Korean J. Plant Res. 27(3):209-214(2014)
http://dx.doi.org/10.7732/kjpr.2014.27.3.209

Print ISSN 1226-3591
Online ISSN 2287-8203

Original Research Article

Anti-Inflammatory Effect of the Extracts from *Abeliophyllum distichum* Nakai in LPS-Stimulated RAW264.7 Cells

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Abstract - In this study, we investigated whether *A. distichum* decreases the production of inflammatory mediators through downregulation of the NF-κB and ERK pathway. Our data indicated that *A. distichum* leaf inhibits the overexpression of iNOS in protein and mRNA levels, and subsequently blocked LPS-mediated NO overproduction in RAW264.7 cells. *A. distichum* leaf inhibited IκB-α degradation and p65 nuclear translocation, and subsequently suppressed transcriptional activity of NF-κB in LPS-stimulated RAW264.7 cells. In addition, *A. distichum* leaf suppressed LPS-induced ERK1/2 activation by decreasing phosphorylation of ERK1/2. These findings suggest that *A. distichum* leaf shows anti-inflammatory activities through suppressing ERK-mediated NF-κB activation in mouse macrophage.

Key words - *Abeliophyllum distichum* Nakai, Anti-inflammation, ERK1/2, NF-κB

Introduction

Inflammation including acute and chronic inflammation presents double-edged sword. In other words, acute inflammation exerts beneficial effects on the maintenance of homeostasis. However, chronic inflammation by uncontrolled active immune cells under certain circumstances can induce carcinogenesis. For example, inflammation-induced oxidative stress induces the mutation of genetic and epigenetic aberrations, which finally leads to the malignant transformation of normal cells into cancer cells (Mannick *et al.*, 1996; Zhang et al., 2013). Under inflammatory response, macrophages produce excess amounts of mediators such as nitric oxide (NO) and pro-inflammatory cytokines (Laskin and Laskin, 2001; Lee *et al.*, 2005). NO has been reported to play a key role in the various forms of inflammation and carcinogenesis (Kroncke *et al.*, 1998; Ohshima and Bartsch, 1994). In addition, there is growing evidence that NO overproduction can induce various harmful responses including apoptosis and necrosis as well as organ failure in autoimmune diseases (Kleemann *et al.*, 1993; Nagai *et al.*, 2003). Thus, NO has been regarded as a ubiquitous mediator of a wide range of inflammatory conditions and reflects degree of inflammation (Kwon *et al.*, 2010), which indicates that NO is a major target for the treatment of inflammation-induced human diseases such as cancer, atherosclerosis, arthritis and septic shock.

Although non-steroidal anti-inflammatory drugs (NSAIDs) have been broadly used for treating inflammation-related diseases, the long-term administration of NSAIDs can induces various and severe adverse effects (Bjarnason *et al.*, 1993). Therefore, plant-derived natural compounds have been targeted for the treatment of various inflammatory diseases due to high effectiveness and very few side effects.

Abeliophyllum distichum Nakai (*A. distichum*) as the deciduous shrub is a monotypic taxon of Pleaceae and endemic to Korea (Ahn and Park, 2013). Recently, the flower of *A. distichum* has a protective effect on oxidative DNA damage via its anti-oxidant activity (Ahn and Park, 2013). However, the biological mechanisms for anti-inflammatory effect of *A. distichum* have not been studied. Here, for the first time we report that A. distichum reduces inflammatory

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activation through inhibiting ERK-mediated NF-κB activation and subsequently down-regulating iNOS-mediated NO production in mouse macrophage.

Materials and Methods

Materials

Cell culture media, Dulbecco's Modified Eagle medium (DMEM) was purchased from Gibco Inc. (NY, USA). LPS (Escherichia coli 055:B5) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibody against iNOS was purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA, USA). Other antibodies against phospho- IκB-α, IκB-α, p65, ERK1/2, phospho-ERK1/2 (Thr202/Tyr204) and β-actin were purchased from Cell Signaling (Bervely, MA, USA). All chemicals were purchased from Sigma-Aldrich, unless otherwise specified.

Sample preparation

The plant parts of *A. distichum* including flower, leaf and branch were collected at Jangyyeon-myeon Goesan-gun Korea. Five hundred grams of fresh parts was extracted with 1,000 mL of 80% methanol with shaking for 24 h. The methanol-soluble fraction was filtered and concentrated to approximately 20 mL volume using a vacuum evaporator and a fraction was placed in a separating funnel. The ethyl acetate fraction was separated from the mixture, evaporated by a vacuum evaporator and prepared aseptically and kept in a refrigerator until use.

Cell culture and treatment

Mouse macrophage cell line, RAW264.7 cells was purchased from American Type Culture Collection (Manassas, VA, USA) and grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells were maintained 37℃ under a humidified atmosphere of 5% CO_2. Ethyl acetate fraction from *A. distichum* (EAFAD) was dissolved in dimethyl sulfoxide (DMSO) and then treated to cells. DMSO was used as a vehicle and the final DMSO concentration was no exceeded 0.1% (v/v).

Measurement of nitric oxide (NO)

RAW264.7 cells were plated in 12-well plate for overnight. Cells were pre-treated with the extracts at the indicated concentrations for 2 h and then co-treat with LPS (1 μg/mL) for the additional 18 h. After 18 h, 200 μL of the media was mixed with equal amount of Griess reagent (1% sulfanilamide and 0.1% N-1-(naphthyl) ethylenediamine-diHCl in 2.5% H_3PO_4). The mixture was incubated for the additional 5 min at the room temperature and the absorbance was measured at 540 nm.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was prepared using a RNeasy Mini Kit (Qiagen, Valencia, CA, USA) and total RNA (1 μg) was reverse-transcribed using a Verso cDNA Kit (Thermo Scientific, Pittsburgh, PA, USA) according to the manufacturer's protocol for cDNA synthesis. PCR was carried out using PCR Master Mix Kit (Promega, Madison, WI, USA) with primers for mouse iNOS and mouse GAPDH as follows: mouse iNOS: forward 5'-gtgctgcctctggtcttgcaagc-3' , and reverse 5'-agggg caggctgggaattcg-3' , mouse GAPDH: forward 5'-caggagcgag accccactaacat-3' and reverse 5'-gtcagatccacgacggacacatt-3'.

Isolation and cytosol and nucleus fraction

Nuclear and cytosolic fractions were prepared following the manufacturer's protocols of nuclear extract kit (Active Motif, Carlsbad, CA, USA). Briefly, RAW264.7 cells were washed with ice-cold PBS containing phosphatase inhibitors and harvested with 1xhypotonic buffer for 15 min at 4℃. After adding detergent, the cells were centrifuged at 15,000 rpm for 30 min and then the supernatants were collected as cytoplasmic fraction. Nuclear fractions were collected by suspending nuclear pellet with lysis buffer and centrifugation.

SDS-PAGE and Western blot

Cells were washed with 1 × phosphate-buffered saline (PBS), and lysed in radioimmunoprecipitation assay (RIPA) buffer (Boston Bio Products, Ashland, MA, USA) supplemented with protease inhibitor cocktail (Sigma Aldrich) and phosphatase inhibitor cocktail (Sigma Aldrich), and centrifuged at 12,000 × g for 10 min at 4℃. Protein concentration was determined by the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA) using bovine serum albumin (BSA) as the standard. The proteins were separated on SDS-PAGE and

transferred to PVDF membrane (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The membranes were blocked for non-specific binding with 5% nonfat dry milk in Tris-buffered saline containing 0.05% Tween 20 (TBS-T) for 1h at room temperature and then incubated with specific primary antibodies in 5% nonfat dry milk at 4℃ overnight. After three washes with TBS-T, the blots were incubated with horse radish peroxidase (HRP)-conjugated immunoglobulin G (IgG) for 1 h at room temperature and chemiluminescence was detected with ECL Western blotting substrate (Amersham Biosciences) and visualized in Polaroid film.

Transient transfection

Transient transfection was performed using PolyJet DNA transfection reagent (SignaGen Laboratories, Ijamsville, MD, USA) according to the manufacturer's instruction. Briefly, RAW264.7 cells were seeded in 12-well plates and incubated overnight. Then, plasmid mixtures containing 1 μg of pNF-κB-Luc plasmid and 0.1 μg of pRL-null vector were transfected for 24 h. After transfection, cells were pre-treated with the extracts for 2 h and then co-treated with LPS for an additional 15 h. The cells were harvested in 1 x luciferase lysis buffer, and luciferase activity was measured and normalized to the *pRL*-null luciferase activity using a dual-luciferase assay kit (Promega, Madison, WI, USA).

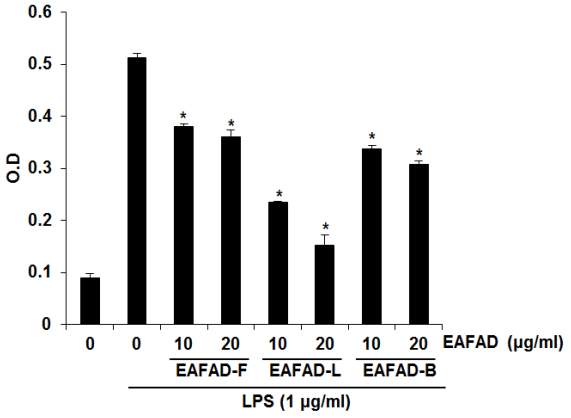

Fig. 1. Effect of EAFAD on NO production in LPS-stimulated RAW264.7 cells. RAW264.7 cells were pre-treated with EAFAD at the indicated concentrations for 2 h and then co-treated with LPS (1 μg/mL) for the additional 18 h. After treatment, NO production was measured using the media and Griess reagent. DMSO was used as a vehicle. Values given are the mean ± SD (n = 3). *p < 0.05 compared to LPS treatment without EAFAD. EAFAD-F, EAFAD-L and EAFAD-B means the ethyl acetate fraction from the flower (EAFAD-L), leaf (EAFAD-L) and branch (EAFAD-B) of *A. distichum*.

Statistical analysis

A statistical analysis was performed with the Student's unpaired t-test, with statistical significance set at * $P < 0.05$.

Results

Effect of the part of *A. distichum* on NO production in LPS-stimulated RAW264.7 cells

Under stimulation, macrophages-generated NO plays an important role in inflammatory response. Thus, we used the mouse macrophage cell line RAW264.7 cells for evaluating anti-inflammatory effect of the part of *A. distichum*. To determine if the parts of *A. distichum* could reduce NO generation by LPS, RAW264.7 cells were pretreated with the extracts from the parts of *A. distichum (EAFAD) for 2 h and then co-treated with LPS (1 μg/m*L) for the additional 18 h. As shown in Fig. 1, treatment of LPS without EAFAD induced NO overproduction in LPS-stimulated RAW264.7 cells, while pretreatment of all EAFADs suppressed LPS-mediated NO overproduction. Among EAFADs, the inhibitory effect of NO production was highest in the treatment of EAFAD-L (extracts from the leaf of *A. distichum*). Thus, we chose EAFAD-L for further studies.

Effect of EAFAD-L on iNOS expression in LPS-stimulated RAW264.7 cells

Since NO production is regulated by iNOS expression, the effect of EAFAD-L on iNOS expression was evaluated by Western blot and RT-PCR. As shown in Fig. 1A, LPS overexpression was detected in the cells stimulated LPS alone. However, EAFAD-L inhibited iNOS expression in LPS-stimulated RAW264.7 cells. For the further study for evaluating whether inhibitory effect of EAFAD-L against the expression of iNOS protein is mediated from regulating iNOS transcription, we also determined the mRNA level of iNOS regulated by EAFAD-L in LPS-stimulated RAW264.7 cells. As shown in Fig. 2B, the treatment of EAFAD-L

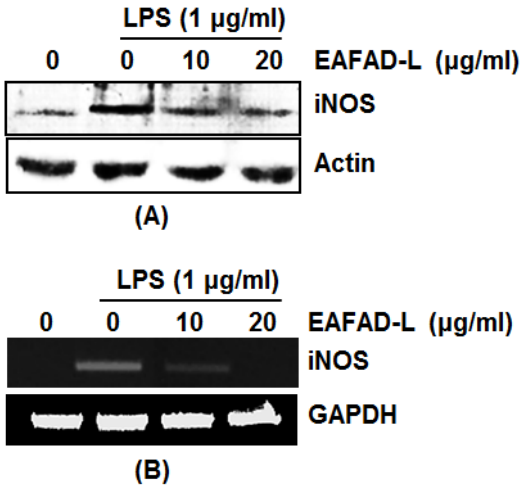

Fig. 2. Effect of EAFAD-L on iNOS expression in LPS-stimulated RAW264.7 cells. For Western blot (A), cells were pre-treated with indicated concentrations of EAFAD-L for 2 h and then co-treated with LPS (1 μg/mL) for an additional 15 h. DMSO was used as a vehicle. Cell lysates (30 μg) were resolved by 10% SDS-PAGE, transferred to nitrocellulose membranes, and probed with antibodies against iNOS. The proteins were then visualized using ECL detection. β-actin was used as an internal control. For RT-PCR (B), cells were pre-treated with indicated concentrations of EAFAD-L for 2 h and then co-treated with LPS (1 μg/mL) for an additional 15 h. Total RNA was isolated and RT-PCR was performed for iNOS. GAPDH was used as an internal control for RT-PCR.

attenuated the mRNA expression of iNOS. These data indicates that EAFAD-L may reduce iNOS expression through suppressing iNOS transcription.

Effect of EAFAD-L on NF-κB activation in LPS-stimulated RAW264.7 cells

To elucidate the effect of EAFAD-L on NF-κB activation, we firstly carried out a Western blot for IκB-α degradation in LPS-stimulated RAW264.7 cells. As shown in Fig. 3A, LPS induced IκB-α phosphorylation and subsequent IκB-α degradation at 15 min after the stimulation. However, pretreatment of EAFAD-L blocked LPS-induced IκBα degradation in a dose-dependent manner.

p65 translocation from cytosol to nucleus resulted from IκB-α degradation by various stimuli are essential in NF-κB activation. Thus we examined whether EAFAD-L suppresses nuclear translocation of p65. As shown in Fig. 3B, LPS

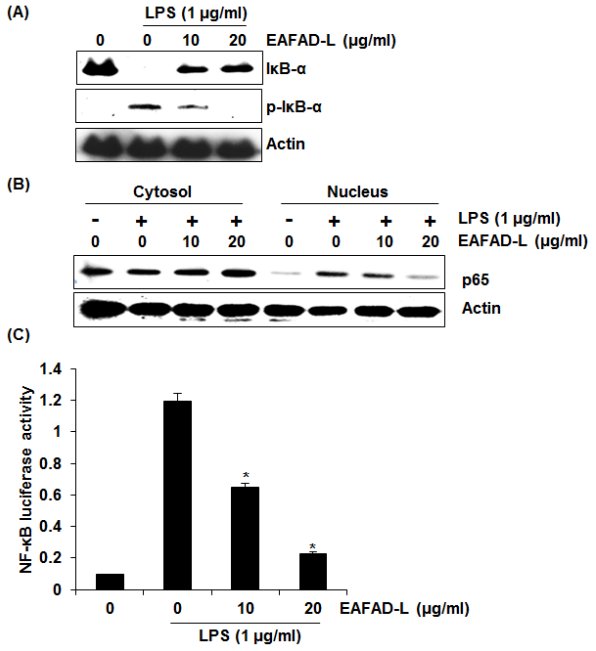

Fig. 3. Inhibitory effect of EAFAD-L against NF-κB activation. (A, B) Cells were pre-treated with indicated concentrations of EAFAD-L for 2 h, and then co-treated with LPS (1 μg/mL) for an additional 15 min (for Western blot of IκB-α and p-IκB-α) or 30 min (for Western blot of p65). DMSO was used as a vehicle. Cell lysate (30 μg) were resolved by 12% SDS-PAGE, transferred to nitrocellulose membranes, and probed with antibodies against IκB-α and p65. The proteins were then visualized using ECL detection. β-actin was used as an internal control. (C) pNF-κB -Luc plasmid-transfected cells were pre-treated with EAFAD-L for 2 h, and then co-treated with LPS (1 μg/mL) for an additional 15 h. DMSO was used as a vehicle. The cells were harvested in 1 x luciferase lysis buffer, and luciferase activity was measured using a dual-luciferase assay kit. Values given are the mean ± SD (n = 3). *p < 0.05 compared to LPS-stimulated cells.

increased an amount of p65 in the nucleus of RAW264.7 cells. However, pretreatment of EAFAD-L dose-dependently suppressed LPS-induced p65 translocation.

Translocate p65 into the nucleus binds to the NF-κB binding site and increases NF-κB transcriptional activity. Thus, we determined whether EAFAD-L inhibits transcriptional activity of NF-κB using pNF-κB -Luc-cis-reporter plasmid. As shown in Fig. 3C, EAFAD-L dose-dependently inhibited LPS-induced transcriptional activity of NF-κB in RAW264.7 cells. These data suggest that EAFAD-L may inhibit NF-κB

activation by suppression of p65 translocation into the nucleus via blocking the IκB-α degradation.

Effect of EAFAD-L on ERK1/2 activation in LPS-stimulated RAW264.7 cells

To further investigate whether decrease of NF-kB activation by EAFAD-L treatment is associated with the modulation of ERK1/2 activation, we evaluated the effects of EAFAD-L on phosphorylation of ERK1/2 in LPS-stimulated RAW264.7 cells. As shown in Fig. 4, Increase of ERK1/2 phosphorylation was observed in RAW264.7 cells by LPS. However, EAFAD-L inhibited phosphorylation of ERK1/2, indicating that EAFAD-L suppresses the inflammatory response by inhibiting ERK1/2 activation in LPS-stimulated RAW264.7 cells.

Discussion

Recently, it has been reported that the flower of *Abeliophyllum distichum* Nakai (*A. distichum*) has a protective effect against oxidative DNA damage induced by reactive oxygen species (Ahn and Park, 2013). However, other pharmacological properties of *A. distichum* have not been studied. Thus, we report that anti-inflammatory effect of *A. distichum* and the potential mechanisms by which *A. distichum* regulates an inflammatory response.

Nitric oxide (NO) produced by nitric oxide synthase (NOS) has beneficial effects such as anti-cancer, anti-virus replication and anti-inflammation (MacMicking *et al.*, 1997). However,

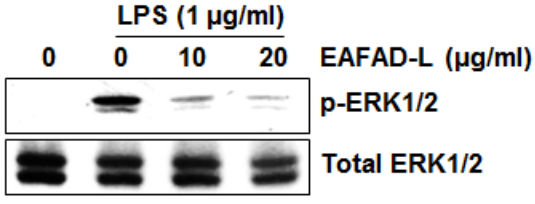

Fig. 4. Effect of EAFAD-L on phosphorylation of ERK1/2 in LPS-stimulated RAW264.7 cells. Cells were pre-treated with indicated concentrations of EAFAD-L for 2 h, and then co-treated with LPS (1 μg/mL) for an additional 15 min. DMSO was used as a vehicle. Cell lysate (30 μg) was subjected to 12% SDS-PAGE, followed by Western blot using anti-p-ERK1/2 and ERK1/2. The proteins were then visualized using ECL detection. β-actin was used as an internal control.

the overproduction of NO generated by iNOS, one of the NOSs leads to various inflammatory diseases (Bogdan, 2001). Therefore, the inhibition of NO generation through down-expression of iNOS has been regarded as a chemopreventive target for treating inflammation. Among the part of *A. distichum*, the inhibitory effect against NO generation was highest in the treatment of *A. distichum* leaf. In addition, *A. distichum* leaf inhibited the over-expression of iNOS in protein and mRNA levels. These results suggest that the *A. distichum* leaf may exert an anti-inflammatory effect.

In inflammatory response, NF-κB controls expression of various genes associated with chronic inflammation (Gilmore, 2006). In absence of stimuli, NF-κB activity formed by p50 and p65 is suppressed in the cytoplasm by forming a complex with IκB-α. However, the external stimuli such as LPS activate the IκB-α kinase (IKK), resulting in the phosphorylation of Iκ B-α. Phosphorylated IκB-α is then ubiquitinated and subsequently degraded by the 26S proteasome, which thereby releases NF-κB from the cytoplasmic NF-κB-IκBα complex and allows NF-κB to translocate to the nucleus. Translocated NF-κB activates inflammation genes such as iNOS. Thus, NF-κB has been shown to be the most influencing transcription factor inducing inflammatory response and a promising target for anti-inflammatory therapies (Mankan *et al.*, 2009; Sarkar *et al.*, 2008). In this study, we determined that the *A. distichum* leaf inhibits NF-κB activation through blocking the nuclear translocation of NF-κB p65 via suppressing IκB-α degradation.

There is growing evidence that MAPKs play a critical role in an inflammatory response (Vanden Berghe *et al.*, 1998). The activation of MAPKs such as JNK, p38, and ERK1/2 protein is known to be involved in the regulation of iNOS. In addition, they play a critical role in the modulation of NF-κB activity (Surh *et al.*, 2001). We found that the *A. distichum* leaf suppress phosphorylation of ERK1/2 induced by LPS. These results suggest that suppression of NF-κB and ERK pathway is a major mechanism by which *A. distichum* leaf possesses anti-inflammatory activity.

In conclusion, *A. distichum* leaf possesses an anti-inflammatory activity in macrophages. These anti-inflammatory effects may result from a decrease of ERK-mediated NF-κB activation and subsequent down-regulation of iNOS-induced NO

production. We anticipate that *A. distichum* leaf could be used an as effective natural anti-inflammatory agent. However, further studies are required to estimate the physiological relevance of application of *A. distichum* leaf to anti-inflammatory benefit.

Acknowledgement

This study was supported by the BK21 PLUS program of Ministry of Education and by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2011-0025272).

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(Received 6 March 2014 ; Revised 9 May 2014 ; Accepted 16 May 2014)