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Anti-inflammatory Effect of α-Linolenic Acid and Its Mode of Action through the Inhibition of Nitric Oxide Production and Inducible Nitric Oxide Synthase Gene Expression via NF-κB and Mitogen-Activated Protein Kinase Pathways

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α-Linolenic acid (ALA) isolated from *Actinidia polygama* fruits exhibits potent anti-inflammatory activity with an unknown mechanism. To elucidate the molecular mechanisms of ALA on pharmacological and biochemical actions in inflammation, we examined the effect of ALA on lipopolysaccharide (LPS)-induced nitric oxide (NO) production in the murine macrophages cell line, RAW 264.7. We found that ALA has a strong inhibitory effect on the production of NO. ALA also inhibited inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2), and tumor necrosis factor-α (TNF-α) gene expressions induced by LPS. To explore the mechanisms associated with the inhibition of iNOS gene expression by ALA, we investigated its effect on LPS-induced nuclear factor-κB (NF-κB) activation. Treatment with ALA reduced a translocation of NF-κB subunit and NF-κB-dependent transcriptional activity. The activation of NF-κB was inhibited by prevention of the degradation of inhibitory factor-κBα. We also found that ALA inhibited LPS-induced phosphorylation of mitogen-activated protein kinases (MAPKs). In addition, the antinociceptive effect of ALA was also assessed by means of the acetic acid-induced abdominal constriction test and Randall−Selitto assay. ALA (5 and 10 mg/kg) showed the potent antinociceptive effects in these animal models. Taken together, these results suggest that ALA downregulates inflammatory iNOS, COX-2, and TNF-α gene expressions through the blocking of NF-κB and MAPKs activations in LPS-stimulated RAW 264.7 cells, which may be the mechanistic basis for the anti-inflammatory effect of ALA.

KEYWORDS: ***Actinidia polygama*; α-linolenic acid; anti-inflammation; nitric oxide; iNOS; TNF-α; NF-κB; MAPKs**

INTRODUCTION

Nitric oxide (NO) is produced by inducible NO synthase (iNOS) in activated macrophages and is one of the most important inflammatory mediators (*1*). Physiologically, it induces various harmful responses including tissue injury, septic shock, and apoptosis (*2*), which has been attributed to the iNOS-mediated production of NO and the associated generation of potent reactive radicals such as peroxynitrite (*3*). Thus, the inhibition of NO production by suppressing iNOS expression is an important target in the treatment of inflammatory diseases. Expression of cyclooxygenase-2 (COX-2) in various tissue preparations following lipopolysaccharide (LPS) treatment has also been reported (*4*). This enzyme is considered to play a major role in the inflammatory process by catalyzing the production of prostaglandins (PGs). Therefore, there is an increasing interest in the applicability of COX-2 inhibitors.

The promoter region of the murine gene encoding iNOS contains two nuclear factor (NF)-κB binding sites, located 55 and 971 bp upstream of the TATA box (*5*). The binding of the potentially relevant transcription factor, nuclear factor-kB (NF-κB), to the κB sites has been shown to be functionally important for iNOS induction by LPS. The NF-κB family of transcription factors regulates various inflammatory cytokines, such as IL-1, IL-2, IL-6, IL-8, and tumor necrosis factor-α (TNF-α), as well as genes encoding COX-2 and iNOS. NF-κB is located in the cytoplasm of unstimulated cells in a quiescent form bound to its inhibitor, IκB (*6*). External stimuli such as LPS, a major component of the outer membranes of Gram-negative bacteria, can trigger the phosphorylation of IκBα, leading to its degradation and the simultaneous activation and translocation of NF-κB to the nucleus for binding to the promoter region of immune and inflammatory genes for transcriptional regulations. Moreover, three well-defined mitogen-activated protein kinases (MAPKs), extracellular signal-regulated kinase (ERK), p38 MAP kinase (p38), and c-Jun NH_2-terminal kinase (JNK), have been implicated in the transcriptional regulation of iNOS gene

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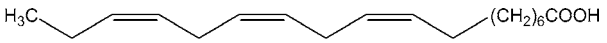

Figure 1. Chemical structure of ALA.

(*7*), and the specific MAPKs inhibitors suppress the expression of iNOS gene (*8*).

In recent decades, ω-3 has become of interest. Among them, α-linolenic acid (ALA) has been suggested to have many pharmacological effects, such as the treatments of arthritis, asthma, eating disorders, and inflammatory bowel disease (IBD), and especially of cardiovascular diseases such as heart attack, hypercholesterolemia, and hypertension (*9*), and possesses a major neuroprotective effect (*10*). Singh and Majumdar reported that mixed fatty acid or the plant extract containing ALA significantly decreased the edema induced by carrageenan, in which the peak edema is characterized by the presence of PGs (*11*).

The recent emphasis on the role of NO in pathological conditions has led to the discovery of new therapeutic agents. In our ongoing study, bioassay-guided fractionation of *Actinidia polygama* (AP) fruits led to separation and identification of a polyunsaturated fatty acid (PUFA), ALA (**Figure 1**), which turned out to show a potent anti-inflammatory activity (*12*). However, the mechanism of action is yet unclear. The following study was designed to evaluate the effects of ALA on LPS-induced NO production and iNOS, COX-2, and TNF-α gene expressions and to clarify its mode of action by which ALA exerts an anti-inflammatory effect in RAW 264.7 cells, a murine macrophage cell line. Moreover, the in vivo antinociceptive effect of ALA is also investigated.

MATERIALS AND METHODS

Reagents. Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), and antibiotics were obtained from Gibco BRL (Grand Island, NY). Rabbit polyclonal anti-iNOS, rabbit polyclonal anti-NF-κB p65, rabbit polyclonal anti-IκBα, mouse monoclonal anti-p-IκBα, mouse monoclonal anti-p-p38, p-JNK, p-ERK and JNK, rabbit polyclonal anti-p38 and ERK, goat polyclonal anti-β-actin, peroxidase-conjugated secondary antibodies, and donkey anti-rabbit IgG FITC were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Enhanced chemiluminescence (ECL) and [γ-^{32}p]dATP and poly(dI-dC) were purchased from Amersham Life Science (Arlington Heights, IL) and Roche Diagnostics Korea Co., Ltd. (Seoul, Korea), respectively. An Easy Blue kit was obtained from Intron Biotechnology (Kyungki-Do, Korea). iNOS, COX-2, COX-1, TNF-α, CPN oligonucleotide primers, and NF-κB oligonucleotide were purchased from Bioneer (Daejeon, Korea). 3-(4,5-Dimethlythiazol-2-yl)-2,5-diphenyl-tertazolium bromide (MTT), Griess reagent, dithiothreitol (DTT), DMSO, SDS, Tween-20, *Escherichia coli* LPS, indomethacin, phenylmethanesulfonyl fluoride (PMSF), EDTA, Nonidet P40 (NP-40), paraformaldehyde, Triton X-100, Hoechst 33258, and all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO).

Plant Material. The AP fruits were obtained from Korea Herbal Science Institute (Seoul, Korea), and authenticated with ethnopharmacologist, J.G. Choi. A voucher specimen of AP fruits was deposited at the Medicinal Plants Herbarium of the College of Pharmacy, Kyung Hee University, with the registration number 151.

Isolation and Identification of ALA. ALA was isolated from AP fruits by activity-directed fractionation and was identified by the comparison of its spectra (^{1}H and ^{13}C NMR, 200 MHz, $CDCl_3$) with the reported spectra (The Aldrich Library of ^{13}C and ^{1}H FTNMR Spectra), as described in our previous study (*12*).

Animals. Male Sprague−Dawley rats (150−200 g) and ICR mice (18−22 g) were purchased from Orient (Kyunggi-do, Korea). All animals were acclimatized to the laboratory environment for 1 week before the experiment. They were housed under standard conditions (25 ± 1 °C, 50 ± 5% humidity, and a 12 h light−dark cycle) and maintained with free access to water and a regular diet. Animal experiments were approved by the Kyung Hee University Animal Care and Use Committee, and all procedures were conducted in accordance with the "Guide for the Care and Use of Laboratory Animals" published by the Korea National Institute of Health.

Acetic Acid-Induced Writhing in Mice. This experiment was carried out in male ICR mice using nine animals in each group according to the method of Witkin (*13*). They were divided into four groups, and ALA and indomethacin (suspended in 0.5% sodium carboxymethyl cellulose) were administered orally 1 h before acetic acid injection. The writhing reaction was induced by an intraperitoneal injection of 0.7% acetic acid (10 mL/kg) into the mice. Five minutes later, the number of writhing was counted for 20 min. The percent inhibition of writhing count of treated group was calculated from the mean writhing count of the control group.

Randall−Selitto Assay in Rats. The analgesic activity of ALA was tested using the Randall−Selitto assay (*14*). In this experiment, force was applied to the right hind paw of experimental animals using an analgesy meter (LE5002, Barcelona, Spain), which exerts a constantly increasing force on the rat paws. The rat was suspended vertically while its right hind paw was placed between the plinth and the finger. As the applied force increases, it gets to a point where the animal struggles to free its paw. This is the level at which the animals feels pain. The weight causing pain before treatment, 30, 60, and 120 min after treatment with ALA, was determined. The antinociceptive effect was calculated according to the following formula:

$$\%\ \text{antinociception} = 100 \times (\text{test pressure threshold}/\text{predrug pressure threshold}) - 100$$

Cell Culture. The murine macrophage cell line, RAW 264.7 cells, was obtained from the American Type Culture Collection (Manassas, VA). Cells were cultured in DMEM supplemented with 2 mM glutamine, antibiotics (100 U/mL of penicillin A and 100 U/mL of streptomycin), and 10% heat-inactivated FBS and maintained in a 37 °C humidified incubator containing 5% CO_2. Cells were incubated with 200 ng/mL of LPS with or without various concentrations of ALA for different indicated times. ALA was dissolved in DMSO at the concentration of 80 mg/mL and kept as a stock solution at −20 °C.

Nitrite Assay. Nitrite accumulation, an indicator of NO synthesis, was measured in the culture medium by Griess reaction (*15*). Briefly, 100 μL of cell culture medium was mixed with 100 μL of Griess reagent [equal volumes of 1% (w/v) sulfanilamide in 5% (v/v) phosphoric acid and 0.1% (w/v) naphtylethylenediamine-HCl] and incubated at room temperature for 10 min. The absorbance at 550 nm was then measured using a microplate reader. Fresh culture medium was used as a blank in all experiments. The amount of nitrite in the test samples was calculated from a sodium nitrite standard curve.

Western Blot Analysis. Protein extracts were prepared in lysis buffer (50 mM Tris, pH 7.4, 1% NP-40, 150 mM NaCl, 1 mM EDTA, 1 mM PMSF, 1 μg/mL each of aprotinin, leupeptin, and pepstatin). Lysates (50 μg) were electroblotted onto a nitrocellulose membrane following separation on a 10 or 15% SDS-polyacrylamide gel electrophoresis. The immunoblot was incubated for 1 h with blocking solution (Tris-buffered saline/Tween 20, TBST) containing 5% skim milk (w/v) at room temperature, followed by incubation overnight at 4 °C with a 1:1000 dilution of primary antibodies against iNOS, NF-κB p65, IκBα, p-IκBα, ERK1, p-ERK, JNK2, p-JNK, p38, p-p38, or β-actin antibodies. The membrane was washed four times with TBST and was incubated for 1 h with blocking solution at room temperature and then incubated with a 1:2000 dilution of horseradish peroxidase-conjugated goat anti-rabbit, donkey anti-mouse, or donkey anti-goat IgG secondary antibody for 1 h at room temperature. The membrane was washed four times in TBST and then developed by ECL.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Total mRNA was isolated using an Easy Blue kit according to the manufacturer's instructions. From each sample, 1 μg of RNA was reverse-transcribed using 200 units/μL M-MLV reverse transcriptase, 10 mM dNTP, and 0.5 μg/μL of oligo dT_{15}. PCR analysis was then performed on the aliquots of the cDNA preparations to detect iNOS, COX-2, COX-1, TNF-α, or cyclophilin (CPN) gene expression using a thermal cycler (PTC-200, MJ Research Inc., United States). The

reactions were carried out in a volume of 25 μL containing 1 unit of Taq DNA polymerase, 0.2 mM dNTP, 10 × reaction buffer, and 0.2 μmol of 5′- and 3′-primers. After initial denaturation for 5 min at 95 °C, 30 amplification cycles were performed [1 min of denaturation at 95 °C, 1 min of annealing at 56 (for iNOS, COX-2, and CPN) or 53 °C (for COX-1 and TNF-α), and 2 min of extension at 72 °C]. The PCR primers used in this study were as follows: iNOS sense 5′-ATGGCTTGCCCCTGGAAGT-3′, anti-sense GTACTTGGGATGCTCCATGGTCA; COX-2 sense 5′-ATGCTCTTCCGAGCTGTGCT-3′, anti-sense 5′-TTACAGCTCAGTTGAACGCCTTTT-3′; COX-1 sense 5′-ATGAGTCGAAGGAGTCTCTC-3′, anti-sense 5′-TCAGAGCTCAGTGGAGCGTC-3′; TNF-α sense 5′-ATGAGCACAGAAAGCATGATC-3′, anti-sense 5′-TACAGGCTTGTCACTCGAATT-3′; and CPN sense 5′-ATGGTCAACCCCACCGTG-3′, anti-sense 5′-TTAGAGTTGTCCACAGTCGGAGA-3′. After the amplification, PCR products were electrophoresed on 1% agarose gel and visualized by ethidium bromide staining and UV irradiation. Scanning densitiometry was performed with a model GS-700 Imaging Densitometer (Bio-Rad Laboratories, CA).

Electrophoretic Mobility Shift Assay (EMSA). Nuclear extracts were prepared according to a modified procedure of Lo et al. (*16*). Briefly, cells were scraped, washed with PBS (pH 7.4), resuspended in hypotonic buffer containing 10 mM Hepes (pH 7.9), 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF, 1.5 mM $MgCl_2$, and 1.2% NP-40, and allowed to swell on ice for 10 min. Lysates were separated by centrifuging at 3300*g* for 5 min at 4 °C, and the supernatant was used as a cytosolic extract. The pellets were extracted with nuclear extraction buffer containing 20 mM Hepes (pH 7.9), 0.4 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 0.5 mM PMSF, and 0.5 mM DTT for 30 min on ice and centrifuged at 12000*g* for 30 min. The supernatant was used as a nuclear extract and stored at −70 °C prior to use. Nuclear extract (10 μg) was mixed with the double-stranded NF-κB oligonucleotide 5′-AGTTGAGGGGACTTTCCCAGGC-3′ end-labeled by [γ-^{32}P]-dATP (underline indicates a κB consensus sequence or a binding site for NF-κB/cRel homodimeric and heterodimeric complex). The binding reactions were performed at 37 °C for 30 min in 30 mL of reaction buffer containing 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 4% glycerol, 1 mg of poly(dI-dC), and 1 mM DTT. The specificity of binding was examined by competition with the 80-fold unlabeled oligonucleotide. DNA/nuclear protein complexes were separated from the unbound DNA probe on native 5% polyacrylamide gels at 100 V in 0.5 × TBE buffer. The gels were vacuum-dried for 1 h at 80 °C and exposed to X-ray film at −70 °C for 24 h.

Transient Transfection and Luciferase Assay. The luciferase assay was performed as described by George et al. (*17*) with some modifications. When the cells reached confluence, the medium was replaced with serum-free DMEM. The cells were then transfected with the pNF-κB-luc plasmid reporter gene using GenePORTER (Genlantis, CA). After another 4 h of incubation, the medium was replaced with complete medium. After 20 h of incubation, the cells were pretreated with various concentrations of ALA for 1 h and further incubated with 200 ng/mL of LPS for 18 h. Cells were then washed twice with cold PBS and harvested in 90 μL of lysis buffer [1% Triton X-100, 2 mM EGTA (pH 8.0), 25 mM GLY-GLY, and 15 mM $MgSO_4$ (pH 7.5)]. Luciferase assay was conducted using a luciferase assay system (Promega BioSciences, CA) with 50 μL of cell lysate in each assay on a TD-20/20 Luminometer (Turner BioSystems, CA). Luciferase activities were determined and normalized to the same protein concentration.

Immunofluorescence Assay. A standard immunocytochemical method was used to detect nuclear translocation of p65 subunit of NF-κB (*18*). RAW 264.7 cells were cultured directly on glass coverslips in 24 well plates. After stimulation with 200 ng/mL LPS for 1 h, cells were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature and permeabilized with 0.2% Triton X-100 in PBS for 10 min. To investigate the cellular localization of NF-κB, cells were treated with a polyclonal antibody (1:100) against NF-κB p65 for 1.5 h. After extensive washing with PBS, cells were further incubated with a secondary FITC-conjugated donkey anti-rabbit IgG antibody diluted at 1:100 in PBS for 1 h at room temperature. Nuclei were stained with 5 μg/mL of Hoechst 33258, and after extensive washing, cells were examined by fluorescence microscopy (Olympus IX51, Dulles, VA).

Table 1. Analgesic Effect of ALA on Acetic Acid-Induced Writhing Test in Mice[a]

treatment	dose (mg/kg)	writhing times	inhibition (%)
control		35 ± 15	
ALA	5	18 ± 10	48.5**
	10	16 ± 8	54.5**
indomethacin	10	15 ± 8	57.6**

[a] Values represent means ± SD ($n = 9$). **$P < 0.01$ significantly different from the control.

Table 2. Effect of ALA on Paw Pressure in Rats[a]

		pain threshold (g)			
treatment	dose (mg/kg)	0 min	30 min	60 min	120 min
control		131.5 ± 27.8	136.1 ± 29.4	93.2 ± 22.4	105.1 ± 22.1
ALA	5	145.0 ± 32.8	134.2 ± 25.0	132.8 ± 26.1*	126.6 ± 17.7
	10	138.1 ± 29.7	148.8 ± 27.8	159.9 ± 42.5**	112.2 ± 21.6
indomethacin	10	135.8 ± 37.2	148.4 ± 39.3	142.1 ± 43.8**	134.9 ± 28.2

[a] Values represent means ± SD ($n = 6$). *$P < 0.05$ and **$P < 0.01$ significantly different from the control.

Statistical Analysis. All data are expressed as means ± standard deviations (SD) from three independent experiments. The data were analyzed using Student's *t* test. Mean values were considered significantly different when $P < 0.05$.

RESULTS

In Vivo Antinociceptive Activity. The antinociceptive effect of ALA was evaluated using two different models, acetic acid-induced writhing in mice and the Randall−Selitto assay in rats. ALA was found to reduce the number of abdominal constrictions that had been induced by a 0.7% acetic acid solution. The level of protection afforded by ALA at 5 and 10 mg/kg was 48.5 and 54.5%, respectively (**Table 1**). Indomethacin (10 mg/kg) was used as a positive control in this test with an effect (57.6%) comparable to that of ALA (10 mg/mL). In the Randall−Selitto assay, the peak antinociceptive response by ALA was reached at 1 h after oral administration. ALA at 5 and 10 mg/kg caused a 21.1 and 44.9% increase in the pain threshold at 1 h, respectively. The indomethacin (10 mg/kg) treatment increased the pain threshold by 33.7% as compared with the control group (**Table 2**).

Inhibitory Effect on iNOS Protein and mRNA Expressions. The effect of ALA on NO production was determined by measuring the level of nitrite accumulation (the stable metabolite of NO) in culture media. LPS (200 ng/mL) induced significant nitrite production as compared with the naive control. This was inhibited by an ALA treatment in a dose-dependent manner with an IC_{50} value of 8.0 μg/mL (**Figure 2A**). An examination of the cytotoxicity of ALA in RAW 264.7 macrophages by the MTT assay indicated that even 80 μg/mL ALA did not affect the viability of the RAW 264.7 cells (data not shown). Therefore, the inhibition of LPS-induced nitrite production by ALA was not the result of a cytotoxic effect on these cells. In view of the involvement of iNOS in the inflammatory process, the levels of iNOS protein and mRNA gene expression in the macrophages exposed to ALA were monitored. As shown in **Figure 2B**, the expression of the iNOS protein was barely detected in the nonstimulated cells. However, the level increased markedly 24 h after the LPS treatment. ALA exerted a concentration-dependent inhibition of iNOS protein

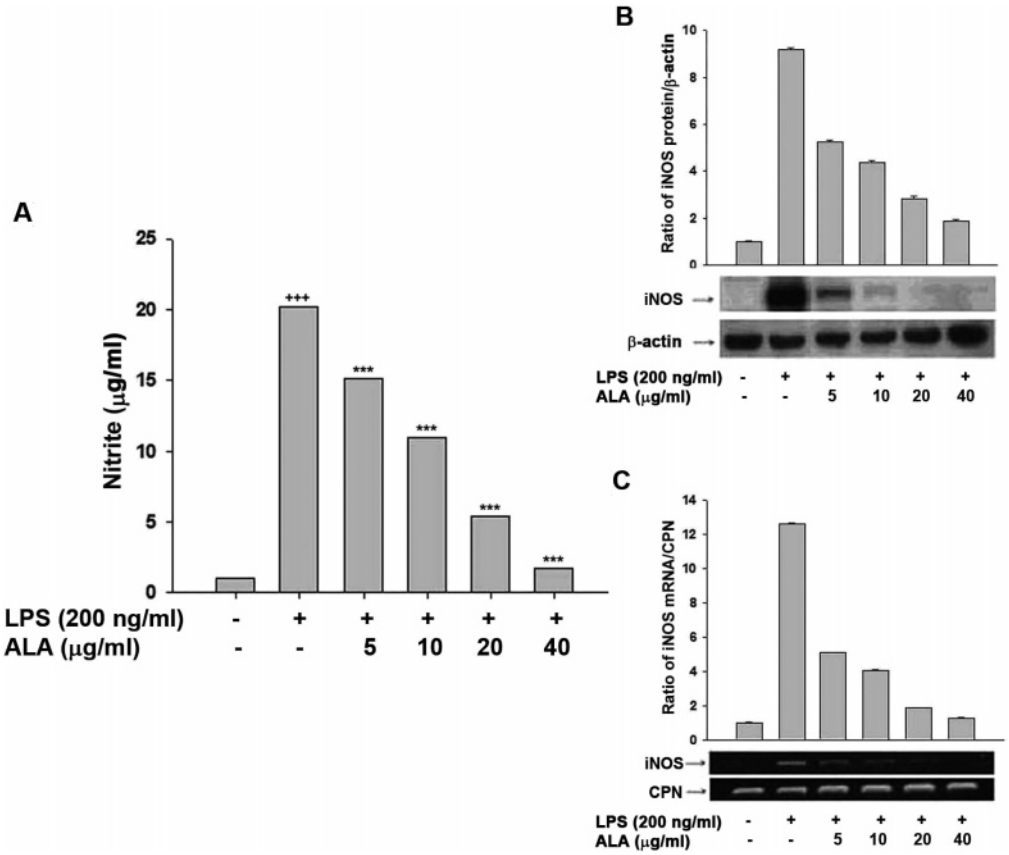

Figure 2. Effects of ALA on iNOS protein and gene expressions in LPS-stimulated RAW 264.7 cells. RAW 264.7 macrophages were pretreated with various concentrations of ALA 1 h prior to LPS (200 ng/mL) treatment. Control cells were incubated with vehicle alone. (**A**) Level of nitrite. The concentrations of nitrite in the medium of RAW 264.7 macrophages cultured with LPS (200 ng/mL) for 24 h were monitored by Griess reaction. (**B**) iNOS protein expression. iNOS protein expression was monitored 24 h after treatment of cells with LPS (200 ng/mL). The relative iNOS protein levels were measured by scanning densitometry of the band. (**C**) iNOS mRNA expression. iNOS mRNA expression was assessed by RT-PCR in RAW 264.7 macrophages exposed to LPS for 4 h. The amount of RNA loaded in each lane was confirmed by CPN mRNA. Data shown are the means ± SD from three independent experiments. $^{+++}p < 0.001$ vs control and $^{***}p < 0.001$ vs LPS-treated group.

expression in the LPS-stimulated RAW 264.7 macrophages. To assess the effect of ALA on iNOS mRNA expression, the mRNA level was measured by RT-PCR analysis. iNOS mRNA expression was also hardly detectable in nonstimulated cells. On the other hand, the RAW 264.7 macrophages expressed high levels of iNOS mRNA when stimulated with LPS for a 4 h period. Consistent with protein expression, ALA inhibited the LPS-stimulated expression of iNOS mRNA in a dose-dependent manner (**Figure 2C**).

Effect on COX-2 and COX-1 mRNA Expressions. COX-2 is induced by a wide variety of stimuli such as LPS, certain serum factors, cytokines, and growth factors and is expressed predominantly at the sites of inflammation (*19*). COX-1 is known as a constitutive isoform and is expressed typically at constant levels throughout the cell cycle in many tissues. Therefore, there is increasing interest in the use of COX-2 inhibitors for treating inflammatory diseases. We further evaluated the effects of ALA on the LPS-induced expression of the COX-2 and COX-1 mRNA genes in macrophages. The expression of COX-2 and COX-1 mRNA was monitored in RAW 264.7 macrophages exposed to LPS for 4 h. ALA effectively suppressed the induction of COX-2 mRNA by LPS in a dose-dependent manner. However, the expression of COX-1 mRNA was unaffected (**Figure 3**). Therefore, ALA selectively inhibits COX-2 and decreases the COX-2/COX-1 mRNA expression ratio in a dose-dependent manner.

Inhibitory Effect on TNF-α mRNA Expression. TNF-α is the principal mediator of the response to LPS and may play a role in the innate immune response (*20*). Therefore, the effect of ALA on TNF-α gene expression was examined. The level of TNF-α mRNA expression was monitored in RAW 264.7 macrophages exposed to LPS (200 ng/mL) in the presence or absence of ALA for 3 h. The ALA treatment inhibited TNF-α mRNA expression in a dose-dependent manner (**Figure 4**).

Effect on NF-κB Activation. NF-κB is activated in cells challenged with LPS and other inflammatory stimuli, and it is involved in the transcriptional activation of the responsive genes (*21*). To elucidate the mechanism, we next determined whether ALA could inhibit the DNA binding activity of NF-κB in LPS-stimulated macrophages RAW 264.7. Treatment of LPS caused a significant increase in the DNA binding activity of NF-κB. In the presence of ALA, however, LPS-induced NF-κB-DNA binding was markedly suppressed in a dose-dependent manner (**Figure 5A**). In addition, transient transfection with the NF-κB-dependent luciferase reporter plasmid was carried out to confirm whether ALA inhibits the NF-κB transcriptional activity in LPS-induced macrophages. Treatment of LPS to the transfected RAW 264.7 cells increased a luciferase activity to about five-fold, as compared with the resting cells, indicating that cellular NF-κB is transcriptionally functional (**Figure 5B**). ALA also inhibits the LPS-induced NF-κB transcriptional activity in a dose-dependent manner.

Because p65 is the major component of the NF-κB activated by LPS in macrophages, the translocation of p65 into the nucleus was determined using immunocytochemistry. The RAW 264.7 cells were incubated with LPS in the presence or absence of 40

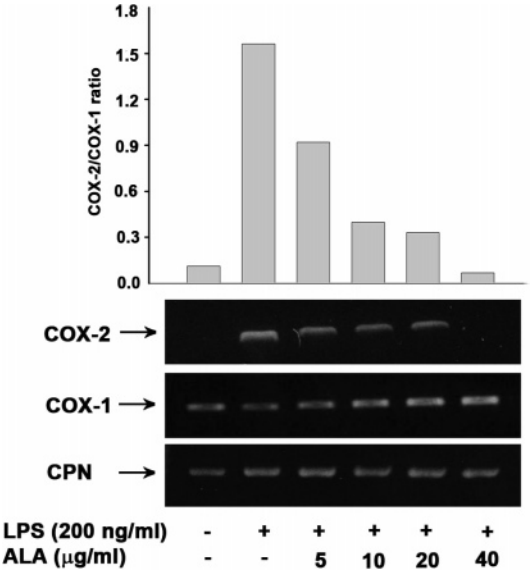

Figure 3. Effects of ALA on COX-2 and COX-1 gene expressions in LPS-stimulated RAW 264.7 cells. COX-2 and COX-1 mRNA expressions were assessed by RT-PCR in RAW 264.7 macrophages exposed to LPS (200 ng/mL) with or without various concentrations of ALA for 4 h. Control cells were incubated with vehicle alone. The COX-2 and COX-1 levels were measured by scanning densitometry of the band intensities.

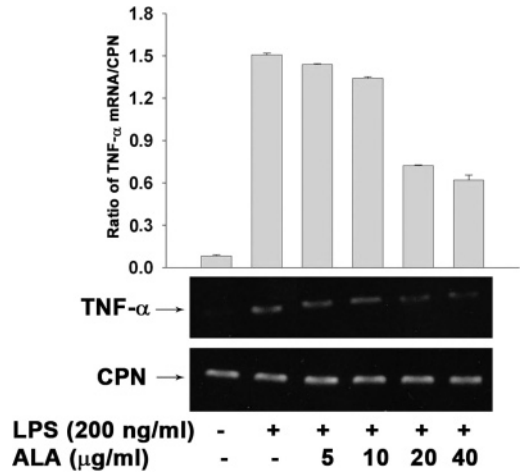

Figure 4. Effect of ALA on TNF-α gene expression in LPS-stimulated RAW 264.7 cells. TNF-α mRNA expression was assessed by RT-PCR in cells cultured with LPS (200 ng/mL) in the presence or absence of ALA for 3 h. The relative TNF-α expression levels were measured by scanning densitometry of the band intensities.

μg/mL ALA for 1 h. The cells were then fixed and permeabilized. The p65 protein was located mainly in the cytoplasm of the control cells. In contrast, the p65 protein moved into the nucleus at 1 h after the LPS treatment. When the cells were treated with 40 μg/mL of ALA for 1 h, the p65 protein was detected mainly in the cytoplasm of the cells exposed to LPS, suggesting that ALA inhibits the nuclear localization of the p65 protein. The nuclear integrity was also confirmed by Hoechst staining of identical cells (**Figure 6**). The level of p65 in the nuclear extract was also determined by Western blot analysis. The RAW 264.7 cells were incubated with LPS in the presence or absence of ALA for 1 h. ALA inhibited both LPS-induced nuclear translocation of p65 (**Figure 7A**) and the NF-κB transcriptional activity (**Figure 5B**) in a dose-dependent manner similar to that observed with NF-κB-DNA binding (**Figure 5A**).

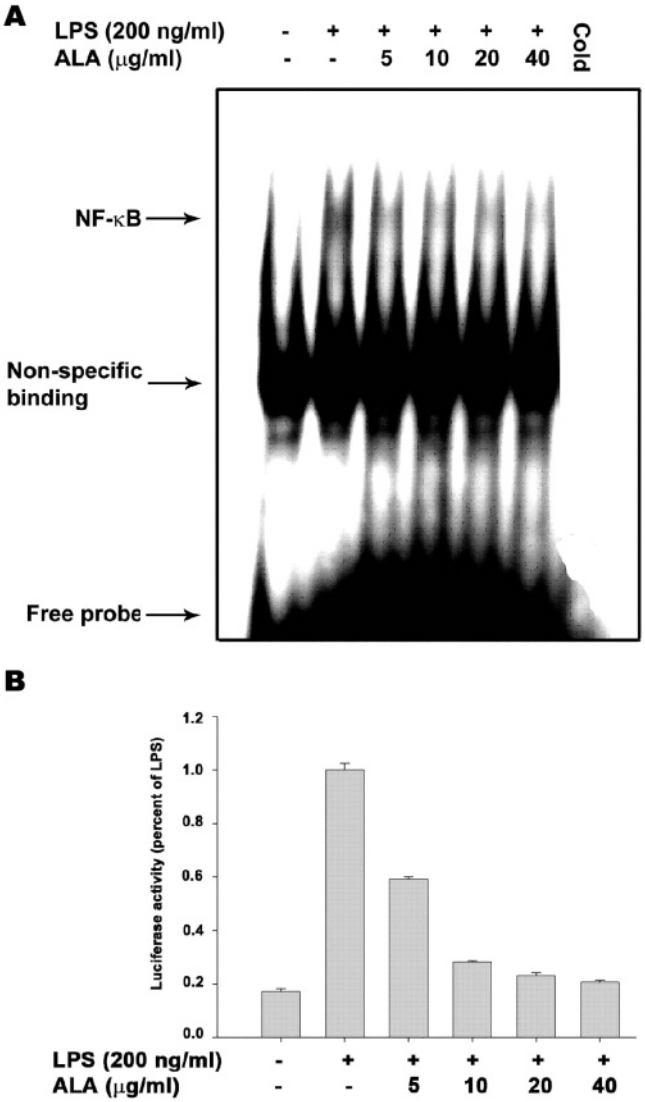

Figure 5. Effect of ALA on LPS-induced DNA binding and transcriptional activity of NF-κB complex in RAW 264.7 cells. (**A**) RAW 264.7 macrophages were pretreated with ALA for 1 h and stimulated with LPS (200 ng/mL) for another 1 h. Nuclear extracts of the cells were prepared, and 5 μg of nuclear extracts for each lane was subjected to gel shift analysis using the consensus sequence of NF-κB. The NF-κB complexes, p65/p50 and p50/p50, are indicated with arrows. (**B**) The cells were transfected with 1 μg of pNF-κB-Luc reporter plasmid. After transfection, cells were pretreated with various concentrations of ALA followed by LPS (200 ng/mL) treatment for another 18 h. Data were expressed as the means ± SD of the percentage of maximal luciferase activity observed with LPS only, as determined in three independent experiments.

Effect on I-κBα Phosphorylation and Degradation. The nuclear translocation and DNA binding of the NF-κB transcription factor are preceded by the degradation of inhibitory factor-κBα (I-κBα) (*22*). The cytoplasmic levels of I-κBα were examined by a Western blot analysis at the indicated times to determine whether inhibition of NF-κB-DNA binding by ALA is related to I-κBα degradation. I-κBα protein expression was barely detected at 15 min after the LPS treatment. However, ALA significantly recovered the level of I-κBα protein (**Figure 7B**). Because NF-κB is activated by I-κBα degradation after the phosphorylation of I-κBα at the serine residues (*23*), ALA also inhibited the LPS-induced phosphorylation of I-κBα (**Figure 7B**). This suggests that ALA inhibits nuclear NF-κB

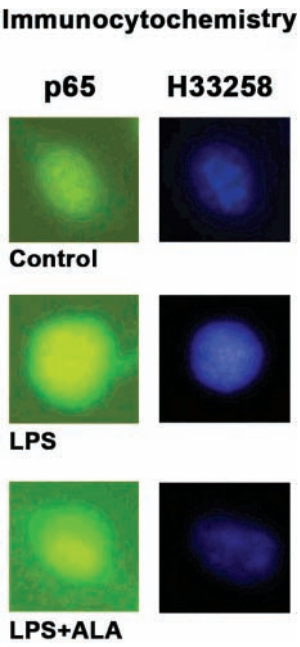

Figure 6. Effect of ALA on immunofluorescence subcellular localization of p65 protein in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were treated with 200 ng/mL LPS for 1 h, and p65 protein localization was immunochemically detected using anti-p65 antibody. LPS caused p65 protein to migrate to the nucleus at 1 h. ALA (40 μg/mL) prevented LPS-induced nuclear translocation of p65 protein. The same fields were stained with Hoechst for the location of nuclei.

binding by preventing I-κBα phosphorylation and the subsequent nuclear translocation of the p65 protein.

Effect on MAPKs Phosphorylation. The MAPKs play an important role in regulating cell growth and differentiation as well as in the control of the cellular responses to cytokines and various stresses. Moreover, they are also important for activating NF-κB. The effect of ALA on the LPS-stimulated phosphorylation of Erk1/2, SAPK/JNK, and p38 MAPK in RAW 264.7 cells was examined using Western blot analyses to determine whether the ALA-induced inhibition of NF-κB activation is mediated by the MAPKs pathway. As shown in **Figure 8**, LPS treatment induced strong increases in the levels of phosphorylated ERK, JNK, and p38 at different indicated times. However, ALA significantly suppressed the LPS-induced activations of Erk1/2 and SAPK/JNK MAPKS, and p38 MAPK was also slightly affected by the ALA treatment.

DISCUSSION

AP fruits have long been used in Korean folk medicine to treat abdominal pain, rheumatoid arthritis, and stroke. We previously reported the anti-inflammatory activities of the 70% ethanol extract of AP fruits using several inflammatory models (*24*). Recently, we isolated the most active anti-inflammatory compound, ALA, from AP fruits through a nitrite assay (*12*). ALA is an ω-3 (n-3) PUFA found mainly in plant sources, including flaxseed oil, canola oil, and walnuts. There is evidence that a diet rich in PUFAs, in particular the n-3, is associated with lower cardiovascular morbidity and mortality and reduced risk of sudden death, independent of other known cardiovascular risk factors (*25*). Studies have suggested that the protective effects of n-3 PUFA are mediated by multiple mechanisms, including their anti-inflammatory properties (*26*).

Inflammation is part of the normal host response to infection and injury. However, excessive or aberrant inflammation

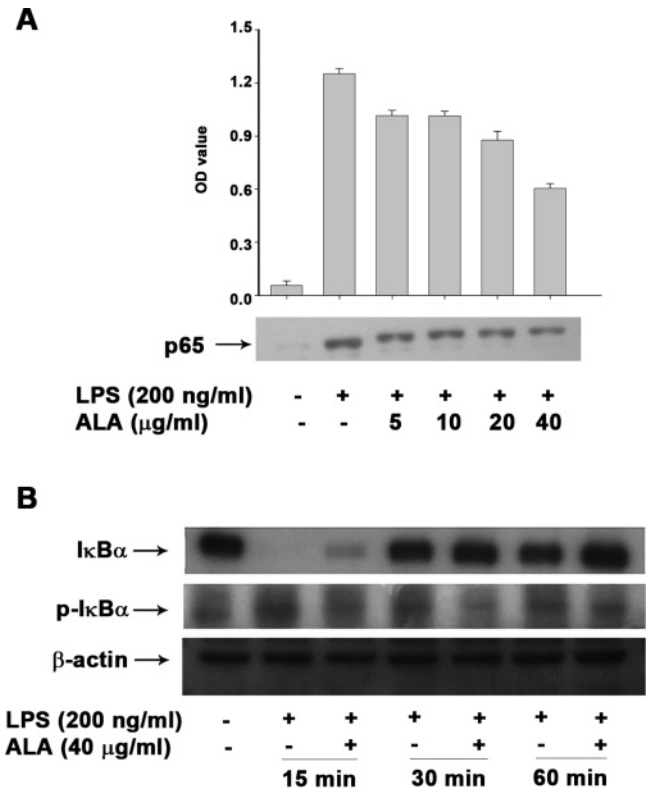

Figure 7. Effects of ALA on LPS-induced NF-κB activation and I-κBα degradation and phosphorylation in RAW 264.7 cells. (**A**) Effect of ALA on LPS-induced NF-κB activation. RAW 264.7 cells were treated with 200 ng/mL LPS for 1 h, and p65 protein was immunochemically detected using anti-p65 antibody. The result presented is representative of three independent experiments. p65 protein expression levels were measured by scanning densitometry of the band intensities. (**B**) Effects of ALA on LPS-induced I-κBα degradation and phosphorylation. The effect of ALA on I-κBα degradation and phosphorylation was immunochemically assessed for cytosol extract of RAW 264.7 cells at the indicated time. Equal loading of proteins was verified by actin immunoblotting.

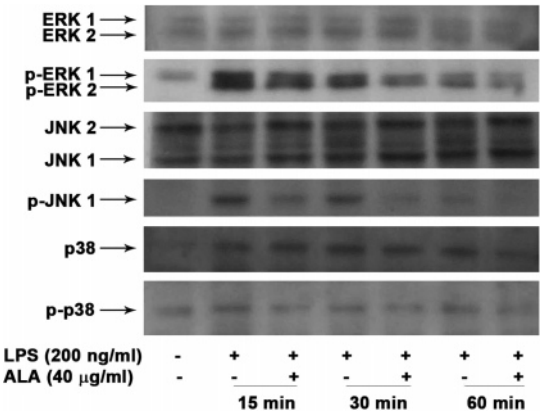

Figure 8. Effects of ALA on LPS-induced phosphorylations of MAPKs in RAW 264.7 cells. RAW 264.7 cells were stimulated with LPS (200 ng/mL) alone or LPS plus ALA (40 μg/mL) for the indicated time points. The whole-cell lysates were analyzed by immunoblot analysis using various antibodies against the activated MAPKs (dually phosphorylated on Tyr/Thr). To ascertain that the total level of each MAPK did not change, blots were stripped and reprobed with the antibodies raised against the corresponding phosphorylation-independent MAPKs. The results presented are representative of three independent experiments.

contributes to a range of acute and chronic human diseases and is characterized by the production of inflammatory cytokines,

arachidonic acid-derived eicosanoids, reactive oxygen species, and adhesion molecules. At sufficiently high intakes, long-chain n-3 PUFAs decrease the production of inflammatory eicosanoids, cytokines, and reactive oxygen species and the expression of adhesion molecules. Long-chain n-3 PUFAs are known to act both directly (e.g., by replacing arachidonic acid as an eicosanoid substrate and inhibiting arachidonic acid metabolism) and indirectly (e.g., by altering the expression of inflammatory genes through effects on transcription factor activation). The in vivo anti-inflammatory activity of ALA has already been reported using two different animal models, that is, the paw edema induced by carrageenan in rats and the acetic acid-induced vascular permeability in mice (*11*, *12*). Recently, Munsterman et al. also demonstrated anti-inflammatory effect of ALA on equine explants challenged with LPS (*27*).

To explore the anti-inflammatory mechanism(s) of ALA, RAW 264.7 murine macrophage cell line was used in this study, and effects of ALA on the expression levels of iNOS, COX-2, and TNF-α were examined. Our results suggest that ALA exerts its anti-inflammatory effect by inhibiting iNOS, COX-2, and TNF-α mRNA transcription steps in a dose-dependent fashion (**Figures 2**−**4**).

The promoter of the iNOS gene is known to contain two transcriptional regions, an enhancer and a basal promoter (*28*). There are a number of binding sites for transcription factors, including κB sites, which are located in both the enhancer and the basal promoter (*5*), and NF-κB, which is essential for LPS-mediated NO production. In nonstimulated cells, NF-κB is present in the cytosol as either a homodimer or a heterodimer and is linked to the IκB protein. The activation of NF-κB results in the phosphorylation, ubiquitination, and proteasome-mediated degradation of the IκB proteins, followed by the nuclear translocation and DNA binding of NF-κB (*29*). Our results suggest that ALA inhibits the nuclear translocation of the p65 protein and the LPS-induced DNA activation of NF-κB (**Figures 5** and **6**). In addition, because NF-κB is activated by I-κBα degradation after phosphorylation of I-κBα at the serine residues by IκB kinase (*23*), inhibition of NF-κB activity by ALA might be the result of the inhibition of I-κBα degradation and phosphorylation, followed by decrease in the translocation of the NF-κB subunits (**Figure 7**).

Another signaling pathway that controls inflammation-associated gene expression also leads to the activation of the transcription factor, NF-κB. Hence, considerable attention has also been paid to MAPKs, which are also likely targets for the development of novel anti-inflammatory drugs. MAP kinases (ERK, JNK, and p38 MAPK) play a pivotal role in regulating cell growth and differentiation, particularly in response to cytokines and stress (*30*). Several studies have implicated MAPKs in the LPS-induced expression of iNOS (*8*, *31*) and the activation of NF-κB (*32*). Cell stimulation induces a signaling cascade that leads to the activation of MAPKs through the phosphorylation of both tyrosine and threonine residues (*33*), which in turn induces a conformational change that exposes the active site to substrate binding. The effects of ALA on the LPS-induced phosphorylation of ERK, JNK, and p38 were assessed to further confirm that ALA affects AP-1 activation. Interestingly, ALA treatment decreased the levels of ERK, JNK, and p38 phosphorylation in response to LPS (**Figure 8**). This supports the view that ERK, p38, JNK, NF-κB, and AP-1 are likely to be responsible for the suppressive effect of ALA on iNOS induction.

In this study, the analgesic activity of ALA was also assayed using two different models, the acetic acid-induced abdominal constriction test and the Randall−Selitto assay. Regarding the analgesic activity of ALA on the mice writhing assay, studies have shown that ALA inhibited the acetic acid-induced abdominal constriction response in mice at doses of 5 and 10 mg/kg (**Table 1**). Although the abdominal constriction response induced by acetic acid is a very sensitive procedure that enables the peripheral antinociceptive activity of compounds to be detected using animal protocols, it is not a specific model (*34*). This model involves different nociceptive mechanisms, such as sympathetic system (biogenic amines release), cyclooxygenases and their metabolites (*35*), as well as opioid mechanisms (*36*). Therefore, the Randall−Selitto assay was also carried out to determine if ALA has any specific analgesic effect. ALA elevated the pain threshold toward a mechanical force at doses of 5 and 10 mg/kg, which could contribute to its peripheral analgesic effects (**Table 2**).

Our findings are consistent with the fact that n-3 PUFAs are potentially potent anti-inflammatory agents (*37*), and to our knowledge, this is the first investigation showing that ALA markedly inhibits LPS-induced NO production and iNOS, COX-2, and TNF-α gene expressions by blocking NF-κB activation and inhibiting the phosphorylation of MAP kinases in RAW 264.7 macrophages. These anti-inflammatory and antinociceptive effects were also evident in vivo. Because NF-κB is one of the critical transcription factors that regulate the transcription of many genes associated with inflammation, the inhibition of this transcription factor by ALA presents a potential therapeutic approach for the treatment of various inflammatory diseases.

ABBREVIATIONS USED

ALA, α-linolenic acid; AP, *Actinidia polygama*; COX-2, cyclooxygenase-2; CPN, cyclophilin; DMEM, Dulbecco's modified Eagle medium; DTT, dithiothreitol; ECL, enhanced chemiluminescence; EMSA, electrophoretic mobility shift assay; ERK, extracellular signal-regulated kinase; FBS, fetal bovine serum; IBD, inflammatory bowel disease; I-κBα, inhibitory factor-κBα; iNOS, nitric oxide synthase; JNK, c-Jun NH_2-terminal kinase; LPS, lipopolysaccharide; MAPKs, mitogen-activated protein kinases; MTT, 3-(4,5-dimethlythiazol-2-yl)-2,5-diphenyl-tertazolium bromide; NF-κB, nuclear factor-κB; NO, nitric oxide; NP-40, Nonidet P40; p38, p38 MAP kinase; PGs, prostaglandins; PMSF, phenylmethanesulfonyl fluoride; PUFA, polyunsaturated fatty acid; RT-PCR, reverse transcription-polymerase chain reaction; TNF-α, tumor necrosis factor-α.

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