대식세포 Raw 264.7에서 두충의 항염증효과

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Anti-inflammatory Effects of Water Extract of *Eucommia ulmoides* Oliver on the LPS-induced RAW 264.7 Cells

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ABSTRACT : *Eucommia ulmoides* Oliver (EU) is a traditional Korean herbal used for the treatment of rheumatoid arthritis (RA). In the present study, the molecular pharmacology basis of its anti-inflammatory effect is revealed in this work, EU was studied in lipopolysaccharide (LPS)-activated macrophage cells (RAW 264.7) as an established inflammation model. After activation, nitric oxide (NO) production and iNOS mRNA were measured by using a colorimetric assay (Griess reagent), and reverse transcription polymerase chain reaction (RT-PCR), respectively. The change in the content of PGE_2, TNFα, and IL-6 was concurrently monitored by ELISA. In results, we found that in the concentration range without showing cytotoxicity, EU produced a remarkable anti-inflammatory effect and showed a dose-dependent inhibition of LPS-induced NO production. Compared with indomethacin, EU has more potency and a specific action of NO inhibition, PGE_2, IL-6, and TNF-α inhibition. These results suggest that EU may be a suitable herbal medicine to yield the greatest anti-inflammatory activity for food additives and medicine.

Key Words : *Eucommia ulmoides* Oliver, NO, iNOS, PGE_2, IL-6, TNF-α

INTRODUCTION

Nitric oxide (NO), made by inducible NO synthase (iNOS) and excessive NO would produce injurious effects to individuals with chronic inflammation (Jeoung *et al*., 2009, Hesslinger *et al*., 2009). In 1998, Drs. Furchgott, Ingnarro and Murad received the Noble Prize in Physiology and Medicine for 'the first discovery that a gas can act as a signal molecule', emphasizing that nitric oxide (NO) produced by mammalian cells is an important signaling mediator in immunology, physiology, and neuroscience. The inhibitory activiry of NO production in lipopolysccharide (LPS) activated macrophage cell line, RAW 264.7 from the ethanolic extract of EU. Collectively, NO plays a essential role in endotoxin induced septic shock and death. Thus, iNOS-derived NO overproduction appears to be a

ubiquitous mediator of a wide range of inflammatory conditions (Sacco *et al*., 2006).

Marcrophages are immune cells usually dispersed throughout the body. Macrophage detect pathogenic substances control inflammatory responses (Medzhitov R *et al*., 1997) using pro-inflammatory mediators such as NO, TNF-α, interleukins (ILs), bacterial endotoxin, lipopolysaccharide (LPS), ionizing radiation, and carcinogens that are often associated with inflammatory diseases or tumorigenesis. LPS is the primary component of endotoxin and is formed by a phosphoglycolipid (Lee and Cho, 2007) and prohibits macrophage proliferation and activates them to produce proinflammatory factors, which play principal roles in the immune response (Morrison *et al*., 1987).

EU also called Du-zhong, a dioecious perennial angiosperm, is one of the oldest tonic herbs. The main

components of EU Cortes are 6-10% gutta percha (plant gum), glycoside, alkaloid, pectin, chlorogenic acid, iridoid, cyclopentanoid and dilignan-glycoside (Deyama *et al.*, 2001), The pharmacological studies of EU is various activities such as antioxidant (Hsieh and Yen, 2000) antimicrobial, anti-inflammatory (Kim *et al.*, 2009), antihypertensive (Gu *et al.*, 2011), and immunosuppressive (Zhu *et al.*, 2009) neuroprotective (Kwon *et al.*, 2011) or inhibitory of osteoclast and osteoblastic apoptosis (Lin *et al.*, 2011). Du-zhong tea, the aqueous extract of EU leaves, has been already known as a functional food and commonly used for reduction of hypertension (Greenway *et al.*, 2011). EU leaves have also been used in foods and contain the following active ingredients (Lie *et al.*, 2005), geniposidic acid and asperuloside as iridoid glycosides, and chlorogenic acid as a coffeic acid derivative. Based on the traditional uses and known functionalities of EU, we examined for its potential anti-inflammatory activities *in vitro* assay models evaluating effects on NO, iNOS, PGE2 IL-6 and TNF-α production.

MATERIALS AND METHODS

1. Materials

Indomethacin, and LPS purified from *Escherichia coli* O111 : B4 were purchased from Sigma-Aldrich (St. Louis, MO, USA). 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazoliumbromide (MTT, Sigma, USA), Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were from Invitrogen (Carlsbad, CA, USA). The prostaglandin E_2 (PGE_2) ELISA assay was from R & D Systems (Minneapolis, MN, USA). Lipopolysacharide (LPS, Sigma, USA), Griess reagent (Sigma, USA), Dimethyl sulfoxide (DMSO, Sigma, USA), RIPA buffer (Pierce, Rockford, IL, USA), protein assay kit (Bio-Rad Laboratories, USA), all other reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Filtered ethanol EU extracts were air dried using a rotary evaporator and powdered.

2. Plant materials

The leaves of EU was purchased from a store at the Gyung Dong herbal market in Seoul, Republic of Korea, and then specimens were taxonomically identified by a oriental doctor, S.W. Lee at the National Institute of Horticultural & Herbal Science, RDA. The voucher specimen (HPR-208) was deposited at the herbarium of Herbal Crop

Research Institute (Eumsung, Republic of Korea). We used water extraction because most traditional oriental herbal materials are decocted with boiling water and because the iridoid glycosides are more soluble in water than in organic solvents. The crushed plant materials (200 g for each) were extracted under reflux with distilled water three times. The combined water extracts were lyophilized, and yields were 23.5% (wt = wt) for EU in the dried state. They were kept at 80℃ until use.

3. Cell culture

Murine macrophage RAW 264.7 cells (ATCC) were cultured at 37℃ in Dulbecco's modified Eagles's medium containing 10% fetal bovine serum, 2 mM glutamate, 100 unit/mℓ of penicillin, and 100 μg/mℓ of streptomycin in a humidified incubator with 5% CO2. Cells were incubated with 1 μg/mℓ LPS along with various concentrations of plant extracts for 24 hrs as indicated.

4. MTT cell viability assay

The cytotoxicity of EU was assessed using the microculture tetrazolium [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)]-based colorimetric assay. The remaining cells after the Griess reaction were used for MTT assay. MTT solution (5 ㎎/mL) was added to each well (final concentration, 62.5 μg/mL). The degree of mitochondrial respiration, as an indicator of cell viability, was assayed by measuring the mitochondria dependent reduction of MTT to formazan crystals. The absorbance of each well was then read at 570 ㎚ using the microplate reader (model EL808, Bio-Tek Instrument Inc., Winooski, VT). The details of this experiment were reported elsewhere (Park *et al.*, 2004).
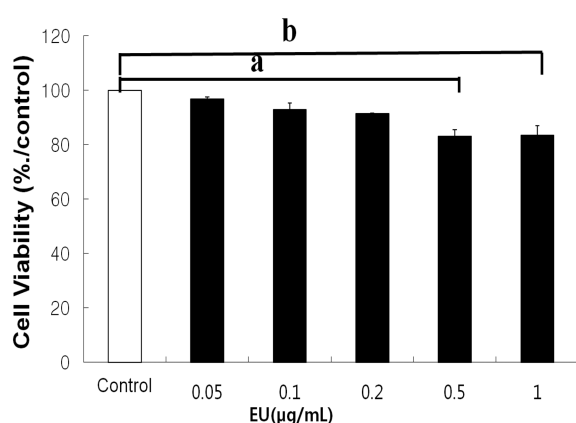
5. RNA preparation and iNOS mRNA analysis by RT-PCR

Cells (6×10^5 cells/well) in 6-well plates were treated with and without EU and indomethacin in the presence of LPS for an optimal incubation time of 9 h. The total RNA was isolated by using a Total RNA Miniprep System (Viogene). One microgram of total RNA was used to produce the first strand cDNA pool using the Access RT-PCR System. PCR primer sequences for iNOS gene were 5-CCCTTCCGAAGTTTCTGGCAGCAGC-3 (sense) and 5-GGCTGTCAGAGAGAGCCTCGTGGCTTTGG-3 (antisense). Primers for β-actin (as a house keeping gene) were 5-

ATGCCATCCTGCGTCTGGACCTGGC-3 (sense) and 5-AGCATTTGCGGTGCACGATGGAGGG-3 (antisense). The expected sizes of PCR products were 499 bp for iNOS gene and 607 bp for β-actin gene. The amplification profile consisted of denaturation at 94℃ for 45 s, annealing at 65℃ for 45 s, and extension at 75℃ for 2 min (15 cycles), and then another denaturation at 94℃ for 45 s, annealing at 67℃ for 45 s, and extension at 75℃ for 2 min (10 cycles). The products were analyzed by electrophoresis on 2% agarose gels containing ethidium bromide and visualized with an ultraviolet transilluminator.

6. NO, PGE$_2$, TNF-α, and IL-6 assays

To investigate NO formation, nitrite (NO$_2^-$) is measured since it is a stable, nonvolatile breakdown product of NO. The determination of nitrite relies on a diazotization reaction with the Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 2% phosphoric acid). Therefore, the nitrite accumulated in culture medium is a direct indicator of NO production (Green *et al.*, 1982). A 100-μl aliquot of each supernatant from the 96-well plate was mixed with 100 μl Griess reagent and incubated at room temperature for 15 min. The concentration of total nitrite was determined by reading the absorbance at 550 nm and then calculated by a NaNO$_2$ dilution standard curve. The culture medium was collected after LPS treatment for PGE$_2$, TNF-α, and IL-6 assays, respectively.

The time points for PGE$_2$, TNF-α, and IL-6 level were determined by a time course assay (data not shown). Levels of PGE$_2$, TNF-α, and IL-6 in the culture media were determined with commercial ELISA assay kits (Park et al., 2004).
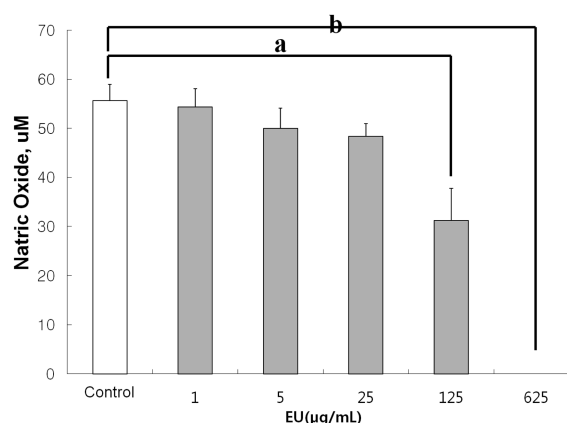
7. Statistical Analysis

All data were expressed as mean ± SD values which were made in triplicate experiments. Statistical significance was tested by one-way ANOVA (version 20.0 Chicago, IL), and $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Numerous previous studies have shown that certain traditional herbal medicines have therapeutic effects on inflammation. Here, we explored the potential of UE as an anti-inflammatory herbal medicine. Our data demonstrate that EU exhibits pharmacological activity via significant inhibitory effects on the LPS-induced inflammatory mediators and cytokines such as NO, iNOS, PGE$_2$, IL-6, and TNF-α, and in RAW 264.7 cells.

1. Effect of EU extracts on cellular cytotoxicity

Cell viability was tested throughout the experiments using the MTT assay. As shown in Fig. 1, EU did not decrease cell viability but rather protected the toxicity of

Fig. 1. Effect of EU on cell viability. RAW 246.7 cells macrophages were incubated with various concentrations of EU in the presence of 1 μg/mL LPS for 24 hours. Then cell viability was measured by the MTT assay as described in Materials and methods. Values represent the relative viability relative to the control group and are mean ± SD values of three independent experiments. Different letters indicate significantly different values.

Fig. 2. Inhibitory effect of EU on nitric oxide production in LPS-stimulated RAW 264.7 cells. The cells were stimulated with 1 μg/mL of LPS or with LPS plus various concentrations (1 μg/mL, 5 μg/mL, 25 μg/mL, 125 μg/mL, 625 μg/mL) of ethanol-based Eucommia ulmoides OLIVER extract for 16 h. Nitric oxide production was determined using the Griess reagent method. Different letters indicate significantly different values.

LPS. This result suggests that EU inhibits NO and IL-6 release without affecting cell viability.

2. Effects of EU on NO inhibition

In the EU concentration range showing cytotoxicity from 0.5 to 2 ㎎/㎖ (Fig. 1), it produced a dose-dependent inhibition of LPS-induced NO production (Fig. 2). Clearly, the remarkable effect on NO inhibition indicates that EU has a potency against inflammation. Over NO production lead to serious diseases like septic shock, stroke, arthritis chronic inflammatory diseases, and autoimmune disease (Bak *et al.*, 2011). It has been reported that NO synthesis is greatly diminished by neutralization with an anti-TNF-α antibody (Kim *et al.*, 1999).

3. Effects of EU on LPS-induced iNOS expression levels in Raw 264.7 cells

LPS is the principal component of endotoxin, arrests macrophage proliferation and activates them to produce

pro-inflammatory factors (Morrison and Ryan, 1987). Our results showed that LPS enhanced iNOS expression in Raw 264.7 cells and EU suppressed LPS-induced iNOS expression. Therefore, we assumed the effect EU on iNOS expression levels with LPS exposure. Raw 264.7 cells were incubated for 12 h with LPS (1 ㎍/mL) in the absence or presence of EU (10-100 ㎍/mL). Our results showed that EU suppresses LPS-induced iNOS expression levels in a dose-dependent manner (Fig. 3). These results suggests that the inhibition of iNOS expression is important for alleviating inflammation (Lee *et al.*, 2010). Therefore, the inhibition of iNOS expressions may establish an effective new therapeutic vision for the remedy of inflammation and the prevention of inflammatory disease.
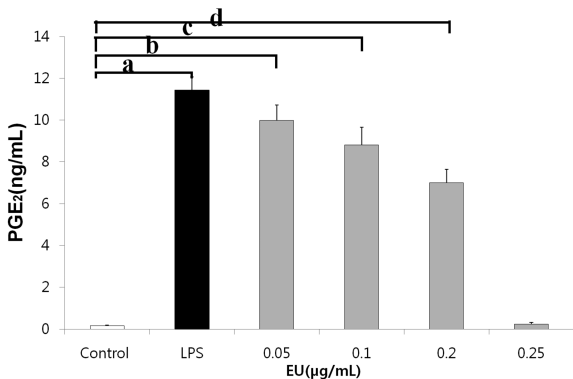
Fig. 3. Inhibitory effect of EU on LPS-induced iNOS mRNA levels in Raw 264.7 cells. The cells were pretreated with the EU for 1 h before incubation with LPS (1 ㎍/mL) for 12 h and total RNA was extracted. iNOS mRNA were analyzed by RT-PCR using specific primers.
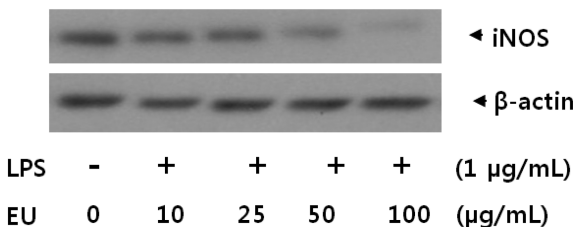
Fig. 4. Effect of EU on PGE$_2$ inhibition. RAW 246.7 cells were treated with LPS (1 ㎍/mL) without and with EU (50, 100, and 200 ㎍/mL), and indomethacin (0.25 mM) for 12 h prior to PGE$_2$ concentration being measured. Different letters indicate significantly different values.
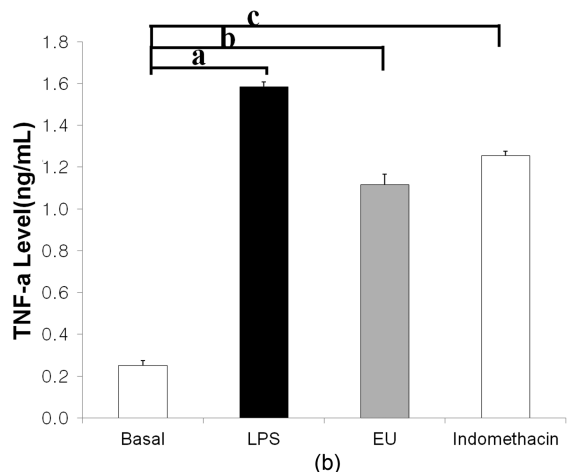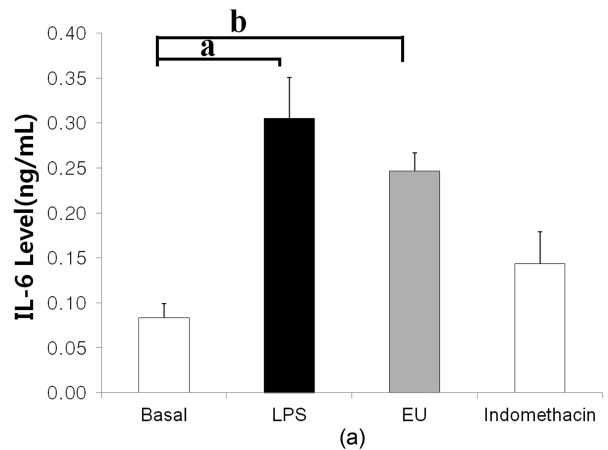
Fig. 5. Effects of Eu on IL-6 (a) and TNF-α (b) production. RAW 246.7 cells were treated with LPS (1 ㎍/mL) with and without EU (50 ㎍/mL), or indomethacin (0.25 mM) for 3 h prior to TNF-α and IL-6 concentration being measured. Different letters indicate significantly different values.

4. Effects of EU on the PGE$_2$ inhibition

And 0.25 mM of indomethacin was used as a positive control in further studies to allow a comparison with EU anti-inflammatory activity (Shin *et al*., 2010). An abnormal level of PGE$_2$ via COX activity is known to mediate inflammation. LPS stimulation significantly increased PGE$_2$ production ($p < 0.05$, Fig. 4). A dose of EU (0.05 mg/mL) did not affect LPS-induced PGE$_2$ production. The induction of PGE$_2$ was decreased by dose dependent of EU but can be mostly abolished by 0.25 mg/mL of indomethacin. Plainly, EU was less potent than indomethacin in the means of production.

5. Effects of EU on IL-6 and TNF-α production

The cellular IL-6 and TNF-α levels were also assessed using a IL-6 and TNF-α specific ELISA experiment. IL-6 and TNF-α are early secreted cytokines (proinflammatory signals). IL-6 is related to a variety of inflammatory processes, particularly acute inflammation (Diehl *et al*., 2002) and mediates autoimmune conditions so IL-6 has become a promising new target for immunomodulatory anti-rheumatic therapy (Nishimoto *et al*., 2006). The inhibition LPS-induced increases in IL-6 and TNF-α has been used to assess the potential anti-inflammatory effects of drug (Kumar *et al*., 2004) and medicinal herb (Kim *et al*., 2005). Fig. 5 a, and b showed that LPS significantly stimulated IL-6 and TNF-α production.

EU (50 μg/mL) significantly inhibited the production of IL-6 and TNF-α in LPS-induced RAW264.7 cells ($p < 0.05$). The administration of 50 μg/mL EU could produce a greater suppression of IL-6 production ($p < 0.05$ concentration of EU(50 μg/mL) required to lower IL-6 and TNF-α by 30% and 20%, respectively, they could be very interesting for rheumatological inflammation treatments.

EU has long been used as traditional oriental herbs as well as ingredients for medicinal food like tea-type beverages. Furthermore, when conducting *in vivo* animal experiments with the iridoid-containing herbal samples, the pharmacokinetic properties of iridoid glycosides should be considered. Therefore, the extract of EU could be a new selective NO inhibitor in the future treatment of arthritis inflammation.

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