Thesis for the Degree of Master of Science

Anti-inflammatory effect of phlorofucofuroeckol A on lipopolysaccharide-induced RAW 264.7 macrophages

By

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August 2009

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LPS로 유도된 RAW 264.7 대식세포에서 phlorofucofuroeckol-A의 항염증 효과

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By

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A thesis submitted in partial fulfillment of requirements
For the degree of

Master of Science

in Department of Food and Life Science, The Graduate School,
Pukyong National University

August 2009

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A dissertation

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August 2009

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Abstract

Phlorofucofuroeckol A (PFF-A) is a phloroglucinol derivative isolated from the edible brown algae *Ecklonia stolonifera* and has been shown to possess various biological activities. In this study, the effect of PFF-A on the regulation of iNOS and COX-2 which are key inflammatory proteins responsible for excessive production of NO and PGE_2, respectively, was investigated using the LPS-treated RAW 264.7 cells. Treatment with 20 μM of PFF-A led to strong inhibition of iNOS and COX-2 protein and mRNA expressions in the LPS-treated RAW 264.7 cells. Data of luciferase assays demonstrated that PFF-A treatment largely suppressed the iNOS or COX-2 promoter-driven luciferase expression in the LPS-treated RAW 264.7 cells. PFF-A also had an inhibitory effect on the NF-κB-promoter driven expression. Moreover, PFF-A had an ability to inhibit activation of Akt, p38 MAPK, ERK-1/2 and JNK-1/2, and to reduce cellular ROS in the LPS-treated RAW 264.7 cells. Taken together PFF-A inhibits iNOS and COX-2 expression in the LPS-treated RAW 264.7 cells through the NF-κB-dependent transcriptional down-regulation in association with inhibition of ERK-1/2, JNK-1/2, p38 MAPK, and Akt, and with reduction of cellular ROS. These results suggest an important implication for using PFF-A toward the development of an effective anti-inflammatory agent.

1. Introduction

Macrophages play an important role in response to inflammation by producing nitric oxide (NO), superoxide anions, and cytokines [1]. NO is a short-lived free radical and intercellular messenger that mediates a variety of biological functions, including vascular homeostasis, neurotransmission, antimicrobial defense, and antitumor activities [2]. Production of NO is controlled by nitric oxide synthase (NOS), which converts L-arginine to L-citrulline [3]. In mammals, NO is synthesized by three different isoforms of NO synthase (NOS), namely, neuronal NOS (nNOS), endothelial NOS (eNOS) and inducible NOS (iNOS). nNOS and eNOS are constitutively expressed, however, iNOS is expressed in response to interferon-γ, LPS, and variety of pro-inflammatory cytokines [4]. Physiologically, iNOS induces various harmful responses including tissue injury, septic shock, and apoptosis [5,6].

COX converts arachidonic acid to PGs, and has two isoforms [4]. COX-1 is constitutively expressed in most cells, and COX-1-derived PGs are involved in the maintenance of normal physiological functions. On the other hand, COX-2 is inducible by pro-inflammatory cytokines, tumor promoters, and bacterial toxins in certain types of tissues [7,8]. Evidence that nonsteroidal anti-inflammatory drugs or compounds that target COX-2 lessen major inflammatory symptoms such as fever and pain suggests a role for COX-2 in inflammation [9].

The expression of iNOS and COX-2 genes is regulated by nuclear factor-κB (NF-κB), which performs pivotal roles in the immediate early stages of immune, acute phase, and inflammatory responses, as well as in cell survival [10,11]. NF-κB has been identified as a heterodimer of

p50 and p65 subunits. In the unstimulated condition, NF-κB is located in the cytoplasm as an inactive complex bound IκB-α. However, in the stimulated condition by LPS, activate the IκB kinase complex, resulting in the phosphorylation, ubiquitination, and degradation of IκB, and NF-κB is then translocated to the nucleus and promotes the transcription of target genes and initiates gene expression.

The activation of NF-κB is regulated by cellular kinases such as mitogen-activated protein kinases (MAPKs) [12]. The MAPKs are serine/threonine-specific protein kinases that play a critical role in the regulation of cell survival/apoptosis, and differentiation as well as in the control of cellular response to cytokines and stresses. These classical MAPKs, extracellular signal regulated kinase (ERKs), p38 MAPK, and c-Jun NH_2-terminal kinase (JNKs) have been implicated in the transcriptional regulation of inflammatory gene [13]. Especially, p38 MAPK signaling pathways constitute an additional level of gene regulation by NF-κB, more particularly of the p65 subunit [14]. In addition, inhibition of p38 MAPK has been demonstrated to prevent stabilization of IκB-α and delay the re-emergence of IκB-α following TNF-α-induced degradation [15].

PI3K is involved in cytokine expression and NF-κB activation in mouse macrophage [16]. Moreover, Akt, a down-stream regulator of PI3K, was reported to be implicated in the PI3K-mediated regulation of NF-κB [17]. PI3K activation leads to phosphorylation of phosphatidylinositides, which then activate the downstream main target, Akt, which appears to play various important role in regulating cellular growth, differentiation, adhesion, and the inflammatory reaction [18]. Activation of PI3K/Akt plays an important role in the expression of

iNOS and COX-2 in vascular smooth muscle cells, peritoneal macrophages, and mesangial cells [19,20].

Ecklonia stolonifera OKAMURA (Laminariaceae), a perennial brown alga growing in a water depth of 2-10 m, is distributed in Korea and Japan, and is commonly used as a foodstuff along with *Laminaria japonica* and *Undaria pinnatifida* [21]. Several components from *Ecklonia* species have been isolated and investigated on the radical scavenging activity [22,23], anti-plasmin inhibiting activity [24,25], antimutagenic activity [26-28], anti-bactericidal activity [29], HIV-1 reverse transcriptase and protease inhibiting activity [30], and tyrosinase inhibitory activity [31]. However, little is known about the mechanism responsible for anti-inflammatory effects of phlorotannins isolated from *E. stolonifera*.

In the present study, phlorofucofuroeckol A (PFF-A) isolated from *E. stolonifera* was characterized in LPS-induced iNOS and COX-2 expressions in macrophages via inhibition of NF-κB, Akt, p38 MAPK, and ROS production.

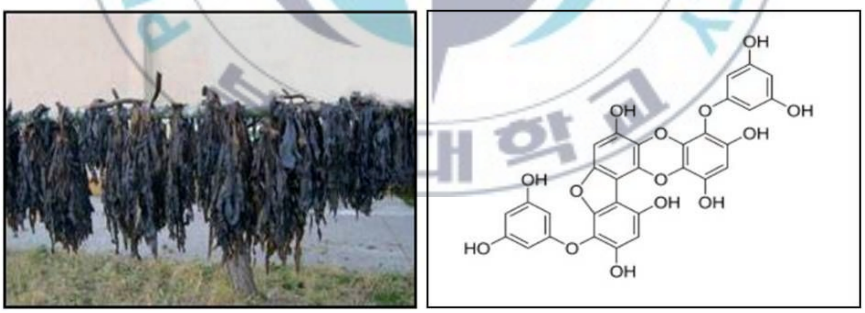


Fig. 1. Photograph of *E. stolonifera* and chemical structure of PFF-A.

2. Materials and Methods

2-1. Materials

DMEM (Dulbecco's modified Eagle's medium), penicillin-streptomycin mixture, and fetal bovine serum (FBS) were purchased from HyClone Laboratory Inc. (Logan, Utah). LPS (*Escherichia coli* O55:B5) was obtained from Sigma (St. Louis, MO). Celltiter 96 AQ one solution Cell Proliferation Assay kit was purchased from Promega (Madison, WI), and DMSO (dimethyl sulfoxide) was purchased from Sigma Chemical (St. Louis, MO). Protein standard marker was purchased from Amersham Pharmacia (Piscataway, NJ) and the enhanced chemiluminescence (ECL) detection kit was purchased from Perkin Elmer Life Science (Wellesley, MA). Polyclonal iNOS, COX-2, PARP, phospho-Akt, Akt, ERK, phospho-IκB, IκB, and NF-κB were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal phospho-ERK, phospho-JNK, phospho-p38 and actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary antibodies, horse radish peroxidase conjugated anti-mouse IgG, anti-rabbit IgG, and anti-goat IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

2-2. Methods

2-2-1. Cell culture and sample treatment

Murine macrophage RAW 264.7 cell line was obtained from American Type Culture Collection (ATCC, Rockville, MD). The cells were grown at 37°C in DMEM medium supplemented with 10% FBS, penicillin (100 units/ml), and streptomycin sulfate (100 μg/ml) in a

humidified atmosphere of 5% CO_2. Cells were stimulated with LPS (1 μg/ml) in the presence or absence of PFF-A isolated from *E. stolonifera* for the indicated periods. The stock solutions of PFF-A were prepared in dimethyl sulfoxide (DMSO).

2-2-2. Cell viability and proliferation

Preconfluent RAW 264.7 cells were seeded onto 96-well plates at a density of 5×10^4 cells/well in DMEM plus 10% FBS. After 24 h, the culture medium was replaced by 200 μl of serial dilution (0-20 μM) of PFF-A and the cells were incubated for 24 hr. Cells were then treated without or with LPS (1 μg/ml) in the absence or presence of PFF-A in different concentrations for 24 hr. The final concentration of solvent was less than 0.1% in the cell culture medium. The culture medium was removed and replaced by 95 μl of fresh culture medium and 5 μl of MTS solution. After 3 h, the absorbance was measured using microplate reader (Ultraspec 2100 pro, Amersham Biosciences, Piscataway, NJ) at 490 nm.

2-2-3. Determination of ROS production

The intracellular ROS level of the sample was measured using the oxidant-sensitive fluorescent probe DCFH-DA. The cells were incubated with 20 μM of PFF-A in the absence or presence of LPS (1 μg/ml) for 2 hr. Two hours later, 20 μM DCFH-DA was added for 30 min at 37°C and the cells were measured with spectrofluorometry. All measurements were made with the instrument excitation wavelength set at 485 nm and emission wavelength at 523 nm (Dual Scanning SPECTRAmax, Molecular Devices Corporation, Sunnyvale, CA).

2-2-4. Measurement of NO and PGE_2

The nitrite concentration in the culture medium was measured as an indicator of NO production, according to the Griess reaction [32]. The cells were incubated with 0-20 μM of PFF-A in the absence or presence of LPS (1 μg/ml) for 24 hr. Briefly, cells were dispensed into 96-well plates, and 100 μl of cell culture medium was mixed with the same volume of Griess reagent (1% sulfanilamide and 0.1% naphthylethylenediamine dihydrochloride in 5% phosphoric acid) and incubated at room temperature for 10 min. Absorbance of the mixture at 540 nm was measured with ELISA reader. The amount of nitrite in the samples was measured with standard curve made with serially diluted sodium nitrite and nitrite concentration was calculated.

RAW 264.7 cells were plated in 7×10^5 cells per well in 24 well-plates overnight. After treatments for 24 hr, the conditioned medium was collected and subjected to PGE_2 assay in a 96-well plate according to the manufacturer's instructions. Principally, the product of this enzymatic reaction has a blue color that absorbs at 450 nm. The extent of color is inversely proportional to the amount of free PGE_2 present in the well during the incubation.

2-2-5. Western Blot analysis

2-2-5-1. Preparation of total cell lysates

RAW 264.7 cells were washed twice with ice-cold PBS, and added 100 μl of lysis buffer [50 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 1% Nonidet-40, 1% Tween-20, 0.1% SDS, 1mM Na_3VO_4, 10 μg/ml leupeptin, 50 mM NaF, and 1 mM phenylmethylsulfonyl fluoride (PMSF)] and stood on ice for 1 hr for lysis. After centrifuge at 18,000 ×

g for 10 min, protein content of supernatant was measured, and aliquots (20 μg) of protein were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was blocked with 5% non-fat dried milk in TBST buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.06% Tween-20) for 1 hr and incubated for 2 hr with primary antibody in TBST buffer containing 5% non-fat dried milk. The blots were treated with horseradish peroxidase-conjugated secondary antibody in TBST buffer containing 5% non-fat dried milk for 1 hr, and immune complex was detected using ECL detection kit (Amersham Pharmacia, Piscataway, New Jersey).

2-2-5-2. Purification of cytosolic and nuclear extracts

Cells were washed twice with PBS and scraped into 1 ml of cold PBS, and pelleted by centrifugation (300 × g for 5 min). Cell pellets were resuspended in hypotonic buffer (10 mM HEPES/KOH, 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 1 mM DTT, and 0.5 mM PMSF, pH 7.9), and incubated on ice for 15 min. After vortexing for 10 s, and homogenates were centrifuged at 13,000 × g for 1 min. The supernatant was collected as cytosolic extract. The pellet was gently resuspended in 20 μl of ice cold dissolving buffer (50 mM HEPES/KOH, 50 mM KCl, 1 mM DTT, 300 mM NaCl, 0.1 mM EDTA, 10% glycerol, and 0.5 mM PMSF, pH 7.9), and centrifuged at 13,000 × g for 20 min at 4°C. The supernatant used as nuclear extract.

2-2-6. Reverse transcriptase PCR (RT-PCR) analysis

RAW 264.7 cells were lysed with TRIzol reagent in microcentrifuge tubes. Tubes were shaken vigorously for 15 s and

incubate the homogenized samples for 5 min at room temperature and then add 0.2 ml of chloroform per 1 ml of TRIzol reagent. Mixture was centrifuged at no more than 12,000 × g for 15 min at 2 to 8°C. The aqueous phase was transferred to a fresh tube and the RNA was precipitated by addition of 0.5 ml of isopropanol and centrifugation at no more than 12,000 × g for 15 min at 2 to 8°C. The supernatant was removed and the RNA pellet was washed once with 75% ethanol by shaking. After centrifugation at 7,500 × g for 5 min at 2 to 8°C, the RNA pellet was briefly dried and dissolved into DEPC treated water.

Three micrograms RNA were reverse-transcribed using M-MLV reverse transcriptase in a final volume of 25 μl. RT reactions were performed at 42°C, 50 min, and 70°C, 15 min, in a GeneAmp PCR system 2700 thermocycler (Applied Biosystems, USA). cDNA was amplified in 25 μl of PCR mixture containing 2.5 μl 10 × buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$), 2.5 mM dNTP, 0.5 μl of Taq DNA polymerase (5U/ μl), and 10 pmole of forward primer and reverse primer. PCR was performed using 30 cycles of 95°C for 30 s, 60°C for 30 sec and 72°C for 1 min. Values for each gene were normalized against the expression levels of GAPDH bands, and all experiments were repeated for triplicate. Primers used in this study were as follows **Table. 1**.

Table. 1. Primer sequences used in this study.

Target gene		Sequences
Mouse iNOS	Forward	5'-CACCTTGGAGTTCACCCAGT-3'
	Reverse	5'-ACCACTCGTACTTGGGATGC-3'
Mouse COX-2	Forward	5'-CCTAGGCTTCAGCCTCACAC-3'
	Reverse	5'-CAGCCTAATGTTCAGCGACA-3'
Mouse GAPDH	Forward	5'-TGGCACAGTCAAGGCTGAGA-3'
	Reverse	5'-CTTCTGAGTGGCAGTGATGG-3'

2-2-7. COX-2, iNOS, NF-κB or AP-1 promoter/luciferase assay

Briefly, 1 μg of murine COX-2 (Dr. H. Herschman, UCLA, CA), iNOS (Dr. C.J. Lowenstein, John's Hopkins School of Medicine, WA), NF-κB, or pAP-1 promoter/luciferase DNA (Stratagene) along with 20 ng of control pRL-TK DNA (Promega) was transiently transfected into 1.5×10^6 cells/well in a six-well plate using LipoFectamine/Plus reagents (Invitrogen) for 24 hr. Cells were treated with PFF-A in the absence or presence of LPS (1 μg/ml) for 6 hr. Cell lysates were prepared, and used for the measurement of luciferase activity using a luciferase assay kit (Promega). The luciferase activity was normalized with expression of control pRL-TK.

2-2-8. Statistical analysis

All data are expressed as the mean ± standard deviations (SDs). Data were analyzed using one-way ANOVA, followed by Student's t tests. Differences were considered significant of $P < 0.05$. All analyses were performed using SPSS for Windows, version 10.07 (SPSS, Chicago, IL).

3. Results and Discussion

3-1. Cell viability of PFF-A on RAW 264.7 cells

To examine the cell viability of PFF-A on RAW 264.7 cells, cells were cultured in 96-well plates at a density of 5×10^4 cells/well. Twenty four hours later, Vehicle or 1, 5, 10, 20 μM PFF-A were treated to cells in serum free DMEM medium. After 24 hr incubation, cell viability was determined by MTS assay. **Fig. 2** shows that there were not any decreases of cell viability in PFF-A treated RAW 264.7 cells. Furthermore, MTS assays demonstrated no cytotoxicity effect of PFF-A at the concentrations used on RAW 264.7 cells treated without or with LPS (1 μg/ml). This result suggests that there were no inhibitory effects of PFF-A at concentration applied in this study on RAW 264.7 cells proliferation.

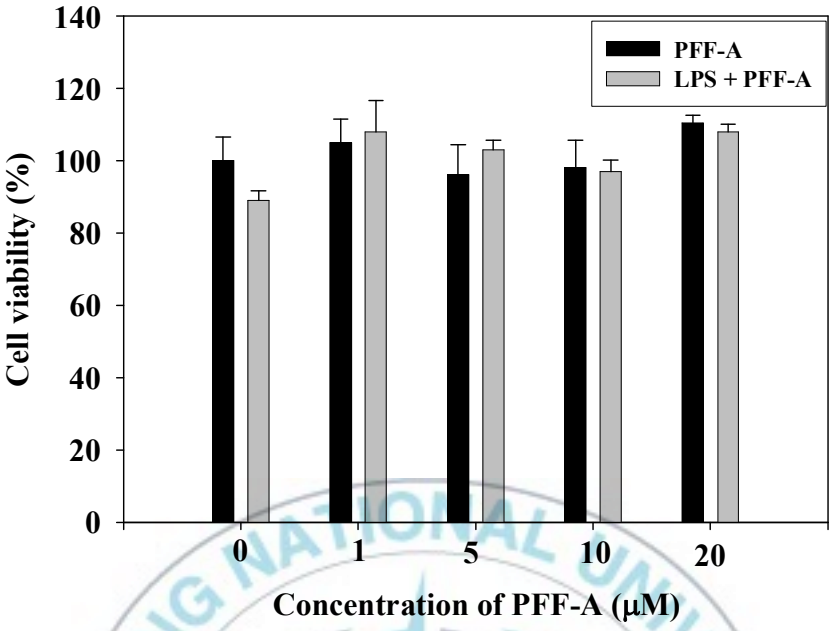


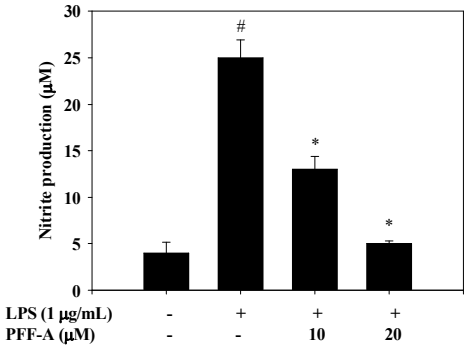
Fig. 2. Cytotoxic effect of PFF-A in RAW 264.7 cells. RAW 264.7 cells were incubated with PFF-A (0-20 μM) in the absence or presence of LPS (1 μg/ml) for 24 hr. Cell viability was measured by MTS assay. Data are presented as means ± SDs of three independent experiments.

3-2. Effect of PFF-A on LPS-induced NO and PGE_2 production

NO is synthesized from the amino acid arginine by nitric oxide synthase (NOS). Under the pathological conditions, NO production is increased by the inducible NOS (iNOS) and, subsequently, brings about cytotoxicity and tissue damage [33]. **Fig. 3A** shows the effect of PFF-A on nitrite production in LPS-stimulated RAW 264.7 cells. Cells were treated with LPS (1 μg/ml) in the presence or absence of the PFF-A for 24 hr. Both LPS and sample were not treated in control group. The NO production, measured as nitrite, increased remarkably up to 25.1±1.9 μM when 1 μg/ml LPS was added to RAW 264.7 cells, compared to 4.2±1.1 μM of the control group. The inhibition of NO production in RAW 264.7 cells resulted in a dose-dependent manner when cells were exposed to PFF-A in the concentration range of 0-20 μM.

PGE_2 is produced by macrophages and contributes to physiological responses such as vasodilatation, pain, and fever [34]. The effect of PFF-A on PGE_2 production in LPS-induced RAW 264.7 cells shown in **Fig. 3B**. Cells were treated with LPS (1 μg/ml) in the presence or absence of the PFF-A for 24 hr. After cell culture media were collected, PGE_2 levels were determined, and PFF-A was found to reduce PGE_2 production in a dose-dependent manner. This results suggest that PFF-A could suppress LPS-induced NO and PGE_2 production in LPS-stimulated RAW 264.7 cells.

(A)



(B)

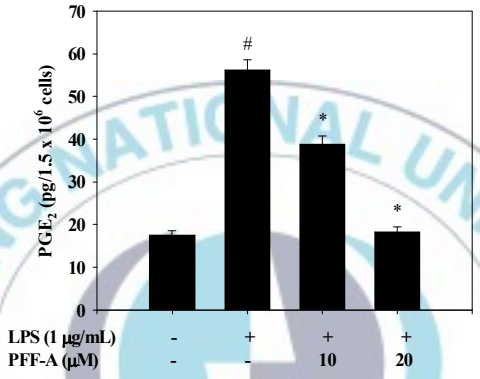


Fig. 3. Inhibition of nitrite (A) and PGE_2 (B) production by PFF-A in LPS-induced RAW 264.7 cells. RAW 264.7 cells were treated without or with LPS (1 μg/ml) in the absence or presence of PFF-A for 24 hr. The conditioned culture medium was used to measure the amount of nitrite, a stable NO metabolite, to evaluate NO production using 100 μl of Griess reagent (A). At the end of incubation time, 100 μl of culture medium was collected for the PGE_2 assay (B). Data are mean ± SDs of three independent experiments. $^{\#}P<0.05$ indicates significant differences from the control group. $^{*}P<0.05$ indicates significant differences from the LPS-treated group.

3-3. Effect of PFF-A on LPS-induced iNOS and COX-2 protein and mRNA expressions

Western blot and RT-PCR analyses were performed to determine whether the inhibitory effects of PFF-A on the pro-inflammatory mediators (NO and PGE_2) were related to the modulation of the expressions of iNOS and COX-2. RAW 264.7 cells were treated with LPS (1 μg/ml) in the presence or absence of the PFF-A for 16 hr. As shown in **Fig. 4A**, PFF-A strongly and concentration-dependently suppressed the protein levels of both iNOS and COX-2. These data suggest that translational events are involved in PFF-A inhibition of LPS-induced expressions of iNOS and COX-2.

Reverse transcription PCR analysis was done to investigate whether PFF-A suppressed LPS-mediated induction of iNOS and COX-2 via a pre-translational mechanism. Cells were treated without or with LPS (1 μg/ml) in the absence or presence of PFF-A for 6 hr. From the result of this experiment, we found that PFF-A has inhibitory activities on iNOS and COX-2 (GAPDH as control gene) gene expression in a dose-dependent manner in LPS-activated macrophages (**Fig. 4B**).

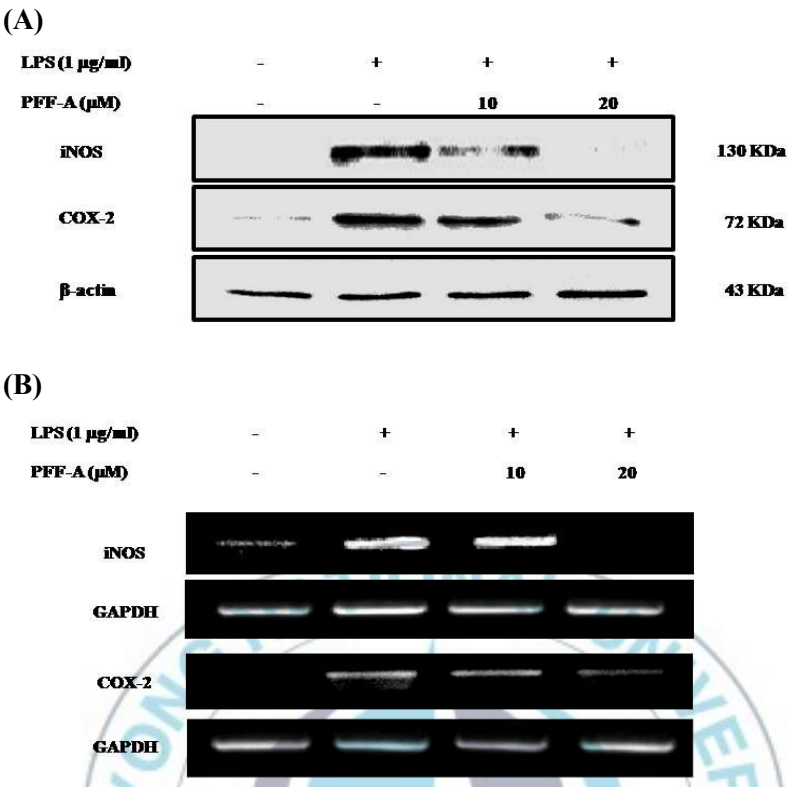
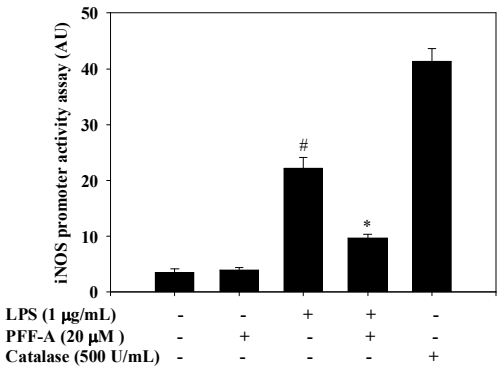


Fig. 4. Effects of PFF-A on LPS-induced iNOS and COX-2 protein (A) and mRNA (B) expressions in RAW 264.7 cells. (A) Cells were treated with LPS (1 μg/ml) with and without PFF-A for 16 hr. Equal amounts of total proteins were subjected to 10% SDS-PAGE. The expression of iNOS, COX-2 and β-actin protein was detected by Western Blot using specific antibodies. (B) Total RNA was prepared for RT-PCR of iNOS and COX-2 gene expression. Cells were treated without or with LPS (1 μg/ml) in the absence or presence of PFF-A for 6 hr. PCR using housekeeping gene GAPDH mRNA was carried out in parallel to confirm equivalency of cDNA preparation.

3-4. Suppressive effects of PFF-A on iNOS and COX-2 promoter activities in the LPS-stimulated RAW 264.7 cells

To better understand the PFF-A-mediated iNOS and COX-2 transcriptional down-regulations, we next investigated the effect of PFF-A on iNOS and COX-2 promoter activities in the LPS-treated RAW 264.7 cells. For this, RAW 264.7 cells were transiently transfected with a luciferase construct containing the murine iNOS or COX-2 promoter, and the transfected cells were then treated for 6 hr with 20 μM of PFF-A. Catalase (500 U/ml) was used as a positive control. Data of the luciferase assays clearly demonstrated that PFF-A (20 μM) largely inhibited the iNOS or COX-2 promoter-driven luciferase activity in the LPS-treated RAW 264.7 cells (**Fig. 5A and B**). This result suggests that the PFF-A-mediated down-regulation of iNOS and COX-2 expressions in the LPS-treated RAW 264.7 cells is due to transcriptional repression of iNOS and COX-2.

(A)



(B)

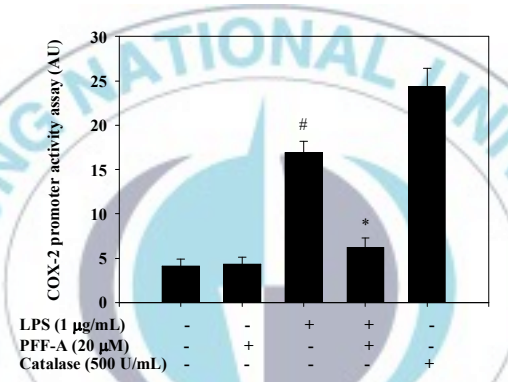


Fig. 5. Effects of PFF-A on the LPS-induced iNOS and COX-2 promoter driven luciferase expression in RAW 264.7 cells. RAW 264.7 cells were co-transfected with 1 μg of the murine iNOS (A) or COX-2 (B) promoter-containing luciferase DNA along with 20 ng of control pRL-TK DNA for 24 hr. Transfected cells were then treated without or with LPS in the absence or presence of PFF-A for 6 hr. Data are mean ± SDs of three independent experiments. $^{\#}P<0.05$ indicates significant differences from the control group. $^{*}P<0.05$ indicates significant differences from the LPS-treated group.

3-5. Effect of PFF-A on LPS-induced phosphorylation and degradation of IκB-α in RAW 264.7 cells

NF-κB is inactive in the cytosol via binding to IκB-α and IκB-α becomes active through phosphorylation of IκB-α and subsequent nuclear translocation of NF-κB preceded by LPS [35]. To examine whether PFF-A inhibits LPS-induced IκB-α phosphorylation, we prepared cytosolic extracts of RAW 264.7 cells and determined IκB-α levels by Western blotting. Moreover, to determine whether this IκB-α degradation is related with IκB-α phosphorylation, we examined the effect of PFF-A on LPS-induced p-IκB-α by Western blotting. As shown in **Fig. 6A**, IκB-α was phosphorylated after treatment with LPS in RAW 264.7 cells for 30 min, and this phosphorylation was markedly inhibited by pre-treatment with PFF-A (10, 20 μM) for 1 hr. These results suggest that PFF-A inhibited the NF-κB translocation to the nucleus through prevention of IκB-α phosphorylation.

3-6. Effect of PFF-A on LPS-induced translocation of NF-κB in RAW 264.7 cells

iNOS or COX-2 gene expression is primarily controlled at the transcriptional level. iNOS gene expression is regulated mainly at the transcriptional level in macrophages and its major transcriptional regulators are the NF-κB family of transcription factors, which are also key regulators of a variety of genes involved in immune and inflammatory responses [36]. Importantly, the NF-κB binding site has

been identified on the murine iNOS promoter and plays a role in the LPS-mediated induction of iNOS in RAW 264.7 cells [37]. In unstimulated cells, NF-κB is sequestered in the cytosol by its inhibitor, IκB. Upon LPS stimulation, IκB is phosphorylated by IκB kinases, ubiquitinated, and rapidly degraded via 26S proteosome, to release NF-κB [38]. We investigated the translocation of p65, a subunit of NF-κB, from the cytosol to the nucleus using Western blot analysis. Cells were treated with indicated concentrations of PFF-A for 1 hr and stimulated with LPS (1 μg/ml) for 30 min. PARP, a nuclear protein, and β-actin, a cytosolic protein, were used as controls to confirm that there was no contamination during extraction of each fraction. A large amount of p65 had translocated into the nucleus at 30 min in the absence of PFF-A, but PFF-A inhibited this translocation. In addition, LPS stimulation decreased cytosolic p65 levels, but PFF-A blocked this decrease (**Fig. 6B**). These results suggest that the inhibition of NF-κB translocation by PFF-A is due to the prevention of IκB-α degradation.

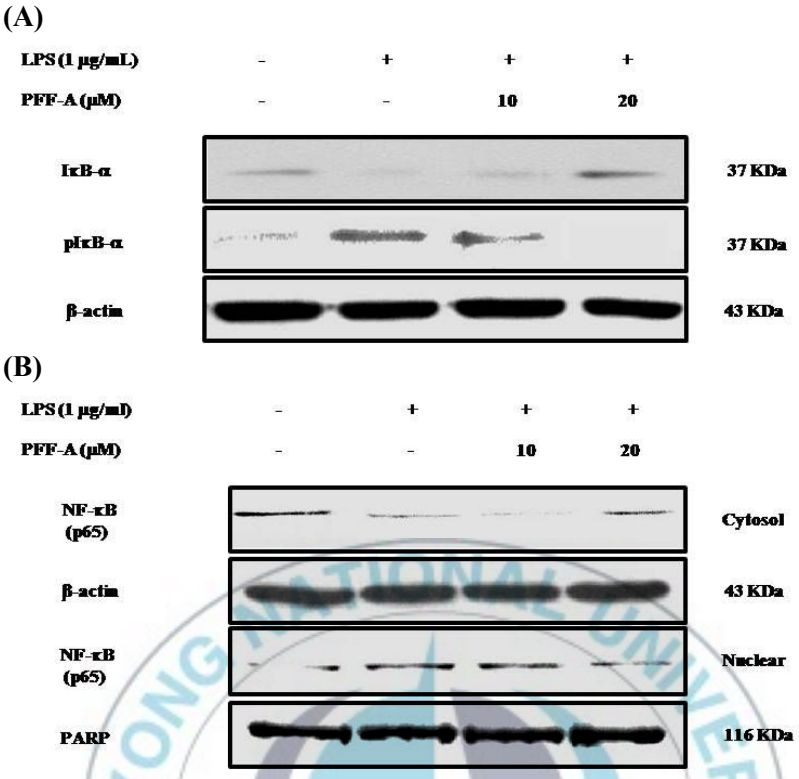


Fig. 6. Inhibition of LPS-inducible IκB-α phosphorylation (A) and NF-κB activation (B) by PFF-A in RAW 264.7 cells. (A) RAW 264.7 cells were incubated with various concentrations of PFF-A for 1 hr, and then stimulated with LPS (1 μg/ml) for 30 min. Cells were harvested and equal cytosolic extracts were analyzed by Western blotting with anti-IκB-α or anti-phospho-IκB-α antibody. (B) RAW 264.7 cells were preincubated for 1 hr with or without PFF-A at indicated concentrations and then stimulated for 30 min with LPS (1 μg/ml). Cytosolic extract and nuclear extract were isolated and investigated as to whether PFF-A inhibited NF-κB translocation.

3-7. PFF-A inhibits activation of intracellular signaling proteins, including Akt and MAPKs in the LPS-induced RAW 264.7 cells

LPS activates all three types of MAPKs (ERK1/2, JNK1/2 and p38 MAPK) in mouse macrophages [39]. The ERK1/2 pathway is activated by the dual-specific MAPK kinase (MAPKK) known as MEK-1/2 [40]. The activation of ERK1/2 is thought to be involved in LPS-induced macrophage responses, such as the increased production of pro-inflammatory cytokines and inducible nitric oxide synthase (iNOS) [41,42]. LPS stimulation of RAW 264.7 cells rapidly activates the JNK1/2 pathway [43]. p38 MAPK is activated by LPS stimulation and has been postulated to play an important role in the control of iNOS [41]. Thus, the activations of ERK1/2, JNK1/2 and p38 MAPK are used as a hallmark of LPS-induced signal transduction in RAW 264.7 cells. To further investigate the mechanisms of NF-κB inactivation and inhibition of NO production by PFF-A, the effects of PFF-A on the LPS-induced phosphorylation of ERK1/2, JNK1/2 and p38 MAPK were examined.

As shown in **Fig. 7**, though PFF-A treatment (20 μM) showed a mild inhibitory effect on the LPS-induced phosphorylation of ERK1/2 and JNK1/2 in RAW 264.7 cells, the same treatment strongly inhibited phosphorylation of Akt, a PI3K downstream effector, and p38 MAPK induced by LPS in the cells. Collectively, these findings suggest the ability of PFF-A to inhibit multiple intracellular signaling protein pathways triggered by LPS in macrophages.

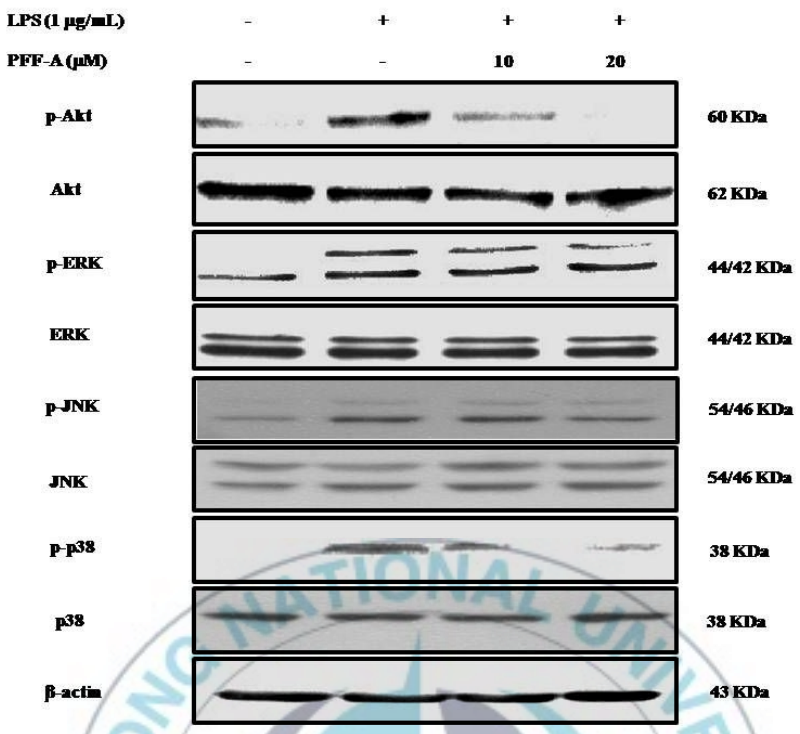
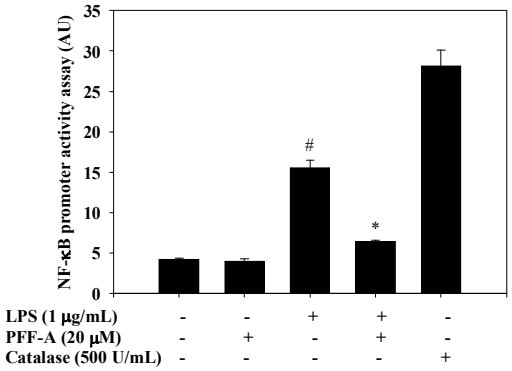


Fig. 7. Effect of PFF-A on LPS-induced phosphorylation of ERK1/2, JNK1/2, p38 MAP kinase, and Akt in RAW 264.7 cells. RAW 264.7 cells were treated with indicated concentrations of PFF-A for 1 hr and stimulated with LPS (1 μg/ml) for 30 min at 37°C. Whole cell lysates were prepared and analyzed by Western blotting for measuring the phosphorylation of Akt, ERK, JNK, or p38 MAPK using corresponding antibody. The membranes used for the phosphorylation of Akt, ERK, JNK, and p38 MAPK were stripped and reprobed for measuring total amount of Akt, ERK, JNK, and p38 MAPK, respectively, antibody.

3-8. PFF-A inhibits activities of NF-κB and AP-1 transcription factor

Activation of transcription factors, including NF-κB or AP-1, is critical for the transcriptional induction of COX-2 and iNOS [44,45]. AP-1 is another transcription factor that plays a role in the iNOS or COX-2 transcriptional induction [46,47]. AP-1 is composed of two subunits of transcription factors, c-fos and c-jun whose activations are influenced by ERKs and JNKs, respectively [48]. Considering that PFF-A showed inhibitory effects on the LPS-induced proteolytic degradation of IκB-α (an indicator of NF-κB activation) and activation of ERK1/2 and JNK1/2 (upstream activators of AP-1) in RAW 264.7 cells, we next determined the effect of PFF-A on activities of NF-κB and AP-1 in the LPS-treated RAW 264.7 cells. To this end, RAW 264.7 cells were transiently transfected with a luciferase construct containing the murine NF-κB or AP-1 promoter, and the transfected cells were then treated for 6 hr with PFF-A (20 μM). Catalase (500 U/ml) was used as a positive control. Data of the luciferase assays showed that while PFF-A treatment (20 μM) showed a mild suppressive effect on the LPS-induced AP-1 promoter-driven luciferase expression in RAW 264.7 cells (**Fig. 8B**), it largely inhibited the NF-κB promoter-driven luciferase expression by LPS (**Fig. 8A**). From these, it is likely that the PFF-A-mediated down-regulation of the LPS-induced iNOS and COX-2 expressions in RAW 264.7 cells is largely associated with the ability of PFF-A to inhibit NF-κB and AP-1 pathway. This is, to be to our best knowledge, the first report addressing the PFF-A's negative regulation of NF-κB and AP-1 pathway in response to LPS.

(A)



(B)

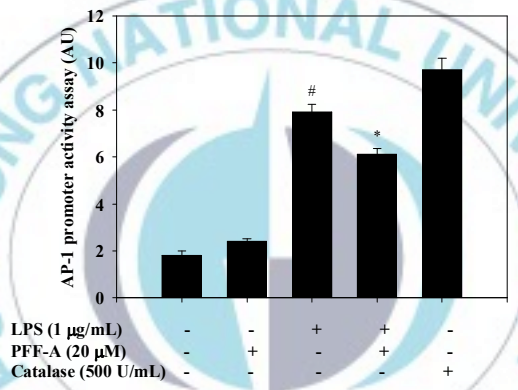


Fig. 8. Effects of PFF-A on NF-κB or AP-1 promoter driven luciferase expression. RAW 264.7 cells were co-transfected with 1 μg of NF-κB (A) or AP-1 (B) promoter-containing luciferase DNA along with 20 ng of control pRL-TK DNA for 24 hr. Transfected cells were prepared, and used for reporter gene activity. Data are mean ±SDs of three independent experiments. $^{\#}P<0.05$ indicates significant differences from the control group. $^{*}P<0.05$ indicates significant differences from the LPS-treated group.

3-9. PFF-A suppress ROS in the LPS-induced macrophage

The exposure of LPS into cells leads to increase in the amount of cellular ROS. Role of oxidative stress in activation of the redox-sensitive NF-κB and AP-1 [49,50] and the stress-activated protein kinases p38 MAPK and JNK1/2 [51], and in inflammation [52] has been reported. Macrophage activated by LPS generated the ROS and promotes the secretion of pro-inflammatory mediators, such as tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), PGs, and NO [53]. ROS play a key role in enhancing the inflammation through the activation of NF-κB and AP-1 transcription factors, and nuclear histone acetylation and deacetylation in various inflammatory diseases [49]. Such undesired effects of oxidative stress induce oxidative damage in biomolecules causing atherosclerosis, hypertension, diabetes, and cancer [54]. Compounds derived from botanic sources, such as phenolic compounds, have suppressed inflammatory protein and cytokine expression through the removal of ROS by antioxidant activities [55].

The antioxidant activity of PFF-A was determined in cellular systems to investigate whether PFF-A can affect the free radical-mediated oxidation in cellular systems or not. As shown in **Fig. 9**, the relative content of intracellular ROS level was decreased by PFF-A in LPS-stimulated RAW 264.7 cells. Treatment of cells with PFF-A at the concentration of 20 μM significantly inhibited LPS to induce ROS generation.

These results may collectively suggest that the PFF-A-mediated transcriptional down-regulation of iNOS and COX-2 in RAW 264.7 cells may be in part due to the PFF-A's antioxidative effect associated with

inhibition of NF-κB and p38 MAPK. An interesting finding of the present study is the ability of PFF-A to block activation of Akt, also called PKB, a downstream of PI3K, in response to LPS signal in RAW 264.7 cells.

In conclusion, PFF-A inhibits iNOS and COX-2 expressions at the transcriptional level in the LPS-treated RAW 264.7 cells and the inhibition seems to be associated with the ability of PFF-A to inactivate multiple cellular transcription factors and signaling proteins, including NF-κB, p38 MAPK and Akt, and to reduce cellular ROS (**Fig. 10**). Considering that NO and PGE_2 are key inflammatory mediators, the present findings suggest that PFF-A may be useful as an anti-inflammatory agent against inflammatory diseases in which high expressions (and activities) of COX-2 and iNOS are problematic.

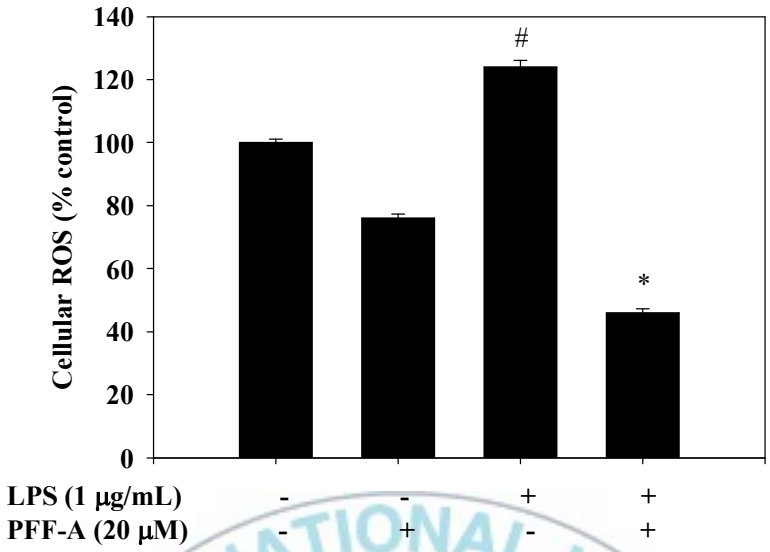


Fig. 9. Effect of PFF-A on LPS-induced ROS production in RAW 264.7 cells. The cells were incubated with 20 μM of PFF-A in the absence or presence of LPS (1 μg/ml) for 2 hr. Data are mean ± SDs of three independent experiments. $^{\#}P<0.05$ indicates significant differences from the control group. $^{*}P<0.05$ indicates significant differences from the LPS-treated group.

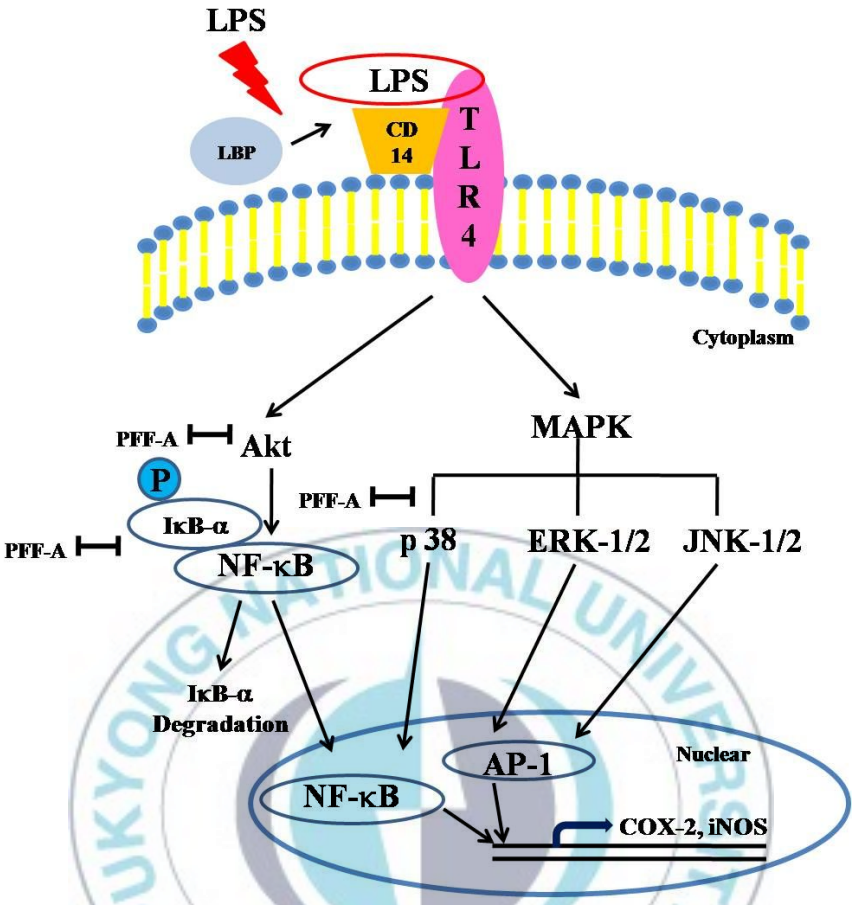


Fig. 10. Possible regulatory mechanisms of PFF-A in the NF-κB pathway.

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