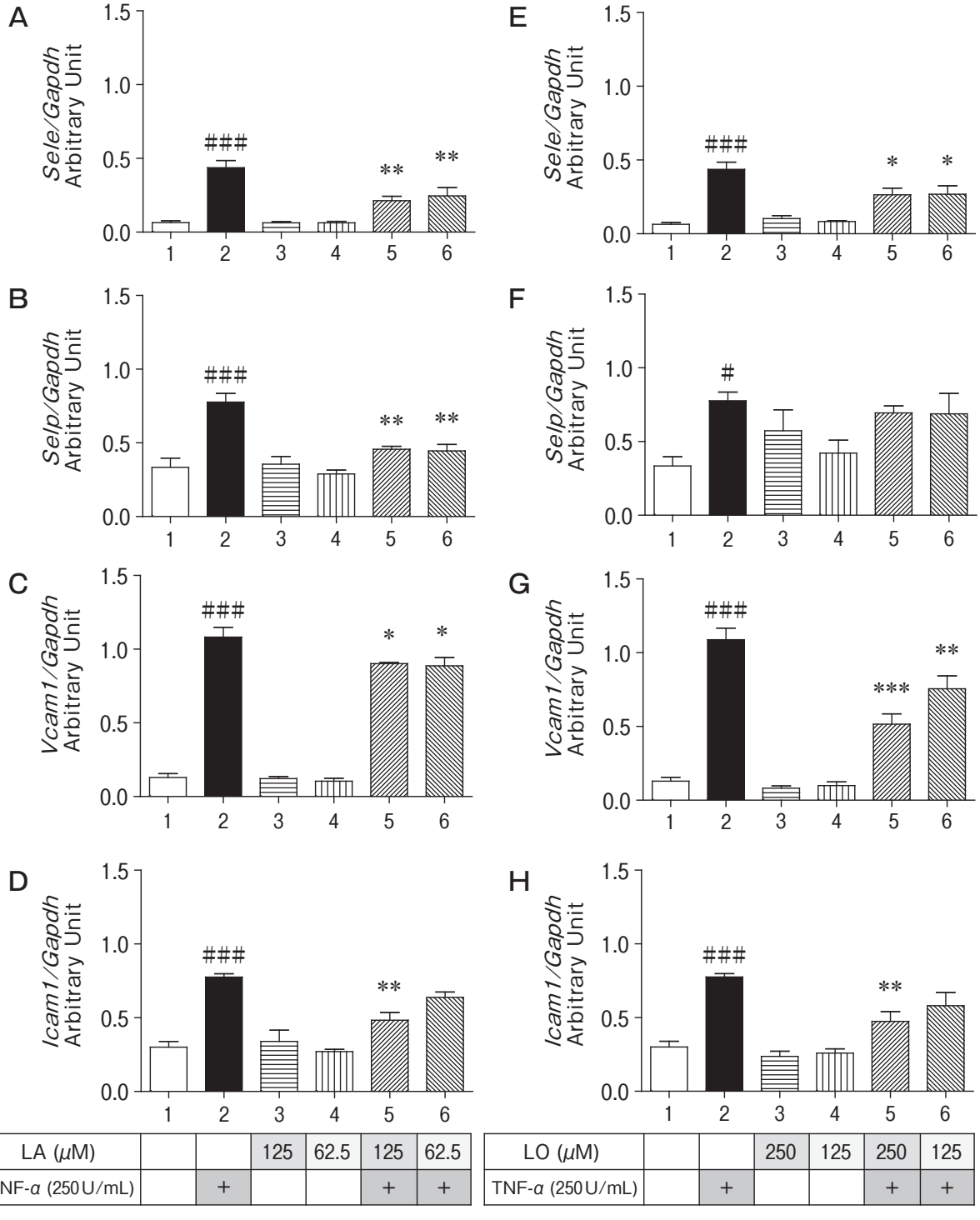
LA inhibited the TNF-α-induced NF-κB activation in bEnd.3 cells. To determine whether LA or LO inhibits NF-κB activation in bEnd.3 cells, we performed western blotting for phosphorylated-NF-κB p65 in the nucleus (Fig. 4). The phosphorylated-NF-κB p65 was significantly increased by TNF-α stimulation at 30 and 60 min (Fig. 4A, B, lane 2, $p < 0.01$, both; lane 3, $p < 0.001$, both) relative to the non-stimulated cells (lane 1). The highest activation of NF-κB p65 was observed at 60 min after TNF-α stimulation. In the LA-treated cells, the phosphorylated-NF-κB p65 in the nucleus was significantly suppressed at 60 min after TNF-α stimulation (Fig. 4A, lane 5, $p < 0.01$). LO did not significantly suppress the TNF-α-induced phosphorylated-NF-κB p65 in the nucleus (Fig. 4B). These results indicate that compared to LO, LA more potently inhibited the TNF-α-induced expression levels of cell adhesion molecules through a suppression of NF-κB activation.

Lvn inhibited the TNF-α-induced E-selectin mRNA expression in HUVECs. We also examined the effects of Lvn on the TNF-α-induced expression levels of *SELE*, *VCAM1*, and *ICAM1* in HUVECs (Fig. 5). The expression levels of *SELE*, *VCAM1*, and *ICAM1* were significantly increased by TNF-α stimulation (lane 2). At 0.01% and 0.005%, Lvn inhibited TNF-α-induced E-selectin mRNA expression (Fig. 5A, lane 5, $p < 0.001$; lane 6, $p < 0.01$) relative to the TNF-α-stimulated cells. However, Lvn did not inhibit the TNF-α-induced mRNA expression of *VCAM1* or *ICAM1* (Fig. 5B, C).

Discussion

Essential oils are used for various symptoms of not only illness but also mood and emotion. Here we verified the anti-inflammatory effects of Lvn and its main constituents, LA and LO, on cell adhesion molecules in endothelial cells.

The expression of cell adhesion molecules in endothelial cells plays a key role in recruiting leukocytes and initiating inflammation [18]. The curdlan-induced accumulation of neutrophils and myeloperoxidase



LA (μM)			125	62.5	125	62.5
TNF-α (250 U/mL)		+			+	+

LO (μM)			250	125	250	125
TNF-α (250 U/mL)		+			+	+

Fig. 3 Effects of LA and LO on TNF-α-induced E-selectin, P-selectin, VCAM-1, and ICAM-1 mRNA expression in bEnd.3 cells. The expression levels of *Sele* (**A**, **E**), *Selp* (**B**, **F**), *Vcam1* (**C**, **G**), and *Icam1* (**D**, **H**) were quantified by RT-PCR. *Gapdh* was used as an internal control. The cells were stimulated with 250 U/mL murine TNF-α after treatment with LA or LO for 2 h. $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$ vs. non-stimulated cells; $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ vs. TNF-α-stimulated cells.

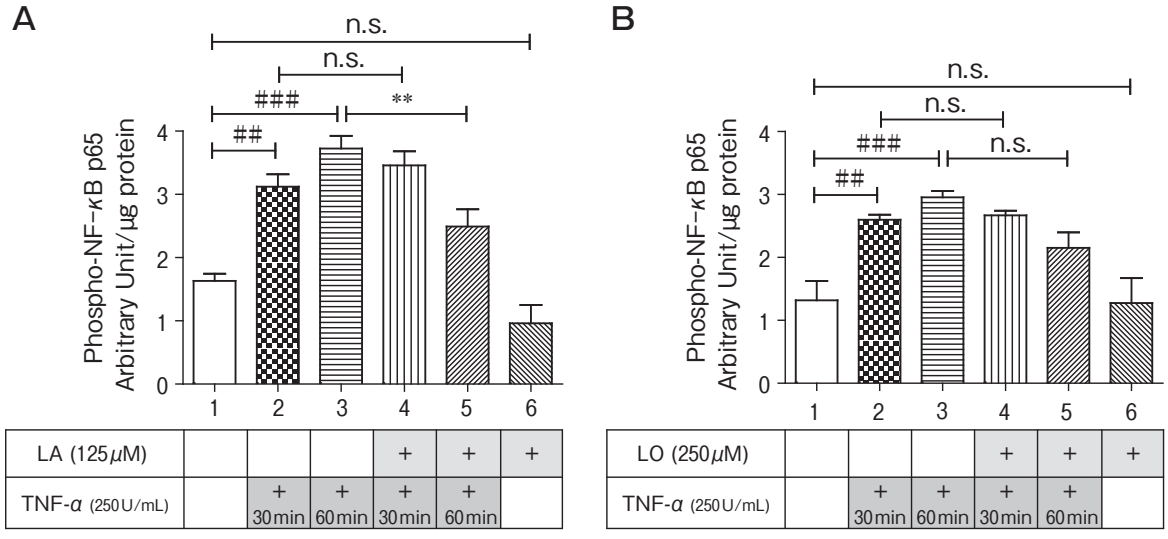


Fig. 4 Effects of LA and LO on NF-κB activation in bEnd.3 cells stimulated with TNF-α. The cells were treated with or without 125 μM LA (**A**) and with or without 250 μM LO (**B**), for 2 h, and stimulated with 250 U/mL murine TNF-α. The phosphorylated-NF-κB p65 in the nucleus was measured by western blotting. $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ vs. non-stimulated cells; $^{**}p < 0.01$ vs. TNF-α-stimulated cells.

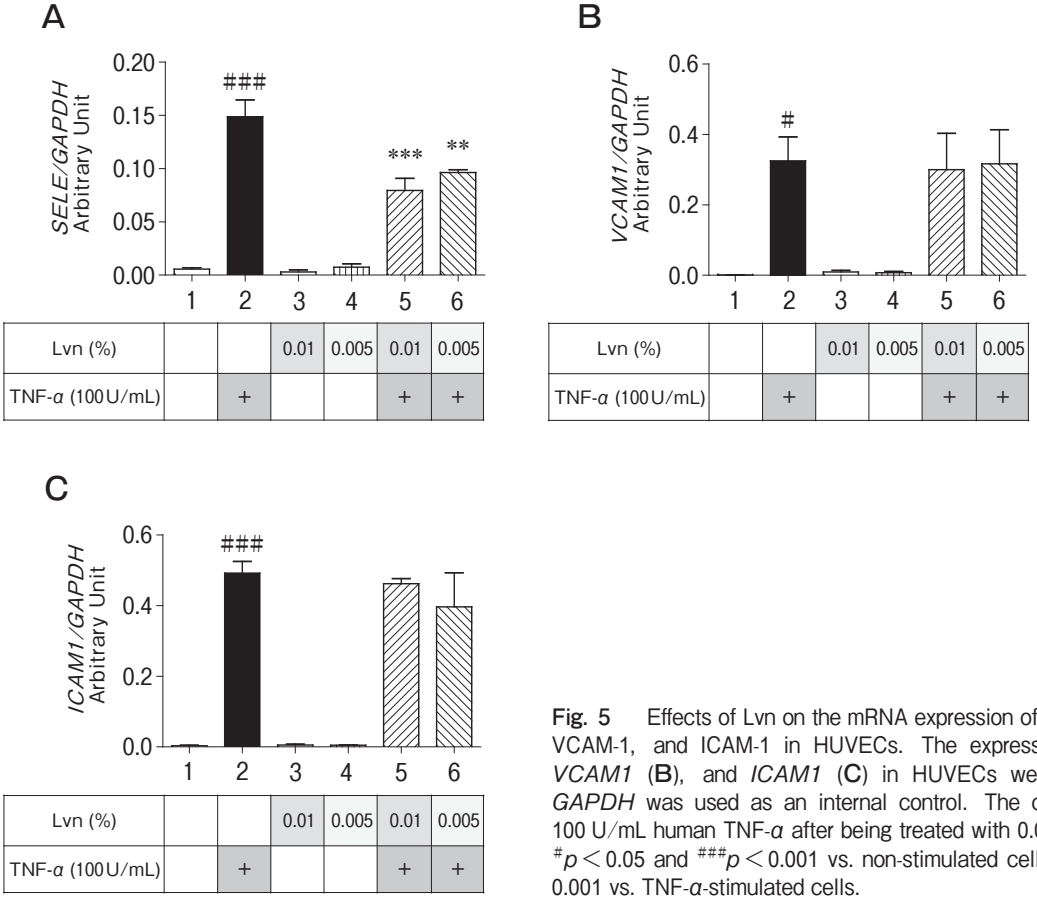


Fig. 5 Effects of Lvn on the mRNA expression of TNF-α-induced E-selectin, VCAM-1, and ICAM-1 in HUVECs. The expression levels of *SELE* (**A**), *VCAM1* (**B**), and *ICAM1* (**C**) in HUVECs were quantified by RT-PCR. *GAPDH* was used as an internal control. The cells were stimulated with 100 U/mL human TNF-α after being treated with 0.01% or 0.005% Lvn for 2 h. $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$ vs. non-stimulated cells; $^{**}p < 0.01$ and $^{***}p < 0.001$ vs. TNF-α-stimulated cells.

activity of skin lesions are suppressed dose-dependently by the cutaneous application of essential oils such as *Pelargonium asperum*, *Lavandula angustifolia* (Lvn), *Eucalyptus globulus*, and *Melaleuca alternifolia* [23]. TNF-α-induced cell adhesion molecules on vascular endothelial cells promote the transmigration of leukocytes into the tissues, thereby causing inflammation.

To the best of our knowledge, there are no previous reports that Lvn and LA inhibit TNF-α-induced cell adhesion molecules in endothelial cells. In bEnd.3 cells, we observed that Lvn inhibited the TNF-α-induced mRNA expression levels of E-selectin, VCAM-1, and ICAM-1, in a dose-dependent manner. Although Lvn did not inhibit the TNF-α-induced P-selectin mRNA expression, western blotting revealed that Lvn inhibited the TNF-α-induced E- and P-selectin expression levels. As E- and P-selectin are involved in the adhesion of granulocytes to vascular endothelial cells, Lvn may inhibit the adhesion of granulocytes to the vascular endothelium through the suppression of both selectins [24]. Moreover, adherent leukocytes induce the clustering of ICAM-1 or VCAM-1 on the surface of endothelial cells, and the dissociation of the vascular endothelial protein tyrosine phosphatase/VE-cadherin complex is implicated in endothelial cell-to-cell adhesion, leading to their enhanced transmigration into the underlying tissues [25, 26]. Thus, the inhibition of the expression of these cell adhesion molecules by treatment with Lvn or its constituents may have a significant impact on the alleviation of inflammation symptoms through the suppression of leukocyte adhesion on endothelial cells and transmigration across the endothelium.

The NF-κB family of transcription factors includes p50, p52, p65 (RelA), c-Rel, and RelB [27]. NF-κB p65 plays an important role in the expression of cell adhesion molecules in endothelial cells. NF-κB p50/p65 heterodimer binds to the NF-κB binding sites of E-selectin [28], VCAM-1 [29], and ICAM-1 genes [30]. In murine P-selectin gene, the p50/p65 heterodimer and p65 homodimer bind to the NF-κB response elements [31]. The activity of NF-κB is regulated by its interaction with the inhibitory protein IκBα in the cytoplasm [27]. The canonical NF-κB pathway is activated through proinflammatory receptors such as the TNF receptor, Toll-like receptor, and cytokine receptors for the interleukins [27, 32]. TNF-α or other cytokines activate this pathway, cause the phosphorylation and ubiquitination of IκBα, and allow the nuclear translocation of NF-κB p50/p65 heterodimer and subsequent DNA binding.

In the present study, Lvn inhibited TNF-α-induced E-selectin, P-selectin, VCAM-1, and ICAM-1 expression in bEnd.3 cells, and suppressed the TNF-α-induced phosphorylated-NF-κB p65 in the nucleus. These results show that the suppression of NF-κB activation by Lvn may be involved in the inhibition of TNF-α-induced cell adhesion molecules, such as E-selectin, P-selectin, VCAM-1, and ICAM-1 in bEnd.3 cells.

LA is the acetate ester of LO [33] and a main constituent of many essential oils from herbal plants such as lavender [9], bergamot [34], and clary sage [35]. The cytotoxicity of LA is higher than that of Lvn in human dermal microvascular endothelial cells and fibroblasts [36]. LA in combination with terpeniol and camphor was also reported to be effective against a human colon cancer cell line [37]. In our study, LA inhibited the TNF-α-induced mRNA expression of E-selectin, P-selectin, VCAM-1, and ICAM-1 in bEnd.3 cells (a murine brain endothelial cell line), and it suppressed phosphorylated-NF-κB p65 in the nucleus. LO inhibited the TNF-α-induced mRNA expression of E-selectin, VCAM-1, and ICAM-1 in bEnd. 3 cells, but it did not significantly suppress the TNF-α-induced P-selectin mRNA expression or phosphorylated-NF-κB p65 in the nucleus. These results suggest that Lvn has anti-inflammatory effects via the suppression of NF-κB activation because of its LA as a main constituent.

The inhibition of TNF-α-induced cell adhesion molecules by Lvn would lessen the attachment of leukocytes to inflammatory sites, potentially exerting anti-inflammatory effects. Our present findings thus suggest a part of the mechanism underlying the anti-inflammatory effects of Lvn, and further studies are necessary to reveal other effects of essential oils. Our model for the roles of LA in Lvn's anti-inflammatory effects might be relevant to the effects of other essential oils that include LA.

We also observed that in the HUVECs, Lvn inhibited the TNF-α-induced E-selectin mRNA expression, but it did not inhibit TNF-α-induced VCAM-1 or ICAM-1 mRNA expression. P-selectin mRNA expression was not increased by TNF-α stimulation in HUVECs (data not shown) because TNF-α does not have the ability to activate the human P-selectin gene

[13,38]. There are three NF-κB binding sites in the human E-selectin gene; these binding sites are necessary for the maximal E-selectin induction of TNF-α stimulation [28]. Two NF-κB binding sites, a single GATA binding site, and a single interferon regulatory factor-1 binding site are necessary for TNF-α-induced human VCAM-1 expression [29,39]. The TNF-α response elements consist of single NF-κB and CCAAT/enhancer binding protein binding sites in human ICAM-1 gene [30,40,41]. The different actions of Lvn on TNF-α-induced cell adhesion molecules in HUVECs may be caused by the number of NF-κB binding sites and the relations of other transcription factors.

Given this situation, the three NF-κB binding sites of human E-selectin gene might provide high sensitivity to Lvn's effects. However, in murine bEnd.3 cells, Lvn inhibited four types of TNF-α-induced cell adhesion molecules. The activation system of transcription factors may differ between mice and humans.

In conclusion, our data suggest that Lvn and LA inhibit the TNF-α-induced expression of cell adhesion molecules in vascular endothelial cells through a suppression of the activation of NF-κB. Therefore, essential oils containing LA as a main constituent might exert anti-inflammatory activity.

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