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Research Article

NF-κB and MAPK-Targeted Anti-Inflammatory Activity of *Andrographis Paniculata* Extract

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Abstract

Andrographis paniculata (Burm.f.) is in the Acanthaceae family. There have been reports on the various medical effects of extracts of *A. paniculata* and the active ingredient, andrographolide (AG). Previous study confirmed that the standardized *A. paniculata* extract (APE, ParActin®) inhibited the expression of MMPs and inflammatory cytokines (IL-1β and IL-6) in monosodium iodoacetate (MIA)-induced cartilage degradation in rats, and relieved acetic acid-induced writhing responses in mice. To investigate the molecular actions of APE, we treated APE and AG, and examined the level of inflammatory mediators in LPS-treated RAW264.7 cells. APE and AG inhibited NO and PGE_2 production in LPS-treated RAW264.7 macrophages. APE inhibited MAPK pathways (p38, JNK, and ERK) and IκBα phosphorylation, and increased IκBα degradation. The results revealed that anti-inflammatory activity of APE were derived from AG by inhibition of MAPKs and NF-κB pathways.

Abbreviations: NO: Nitric Oxide; MAPK: Mitogen-Activated Protein Kinase; ERK: Extracellular Signal-Related Kinase; JNK: c-Jun N-Terminal Kinase; MMP: Matrix Metalloproteinase; iNOS: Inducible Nitric Oxide Synthase; COX: Cyclooxygenase; NF-κB: Nuclear Factor Kappa B; IκBα: Inhibitor Kappa B Alpha; IKK: I Kappa B Kinase; TLR: Toll-like Receptor

Introduction

Andrographis paniculata (Burm.f.) (synonym Justicia paniculata, common name "king of bitter") is in the Acanthaceae family. A lot of research has proven that extracts of *A. paniculata* display wide spectrum of pharmacological activity such as anti-cancer [1, 2], anti-diarrheal [1], anti-viral [1], anti-malarial [3], anti-oxidant [4], anti-inflammatory [5], hepatoprotective [6], cardiovascular [7], and immunostimulatory activities [8].

In a prospective, randomized, and placebo-controlled trial, *A. paniculata* composition, ParActin® showed some beneficial effects on osteoarthritis patient symptoms [9]. Furthermore, treatment with *A. paniculata* showed a reduction in the stiffness score, fatigue, and pain in RA patients [10]. In human osteoarthritic chondrocytes, andrographolide reduces matrix metalloproteinases (MMPs) and inducible nitric oxide synthase (iNOS) [11].

There have been reports on andrographolide's anti-inflammatory efficacy in several literatures. The major diterpenoid constituent of *A. paniculata*, andrographolide has received wide attention of many research groups for its diverse pharmacological properties by inhibition of NF-κB and MAPK pathways [12].

Previous study confirmed that the standardized *A. paniculata* extract (APE, ParActin®) inhibited the expression of MMPs and inflammatory cytokines (IL-1β and IL-6) in monosodium iodoacetate (MIA)-induced cartilage degradation in rats, and relieved acetic acid-induced writhing responses in mice [13]. Through this study, the anti-inflammatory mechanisms of APE using LPS-treated RAW264.7 cells will be discussed further.

Materials and Methods

Materials

High Performance Liquid Chromatography (HPLC) grade methanol and distilled water (DW) used Samchun Chemical (Incheon, Korea). Analytical standard of andrographolide and LPS (*E. coli* 0111:B4) were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Fetal bovine serum (FBS) was obtained from Gibco (Grand Island, NY, USA). DMEM was purchased from Hyclone (Logan, UT, USA). RAW264.7 cells were purchased from ATCC (Rockville, MD, USA). All other chemicals were of analytical grade. Antibodies specific for either the total or phosphorylated forms of extracellular signal-related kinase (ERK), c-Jun N-terminal kinase (JNK), p38, Inhibitor kappa B alpha (IκBα) and β-actin were obtained from either Cell Signaling Technologies (Beverly, MA, USA) or Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Preparation of APE

The leaf extracts of *Andrographis paniculata* Nees. (Acanthaceae) used in the experiment were purchased from HP Ingredients (Bradenton, USA). The dried leaf of *A. paniculata* was extracted in 75% ethanol solution. The extract was filtered and concentrated under decreased pressure, then, it was spray dried to yield a powder. HPLC result showed that level of andrographolide content was 53% in APE (ParActin®).

HPLC Analysis of APE

The chromatographic quantitative analysis, used for APE, was a HPLC analysis system, equipped with a 1200 series HPLC system (Agilent, Palo Alto, USA), as previously described [13]. The chromatic separation was accomplished on C_{18} column, 4.6 × 150 mm, 5 μm (Shiseido capcell pak UG120, Shiseido, Yokohama, Japan) at 35°C. The mobile phase composition was 60% of ethanol and 40% of deionized water which was set in isocratic mode and flow rate was 1 mL/min. An aliquot of 10 μL of the sample was injected and the effluent was monitored at 225 nm and the total run time was fixed as 30 min. Data was obtained and analyzed by Agilent Chemstation Software. A total of 1.5 mg of standard compounds was dissolved in 1 mL of methanol, and then diluted to concentrations (μg/mL) of 12, 23, 47, 94, 188 and 375 each. The obtained coefficients of correlation were 1.000 (y = 12.253x + 15.808). The contents of were quantified by comparing with the standard curve. The 100 and 600 mg of APE were dissolved in 10 mL of DW and dimethyl sulfoxide (DMSO) respectively, and then sonication for 20 min. Additionally, each sample was filtered with a 0.45 μm syringe filter (Waters Corp., Milford, USA). Each sample was diluted for HPLC analysis with DW (from 60,000 to 6,000 ppm) and DMSO (from 10,000 to 100 ppm).

Measurement of NO and PGE_2

RAW264.7 cells seeded at 96 well, 1 × 10^6/ml, were incubated at 37°C, and 5% CO_2 for 24 h. Cells were treated with different concentrations of APE or AG (0.5–16 μg/mL) and LPS (1 μg/mL) and incubated for 24 h. The inhibitory effect of APE on NO and PGE_2 production was determined by analysis of NO and PGE_2, using Griess reagent and an enzyme-linked immunosorbent assay kit (R&D systems, Minneapolis, MN, USA), as described previously with some modification [14].

Cytotoxicity

The cytotoxic effects of APE or AG were then evaluated using a conventional MTT assay, as reported previously with some modification [14]. After 24 h of seeding, 5 mg/mL MTT solution was added to RAW264.7 cells and kept at 37°C, 5% CO_2 for 1 h. The supernatant was removed, 200 μL of DMSO was added, and the absorbance was measured at 550 nm.

Western Blot Analysis

The protein expression of the total or phosphorylated forms of p38, ERK, JNK, IκBα, and β-actin was analyzed through Western blot analysis. RAW264.7 cells seeded at 6 well, 1 × 10^6/ml, were incubated at 37°C, and 5% CO_2 for 24 h. Cells were treated with different concentrations of APE, 2 μg/mL and LPS 1 μg/mL, and incubated for indicated time. The intracellular protein was extracted by RIPA buffer. The protein concentration is measured by bradford assay. The extracted protein was denatured by LDS sample buffer (Thermo Scientific, Massachusetts, USA).

Equal amounts of protein samples were loaded onto SDS-polyacrylamide gel for electrophoresis and the isolated proteins were transferred to PVDF membranes with the Semidry Transfer Cell (Bio-Rad Laboratories, Inc., California, USA) for 7 min at 25 V. Membranes were incubated at room temperature with 3% BSA in TBS-T for 1 h to block non-specific antibody binding. After that, membrane was washed three times with TBS-T and primary antibodies (p38, ERK, JNK, IκBα, and β-actin) were applied to react for 15 h at 4°C. The antibodies were purchased from Cell signaling technology, and Santa Cruz Biotechnology, Inc. The membrane was probed using secondary antibody at room temperature for 1 h and then reacted with D-Plus™ ECL Femto System (Dongin, Seoul, Korea) solution. The Western blot image was identified with LAS-3000 (Fujifilm, Tokyo, Japan). The blot bend densities show mean ± SEM values of three different independent experiments [15].

Statistics

The statistical significance of differences between the control and experimental groups was performed using Student's *t*-test. The

significance was verified at $P < 0.05$ level, and measurements were indicated as mean ± standard error of the mean.

Results and Discussion

Analysis of andrographolide in DW, DMSO solution of the APE and AG standard by HPLC

As shown in Figure 1, it was confirmed that the 6,000 ppm DW and 100 ppm DMSO solution of the APE contained 17.2 and 58.0 ppm AG, respectively. As in a previous study [16], The low solubility of AG on the water is a major limitation of research in cell culture. This limitation shows that the use of organic solvents such as ethanol and DMSO can be an alternative to AG dissolution. DMSO solution of APE was 3.4 times higher in AG content than DW solution. This result suggests that DMSO solution was the optimal extraction solvent. Since APE contains about 50% of AG content, the experiment was carried out by preparing the concentration of APE (1 – 16 μg/mL) and AG (0.5 - 8 μg/mL) to be twice.

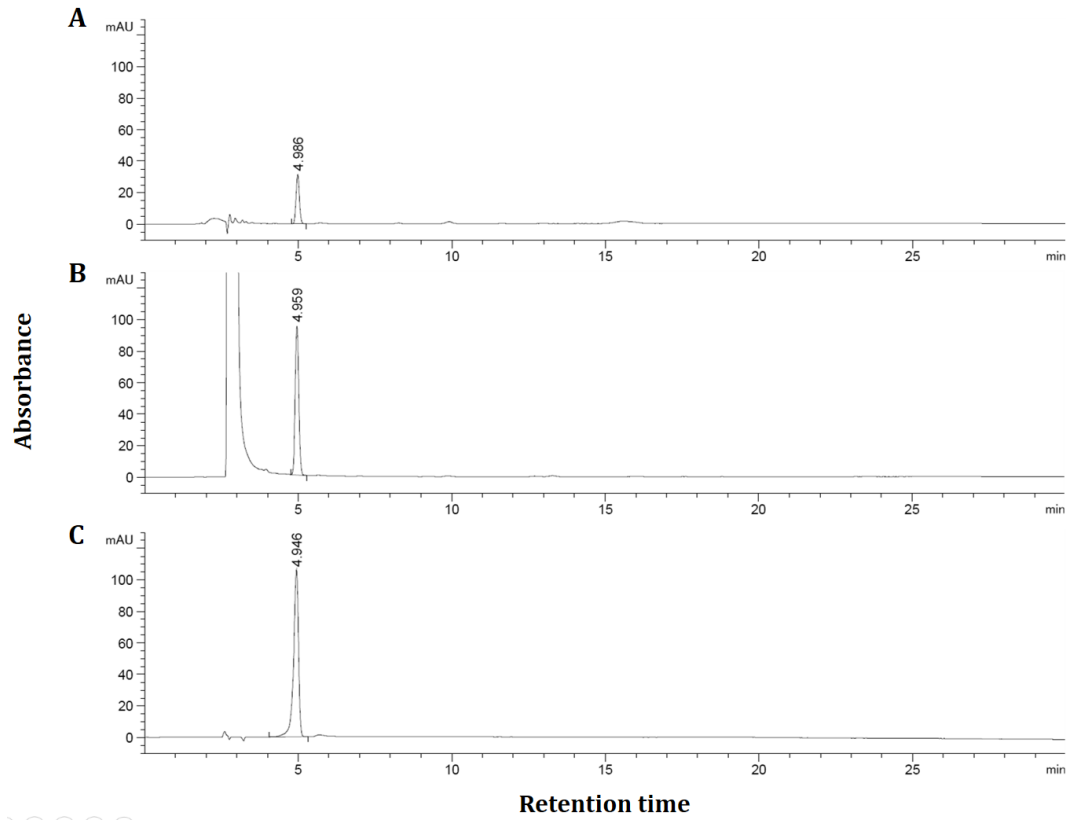

Figure1: HPLC chromatogram of APE re-dissolved in (A)DW, (B) DMSO, (C) standard andrographolide.

Production of Nitric oxide in Macrophages

Nitric oxide (NO) is an important regulatory and effector molecule with various biological functions. [17]. Once iNOS is induced, it produces large amount of NO that profoundly influences and damages cell and tissue function. Large quantity of NO produced by LPS stimulation might play a critical role in LPS-induced tissue damage. NO is a pivotal inflammatory factor which contributes to OA progression by modulation of pro-inflammatory or pro-catabolic factors, including Nos2, Il1b, Mmp13 and Ptgs2. OA chondrocytes overexpress iNOS and its product, NO, which has been considered as a biomarker for OA [18].

Previous studies have shown that AG inhibits NO production in various *in vitro* and *in vivo* models [18,19]. In LPS-induced RAW264.7 cells, APE suppressed the level of NO. APE dissolved in DMSO at different concentrations, the NO production of LPS-induced RAW264.7 cells was dose-dependently decreased by APE at 1-16 μg/mL (IC_{50} 5.3 μg/mL) (Figure 2A). In LPS-induced RAW264.7 cells, AG suppressed the level of NO in a dose-dependent manner (IC_{50} 3.1 μg /mL) (Figure 2B). This is because the content of AG contained in APE is 50%, which is similar to IC_{50}, so AG is predicted to be a major active functional ingredient showing the anti-inflammatory effect of APE. The results of this study are consistent with the results of a study in which AG inhibited iNOS gene expression and NO production in a LPS-induced RAW264.7 cells [20,21].

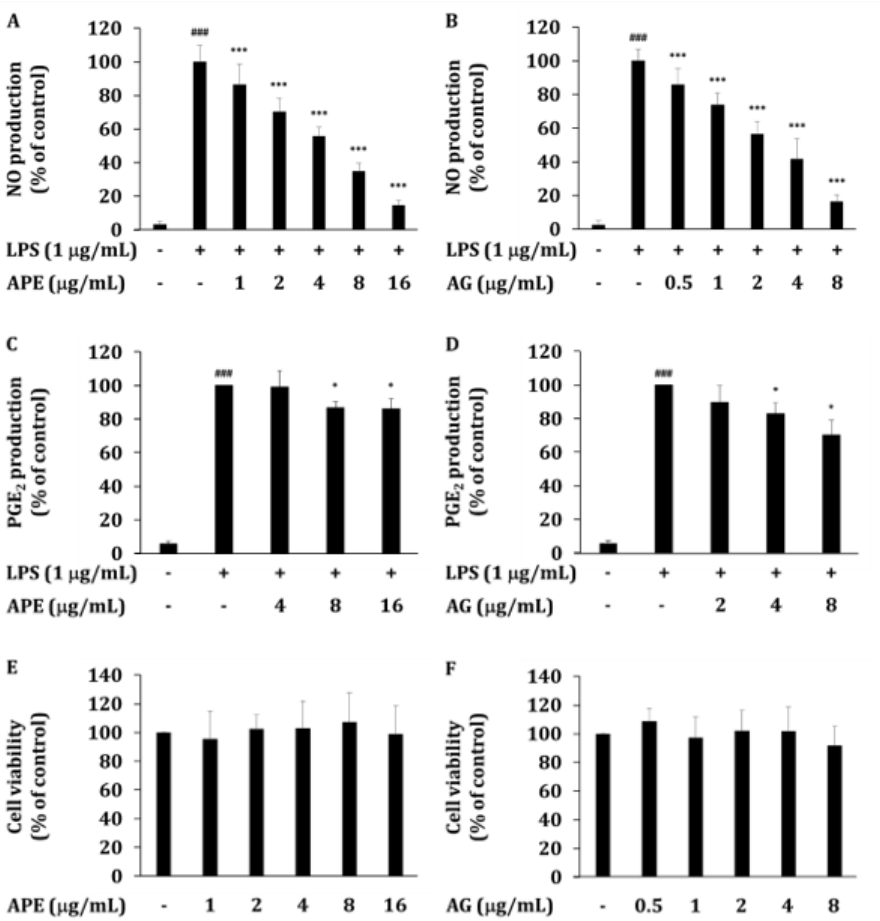

Figure 2: The inhibitory effect of APE or AG on production of inflammatory mediators. (A, B) Levels of NO, and (C, D) PGE_2 were determined in culture supernatants of RAW264.7 cells treated with APE or AG with LPS (1μg/mL) for 24 h. (E, F) The viability of RAW264.7 cells was determined by MTT assay. Data are presented as the mean ± SD of an experiment done with 6 biological replicates (n=6) per treatment. $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$ compared to LPS treated group. $###P < 0.001$ compared to LPS non-treated group.

Our previous study [13], the efficacy was also confirmed in the results of suppressing iNOS expression and NO production in the LPS induction model using APE - water soluble fraction dissolved in water. Through this, it is expected that the inhibitory effect of APE on NO production in this study was revealed by AG, which is contained in APE, suppressing the gene expression of iNOS. Also, inhibition of NO production by AG was confirmed. This result suggested that APE could be a useful complement in the treatment of arthritis symptoms [9,10].

Production of PGE_2 in Macrophages

PGE_2 is synthesized from arachidonic acid by COX-2 and overexpressed after being stimulated by LPS, resulting in sepsis-related inflammatory symptoms and signs [22]. In addition, PGE_2 has been reported to play a role in releasing pro-inflammatory cytokines, such as TNF-α and IL-6 in many cells [23]. Pain is perceived through activation of endings of nociceptive afferent nerves by pain-producing substances, released from tissue, sensitized by locally produced prostaglandins [24]. Because AG has been reported to have an inhibitory effect on PGE_2 production [5,19,20], PGE_2 expression in LPS-induced RAW264.7 cells was observed.

In LPS-induced RAW264.7 cells, APE suppressed the level of PGE_2. The PGE_2 production of LPS-induced RAW264.7 cells was dose-dependently decreased by APE at 4-16 μg/mL (Figure 2C). AG prepared with the concentration of AG contained in APE showed an equivalent inhibitory effect in LPS-induced RAW264.7 cells (Figure 2D).

Our previous study [13], APE administration resulted the analgesic effects on acetic acid-induced writhing responses. This result shows the analgesic effects of APE against peripheral PGE_2 synthesis by AG. It was confirmed that APE inhibited PGE_2 production by AG. This result suggested that APE could be a useful complement in the treatment of arthritis symptoms [9,10].

Activation of MAPKs in Macrophages

LPS treatment in macrophages is well known to activate the MAPK pathway through TLR4. MAPKs regulate the production of

pro-inflammatory cytokines and enzymes such as IL-1β, TNF-α, and IL-6, iNOS COX-2. Previous studies have shown that AG inhibits NO, PGE_2 production and MAPKs activity in various *in vitro*/*in vivo* inflammation models [5].

Because the production of NO and PGE_2 were mainly regulated by MAPKs, we investigated the possible involvement of p38, ERK, and JNK in APE inhibition. Before extracting whole lysates, NO concentration was measured and NO decreases by about 90% after 24 h of APE 2 μg/mL treatment in 6 wells (data not shown). To determine the inhibitory effects of APE on LPS-activated MAPKs activities, total lysates prepared from RAW264.7 cells treated with LPS for each 15, 30, and 45 min were incubated with APE. Phosphorylation of p38 by LPS was significantly inhibited by 74% (P<0.01) or 93% (P<0.001) at concentrations of 2 μg/mL of APE for 15 min and 30 min, respectively (Figure 3A-B). In addition, phosphorylation of ERK was significantly inhibited by 9% (P<0.01) and 64% (P<0.05) at 30 and 45 min treatment, respectively (Figure 3C). Finally, the phosphorylation of JNK was significantly inhibited by 61% (p<0.001) at 45 min treatment (Figure 3D). These results showed a similar pattern to the results of inhibiting the phosphorylation of MAPKs, which was treated with LPS for 15 mins when AG was pretreated at concentrations of 1 μM and 10 μM [21].

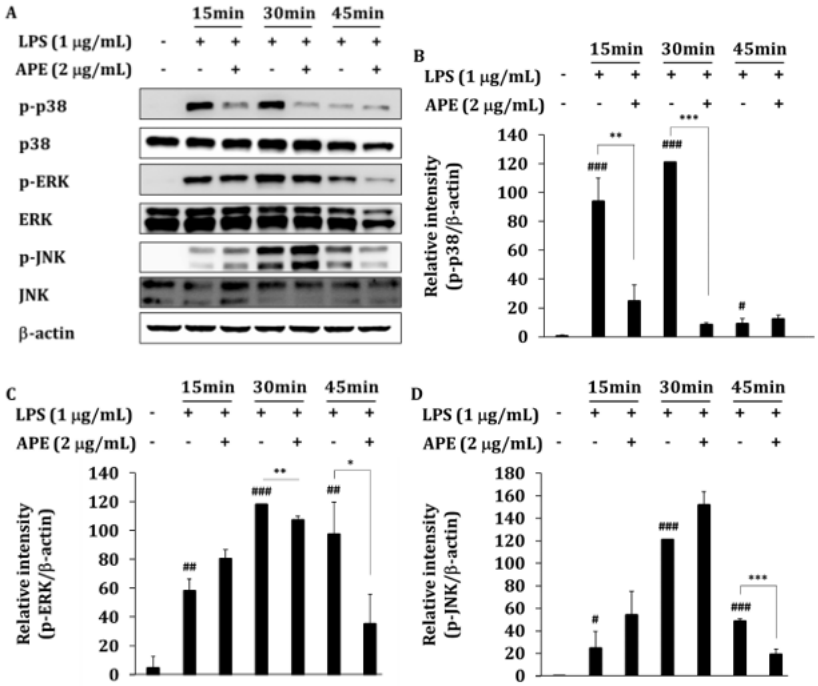

Figure 3: The inhibitory effect of APE on MAPKs activation. (A) RAW264.7 cells (1 × 10^6 cells/mL) were incubated with APE in the absence or presence of LPS (1μg/mL) for the indicated times. After preparing whole cell lysates, levels of total or phospho-forms of upstream signaling enzymes (ERK, p38, and JNK) were identified by immunoblotting. Other data are representative images of three different experiments that had similar results. (B-D) The blot bend densities show mean ± SEM values of three different independent experiments. $*P$ < 0.05, $**P$ < 0.01 and $***P$ < 0.001 APE treated vs. APE non-treated LPS treated group. #P < 0.05, ##P < 0.01 and ###P < 0.001 compared to LPS non-treated group.

Through this, it was confirmed that the extract of APE inhibits the activation of the MAPK pathway. In our previous study [13], APE inhibited the expression of iNOS, COX-2 in an *in vitro* LPS model, and pro-inflammatory cytokines IL-1β, TNF-α, and IL-6 in the blood in rat osteoarthritis (OA) model. Therefore, the MAPK mechanism affects joint damage and bone damage caused by joint inflammation, so it is expected to have a pain relief effect in the arthritis clinical trial of APE [9,10].

Activation of NF-κB in Macrophages

The transcription factor NF-κB is inactivated in the form of a complex with the inhibitory protein IκBα and is present in the cytoplasm. LPS inflammatory stimulation activates I kappa B kinase (IKK) and thereby phosphorylates IκBα. Phosphorylation of IκBα induces ubiquitination, thereby promoting its degradation by the proteasome complex. After decomposition of IκBα, NF-κB migrates to the nucleus, increasing the expression of inflammatory mediator genes such as iNOS and COX-2 and pro-inflammatory cytokines, IL-1β, IL-6, and TNF-α [25]. MMPs are synthesized and secreted by chondrocytes in response to stimulants including IL-1 and TNF. Most evidence suggests that matrix degradation is achieved through the action of MMPs (MMP-1, 3, 8, 13). In OA, the degradation of extracellular matrix is driven mainly by these enzymes from the chondrocytes [26].

Previous report has shown that AG inhibited NF-κB and the production of MMP-1, 3, 13 in IL-1β-treated cartilage-derived chondrocytes of osteoarthritis patients [11]. Similarly to this report, several kinds of *A. paniculata* extracts also inhibited NF-κB pathway in *in vivo* models [27, 28].

To determine the inhibitory effects of APE on LPS-activated NF-κB activities, total lysates prepared from RAW264.7 cells treated with LPS for 15, 30, and 45 min were incubated with APE (Figure 4A). The phosphorylation of IκBα significantly inhibited by 96% (P<0.01) at 15 min, and 35% (P<0.05) at 45 min after exposure of the cells to APE (Figure 4B). Due to the suppression of the phosphorylation of IκBα, the degradation of IκBα was suppressed at 30 min. The amount of IκBα proteins increased significantly by 131% (p<0.05) (Figure 4C).

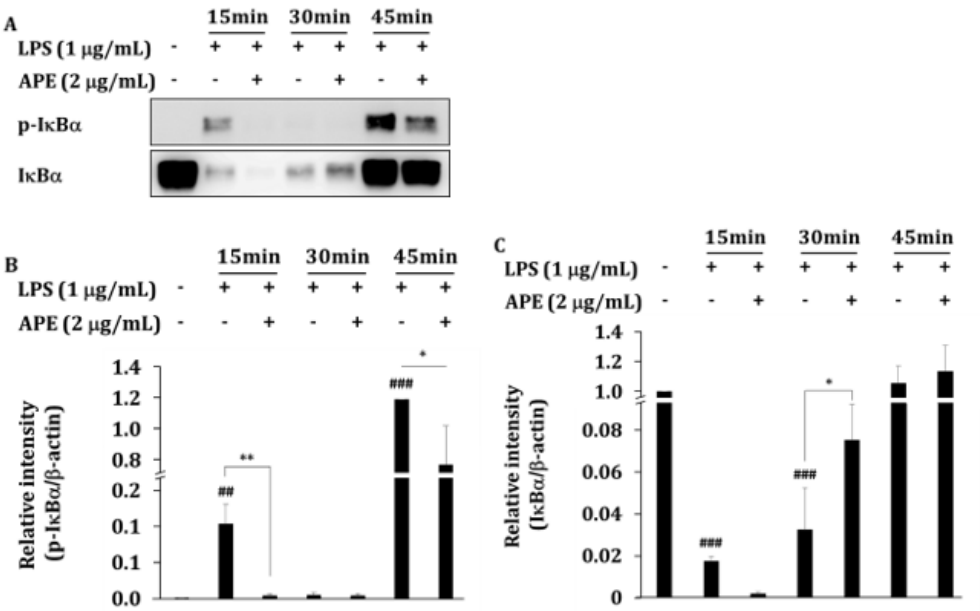

Figure 4: The inhibitory effect of APE on IκBα activation. (A) RAW264.7 cells (1 × 10^6 cells/mL) were incubated with APE in the absence or presence of LPS (1 μg/mL) for the indicated times. After preparing whole cell lysates, levels of total or phospho-form of upstream signalling enzyme, IκBα was identified by immunoblotting. Other data are representative images of three different experiments that had similar results. (B, C) The blot bend densities show mean ± SEM values of three different independent experiments. ∗P < 0.05, ∗∗P < 0.01 APE treated vs. APE non-treated control group. ##P < 0.01 and ###P < 0.001 compared to LPS non-treated group.

The results are similar to the previous study showing that phosphorylation of IκBα was significantly inhibited when 30 μg/mL of *A. paniculata* methanol extract was pretreated for 1 h and then treated with LPS for 15 min [29]. In this study, APE 2 μg/mL was treated at the same time as LPS, and the same effect was shown after 15 min.

In this study, it was confirmed by Western blot that phosphorylation of IκBα decreased and the IκBα protein was degraded by APE. Results are consistent with a mechanism of APE that inhibits the production of IL-1β, IL-6, and TNF-α, pro-inflammatory cytokines produced by NF-κB gene expression in rat osteoarthritis models and *in vitro* LPS models. These results point to APE as the anti-inflammatory ingredient that inhibits the expression of iNOS and COX-2 genes [13].

In addition to APE, it supports the effect of protecting joint cartilage by inhibiting MMP-1, 3, 8, 13 in rat osteoarthritis model. Therefore, it affects joint damage and joint cartilage decomposition due to joint inflammation through the NF-κB mechanism. It is expected to alleviate arthritis symptoms in the arthritis clinical trial [9,10].

Conclusion

The study demonstrated that APE suppresses *in vitro* inflammatory responses in LPS-TLR4-activated macrophages. APE inhibited the expression of NO and PGE_2 increased by LPS in a dose-dependent manner. APE acts as the inhibitor of MAPKs (p38, ERK and JNK) and IκBα which is particularly important in iNOS/COX-2 expression. AG-containing *Andrographis paniculata* has been ethnopharmacologically used for a long time and is orally available [18,24]. Based on the results of the previous clinical trials and toxicity tests on *A. paniculata*, it is expected to be used as a new anti-inflammatory material as a substitute for various inflammatory disease treatments, such as osteoarthritis.

Conflict of Interest

The authors declare no conflict of interest.

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