

Research Article

Extracellular Signal-Regulated Kinase Is a Direct Target of the Anti-Inflammatory Compound Amentoflavone Derived from *Torreya nucifera*

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Amentoflavone is a biflavonoid compound with antioxidant, anticancer, antibacterial, antiviral, anti-inflammatory, and UV-blocking activities that can be isolated from *Torreya nucifera*, *Biophytum sensitivum*, and *Selaginella tamariscina*. In this study, the molecular mechanism underlying amentoflavone's anti-inflammatory activity was investigated. Amentoflavone dose dependently suppressed the production of nitric oxide (NO) and prostaglandin E₂ (PGE₂) in RAW264.7 cells stimulated with the TLR4 ligand lipopolysaccharide (LPS; derived from Gram-negative bacteria). Amentoflavone suppressed the nuclear translocation of c-Fos, a subunit of activator protein (AP)-1, at 60 min after LPS stimulation and inhibited the activity of purified and immunoprecipitated extracellular signal-regulated kinase (ERK), which mediates c-Fos translocation. In agreement with these results, amentoflavone also suppressed the formation of a molecular complex including ERK and c-Fos. Therefore, our data strongly suggest that amentoflavone's immunopharmacological activities are due to its direct effect on ERK.

1. Introduction

Inflammation is a natural defense mechanism that protects the human body from various infections [1]. Inflammatory responses include the production of cytokines, such as interleukin (IL)-1, IL-6, IL-8, and tumor necrosis factor (TNF)- α ; chemokines, such as monocyte chemoattractant protein (MCP)-1; and inflammatory mediators, such as nitric oxide (NO) and prostaglandin E₂ (PGE₂), by various inflammatory cells, including epithelial cells, macrophages, keratinocytes, mast cells, and Langerhans cells [2, 3]. Molecularly, inflammation involves numerous intracellular signaling cascades, including the Src and Syk nonreceptor protein tyrosine kinases, phosphoinositide 3-kinase (PI3K), phosphoinositide-dependent kinase 1 (PDK1), and Akt (protein kinase B) serine-threonine

protein kinases, which contribute to the activation and upregulation of the transcription factors nuclear factor (NF)- κ B and activator protein (AP)-1 [4, 5]. Although inflammation is critical for maintaining health in the face of infection exaggerated immune responses can cause serious diseases, such as cancer, atherosclerosis, and diabetes [6, 7].

Amentoflavone (AF, Figure 1(a)) is a biflavonoid compound isolated from plants such as *Torreya nucifera* Siebold et Zuccarini (Taxaceae), *Biophytum sensitivum*, *Origanum majorana*, *Cnestis ferruginea*, *Calophyllum flavoramulum*, *Byrsonima crassa*, and *Selaginella tamariscina* [8–10]. Like other biflavonoids, this compound has been reported to possess many biological activities, including antioxidant, anticancer, antibacterial, antiviral, anti-inflammatory, and UV-blocking effects [11–13]. The molecular mechanisms

underlying these disparate effects are unknown. Although several pharmacological targets of amentoflavone (such as fatty acid synthase and suppressor of cytokine signaling 3 (SOCS3)) have been described [14, 15], these targets are not enough to explain the multiple activities of amentoflavone.

Recent studies have shown that amentoflavone treatment can decrease the production of cytokines such as tumor necrosis factor- (TNF-) α and inflammatory mediators such as nitric oxide (NO) and arachidonate by tumor-associated macrophages, peritoneal macrophages, and RAW264.7 cells [16, 17]. Though the effect of amentoflavone is very clear, its mechanism of action is not yet fully elucidated. In this study, therefore, we investigated the molecular mechanism underlying the anti-inflammatory activity of amentoflavone.

2. Materials and Methods

2.1. Materials. Amentoflavone (>99% purity), quercetin, phorbol 12-myristate 13-acetate (PMA), 3-4,5-dimethylthiazol-2-yl)-2,5-di-phenyltetrazolium bromide (MTT), pam3CSK, and lipopolysaccharide (LPS; *E. coli* 0111:B4) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Ethyl acetate fraction (Tn-EE-EA) was prepared from 70% ethanol extract of *Torreya nuncifera* leaves (Kyungdong Oriental Medicine Market, Seoul, Republic of Korea; identification by Professor Sukchan Lee (Sungkyunkwan University, Suwon, Republic of Korea); and a voucher specimen number: SKKUMI-0101-Tn-EE-EA), according to general extraction method [18]. U0126, SB203589, and SP600125 were obtained from Calbiochem (La Jolla, CA, USA). Luciferase constructs containing promoters sensitive to CREB and AP-1 were used as reported previously [19, 20]. Enzyme immunoassay (EIA) kits for the determination of PGE₂ levels were purchased from Amersham (Little Chalfont, Buckinghamshire, UK). Fetal bovine serum and RPMI1640 were obtained from Gibco (Grand Island, NY, USA). The murine macrophage cell line RAW264.7 cells and the human embryonic kidney cell line HEK293 cells were purchased from ATCC (Rockville, MD, USA). All other chemicals were of analytical grade and were obtained from Sigma. Phosphospecific and/or total antibodies to c-Jun, c-Fos, ERK, p38, JNK, I κ B α , myelin basic protein (MBP), lamin A/C, and β -actin were obtained from Cell Signaling (Beverly, MA, USA).

2.2. High-Performance Liquid Chromatography (HPLC) Analysis. The content of amentoflavone in Tn-EE-EA was identified by HPLC analysis [21]. The system was equipped with a model K-1001 HPLC pump, model K-2600 fast scanning spectrophotometer, and a model K-500 4-channel degasser (all from KNAUER WellChrom, Berlin, Germany). Elution solvent was buffer A (acetonitrile: 0.5% acetic acid = 40 : 60). A phenomenex Gemini C₁₈ ODS (250 \times 4.60 mm, 5 μ m) column was used as reported previously [22].

2.3. Cell Culture. RAW264.7 and HEK293 cells were cultured in DMEM or RPMI1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gibco, Grand Island, NY, USA), glutamine, and antibiotics (penicillin and streptomycin) at 37°C under 5% CO₂. For each experiment,

cells were detached with a cell scraper. At the cell density used for the experiments (2×10^6 cells/mL), the proportion of dead cells was less than 1%, as measured by trypan blue dye exclusion.

2.4. Cell Viability Test. After incubating RAW264.7 and HEK293 cells (1×10^6 cells/mL) for 18 h, amentoflavone (from 0 to 200 μ M) was added to the cells, which were then incubated for another 24 h. The cytotoxic effect of amentoflavone was then evaluated using a conventional MTT assay as previously described [23]. At 3 h prior to culture termination, 10 μ L of MTT solution (10 mg/mL in phosphate buffered-saline, pH 7.4) was added to each well, and the cells were continuously cultured until the experiment ended. The incubation was halted by the addition of 15% sodium dodecyl sulfate (SDS) to each well, which results in the solubilization of the formazan [24, 25]. Absorbance at 570 nm (OD₅₇₀₋₆₃₀) was measured using a SpectraMax 250 microplate reader (BioTex, Bad Friedrichshall, Germany).

2.5. Determination of NO and PGE₂ Levels. After incubating RAW264.7 cells (1×10^6 cells/mL) for 18 h, the cells were pretreated with amentoflavone (from 0 to 400 μ M) for 30 min and then further incubated with LPS (1 μ g/mL) or pam3CSK (10 μ g/mL) for 24 h. The inhibitory effect of amentoflavone on the production of NO and PGE₂ was determined by analyzing NO and PGE₂ levels using Griess reagent and enzyme-linked immunosorbent assay (ELISA) kits, as described previously [26, 27].

2.6. Semiquantitative Reverse Transcriptase (RT) and Real-Time Polymerase Chain Reaction (PCR) Analysis of mRNA Levels. To determine the mRNA expression levels of various cytokines, total RNA was isolated from LPS-activated RAW264.7 cells using TRIzol Reagent (Gibco BRL), according to the manufacturer's instructions. Briefly, RAW264.7 cells were pretreated with AF (from 0 to 200 μ M) for additional 30 min. After that, LPS (1 μ g/mL) was exposed to the cells for 6 h. Total RNA was stored at -70°C until use. Semiquantitative RT reactions were conducted as reported previously [28, 29]. The primers used (Bioneer, Daejeon, Republic of Korea) are listed in Table 1.

2.7. Luciferase Reporter Assay. HEK293 cells (1×10^6 cells/mL) in a 12-well plate were transfected with 1 μ g of plasmid containing CREB-Luc or AP-1-Luc along with β -galactosidase using the calcium phosphate method according to the manufacturer's protocol [30]. In case of RAW264.7 cells, the plasmids were transfected to the cells (1×10^6 cells/mL) with Lipofectamine 2000 (Invitrogen, Grand Island, NY, USA) to enhance transfection efficiency of the plasmids. After transfection, cells were further incubated for 24 h. Then, AF was treated to the cells in the presence or absence of PMA (100 nM), TNF- α (15 ng/mL), forskolin (2 μ M), or LPS (1 μ g/mL). 18 h later, the cells were lysed to measure luciferase activities. Luciferase assays were performed using the Luciferase Assay System (Promega) as reported previously [31]. Luciferase activity was normalized to β -galactosidase activity,

TABLE 1: Primer sequences used in RT-PCR analysis.

Name		Sequence (5' to 3')
Real-time PCR		
iNOS	F	GGAGCCTTTAGACCTCAACAGA
	R	TGAACGAGGAGGGTGGTG
COX-2	F	CACTACATCCTGACCCACTT
	R	ATGCTCCTGCTTGAGTATGT
IL-1 β	F	GTTGACGGACCCCAAAAAGAT
	R	CCTCATCCTGGAAGGTCCAC
TNF- α	F	TGCCTATGTCTCAGCCTCTT
	R	GAGGCCATTTGGGAACCTCT
GAPDH	F	CAATGAATACGGCTACAGCAAC
	R	AGGGAGATGCTCAGTGTGG
Semiquantitative PCR		
iNOS	F	CCCTTCCGAAGTTTCTGGCAGCAG
	R	GGCTGTCAGAGCCTCGTGGCTTTGG
TNF- α	F	TTGACCTCAGCGCTGAGTTG
	R	CCTGTAGCCACGTCGTAGC
COX-2	F	CACTACATCCTGACCCACTT
	R	ATGCTCCTGCTTGAGTATGT
GAPDH	F	CACTCACGGCAAATTCAACGGCA
	R	GACTCCACGACATACTCAGCAC

measured at 405 nm, by enzymatic reaction with X-gal and lysate for 5 min at 37°C.

2.8. Preparation of Cell Lysates, Nuclear Fractionation, Immunoblotting, and Immunoprecipitation. RAW264.7 cells (5×10^6 cells/mL) were washed three times in cold PBS with 1 mM sodium orthovanadate, lysed using a sonicator in lysis buffer (20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 2 mM ethylene glycol tetraacetic acid, 50 mM β -glycerophosphate, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 1% Triton X-100, 10% glycerol, 10 μ g/mL aprotinin, 10 μ g/mL pepstatin, 1 mM benzamide, and 2 mM phenylmethylsulfonyl fluoride), and then incubated for 30 min with rotation at 4°C. The lysates were clarified by centrifugation at 16,000 \times g for 10 min at 4°C and stored at -20°C until needed.

Nuclear lysates were prepared using a three-step procedure [32]. After treatment, the cells were collected with a rubber policeman, washed with PBS, and lysed in 500 μ L of lysis buffer containing 50 mM KCl, 0.5% Nonidet P-40, 25 mM HEPES (pH 7.8), 1 mM phenylmethylsulfonyl fluoride, 10 μ g/mL leupeptin, 20 μ g/mL aprotinin, and 100 μ M 1,4-dithiothreitol (DTT) on ice for 4 min. Cell lysates were then centrifuged at 19,326 \times g for 1 min in a microcentrifuge. In the second step, the nuclear fraction pellet was washed once in washing buffer, which was the same as the lysis buffer without Nonidet P-40. In the final step, nuclei were treated with an extraction buffer containing 500 mM KCl, 10% glycerol, and the other reagents in the lysis buffer. The nuclei/extraction buffer mixture was frozen at -80°C and then thawed on ice and centrifuged at 19,326 \times g for 5 min. The

supernatant was collected as a nuclear extract. Soluble cell lysates were immunoblotted, and protein levels were visualized as previously reported [33]. For immunoprecipitation, cell lysates containing equal amounts of protein (500 μ g) from RAW264.7 cells (1×10^7 cells/mL) treated with or without LPS (1 μ g/mL) for 2.5 min were precleared with 10 μ L protein A-coupled Sepharose beads (50% v/v) (Amersham, UK) for 1 h at 4°C. Precleared samples were incubated with 5 μ L of anti-c-Fos antibody overnight at 4°C. Immune complexes were mixed with 10 μ L protein A-coupled Sepharose beads (50% v/v) and rotated for 3 h at 4°C.

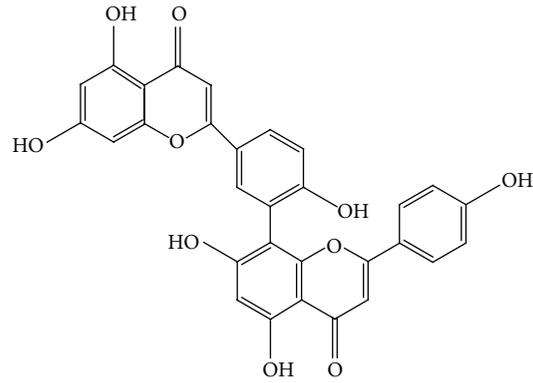
2.9. Enzyme Assay. To evaluate the inhibition of the kinase activity of purified ERK, a kinase profiler service from Millipore was used. In a final reaction volume of 25 μ L, ERK1 (human), ERK2 (human), or MAPK kinase (MEK) 1 (human) (1–5 mU) was incubated with reaction buffer. The reaction was initiated by the addition of MgATP. After incubating the mixture for 40 min at room temperature, the reaction was stopped by the addition of 5 mL of a 3% phosphoric acid solution. Ten microliters of the reaction product was then spotted onto a P30 Filtermat and washed three times for 5 min in phosphoric acid (75 mM) and once in methanol prior to drying and scintillation counting. To determine the effect of amentoflavone on LPS-activated ERK activity, immunoprecipitated ERK (prepared from RAW264.7 cells (5×10^6 cells/mL) that had been treated with LPS in the presence or absence of amentoflavone) was incubated with MBP according to the manufacturer's instructions. ERK kinase activity was determined using an anti-phospho-MBP antibody after immunoblotting analysis, as reported previously [34].

2.10. Statistical Analysis. Data (Figures 1(b), 1(c), 1(d), 1(e), 2(b), 3(a), 3(b), 3(c), 4(b), and 4(c)) are expressed as the means \pm standard deviations (SD) calculated from one ($n = 6$) of two independent experiments. Other data are representative of three different experiments with similar results. For statistical comparison, results were analyzed using an analysis of variance/Scheffe's post hoc test and the Kruskal-Wallis/Mann-Whitney tests. All P values < 0.05 were considered statistically significant. All statistical tests were conducted using SPSS (SPSS Inc., Chicago, IL, USA).

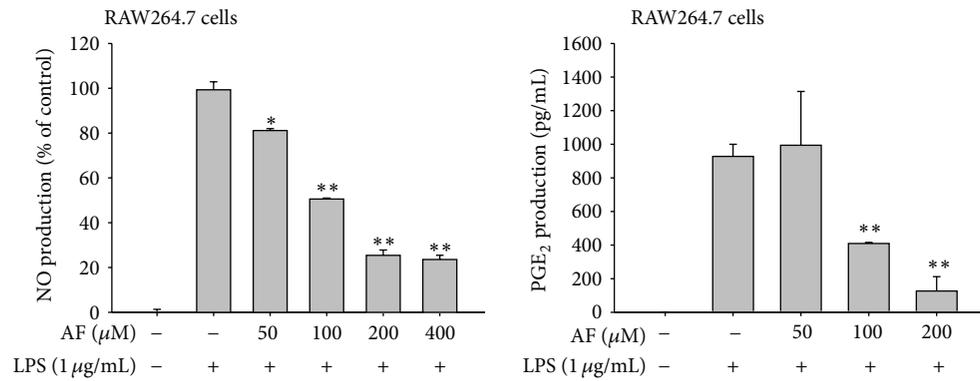
3. Results and Discussion

Amentoflavone is a multipotential biflavonoid compound with antioxidant, anticancer, antiinflammatory, and UV-blocking effects [11–13]. Although the anti-inflammatory activity of amentoflavone has been reported previously [35–37], the molecular targets of this compound have not been fully elucidated. Therefore, in this study, we focused on identifying the molecular target of amentoflavone using LPS-stimulated RAW264.7 macrophage cells.

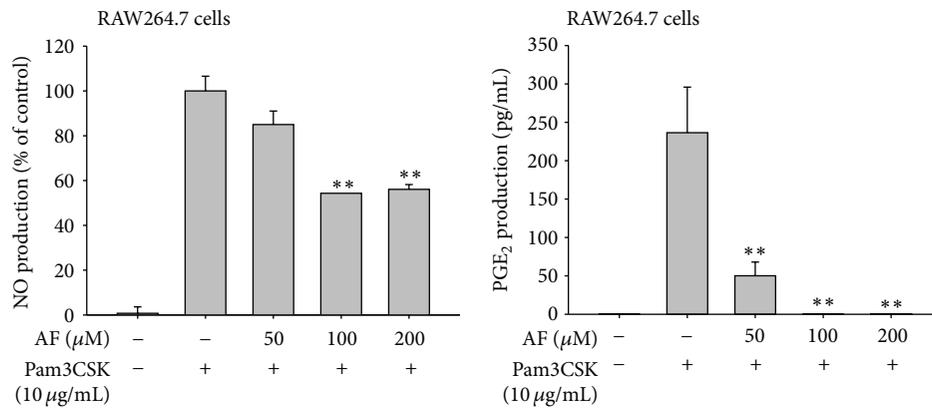
Amentoflavone dose dependently suppressed NO (Figures 1(b) left panel and 1(c) left panel) and PGE₂ (Figures 1(b) right panel and 1(c) right panel) production induced by LPS or pam3CSK in RAW264.7 cells without significantly



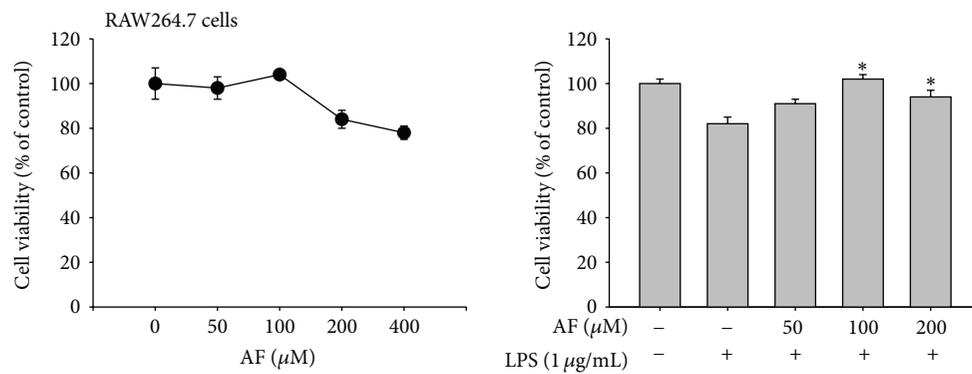
(a)



(b)



(c)



(d)

FIGURE 1: Continued.

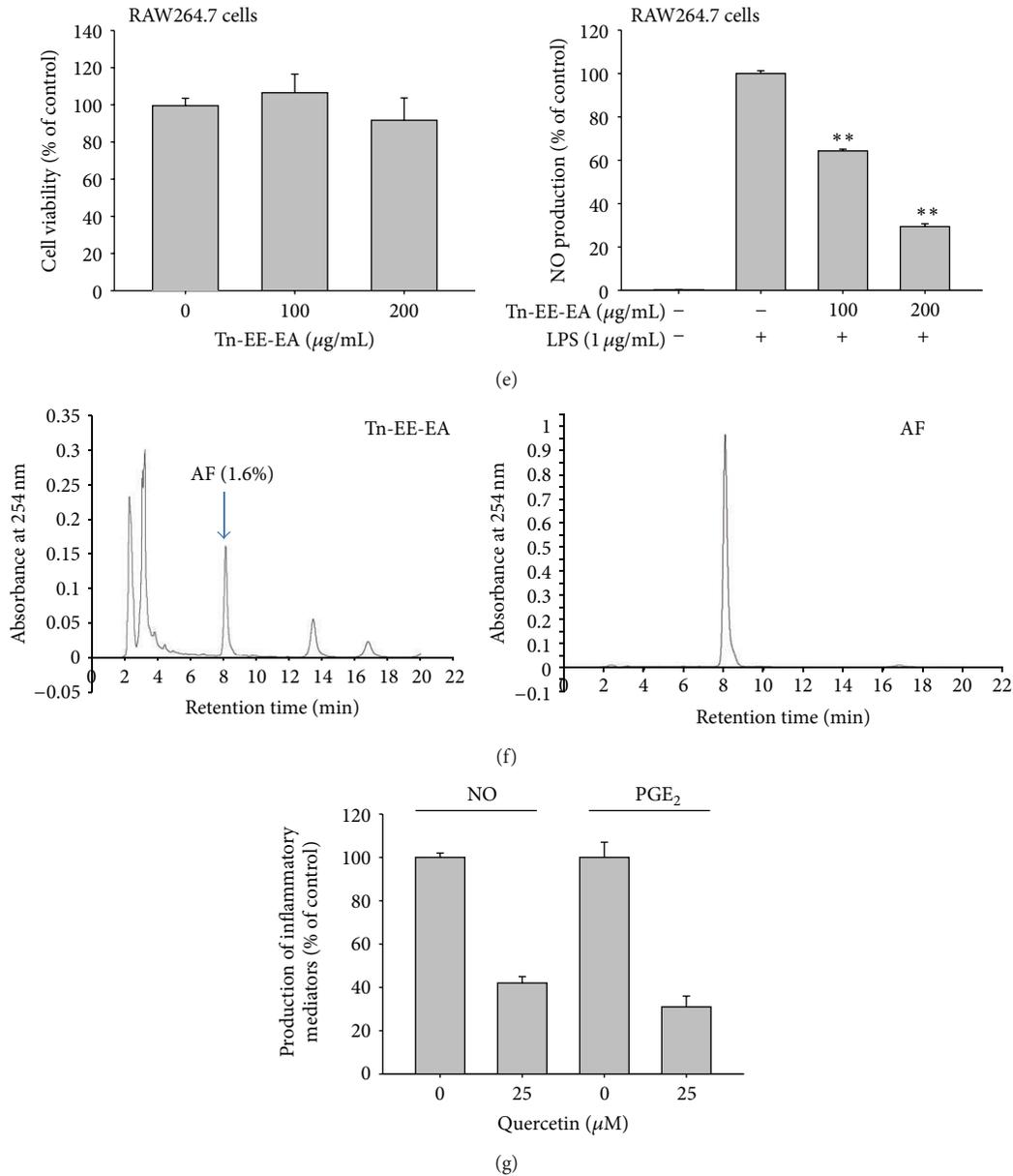


FIGURE 1: Effect of amentoflavone and Tn-EE-EA on the production of inflammatory mediators. (a) Chemical structure of amentoflavone (AF). (b, c, and g) Production of NO and PGE₂ in RAW264.7 cells treated with LPS (1 μg/mL) or pam3CSK (10 μg/mL) in the presence or absence of amentoflavone (from 0 to 200 μM) or quercetin (25 μM) was measured using the Griess assay and EIA. ((d) and (e), right panel) The viability of RAW264.7 cell treated with amentoflavone (from 0 to 400 μM) or Tn-EE-EA (0 to 200 μg/mL) in the presence or absence of LPS was determined using an MTT assay. (e) Production of NO in RAW264.7 cells treated with LPS (1 μg/mL) in the presence or absence of Tn-EE-EA (from 0 to 200 μg/mL) was measured using the Griess assay (left panel). (f) The content of amentoflavone from Tn-EE-EA was analysed using a high-performance liquid chromatograph (HPLC) (Knauer). **P* < 0.05 and ***P* < 0.01 compared to the control.

affecting the viability of RAW264.7 cells in the absence (Figure 1(d) left panel) or presence (Figure 1(d) left panel) of LPS as well as HEK293 cells (data not shown). Furthermore, this compound alone did not increase NO production (data not shown), indicating that there was no contamination with other immunogens. In particular, a well-known flavonoid compound quercetin also showed significant inhibitory activity on NO and PGE₂ production Figure 1(g), as reported previously [38], indicating that our experimental systems are well

established. The mRNA analysis of iNOS and COX-2 as well as TNF-α and IL-1β, which are involved in the production of NO and PGE₂ (Figure 2), demonstrated that amentoflavone-mediated inhibition occurred at the transcriptional level, implying that this compound can block transcriptional activation downstream of LPS-induced TLR4 signaling. Indeed, treatment with amentoflavone dramatically diminished the increase in luciferase activity induced by the activation of AP-1 (Figure 3(a)) but not CREB in HEK293 cells (Figure 3(c)).

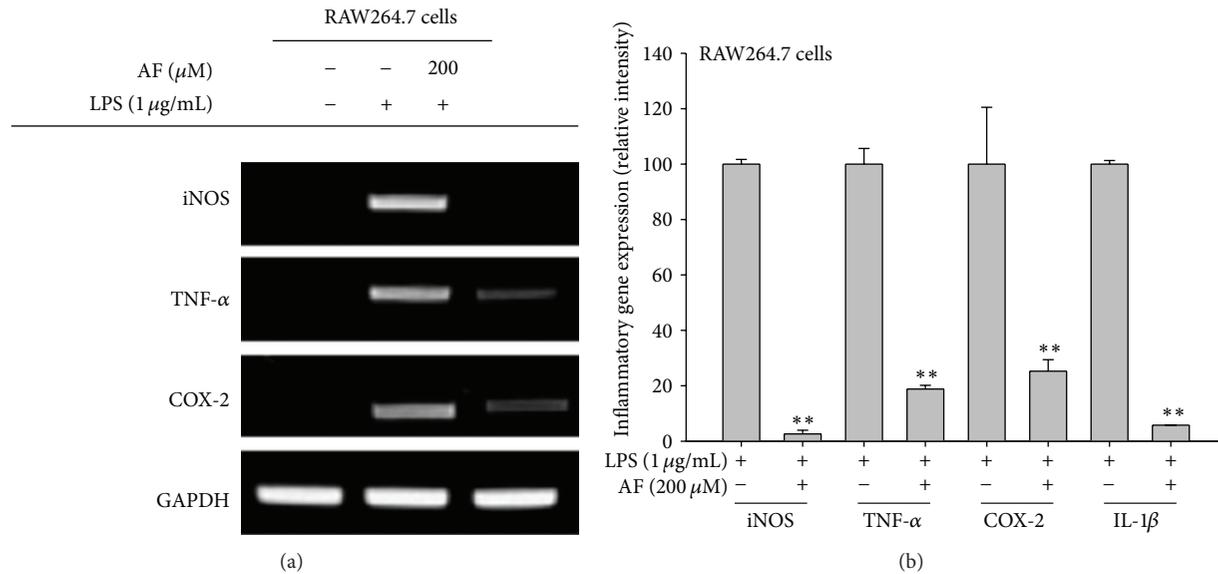


FIGURE 2: Effect of amentoflavone on mRNA expression of proinflammatory genes. (a and b) The mRNA levels of iNOS, COX-2, IL-1 β , and TNF- α in RAW264.7 cells pretreated with or without AF (200 μ M) for 30 min and then exposed to LPS (1 μ g/mL) for 6 h were determined using semiquantitative (a) and real-time (b) PCR. RI: relative intensity. ** $P < 0.01$ compared to the control.

In agreement with this pattern, upregulated luciferase activity mediated by AP-1 in LPS-treated RAW264.7 cells was also significantly reduced by this compound (Figure 3(b)). Furthermore, amentoflavone treatment strongly decreased c-Fos translocation (from 15 to 60 min) and altered the pattern of c-Jun phosphorylation observed in the nucleus at early time points (15 min) (Figure 3(d)). With previous reports that amentoflavone did inhibit the translocation of NF- κ B subunits [35], these data clearly suggest that amentoflavone can also modulate signaling upstream of the translocation of AP-1 (c-Jun and c-Fos).

Next, therefore, we attempted to identify the target of amentoflavone that mediates its inhibitory role. The major enzymes acting upstream of AP-1 include the MAPKs (ERK, p38, and JNK); therefore, we examined the phosphorylation of these proteins using immunoblotting analysis. As shown in Figure 4(a), there was no inhibition in the phosphorylation of any MAPK upon amentoflavone treatment, indicating that the kinases upstream of ERK, p38, and JNK was not suppressed by this compound. Therefore, we examined whether amentoflavone inhibits any MAPK enzyme directly. To determine which MAPK is relevant for inflammatory signaling in these cells, we first tested the effects of specific inhibitors of ERK, p38, and JNK on AP-1-mediated luciferase activity during PMA stimulation at the concentration (20 μ M) with PGE₂ inhibitory activity (data not shown). Interestingly, the ERK inhibitor U0126, but not the p38 and JNK inhibitors SB203580 and SP600125, strongly suppressed AP-1 activity (Figure 4(b)), implying that ERK regulates PMA-induced AP-1 activation. In fact, PMA has been reported to activate ERK in cells such as neutrophils, monocytic U937, RAW264.7, and Jurkat T cells [39–41]. To test whether amentoflavone can directly suppress the enzyme activity of ERK, we performed a kinase assay using immunoprecipitated ERK and MBP protein as a substrate. As expected, amentoflavone strongly

suppressed ERK's kinase activity (Figure 4(c)). Using the same assay, we have previously shown that U0126 strongly inhibits MBP phosphorylation [34]. Here, we also showed that U0126 decreased the translocation of c-Fos (Figure 4(d)). In agreement with these data, amentoflavone disrupted the association between ERK and c-Fos, as assessed by immunoprecipitation and immunoblotting (Figure 4(e)). Interestingly, confocal analysis clearly indicated that LPS treatment induced a condensed localization of ERK in the cytoplasm; under amentoflavone treatment, this pattern was only weakly visible (data not shown). Thus, these results strongly suggest that ERK could be a target of amentoflavone that promotes AP-1 (c-Fos)-mediated inflammatory responses. Since c-Fos is a member of the major AP-1 family [42], AF-mediated inhibition of c-Fos translocation could lead to suppression of AP-1 activity. Moreover, the reduction of c-Jun phosphorylation, an important event for c-Jun binding to DNA [42], could additionally suppress the capacity of c-Jun/DNA binding. Therefore, all of these events could contribute to AF-mediated inhibition of AP-1 activity.

ERK is known to play a pivotal role in inflammatory responses [43]. ERK activity has been clearly shown to be elevated in various inflammatory cells, such as macrophages, monocytic cells, and neutrophils, under inflammatory conditions [44, 45]. In this situation, ERK acts to activate AP-1 and subsequent inflammatory gene expression. For this reason, synthetic and naturally occurring compounds that diminish ERK activity, such as U0126, PD98059, curcumin, resveratrol, and quercetin, have various pharmacological effects in inflammatory diseases [46, 47]. In addition, anti-inflammatory herbal extracts prepared from *Scutellaria baicalensis*, *Sanguisorba officinalis*, Buyang Huanwu, and Cimicifugae rhizome have been shown to exert their anti-inflammatory effects by targeting ERK [28, 48–50]. Because a synthetic method for mass production of amentoflavone is not yet

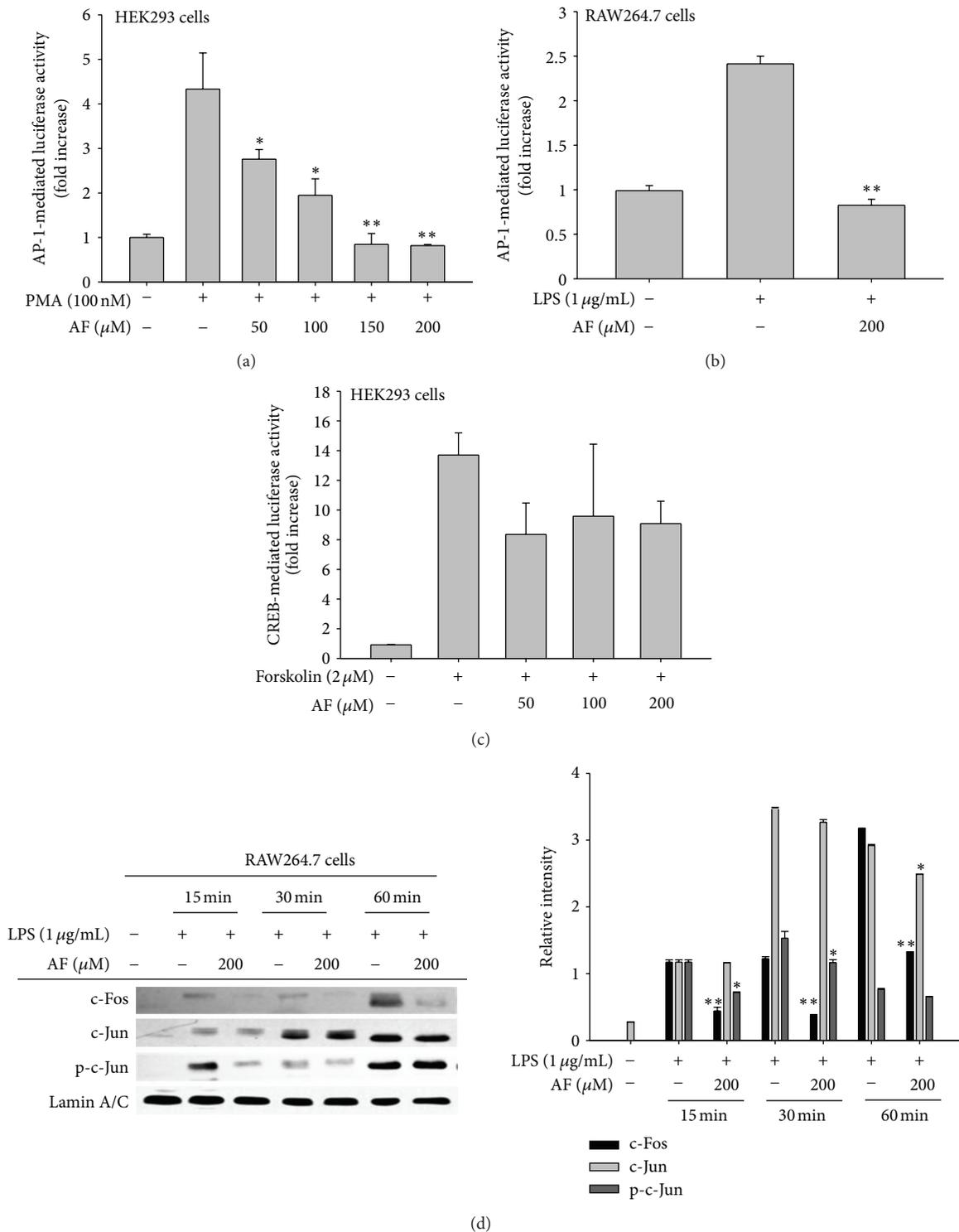


FIGURE 3: Effect of amentoflavone on transcriptional control of inflammatory genes. (a, b, and c) HEK293 or RAW264.7 cells cotransfected with an AP-1-Luc or CREB-Luc plasmid construct (1 μ g/mL each) and β -gal (as a transfection control) were treated with amentoflavone (from 0 to 200 μ M) in the presence or absence of TNF- α (15 ng/mL), PMA (100 nM), forskolin (2 μ M) or LPS (1 μ g/mL). Luciferase activity was measured using a luminometer. ((d), left panel) Total and phosphorylated levels of the AP-1 family proteins c-Jun and c-Fos in the nuclear fractions of LPS-treated RAW264.7 cells were determined by immunoblotting analyses using specific antibodies. Relative intensity (left panel) was calculated by the DNR Bio-Imaging system (a GelQuant software Ver. 2.7). RI: relative intensity. * $P < 0.05$ and ** $P < 0.01$ compared to the control.

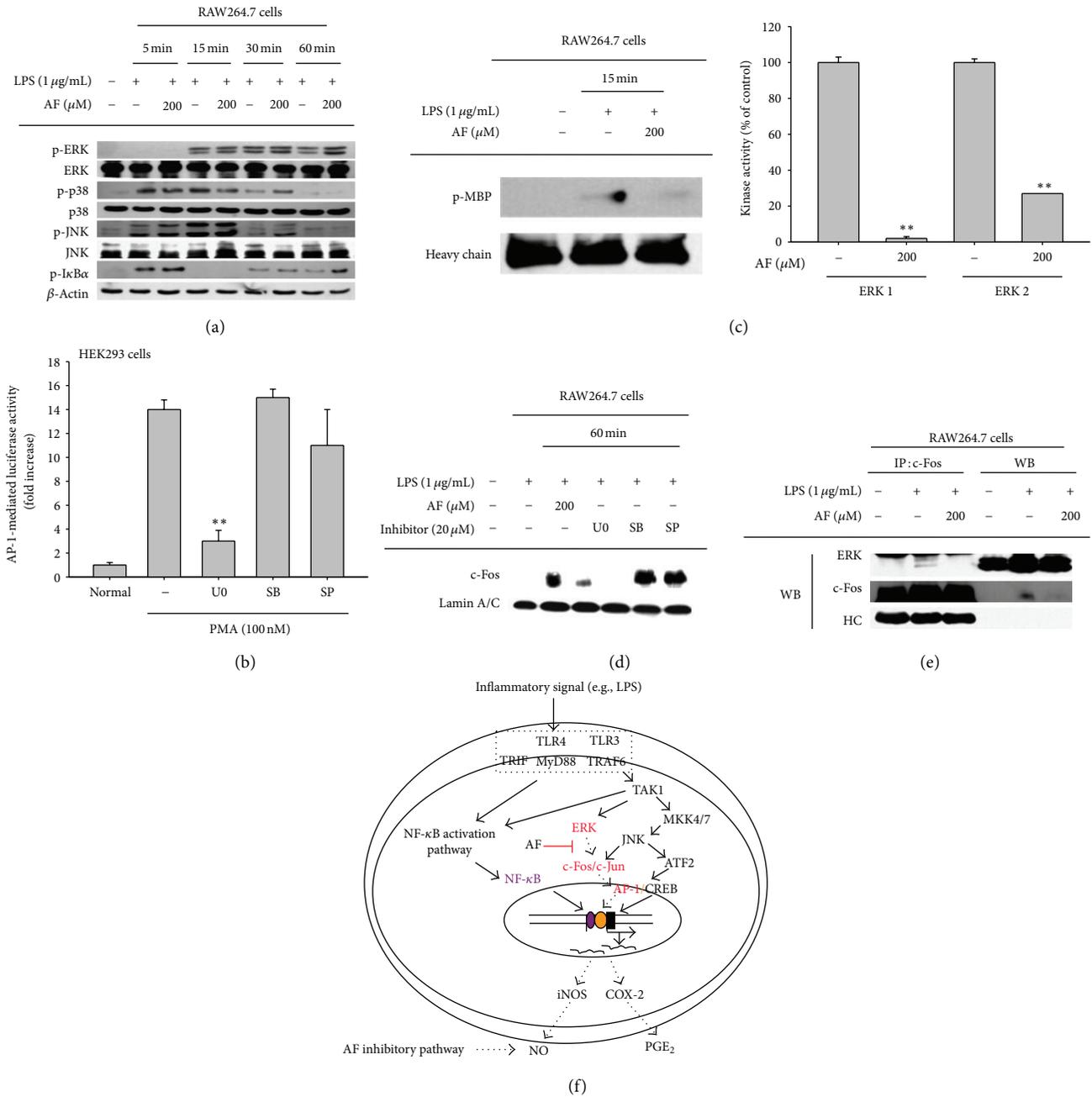


FIGURE 4: Effect of amentoflavone on signaling factors upstream of AP-1. (a) Phosphorylated and total protein levels of ERK, p38, JNK, and β -actin in RAW264.7 cell lysates were determined by immunoblotting analyses using phosphospecific or total protein antibodies. Relative intensities were calculated by densitometric scanning. (b) HEK293 cells cotransfected with an AP-1-Luc plasmid construct (1 μ g/mL) and β -gal (as a transfection control) were treated with U0126 (U0, 20 μ M), SB203580 (SB, 20 μ M), or SP600125 (SP, 20 μ M) in the presence or absence of PMA (100 nM). Luciferase activity was measured using a luminometer. (c) The kinase activities of immunoprecipitated ERK prepared from LPS-treated RAW264.7 cells and purified ERK (ERK1 and ERK2) were determined in a direct kinase assay using purified enzymes or by measuring the level of phospho-MBP. The control (set as 100%) was the activity of each enzyme (ERK1 or ERK2) obtained after treatment with vehicle. The level of phosphorylated MBP was measured by immunoblotting analysis. (d) The total level of c-Fos in the nuclear fraction of LPS-treated RAW264.7 cells after U0, SB, or SP treatment was determined by immunoblotting analyses using specific antibodies. (e) An interaction between ERK and c-Fos was evaluated by immunoprecipitation and immunoblotting analyses. RAW264.7 cells (5×10^6 cells/mL) were incubated with amentoflavone (200 μ M) in the presence or absence of LPS (1 μ g/mL) for 30 min. c-Fos was immunoprecipitated from whole cell lysates using a specific antibody, followed by immunoblotting with antibodies to ERK, c-Fos, and rabbit immunoglobulin heavy chain. (f) Putative anti-inflammatory signaling pathway induced by amentoflavone treatment. ** $P < 0.01$ compared to the control.

established, this compound cannot yet be used as a pharmacological agent. Further study of possible mass production methods and their alternatives will be necessary. In this regard, the plant *Torreya nuncifera*, which contains large amounts (1.6%) of amentoflavone (Figure 1(f)) and has been shown to inhibit NO, could be used as an amentoflavone source; here, we show that an extract from *Torreya nuncifera* mimics the effects of amentoflavone on NO production (Figure 1(e)). Therefore, taken together with previous reports, our data strongly suggest that ERK is a prime target of amentoflavone's anti-inflammatory action and that this compound or *Torreya nuncifera* extracts could be used as an anti-inflammatory medication.

In summary, we have demonstrated that amentoflavone clearly suppresses macrophage-mediated inflammatory responses such as the production of NO and PGE₂. In particular, this compound strongly inhibited nuclear translocation of c-Fos through the inhibition of its upstream signaling enzyme ERK, as summarized in Figure 4(f). ERK is reported to modulate various inflammatory diseases; therefore, the effects of amentoflavone or *Torreya nuncifera* on inflammatory symptoms will be examined in future experiments.

Abbreviations

PGE ₂ :	Prostaglandin E ₂
NO:	Nitric oxide
COX:	Cyclooxygenase
iNOS:	Inducible NO synthase
TNF- α :	Tumor necrosis factor- α
ERK:	Extracellular signal-related kinase
TLR:	Toll-like receptors
MAPK:	Mitogen-activated protein kinase
AP-1:	Activator protein-1
JNK:	c-Jun N-terminal kinase
CREB:	cAMP response element-binding
EIA:	Enzyme immunoassay
ELISA:	Enzyme-linked immunosorbent assay
MTT:	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
LPS:	Lipopolysaccharide
RT-PCR:	Reverse transcriptase polymerase chain reaction
MBP:	Myelin basic protein.

Conflict of Interests

The authors report no conflict of interests. The authors alone are responsible for the content and writing of the paper.

Authors' Contribution

Jueun Oh and Ho Sik Rho equally contributed to this work.

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