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Inhibitory Effect of *Taraxacum mongolicums* (蒲公英) on NO Production in LPS-stimulated Macrophages

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LPS로 자극된 대식세포에서 포공영의 NO 생성 억제 효과

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Abstract

포공영(蒲公英)은 예로부터 淸熱解毒藥으로 사용되어 왔으며 NO가 염증의 한 요인이기 때문에 포공영의 항염증 작용기작을 밝히기 위하여 LPS로 자극된 대식세포주 RAW264.7 세포에서 포공영 열수 추출물 (AETM)의 NO 생성에 미치는 효과를 조사하였다. 포공영은 NO 생성 및 iNOS 단백질 발현, iNOS mRNA 발현을 저해하였으며, 전사인자인 NF-κB의 핵으로의 이동을 억제하였다. 또한 LPS에 의해서 활성화되는 ERK/MAPK 효소의 활성을 현저히 억제하였다. 이 결과들로 보아 포공영의 항염증 작용이 ERK/MAPK 활성 저해 및 NF-κB 활성 저해로 인한 iNOS 발현의 억제 때문인 것으로 사료된다.

Key word : *Taraxacum mongolicum*, Nitric oxide (NO); Inducible nitric oxide synthase (iNOS); Nuclear Factor kappa B (NF-κB); extracellular signal-regulated kinases (ERK)/Mitogen-activated protein kinase (MAPK)

I. Introduction

NO has been known to be an important regulatory molecule in diverse physiological functions such as vasodilation, neural communication, and also toxic for bacteria and

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tumor cells[1,2]. However a large quantity of NO induces an inflammatory response to inhibit the growth of invading microorganisms and tumor cells. This strong inflammatory response to foreign cells could also cause further damage for the neighboring cells and tissues of the host[3]. Therefore, the reduction of the harmful effects is seemed to be important in inflammation therapy.

NO is produced from conversion of L-arginine to citrulline *in vivo* by three distinct isoforms of NO synthase (NOS): neuronal NOS (nNOS or NOS I), inducible NOS (iNOS or NOS II), and endothelial NOS (eNOS or NOS III)[4]. While nNOS and eNOS are constitutively expressed and regulated at post-translational level by Ca^{2+}-calmodulin, the activity of iNOS is regulated at the transcriptional level by mediators such as IL-2, IFN-γ and inflammatory stimuli including bacterial lipopolysaccharide (LPS)[5,6].

The transcriptional activator protein NF-κB plays a critical role in iNOS gene expression[7]. NF-κB is a heterodimeric transcription factor and controls a number of genes that are important for immunity and inflammation. In its unstimulated form, NF-κB is present in the cytosol bound to the inhibitory protein I kappa B (IκB). In response to cell stimulation, IκB becomes phosphorylated and recognized by a specific E3 ubiquitin ligase complex and then degradated by the 26S proteasome. The free NF-κB from IkB, which are spared from degradation, translocates to the nucleus to activate gene transcription[8,9].

Activation of the ERK/MAPK pathway has been correlated with numerous cellular responses, including proliferation, differentiation, and regulation of specific metabolic pathways in different cell types. It is now well established that MAPK is a key regulator of NF-κB activation and iNOS expression[10]. The 44 kDa MAPK (ERK1) and 42 kDa MAPK (ERK2) are phosphorylated and activated by highly specific MEK1 and MEK2[11]. The ERK/MAPK is rapidly activated by LPS in macrophages and has been implicated in both inflammation and immune response[12].

Since the dried whole plant of *Taraxacum mongolicum* is traditionally used for the treatment of many type of inflammation, the effect of this drug is correlated with the function of NO production, one of key parameters of inflammation. In this study, we examined the effects of *Taraxacum mongolicum* on NO production from LPS-stimulated macrophages and investigated possible mechanisms of the effects of the medicine.

II. Materials And Methods

1. Preparation of extract

The dried whole plant of *Taraxacum mongolicum* were purchased from a local herb store, Kwang Myoung Dang (Busan, Korea). The dry roots (200 g) were extracted with distilled water at 100℃ for 2hr. The extract was filtered through 0.45 μm filter and the filterate was freeze-dried (yield, 18 g) and kept at 4 ℃. The dried filterate was dissolved in phosphate buffered saline (PBS) and filtered

through 0.22 ㎛ filter before use.

2. Cell culture

Macrophage cell line RAW264.7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% penicillin-streptomycin and 10% fetal bovine serum (FBS; Gibco BRL). Cells were maintained in a 37℃, 5% CO2, fully humidified incubator, and prepared for experimental procedures when they were in log-phase growth.

3. Measurement of nitrite concentration

NO synthesis in cell cultures was measured by a microplate assay method. After cells plated in 24 well for 24 hr, 100 ㎖ each cultured medium was mixed with the same volume of the Griess reagent (1% sulfanilamide/0.1% *N*-(1-naphthyl)-ethylenediamine dihydrochloride/2.5% H_3PO_4). Nitrite concentration was determined by measuring the absorbance at 540 ㎚ with a Vmax microplate reader (Molecular Devices).

4. Western blot analysis

The cells were washed with PBS three times and scraped off and lysed with lysis buffer [1% Triton X-100, 1% Deoxycholate, 0.1% NaN_3]. Protein concentration of lysates was determined and equal amounts of protein (25 ㎍) were separated electrophoretically using 10% SDS-PAGE, and then the gel was transferred to 0.45 ㎛ polyvinylidene fluoride (PVDF). The blot was incubated with anti-iNOS, -phospho-ERK, -α-tubulin and -TBP antibody at room temperature and secondary antibody, and then was detected by the enhanced chemiluminescence detection system according to the recommended procedure (ECL, Amersham).

5. Preparation of nuclear and cytosolic extract

Nuclear extracts were prepared as described[13] with some modifications. Briefly, cells were incubated in 100 ㎜ dishes and scraped off. Then cells washed with PBS three times, resuspended in 500 ㎖ of ice-cold buffer A [10 mM HEPES-KOH, pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol (DTT), 0.2 mM phenylmethylsulfonyl fluoride (PMSF)] and allowed on ice for 15 min. Then cell extract was added Nonidet P-40 (NP-40), incubated on ice for 5 minand centrifuged at 12000 g for 30 s at 4 ℃. After removal of the supernatant, containing cytosolic proteins, nuclear proteins were extracted by addition of 100 ㎖ of buffer B [20 mM HEPES, pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5mM DTT, 0.2 mM PMSF, protease inhibitor cocktail] for 30 min at 4 ℃ with occasional vortexing. After centrifugation at 13,000 g for 5 min 4 ℃, supernatants were collected and stored at −70 ℃ for use as nuclear extract.

6. Real-time RT-PCR

Total RNA was isolated from cells using RNASPIN mini (Amersham Bioscience) according to the manufacturer's instruction. The cDNAs

were synthesized from 1 ㎍ of total RNA using AMV-RTase (Promega, Mannheim, Germany) at 42℃ for 1 h. The synthesized cDNAs were used for real-time PCR analyses with iNOS and GAPDH primers using DYAD thermocycler (MJ Research, Watertown, MA, USA) and SYBR Green Master Mix (Applied Biosystems, Foster City, CA). The real-time PCR cycling conditions were as follows: 95℃ for 10 min, followed by 45 cycles for 10 sec at 95℃, 10 sec at 55℃ and 10 sec at 72℃ followed by fluorescence measurement. Crossing threshold values for individual genes were normalized to GAPDH expression.

III. Results

1. Suppression of NO production and iNOS expression by AETM in LPS-stimulated macrophages

To determine the effect of AETM on NO generation in LPS-stimulated macrophages, Greiss Method was employed. RAW264.7 cells were pre-incubated in 24-well tissue culture plates ($2X10^5$ cells/well) with AETM for 1 h and stimulated with 1 ㎍/㎖ LPS for 24 hr. LPS alone increased the production of nitrite about 8-fold over basal levels. This induction in nitrite generation by LPS was inhibited by AETM in a dose dependent manner (Fig 1). We next investigated whether AETM could affect iNOS protein levels in LPS-stimulated RAW264.7 cells. Western blot analysis indicated that the level of iNOS was gradually decreased with increasing concentration of AETM (Fig 2A), while the level of α-tubulin was not changed. Moreover, AETM reduced the synthesis of iNOS mRNA in LPS-stimulated RAW264.7 cells (Fig 3) in a dose-dependent manner. This result strongly suggests that the inhibitory effect of AETM on NO release is caused by the gene expression level of iNOS.

2. Suppression of LPS-induced NF-kB activation by AETM

In macrophages, NF-κB is a transcritional factor that is activated in response to stimulation by LPS and also controls the transcriptional initiation of iNOS gene. Thus the inhibitory effect of AETM on NO production might be based on the transcriptional level of iNOS in the participation of NF-κB. To assess whether AETM suppresses NF-κB activation, translocation of NF-κB p65 from cytoplasm into nucleus was examined. RAW264.7 cells were incubated with AETM for 1 h and stimulated with 1 ㎍/㎖ LPS for 30 minutes. Nuclear and cytosolic protein was extracted and Western blotting with anti-p65 antibody was conducted. As shown in Fig 4, p65 was little translocated into nucleus. However, nuclear level of p65 was enormously increased by LPS while cytosolic level of p65 was markedly decreased, which is indicating NF-κB activation by LPS. This increased nuclear level of p65 was inhibited by AETM in dose dependent manner. Therefore this result suggests that the inhibitory effect of AETM on iNOS expression is due to

suppression of NF-κB activity.

3. Inhibition of ERK/MAPK activity by AETM

To elucidate the molecular target of AETM in further upstream signaling pathway, we examined the effect of AETM on ERK/MAPK activity which regulates the function of NF-κB. Since ERK/MAPK has been known to be phosphorylated and activated, ERK/MAPK activity was monitored by its phosphorylation. As shown in Fig 5A, the phosphorylation of ERK/MAPK 42 and 44 kD form reflecting its activation was induced by LPS and reached its peak at 30 min stimulation in RAW264.7 macrophages. So RAW264.7 were treated with indicated concentrations of AETM for 1 h and then stimulated with 1 ㎍/㎖ LPS for 30 min. ERK/MAPK activity was inhibited by AETM in a dose-dependent manner (Fig 5B). This result suggests that the inhibitory effect of AETM on NF-κB activation and iNOS expression is due to suppression of ERK/MAPK activity.

IV. Discussion

Mammals are in contact with Gram-negative bacteria and their LPS[14]. Low dose of LPS are thought to be beneficial for the host, e.g. in causing immunostimulation and enhancing resistance to infections and malignancies. On the other hand, the presence of large amounts of LPS can lead to dramatic pathophysiological reactions such as fever, leukopenia, tachycardia, hypotension, disseminated intravascular coagulation, and multiorgan failure[15]. It has been established that iNOS produces large amount of NO several hours after exposure to LPS in macrophage[16]. In this study, AETM significantly inhibited LPS induced NO production and iNOS expression in RAW264.7 macrophages. These results suggest that AETM could do potent anti-inflammatory action via inhibition of NO release by affecting the iNOS expression level.

The expression of murine macrophage iNOS is regulated at the transcriptional level. NF-κB is activated in response to the stimulation by LPS, and its activation is essential step in inducing iNOS gene expression in macrophage[17]. In nonstimulated cells, NF-κB dimers are maintained in the cytoplasm through interaction with inhibitory proteins, the IκB. However, under LPS exposure, NF-κB is activated by phosphorylation and subsequent degradation of IκB in RAW264.7 mouse macrophage[18,19], and translocated into the nucleus. Our study showed that NF-κB is activated by LPS, and AETM cotreatment significantly inhibited NF-κB translocation in RAW264.7 cells.

MAPKs are critical components of cellular signal transduction cascades. They are directly involved in many diseases, including cancer and inflammation, and have become one of the most important target classes for drug development[20,21]. Thus, in the present study we examined the effect of AETM in LPS-induced ERK/MAPK activation. Our data demonstrated that treatment with AETM greatly inhibited the LPS-induced ERK/MAPK activation at the same range of doses necessary to prevent the translocation of p65 and iNOS expression after LPS application. Several

studies have indicated the participation of this protein kinase in the regulation of inflammatory gene expression, acting both at transcriptional and posttranscriptional levels[22-25].

Taraxacum mongolicum, also known as dandelion, is a herb widely used for its antibacterial activity in the oriental medicine recipe. Traditionally, the dried whole plant of *Taraxacum mongolicum* is used for the treatment of boils, sores, mastitis, lymphadenitis, inflammation of the eye, sore throat, lung and breast abscess, acute appendictis, jaundice, and urinary tract infections[26]. In addition, in vitro antifungal, antileptospiral, and antiviral effects of the herb have also been documented. Since these effects are regarded totally as anti-inflammatory functions, we hypothesize that this drug is correlated with the function of NO production, one of key parameters of inflammation. Therefore we examined the effects of AETM on NO production from LPS-stimulated macrophages. and investigated possible mechanisms of the effects of the medicine.

In summary, AETM inhibited LPS-stimulated NO production and iNOS gene expression, and this biological effect involved the inhibition of NF-κB through negative regulation of ERK/MAPK pathway. These results certify that AETM could be used as an anti-inflammatory drug.

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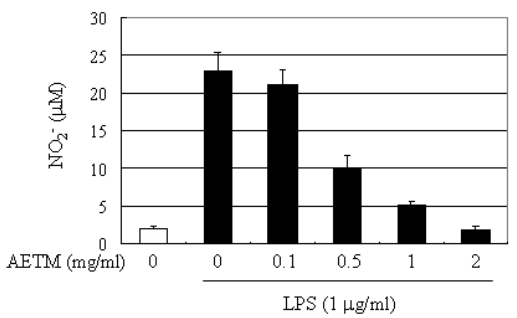

Fig. 1. Effect of AETM on NO production in LPS-stimulated macrophages.

RAW264.7 macrophages were treated with indicated concentrations of AETM for 1 h and stimulated with 1 μg/ml LPS for 24 h at 37oC. At the end of incubation, the culture medium was collected for Griess reaction. Results were presented as the means ± S.E. of four individual experiments performed in duplicate.

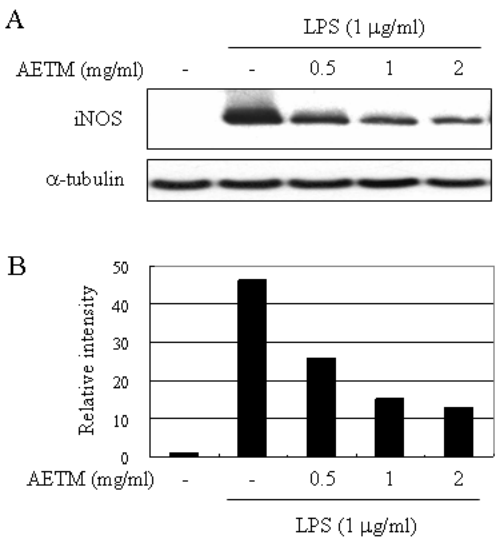

Fig. 2. Inhibition of iNOS expression by AETM in LPS-stimulated macrophages.

(A) RAW264.7 macrophages were treated with indicated concentrations of AETM for 1 h and stimulated with 1 μg/ml LPS for 24 h at 37oC. Whole cell extracts were separated by SDS-PAGE and analyzed by western blotting with anti-iNOS antibody. Western blot detection of α-tubulin was estimated protein-loading control for each lane.

(B) Quantitative analysis for different concenration of AETM was performed using ImageJ and then relative intensity of iNOS divided by that of α-tubulin was presented.

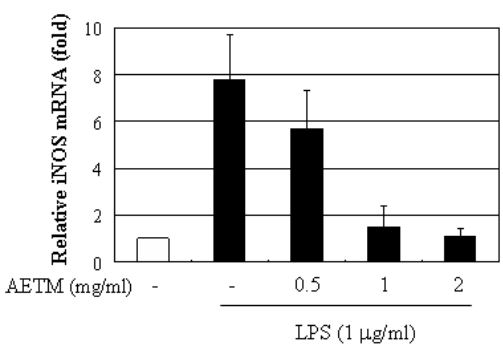

Fig. 3. Effect of AETM on the synthesis of iNOS mRNA in LPS-stimulated macrophages.

RAW264.7 cells were treated with indicated concentrations of AETM for 1 h and stimulated with

1 μg/ml LPS for 6 h at 37oC. Total RNA was extracted and reverse-transcribed into cDNA. Real-time PCR was performed using iNOS- or GAPDH-specific primers, as described in Materials and Methods. Relative content of iNOS mRNA was indicated as folds to control.

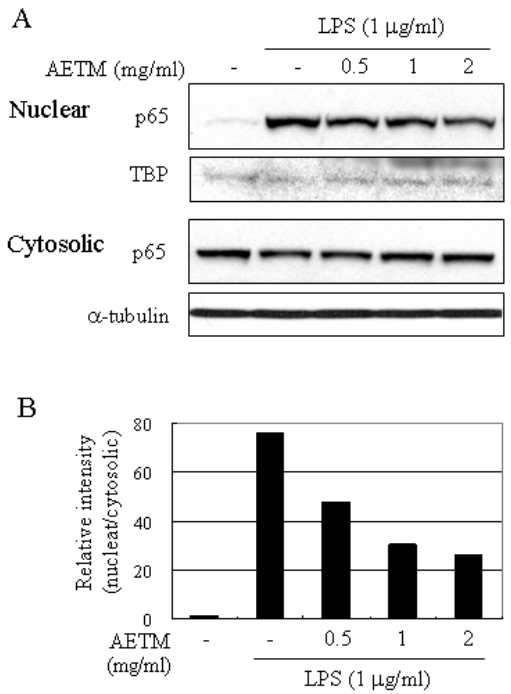

Fig. 4. Effect of AETM on nuclear translocation of NF-κB in LPS-stimulated macrophages.

(A) RAW264.7 cell were treated with indicated concentrations of AETM for 1 h and stimulated with 1 μg/ml LPS for 30 min at 37oC. Nuclear and cytosolic proteins were extracted and separated by SDS-PAGE. Western blotting was performed with anti-NF-κB p65 antibody. Western blot detection of TBP or α-tubulin was estimated protein-loading control for each lane.

(B) Quantitative analysis for nuclear and cytosolic p65 content was performed using ImageJ and then relative intensity of p65 divided by that of TBP (nuclear fraction) or α-tubulin (cytosolic fraction) was presented.

(C) Extent of p65 translocation was represented as relative amount. Relative intensity of p65 in nuclear fraction was divided by relative intensity of p65 in cytosolic fraction, respectively.

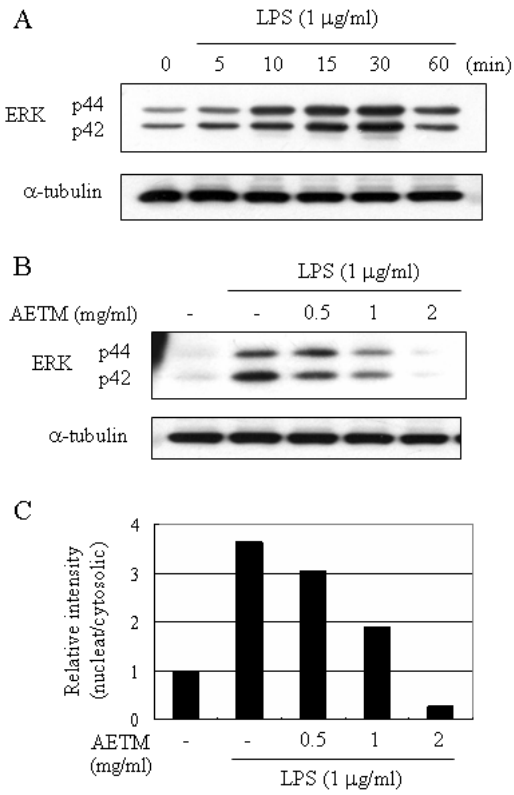

Fig. 5. Effect of AETM on ERK/MAPK activity in LPS-stimulated macrophages.

(A) RAW264.7 macrophages were treated with 1 μg/ml LPS for indicated time and Equal amount of cell extracts was separated by SDS-PAGE and analyzed by western blotting with anti-phospho-ERK antibody.

(B) RAW264.7 cells were treated with indicated concentrations of AETM for 1 h and stimulated with 1 μg/ml LPS for 30 min at 37oC. Equal amount of cell extracts was separated by SDS-PAGE and analyzed by western blotting with anti-phospho-ERK antibody. Western blot detection of α-tubulin was estimated protein-loading control for each lane.

(C) Quantitative analysis for different concenration of AETM was performed using ImageJ and then relative intensity of phospho-ERK divided by that of α-tubulin was presented.