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Anti-inflammatory effects of *Hylomecon hylomeconoides* in RAW 264.7 cells

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Abstract. – BACKGROUND AND OBJECTIVES, Papaveraceae serve as a rich source of various alkaloids which have anti-inflammatory effect.

MATERIALS AND METHODS, In this study, we investigated the effect of *Hylomecon hylomeconoides* ethanol extract (HHE) on lipopolysaccharide (LPS)-induced NO and interleukin-6 (IL-6) production in RAW 264.7 cells.

RESULTS, HHE inhibited LPS-induced NO and IL-6 production. Moreover, HHE suppressed the phosphorylation of ERK1/2 and p38 in LPS-induced RAW 264.7 in a dose-dependent manner. Furthermore, major constituents, dihydrosanguinarine and 6-methoxydihydrosanguinarine, of the chloroform-soluble extract were analyzed.

CONCLUSIONS, Taken together, the results of this study indicate that the anti-inflammatory effects of HHE may occur via the inhibition of NO and IL-6 expression through the down-regulation of MAP kinase (ERK1/2, p38) phosphorylation in RAW 264.7 cells.

Key Words:

Hylomecon vernale, Interleukin-6, Nitric oxide, 6-methoxydihydrosanguinarine, Inflammation

Introduction

Nitric oxide (NO) is free radical that mediates many biological functions, including neurotransmission, vascular homeostasis and host defense. NO known to be synthesized from L-arginine by nitric oxide synthase (NOS) plays an important role in the regulation of many physiological functions[1,2]. Pro-inflammatory mediator such as interleukin-6 (IL-6) is activated through mitogen-actived protein kinases (MAPKs) but they also activate MAPKs, thus amplifying the cytokine cascade and expanding the inflammatory status[3,4]. Because they play a driving role in the inflammatory process, effectively modulating their aberrant production can be beneficial in reducing inflammatory diseases. Therefore, the application of anti-inflammatory drugs has recently become a focus of interest.

Papaveraceae family includes many oriental medicines. *Hylomecon hylomeconoides* also belongs to the Papaveraceae and is widely distributed in Korea. Papaveraceae serve as a rich source of various alkaloids which have anti-inflammatory effect. In recent reports, *Chelidonium majus* and *Corydalis tuber* have anti-inflammatory effect, anti-tumor effect and antioxidant[5-7]. Recent our studies have demonstrated that *H. hylomeconoides* has antibacterial activity[8]. However, little is known about the anti-inflammatory property of *H. hylomeconoides*. There has been no report of phytochemical and pharmacological study for *H. hylomeconoides* so far. Thus, anti-inflammatory effects on the ethanol extract of *H. hylomeconoides* (HHE) were conducted due to its activity in the initial screening using Raw 264.7 cells.

In this study we show that in RAW264.7 cells HHE inhibited the activation of MAPKs, which explains the mechanism of action of HHE. Lipopolysaccharide (LPS)-induced NO and IL-6 productions are inhibited by HHE.

Materials and Methods

Extraction and Isolation of Test Material

Hylomecon hylomeconoides were collected from Hwasun, Korea, in July, 2008. They were

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identified by Dr. D.Y. Kwon. A voucher specimen (No. 08-01) was deposited in the Laboratory of Herbalogy, College of Pharmacy, Wonkwang University, Iksan, Korea. *H. hylomeconoides* air-dried roots (10 g) were then boiled in 30 ml of ethanol for 3 h. The extract was filtered (pore size, 0.45 m) lyophilized. We partitioned 100 g of *H. hylomeconoides* roots with EtOH and it yields 10.7%.

Quantitative Analysis of the Major Constituents in the Ethanol Extract Using High-Performance Liquid Chromatography (HPLC)

The chromatographic system consisted of a pump (Shimadzu LC-10AD) and Shimadzu SPD-10AVP diode array detector. A Optimapak C18 (4.6 × 150 mm, 5 μm) column with the eluent of acetonitrile-phosphate buffer (50 mM, pH 7.0) was used. A flow rate of 1 ml/min. The HPLC was monitored at 280 nm. It was found that the chloroform extract contained 21.9 mg/g of dihydrosanguinarine (tR 15.24 min) and 72.4 mg/g of 6-methoxydihydrosanguinarine (tR 45.85 min) by quantitative analysis (Figure 1).

Chemicals

RPMI 1640, penicillin and streptomycin were obtained from Hyclone (HyClone, Logan, UT, USA). Bovine serum albumin (BSA), LPS, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) were purchased from Sigma (St. Louis, MO, USA). p38, p-p38, ERK, p-ERK, JNK, p-JNK monoclonal antibodies and the peroxidase conjugated secondary antibody were purchased from Santa Cruze Biotechnology Inc (Santa Cruz, CA, USA).

Cell Culture

The murine macrophage cell line, RAW 264.7 cell, was obtained from the Korea Research Institute of Bioscience and Biotechnology (Daejeon, Korea) and grown in RPMI 1640 Medium containing 10% fetal bovine serum and 100 U/ml penicillin/streptomycin sulfate. Cells were incubated in humidified 5% CO_2 atmosphere at 37°C. For stimulation, the medium was replaced with fresh RPMI 1640, and then cells were stimulated with LPS in the presence or absence of HHE for the indicated periods.

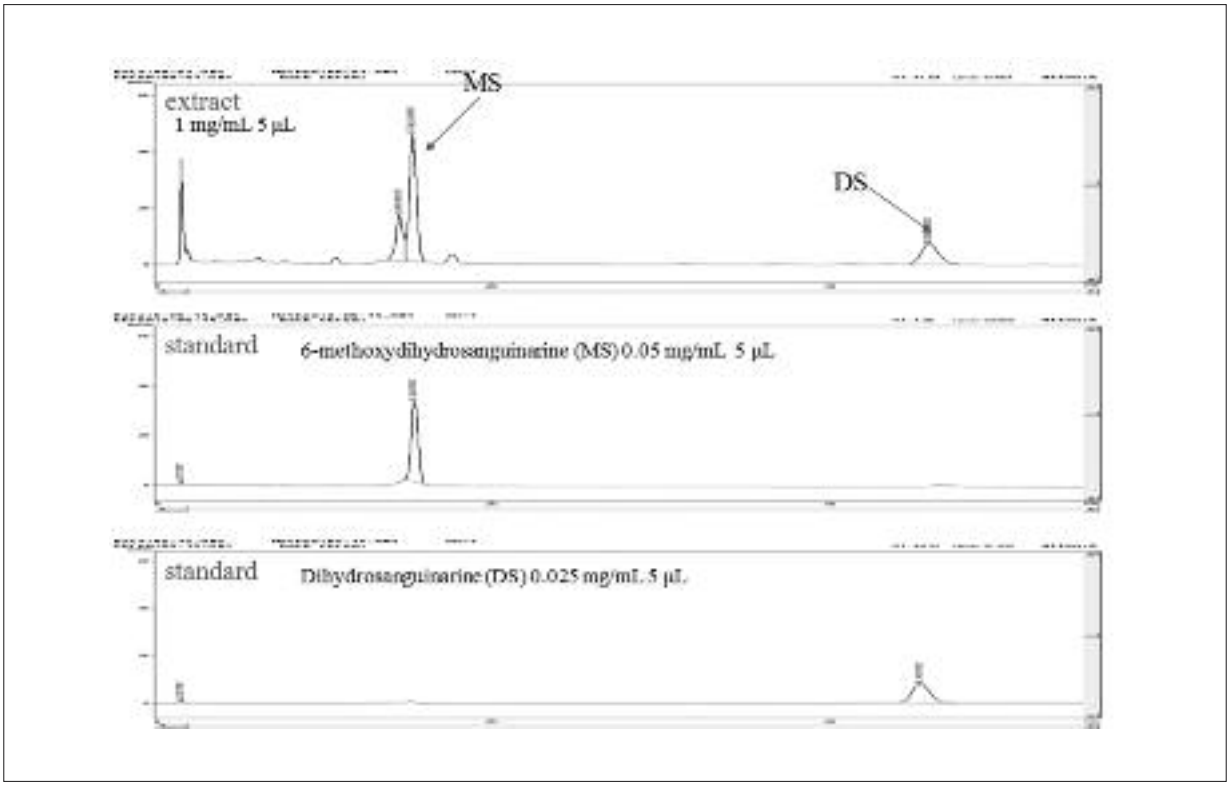

Figure 1. HPLC Chromatograms of HHE ***(A)***, 6-methoxydihydrosanguinarine (MS) ***(B)*** and dihydrosanguinarine (DS) ***(C)*** HPLC indicated that methoxyeugenol and marliolide of CC were detected at tR 15.24 min and 45.85 min.

MTT Assay for Cell Viability

The cell viability was examined by MTT assay. RAW 264.7 cells were seeded at 1×10^5/ml densities in 96 well plates (Nunc, Roskilde, Denmark). Each group had non-treated group as control. HHE (10 and 50 μg/ml) was added to each well and incubated for 24 h at 37°C, 5% CO_2. MTT solutions (5 mg/ml) were added to each well and then cells were cultured for another 4 h. The supernatant was discarded and 100 μl of dimethyl sulfoxide (DMSO) was added to each well. The optical density was read at 590 nm. Cytotoxicity was calculated by subtracting from one the ratio of the mean absorbance value for treated cells over the mean absorbance value for untreated cells.

Measurement of NO Production

NO production was assayed by measuring nitrite in supernatants of cultured RAW 264.7 cells. Cells were seeded at 1×10^6/ml in 96 well culture plates. After pre-incubation of RAW 264.7 cells for 18 hours, cells were pretreated with HHE (10 and 50 μg/ml) and stimulated LPS (1 μg/ml) for 24 hours. The supernatant was mixed with an equal volume of Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 2.5% phosphoric acid) and incubated at room temperature for 5 min. The concentration of nitrite was measured by reading at 570 nm. Sodium nitrite (N_aNO_2) was used as a standard curve.

Enzyme-Linked Immunosorbent Assay (ELISA)

Cells were seeded at 1×10^6/ml per well in 24 well tissue culture plates and pretreated with various concentration of HHE (10 and 50 μg/ml) for 30 min before LPS stimulation (1 μg/mL). The supernatant was decanted into a new microcentrifuge tube, and the amount of IL-6 determined using ELISA kit according to the procedure described by the Manufacturer (BD Bioscience, San Diego, CA, USA). All subsequent steps took place at room temperature, and all standards and samples were assayed in duplicate.

Western Blot Analysis

Protein expression was assessed by Western blot analysis according to standard procedure. RAW 264.7 cells were cultured in 60mm culture dishes (3×10^6/ml), and pretreated with various concentration of HHE (10 and 50 μg/ml). After 30 min, LPS (1 μg/ml) were added to the culture medium and the cells were incubated at 37°C. After incubation, cells were washed twice in ice cold phosphate buffered saline (PBS) (pH 7.4). Cell pellets were resuspended in lysis buffer on ice for 15 min. Cell debris was removed by centrifugation. The protein concentration was determined using the BIO-RAPID protein assay reagent (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. Equal amounts of protein (20 μg) were subjected to sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE), and then transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Membrane was blocked with 5% non fat milk in Tris-Buffered Saline and Tween 20 (TBS/T) buffer (150 mM NaCl, 20 mM Tris-HCl, 0.05% Tween 20, pH 7.4). After blocking, membrane was incubated with primary antibodies for 18 h. Membrane was washed TBS/T and incubated with anti-mouse or anti-rabbit IgG horseradish peroxidase conjugated secondary antibodies. Immuno reactivity was detected by using enhanced chemilumine-science (ECL: Amersham, Milan, Italy).

Statistical Analysis

The data from the experiments are presented as the mean ± S.E.M. The level of statistical significance was determined by analysis of variance (ANOVA) followed by Dunnett's *t*-test for multiple comparisons. p values less than 0.05 were considered to be significant.

Results and Discussion

Hylomecon hylomeconoides has been used as a folk medicine in Korea. However, the anti-inflammatory effect of HHE in RAW 264.7 macrophage is not yet fully understood. Inflammation is a complex process that involves numerous mediators.

It is well known that NO is an important mediator of cellular communication in several systems, including macrophages of the autonomic and central nervous system. However, the overproduction of NO can be harmful and result in septic shock, rheumatoid arthritis, and autoimmune diseases. Therefore, inhibition of NO production may have beneficial therapeutic effects for the treatment of diseases caused by its overproduction[1,9,10]. To examine the effect of HHE

on LPS-induced NO production in RAW 264.7 cells, cells were treated or not treated with HHE for 0.5 h and then treated with LPS (1 μg/ml) for 24 h. Both LPS and sample were not added to control group. The cell culture medium was then harvested and NO levels were determined using the Griess reaction. LPS induced approximately 3-fold more NO than the control group and HHE inhibited this NO production in a concentration-dependent manner (Figure 2). We investigated its effect on IL-6 production using ELISA kit. Pretreatment of cells with HHE at 10, and 50 μg/ml reduced IL-6 production (Figure 3). The cytotoxic effect of HHE was evaluated in RAW 264.7 cells by MTT assay. HHE, even 50 μg/ml, did not affect the viability of RAW 264.7 cells. Moreover, ERK (extracellular signal-regulated-kinase), JNK (c-Jun N terminal kinase) and p38 MAPK are known to be involved in the LPS-mediated induction of IL-6 and NO in mouse macrophages[11,12]. We examined the effect of HHE on phosphorylation of MAPKs in LPS stimulated RAW 264.7 cells by Western blot analysis. HHE suppressed LPS stimulated activation of ERK 1/2 and p38 MAPKs in a dose-dependent manner. The amount of non-phosphorylated ERK 1/2, JNK and p38 MAPKs was not affected by either LPS or HHE treatment. HHE inhibited ERK 1/2 and p38 MAPKs activation, while phosphorylation of JNK MAPK was not affected by HHE treatment (Figure 4).

Furthermore, dihydrosanguinarine and 6-methoxydihydrosanguinarine were identified as main components of HHE. The two isolated compounds were evaluated for their inhibitory activity against NO production in Raw 264.7 cells and found activity (Figure 5).

In conclusion, these results indicate that HHE is a potent inhibitor of LPS induced NO and IL-6 production in RAW 264.7 macrophages. Finally, these results indicate that HHE may block LPS-induced IL-6 and NO expression by inhibiting the ERK and p38 pathway. Therefore, the active constituents found in this study may account, for the

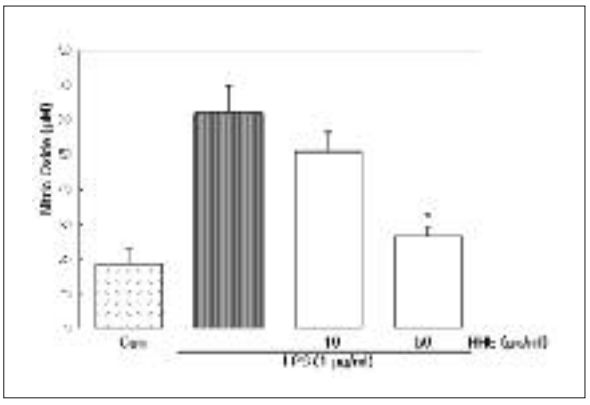

Figure 2. The effect of HHE on LPS-induced NO production in RAW 264.7. NO production. RAW 264.7 cells were pretreated with the indicated concentrations of HHE for 30 min before being incubated with LPS (1 μg/ml) for 24 h. Results are expressed as the mean S.E.M. (n=6). *$p < 0.05$, as compared to the acetic acid treated group and determined by Dunnett's *t*-test.

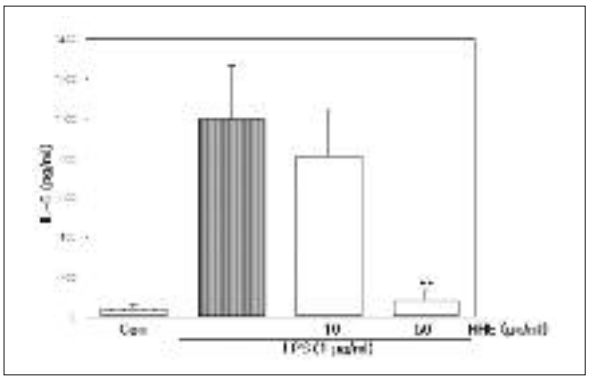

Figure 3. Effect of HHE on the expression of IL-6 in LPS stimulated RAW 264.7 cells. RAW 264.7 cells were pretreated with the indicated concentration of HHE for 30 min prior to being incubated with LPS (1 μg/ml) for 6 hours. The culture supernatant was then isolated and analyzed for IL-6 production. Results are expressed as the mean S.E.M. (n=6). **$p < 0.005$, as compared to the acetic acid treated group and determined by Dunnett's *t*-test.

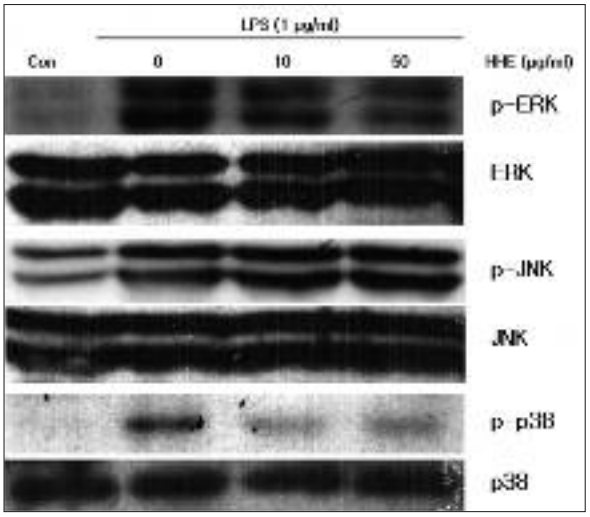

Figure 4. The effects of HHE on the phosphorylation of MAPKs in LPS stimulated RAW 264.7 cells. RAW 264.7 cells were treated with the indicated concentrations of HHE for 30 min prior to being incubated with LPS (1 μg/ml) for 30 min. Whole cell lysates were then analyzed by Western blot.

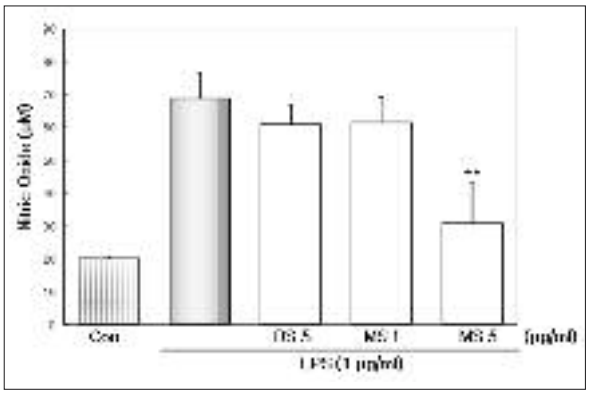

Figure 5. The effect of MS and DS on LPS-induced NO production in RAW 264.7. NO production. RAW 264.7 cells were pretreated with the indicated concentrations of MS and DS for 30 min before being incubated with LPS (1 μg/ml) for 24 h. Results are expressed as the mean S.E.M. (n=6). $^{**}p < 0.05$, as compared to the acetic acid treated group and determined by Dunnett's *t*-test.

anti-inflammatory effects of *H. hylomeconoides* and serve as standard markers for preparations with regard to anti-inflammatory activities.

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