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2014년도 2월
석사학위논문

The Regulatory Mechanisms of Immune Response by Natural Products on Macrophage and Mast cells

조 선 대 학 교 대 학 원

생 명 과 학 과

김 동 환

The Regulatory Mechanisms of Immune Response by Natural Products on Macrophage and Mast cells

대식세포와 비만세포에서 천연물에 의한 면역반응 조절 기전 연구

2014년 2월 25일

조 선 대 학 교 대 학 원

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The Regulatory Mechanisms of Immune Response by Natural Products on Macrophage and Mast cells

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이 논문을 이학석사학위 신청 논문으로 제출함

2013 년 10월

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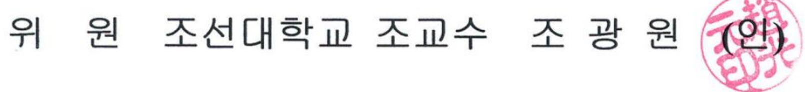
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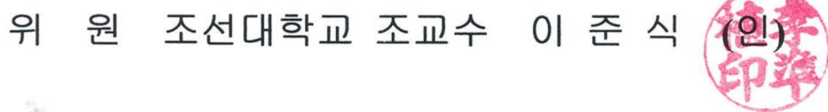
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ABBREVIATIONS

EGL	Extract of *Grateloupia Lanceolata*
MAPK	Mitogen-Activated Protein Kinase
NF-κB	Nuclear Factor-kappa B
TLR	Toll-Like Receptor
LPS	Lipopolysacharride
JNK	c-Jun N-terminal Kinases
ERK	Extracellular signal-Regulated Kinases
iNOS	Inducible Nitric Oxide Synthase
IL	Interleukin
PMA	Phorbol 12-Myristate 13-Acetate
A23187	Calcium Ionophore A23187
TNF	Tumor Necrosis Factor

ABSTRACT

The regulatory mechanisms of immune response by natural products on macrophage and mast cells

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Immune cells participate in the inflammatory response by recognizing and subsequently eliminating unwanted antigens. This response is induced by various inflammatory cytokines secreted by activated immune cells. However, overproduction of these cytokines can cause various inflammatory disorders. Recent studies reported that various natural products can regulate the activation of immune cells such as macrophages and mast cells. In particular, seaweed extract has been demonstrated to inhibit inflammatory cytokines produced by activated macrophages. Therefore, the aim of this study was to determine whether seaweeds inhibit the inflammatory cytokines secreted by activated macrophages.

In recent studies, seaweeds were shown to possess anti-inflammatory activity. However, the effect of *Grateloupia lanceolata*, which inhibits inflammatory cytokine secretion from activated macrophages, remains unknown. Thus, MTT assays were performed to examine the effect of an ethanolic extract of *G.lanceolata* (EGL) on

macrophage cell viability. Moreover, real-time PCR and nitric oxide assays were also conducted to assess inflammatory cytokine expression and nitric oxide (NO) production following EGL treatment. To further elucidate the underlying mechanism of inflammatory cytokine production, protein levels of EGL-pretreated activated macrophages were examined by western blot analysis. Our results demonstrate that EGL was non-cytotoxic to macrophages and decreased inflammatory cytokine production, including IL-1β, and NO. Moreover, EGL inhibited IL-1β and NO expression through the MAPK and NF-κB signaling pathways. These findings provide evidence that EGL may be a candidate therapeutic agent for treating inflammatory diseases. *Grateloupia lanceolata* was first discovered in southern California. Typically, seaweeds reside in the sease of northeastern Asia and North America. The distribution of *G. lanceolata* has been limited to the eastern shores of Asia for a long time. *Grateloupia lanceolata* is a red algae consisting of cirtolline and taurine as major components. This seaweed has a short stalk appressorium with vertical width that can be separated into two or three branches. However, the biological functions of *G. lanceolata* remain poorly understood. Thus, investigation into the biological effect and mechanism of how this seaweed influences anti-inflammation and NO production at the molecular level would be invaluable. In this study, we showed that an extract of *G. lanceolata* inhibited inflammation during LPS-induced macrophage activation by controlling the expression of pro-inflammatory cytokines and genes *via* ERK phosphorylation.

Anaphylaxis is a rapidly occurring allergic reaction to any foreign substance, including venom from insects, foods, and medications, which may cause death. To prevent anaphylaxis, these triggers must be avoided. However, avoidance of many triggers is difficult. For this reason, development of immunotherapeutic adjuvants that

suppress the allergic response is important for anaphylaxis control. Mast cells are one of the major inflammatory cells involved in the inflammatory response, which secrete several inflammatory cytokines such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-1β, and recruits other immune cells. Mast cells are also involved in many diseases such as sinusitis, rheumatoid arthritis, and asthma. Genistein, a phytoestrogen, has been reported to have antioxidative and anti-inflammatory activities. However, the effects of genistein on the anti-inflammatory response of mast cells remain unknown. In this study, we investigated the anti-inflammatory effects of genistein on mast cells. We found that genistein significantly decreased mRNA levels of IL-6 and IL-1β as well as IL-6 production in phorbol 12-myristate 13-acetate (PMA)/A23187-induced mast cells. Moreover, we found that genistein inhibited phosphorylation of ERK 1/2 in PMA/A23187-induced mast cell activation. However, phosphorylation of p38 was not altered. In addition, our findings suggest that genistein inhibited the inflammatory status of mast cells through inhibition of the ERK pathway.

국문초록

대식세포와 비만세포에서 천연물에 의한 면역반응 조절 기전 연구

면역세포는 외부항원에 대한 인식과 제거를 통하여 염증반응을 조절하며, 이러한 반응은 활성화된 면역세포들로부터 분비되는 다양한 염증성 사이토카인에 의해서 유도된다. 그러나, 이들 사이토카인의 과도한 생성은 다양한 염증성 질환을 일으킬 수 있다. 최근 연구에 의하면 다양한 천연물이 대식세포, 비만세포와 같은 면역세포의 활성을 조절 할 수 있다고 보고되어있으며, 특히 해조류 추출물이 활성화된 대식세포에 의해 생성되는 염증성 사이토카인을 억제할 수 있다고 입증되고 있다. 따라서 본 연구의 목적은 해조류로부터 추출된 생리활성 소재가 활성화된 대식세포에 의해 분비되는 염증성 사이토카인을 억제하는지 확인하고자 하였다. 현재까지 활성화된 대식세포로부터 분비되는 염증성 사이토카인 억제에 대한 가는개도박의 효과는 밝혀지지 않았다. 따라서, 가는개도박에 의한 항염증 효능을 검증하기 위하여 가는개도박 에탄올 추출물(EGL)의 세포독성 확인을 위한 MTT assay 를 시행하였고, EGL 처리 후 염증성 사이토카인의 발현과 nitric oxide (NO)생성을 측정하기 위하여 real-time PCR 과 NO assay 를 실시하였다. 더욱이, 염증성 사이토카인 생성의 근본적인 기전을 규명하기 위해 western blot 분석을 통하여 EGL 을 전처리한 활성화된 대식세포에서 신호전달인자의 단백질 수준을 확인하였다. 그 결과, EGL 은 대식세포에 대하여 세포독성을 가지지 않으며, IL-1β를 포함한 염증성 사이토카인과 NO 의 생성을 감소시켰다. 또한, EGL 이 MAPK 와 NF-κB 신호전달 경로를

통하여 IL-1β와 NO 생성을 저해시킴을 확인할 수 있었다. 이러한 결과를 토대로, EGL이 염증성 질환에 대한 치료물질로써의 가능성을 시사하였다.

과민증은 곤충의 독, 음식, 그리고 약물과 같은 특정한 외부 물질에 대해 급성 알러지성 반응을 일컫으며, 심하면 사망에 이르게 할 수 있다. 과민증을 막기 위해서는 이들 유발물질을 피해야 하지만, 다양한 유발물질을 피하는 것은 사실상 어렵다. 이러한 이유로 알러지 반응을 억제하는 면역치료제의 개발은 과민증 조절을 위해 중요하다. 염증성 세포 중 하나인 비만세포는 TNF-α, IL-6, IL-1β와 같은 몇몇 염증성 사이토카인을 분비하여 염증반응에 관여하며 다른 면역세포들을 불러들일 수 있다. 또한 비만세포는 부비강염, 류마티스 관절염, 천식과 같은 많은 질환에 관여한다. Genistein 은 phytoestrogen 으로 항산화와 항염증 효과를 가진다고 보고되어 왔으나, 비만세포의 염증반응에 대한 genistein 의 효과는 아직 알려지지 않았다. 따라서, 본 연구에서는 비만세포에 대한 genistein 의 항염증 효능을 조사하였다. 그 결과, genistein 은 PMA/A23187 에 의해 활성화된 비만세포에서 염증성 사이토카인인 IL-6 와 IL-1β의 mRNA 수준을 현저히 감소시켰을 뿐만 아니라 IL-6 의 생성 또한 감소시켰다. 또한, genistein 이 PMA/A23187 에 유도된 비만세포의 활성에서 ERK 1/2 의 인산화를 억제시키는 것을 확인하였다. 이러한 결과를 종합해보면, genistein 이 ERK 경로를 억제함으로써 비만세포의 염증반응을 조절한다고 제시할 수 있다.

I. Introduction

Inflammation is a complex biological response to harmful stimuli that is associated with many pathophysiological conditions. Macrophages play an essential role in the local host defense and inflammation. In response to inflammatory stimuli, activated macrophages produce various pro-inflammatory molecules, including cytokines and nitric oxide. Synthesized by inducible nitric oxide synthase (iNOS) in mammalian cells, NO plays an important role in many diseases, such as inflammation, hypertension, diabetes, and atherosclerosis (Park et al., 2012; Park et al., 2010). iNOS is induced in response to various inflammatory stimuli, including lipopolysaccharide (LPS), and is responsible for excessive NO production by macrophages during the inflammatory process (Chun et al., 2012). Activation of toll-like receptor by LPS leads to the activation of the MAP kinase (MAPK) and nuclear factor (NF)-κB transcription factors. Activation of NF-κB is important for iNOS activity and cellular responses to extracellular signals and the regulation of pro-inflammatory cytokines, especially IL-1β, IL-6, and TNF-α. Therefore, targeted reduction of NO and pro-inflammatory cytokine production has been proposed as an effective strategy for treating inflammatory diseases (Welters et al., 2000; Gray Pearson and Mahesh Karandikar, 2001; Kawai and Akira, 2006).

Grateloupia lanceolata was first discovered in southern California. Typically, seaweeds reside in the sease of northeastern Asia and North America (Miller et al., 2009). The distribution of *G. lanceolata* has been limited to the eastern shores of Asia for a long time. *Grateloupia lanceolata* is a red algae consisting of cirtolline and taurine

as major components. This seaweed has a short stalk appressorium with vertical width that can be separated into two or three branches. However, the biological functions of *G. lanceolata* remain poorly understood.

This study was performed to identify the anti-inflammatory effect of an EGL. Specifically, we investigated the mechanisms by which EGL modulates NO and pro-inflammatory cytokine production by macrophages stimulated by LPS. Our data demonstrate that EGL reduces LPS-induced NO and pro-inflammatory cytokine production by macrophages through inhibition of ERK phosphorylation.

II. Materials and Methods

II-1. Cell culture

The Raw 264.7 cell line, which was derived from murine macrophages, was obtained from the American Type Culture Collection (Manassas, VA, USA). These cells were maintained in DMEM medium, supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin, at 37°C and 5% CO_2 in humidified incubator. Macrophages were plated in 96-well plates for analysis of cell viability or 6-well plates for other experiments. Pre-treated compounds were dissolved in DMSO and the final concentration of the solvent in the medium was 0.2% by volume. Cells were then stimulated by the addition of 1 μg/ml LPS.

II-2. Purification of total extraction of *G. lanceolata*

The red algae *G.lanceolata* was collected from Korea sea of Wando, Korea, twice on 11th and 12th February in 2012. The algal specimens were maintained at -20°C until use. The marine red algae *G.lanceolata* was washed four times with pure water for removed salt out and homogenized by blender after completely dried. The dried *G.lanceolata* were powdered and incubated in shaking incubator with ethanol (100 g powder per 1 L of 70% ethanol) for 3 days at room temperature twice. The EtOH-extract was filtered using qualitative filter paper, concentrated to a minimum volumn using a rotary evaporator. The remained solvent was removed by lyophilization from concentrated samples and lyophilized powder was dissolved in 100% DMSO.

II-3. Cell viability

Cell viability was evaluated by and MTT assay. Cells were plated into 96-well at 2 × 10^4 cells/well and cultured for 24 h. Cells were then treated with various concentrations of EGL and incubated for 24 h, and then a 4 h incubation with MTT (0.5 mg/ml). Following removal of the culture medium, the cells were dissolved in DMSO and shaken for 10 min. Metabolic activity was quantified by measuring the absorbance of the samples at 570 nm.

II-4. Nitric oxide assay

Raw 264.7 cells (5 × 10^4 cells/well) were seeded in 96-well culture plates in DMEM. Cultured cells were pretreated with various concentrations of EGL (0-100 μg/ml) for 2 h and then cells were incubated for 22 h in presence of LPS. After incubation, the cultured medium was mixed with an equivalent volume of 1× Griess Reagent (Sigma, MO, USA) and incubated for 15 min at room temperature. After incubation, absorbance was measured by ELISA microplate reader at 540 nm of absorbance (Jin et al., 2010).

II-5. Real time PCR and reverse transcription (RT)-PCR

Total RNA was isolated from EGL-treated cells using TRI reagent (Sigma) according to manufacturer's protocol. To synthesize cDNA, 0.5 μg of total RNA was

primed with oligo dT and reacted with mixture of M-MLV RTase, dNTP, and reaction buffer (Promega, WI, USA). To measure the mRNA level of inflammatory cytokine, cDNA was amplified using Gene Atlas G02 gradient thermal cycler system (Astec, Japan) e-Taq DNA Polymerase kit (Solgent, Daejeon, Korea) and to synthesize cDNA, primer, and SYBR® Green master mix, amplified cDNA was measured by Mx3005P QPCR System (Agilent Technologies, CA, USA) indicated primers. (IL-1β primer sequence forward : GTG TCT TTC CCG TGG ACC TT and IL-1β primer reverse : TCG TTG CTT GGT TCT CCT TG).

II-6. Western blot analysis

Cell were harvested, washed twice with ice-cold PBS, and lysed in RIPA buffer (Sigma-Aldrich, MO) supplemented with protease inhibitor cocktail (Sigma) for 30 min on ice with vortexing every 10 min. Lysates were centrifuged at 13000 ×g for 15 min to remove insoluble material, and protein concentration in supernatants were determined using BCA kit (Thermo scientific, PA, USA), according to the manufacturers' instructions. Equivalent amount of protein samples were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to polyvinylidene fluoride (PVDF) membrane (Millipore, MA, USA). The membranes were incubated with blocking (5% skim milk in TBS) for 1 h. After blocking, membranes probed with anti-total ERK, p-38, JNK (Cell Signaling Technology, MA, USA) and anti-p-ERK, p-p38, p-JNK, p-p65, β-actin primary antibodies (Santa Cruz Biotechnology, CA, USA). And then, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbits or anti-mouse secondary

antibodies (Santa Cruz Biotechnology) for 2 h. Band detection were visualized using chemiluminescence (ECL) (Biorad, CA, USA) detection system and exposed to radiographic film.

II-7. Statistical analysis

All results were expressed as the means ± SD of the indicated number of experiments. Statistical significance was estimated using a Student's t-test for unpaired observations, and the differences were compared with regard to statistical significance by one-way ANOVA, followed by LSD *post hoc* test. A P of < 0.05 was considered significant.

III. Results

III-1. Preparation of *G. lanceolata* extract

We collected *G. lanceolata* (Fig. 1) in Wando, South Korea, during February 2012. After removing salt and debris, the seaweed was incubated at 37˚C to eliminate moisture. The collected samples were washed gently with clean water three times and then dried with hot air (40˚C) for 2 days. The extract was isolated with 10 volumes (v/w) of 70% ethanol at room temperature overnight; this procedure was repeated three times. The extracts were filtered through Whatman filter paper No. 2 (Whatman Ltd, Maidstone, Kent, England), concentrated with a vacuum evaporator, and completely dried with a freeze dryer (Fig. 2). Recently, several seaweeds have been reported to possess anti-oxidant and anti-inflammatory activities, as well as inhibition of NO production. For example, Fucosterol from *Undaria pinnatifida*, a green algae, inhibited the production of NO and other pro-inflammatory cytokines (Yoo et al., 2012 ; Chatter et al., 2011; Heo et al., 2010).

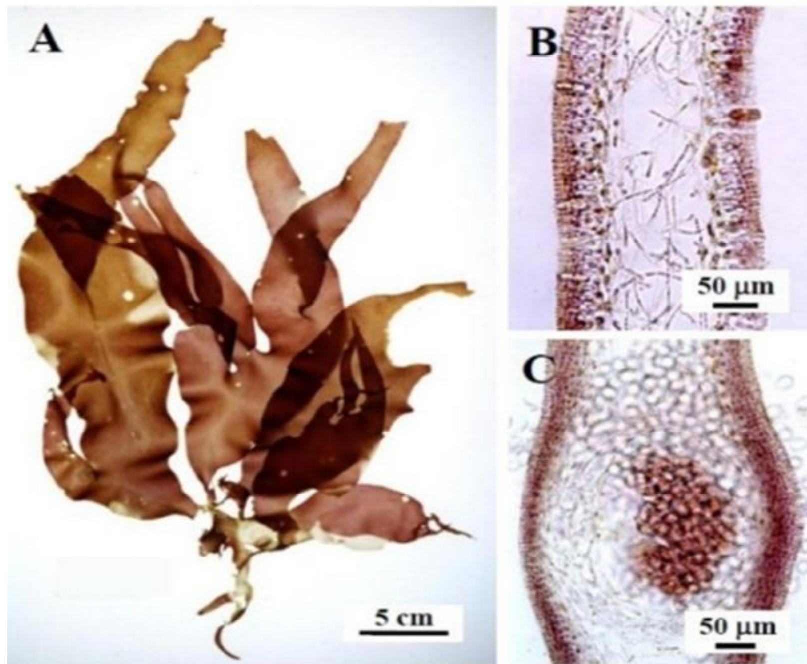

Fig. 1. Morphology of *G. lanceolata.* *Grateloupia lanceolata* was first discovered in southern California. Typically, seaweeds reside in the sease of northeastern Asia and North America.

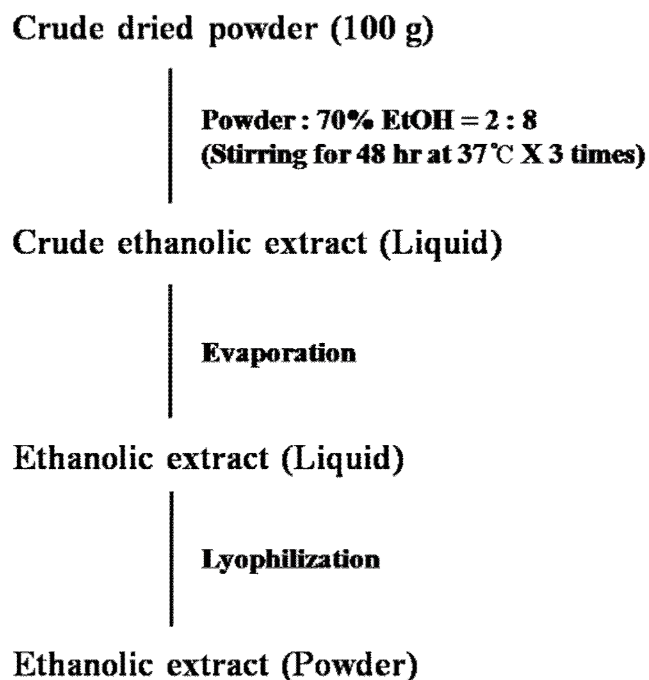

Fig. 2. Process of extraction on obtained sea weeds. Processing of seaweed extracts. Crude dried powder was produced by pulverizing the seaweed. *G. lanceolata* powder (100 g/L) was extracted using 70% ethanol, which was removed using a rotary evaporator. The ethanolic extract was converted into powder form through freeze drying.

III-2. EGL inhibits NO production in LPS-stimulated macrophage

We first examined the cytotoxicity and anti-inflammatory effects of various seaweeds in macrophage using the antioxidant assay (Table .1). We found that EGL has high efficiency of antioxidant activity. Therefore, in this study, we investigated the anti-inflammatory effect of EGL in LPS-induced macrophage activation. We examined the cytotoxicity of EGL in macrophages using the MTT assay. The cells were cultured in the presence of various concentrations of EGL (25, 50, and 100 μg/ml). Treatment with 25 to 100 μg/ml EGL for 24 h did not affect macrophage cytotoxicity (Fig. 3). To investigate whether EGL regulates NO production, we examined NO levels in LPS-stimulated macrophages following treatment with EGL for 2 h. Our data demonstrate that LPS treatment significantly induced NO production compared with control (Fig. 4). However, pre-treatment of macrophages with EGL inhibited LPS-induced NO production significantly in a dose-dependent manner. These results suggest that the concentrations of EGL that inhibit NO production in LPS-stimulated macrophages are non-cytotoxic.

Table 1. Screening of various seaweeds for their effect on cytotoxicity and inhibition of NO production.

Seaweeds Species	Cytotoxicity	Activity	Leading materials potential
Undaria pinnatifida	★	NONE	NONE
Saccharina japonica	NONE	NONE	NONE
Sargassum fulvellum	NONE	★★★	★★★
Sagassum horneri (Turner) C. Agardh	NONE	★★★★	★★★★★
Polyopes affinis (Harvey) Kawaguch et Wang	★	★	★
Laurencia okamurae Yamada	NONE	★	★
Callophyllis japonica Okamura in De Toni & Okamura	**Induce**	★★	★★★
Ulva pertusa Kjellwan	★	★	★
Ecklonia cava Kjellman	**Induce**	★★★	★★★
Sargassum thunbergii (Mertens ex Roth) Kuntze	★★	★★	★★
Hizikia fusiformis	★★★	★★★	NONE
Grateloupia lanceolata (Okamura) Kawaguchi	NONE	★★★★	★★★★★

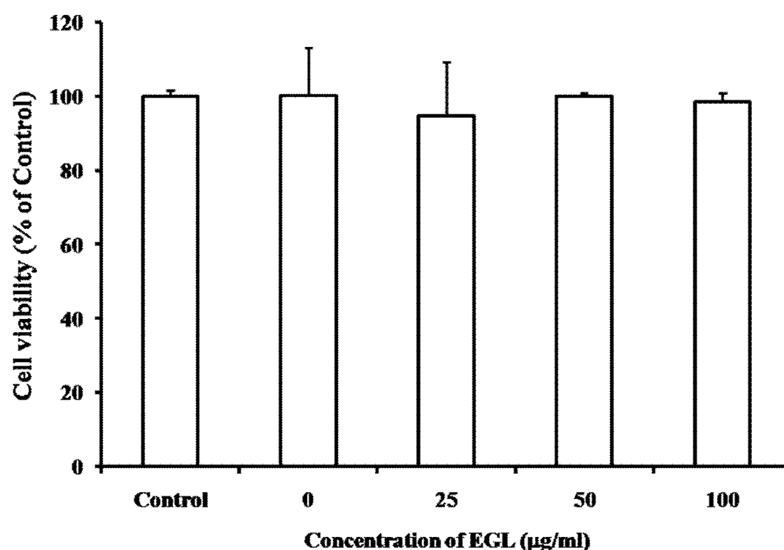

Fig. 3. EGL had no effect on cell viability in Raw 264.7 murine macrophages. Raw 264.7 cells were plated in 96-well cell culture plates. Various concentrations of EGL and control (0.2% DMSO) were added, and viable cell numbers were assessed by MTT assay after 24 h incubation, as described in *Materials and Methods*. Data are reported as viable cells numbers expressed as a percentage of control cells that were exposed to 0.2% DMSO. The data represent the average (±SD) of four replicate wells and are representative of three separate experiments.

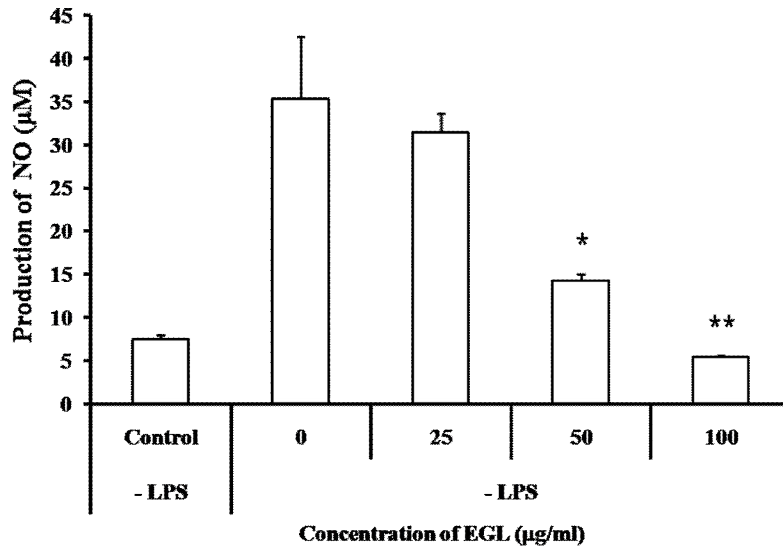

Fig. 4. EGL decreased LPS-induced NO production in Raw 264.7 murine macrophages. Raw 264.7 cells were plated in 96-well cell culture plates. Various concentrations of EGL (0-100 μg/ml) were pretreated on cells for 2 h, and then the cells were incubated for 22 h in presence or absence of LPS (1 μg/ml). Supernatants were mixed with griess reagent and absorbance was measure by ELISA microplate reader. The data represent the average (±SD) of four replicate wells and are representative of three separate experiments. *p<0.05 and **p<0.01 compared LPS only groups.

III-3. EGL suppresses the expression levels of pro-inflammatory genes

IL-1β is a pro-inflammatory cytokine that is released from macrophages upon stimulation with LPS or other inflammatory stimuli. To examine the effects of EGL in IL-1β expression and production, RT-PCR and ELISA were performed to measure IL-1β levels in cell lysate or culture media produced by macrophages pre-treated with for 2 h and then stimulated with LPS for 1 h. As shown in Fig. 5, stimulation of macrophages with LPS increased IL-1β expression, which was significantly reduced by EGL in a dose-dependent manner.

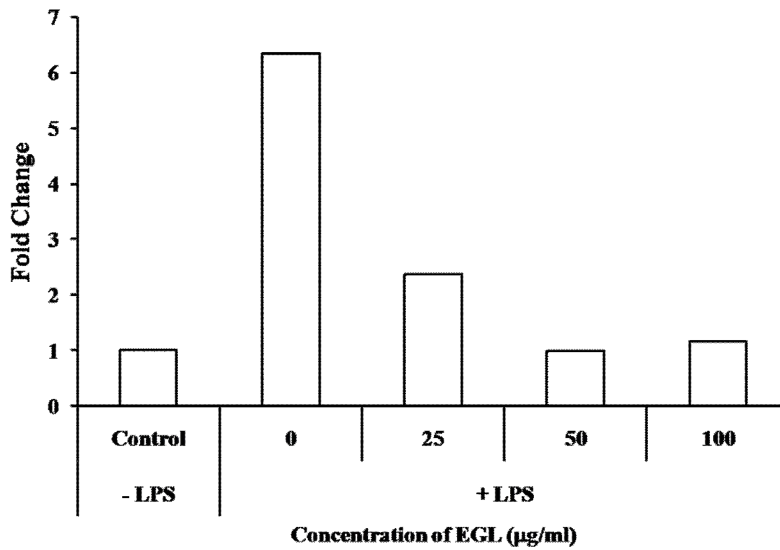

Fig. 5. EGL inhibited LPS-induced the expression level of IL-1β gene on Raw 264.7 murine macrophages. Raw 264.7 cells were pretreated with 0 to 100 μg/ml EGL for 2 h, and stimulated by LPS for 1 h. The production of expression level of IL-1β gene was determined by real-time PCR. The GAPDH was used as the normalizing control

III-4. EGL inhibits LPS-induced ERK and JNK phosphorylation but not p38 MAP kinase

The activation of macrophage was occurred *via* TLR4 recognizing the LPS. After activation of TLR4, a variety of inflammatory cytokines such as IL-1β and IL-6 are expressed though signal transduction. The MAPKs ERK1/2 and p38 are involved in signaling pathways that regulate inflammatory mediators *via* activation of transcription factors, especially NF-κB. To assess the molecular basis of EGL-mediated inhibition of inflammatory mediators, we determined the effect of EGL on LPS-induced MAP kinase phosphorylation. As shown in Fig. 6A, phosphorylation of ERK1/2, JNK1/2, and p38 were increased at 30 min in LPS-induced macrophages. Treatment with EGL strongly inhibited LPS-induced phosphorylation of ERK1/2 and JNK1/2. In contrast, p38 phosphorylation was not affected by treatment with EGL. This result indicates that the ERK and JNK pathway may contribute to EGL-mediated inhibition of pro-inflammatory gene expression in LPS-stimulated macrophages.

Activated NF-κB plays critical roles in LPS-induced expression of inflammatory mediators and cytokines such as iNOS, COX-2, and IL-1β in macrophages. Therefore, we determined the effects of EGL on LPS-stimulated activation of the NF-κB subunit p65. As shown in Fig. 6B, EGL inhibited LPS-stimulated phosphorylation of NF-κB p65 in macrophages, suggesting the NF-κB pathway, along with the ERK and JNK signaling cascade, may contribute to the inhibitory effects of EGL on down-regulating pro-inflammatory mediators such as iNOS and IL-1β (Fig. 7).

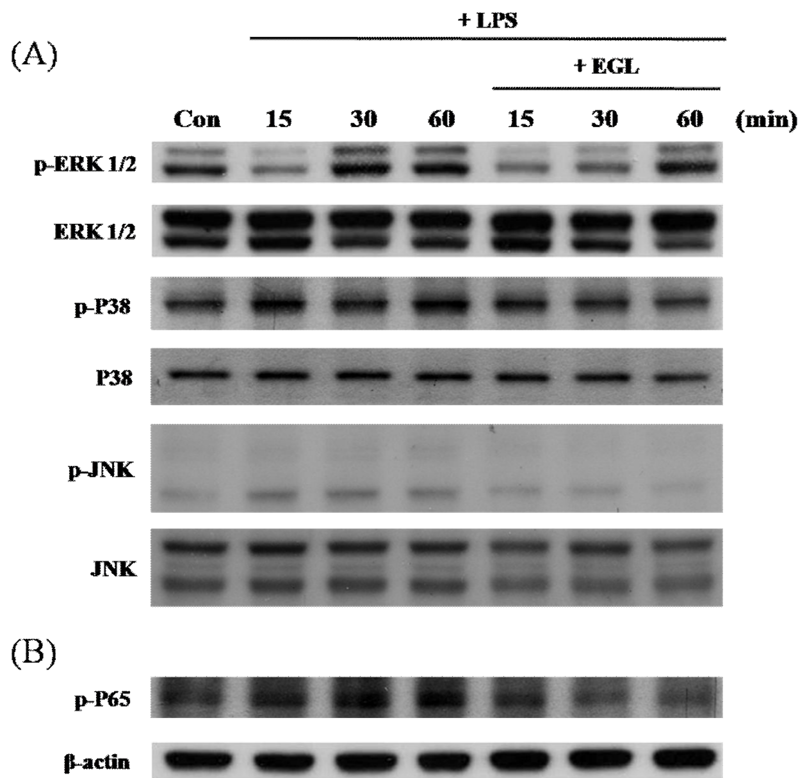
+ LPS
(A)
+ EGL
Con
15
30
60
15
30
60
(min)
p-ERK 1/2
ERK 1/2
p-P38
P38
p-JNK
JNK
(B)
p-P65
β-actin

Fig. 6. EGL inhibited LPS-induced ERK and JNK phosphorylation. Raw 264.7 cells were starved in serum free DMEM and 100 μg/ml EGL was pretreated with cells after 2 h, cells were stimulated with 1 μg/ml LPS for 15, 30, and 60 min. (A) Cells were lysed and equal amount of whole cell proteins were separated by SDS-PAGE and transferred to PVDF membranes. Membranes were probed with anti-ERK, anti-p-ERK, anti-p-38, anti-p-p38, anti-JNK, and anti-p-JNK antibodies. (B) To determine NF-κB translocation, whole cell proteins were probed by anti-p-p-65 antibody. β-actin was used as the internal control. The results are from one experiment representative of four performed that showed similar patterns.

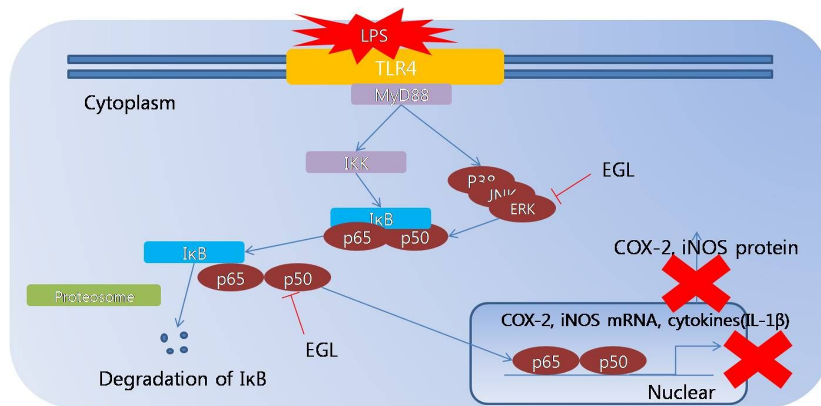

Fig. 7. The simplified depiction of the anti-inflammatory mechanism of EGL on LPS-induced macrophage activation. Treatment of EGL inhibited the production of IL-1β and NO. In addition, EGL suppressed the phosphorylation of ERK 1/2 and JNK 1/2 on the LPS-induced macrophage activation.

IV. Discussion

Macrophages are one of the major cell types that infiltrate the sites of chronic inflammatory disease, such as rheumatoid arthritis, fibrosis, and atherosclerosis (Libby, 2002; Wellen and Hotamisligil, 2005). In this study, we demonstrated that EGL from *G. lanceolata* possesses effective anti-inflammatory properties that inhibit the production of pro-inflammatory mediators such as NO, iNOS, and IL-1β in LPS-stimulated macrophages. Moreover, our results demonstrated that EGL suppressed the expression of iNOS and IL-1β mRNA. EGL also exhibited inhibitory effects on LPS-stimulated phosphorylation of the ERK and JNK, which may mediate its anti-inflammatory activity by suppressing NF-κB activation.

NO is a highly reactive molecule with important roles in several biological processes and inflammation. NO production can be mediated by the products of different NO metabolites, but also iNOS expression. Generated by immune-activated macrophages at sites of inflammation, NO, along with PGE2, plays critical roles in the process of macrophage activation as well as both acute and chronic inflammation (Welters et al., 2000; Kroncke et al., 1997; Evans, 1995). For reason of this, the suppression of NO production by the inhibition of iNOS protein expression can be a very important therapeutic approach in the development of anti-inflammatory agents. In present study, we showed that EGL inhibits the production of NO in LPS-induced macrophage activation (Fig 5).

MAP kinases are a highly conserved family of protein serine/threonine kinases with critical roles in iNOS up-regulation induced by various stimuli. Several studies

have reported that MAP kinase phosphorylation is involved in LPS-induced iNOS expression (Zhou et al., 2008). Moreover, activation of IκB/NF-κB is associated with the MAP kinase signaling pathway. Previous studies have also shown that degradation of IκBα following its phosphorylation and the rapid translocation of the p65 and p50 subunits are essential processes for NF-κB activation in response to various stimuli. Upon activation by LPS, this transcription factor controls the expression of genes associated with cell survival, pro-inflammatory enzymes, and cytokines, such as iNOS, TNF-α, COX-2, IL-1β, and IL-6. In LPS-stimulated macrophages, iNOS and COX-2 expression was dependent on NF-κB activation. Therefore, we examined the effect of EGL on the phosphorylation of MAP kinases and the NF-κB subunit p65. As shown in Fig. 6A and B, we found that phosphorylation of ERK1/2, JNK1/2 and the NF-κB subunit p65 were all significantly inhibited in LPS-stimulated macrophages pretreated with EGL. These results suggest that EGL may not only inhibit the activation of NF-κB, but also upstream signaling proteins such as ERK1/2 and JNK1/2.

In summary, our results demonstrate that EGL isolated from *G. lanceolata* suppresses NO synthesis through the down-regulation of iNOS gene expression in LPS-stimulated macrophages. Moreover, inhibition of iNOS expression is related to the blockade in ERK1/2, JNK1/2, and NF-κB signaling in LPS-stimulated macrophages. Although further investigation is still required to elucidate the exact mechanism, this study provides important information regarding the use of EGL as a candidate therapeutic agent against inflammation.

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I. Introduction

Allergic responses frequently occur in developed nations, anaphylactic shock being particularly deadly (Metcalfe et al., 2009). Anaphylactic shock is a rapidly occurring severe allergic response that may cause death (Oskeritzian et al., 2010). Anaphylaxis can occur in response to any foreign substance, including venom from insects, foods, and medications (Boden and Wesley Burks, 2011; Lee and Vadas, 2011; Worm, 2010). Globally, 0.05-2% of people are estimated to suffer anaphylaxis during their lifetimes, and this rate appears to be increasing (Simons, 2010). It is critically important to avoid the specific triggers such as foods and medications to prevent anaphylaxis (Simons, 2009). However, this is not always possible. Thus, development of immunoadjuvants is essential for preventing anaphylaxis and allergic reactions.

Mast cells derived from hematopoietic cells are associated with tissue immunity and innate immunity and play an important role in allergic and anaphylactic reactions (Metcalfe et al., 2009). Mast cells can secrete pre-formed mediators such as histamine, heparin, and inflammatory cytokines by degranulation (Tchougounova et al., 2003; Zhang et al., 2011). Antigen IgE-dependent-activated mast cells induce degranulation to secrete three kinds of mediators. Several inflammatory and chemotactic cytokines, such as IL-1β, IL-6, and TNF-α, are produced from activated mast cells (Lee et al., 2007). Moreover, these pro-inflammatory cytokines support the well-recognized role of mast cells in allergic

inflammation and hypersensitivity (Lee et al., 2007). Histamine release from mast cells stimulates cardiac contraction, vascular permeability, and anaphylaxis; the IgE-dependent pathway, which results in histamine release, is part of the mechanism related to anaphylaxis (Metcalfe et al., 2009).

The production of these cytokines is transmitted through signal molecules such as the transcription factors MAPK and NF-κB (Kalesnikoff and Galli, 2008; Shin et al., 2012). MAPKs are expressed and activated in a systemic inflammatory disorder and play an important role in the control of cytokines, chemokines, and cell proliferation (Guma et al., 2010). As transcriptional factors, MAPK and NF-κB play a pivotal role in inflammation by virtue of their ability to induce transcription of an array of inflammatory genes, particularly the regulation of pro-inflammatory molecules including IL-1β, IL-6, and TNF-α (Lee and Lim, 2010; Lu et al., 2013).

The phytoestrogen genistein (4′,5,7-trihydroxyisoflavone) is an isoflavonoid compound containing soy beans. It has a variety of biological effects, including anti-inflammatory and antioxidant properties, and inhibits protein tyrosine kinases (PTK) and influences immune responses (Dixon and Ferreira, 2002; Masilamani et al., 2011). Genistein also modulates the activation of NF-κB and Akt during inflammation (Kitaura et al., 2000). Immortalized human mammary epithelial cells demonstrated decreased ERK1/ERK2 phosphorylation when treated with genistein, specifically inhibiting cytokine-induced ERK phosphorylation (Frey and Singletary, 2003). Several animal studies indicate that genistein reduces production of pro-inflammatory molecules such as IL-6 and TNF-α in the plasma of

rats. Moreover, in an acute liver inflammation model, oral administration of soy-derived genistein suppresses IL-6, IL-1β, and TNF-α in RAW264.7 macrophages (Zhao et al., 2006; Palanisamy et al., 2011). However, the signaling pathways involved in the anti-inflammatory effect of genistein on human mast cell activation remain unknown.

In the present study, we sought to investigate the anti-inflammatory effects of genistein on PMA- and A23187-, a calcium ionophore, induced expression of pro-inflammatory cytokines and histamine release, as well as their related regulatory signaling pathways.

II. Materials and Methods

II-1. Cell culture and genistein treatment

Human leukemic mast cell line (HMC)-1 was obtained from Dr Dae Ki Kim at Chunbuk National University. HMC-1 were cultured in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% heat-inactivated Fetal Bovine Serum (FBS), 100 IU/mL penicillin, 100 μg/mL streptomycin (PAA, NJ, USA) at 37°C, 5% CO_2. Genistein was dissolved in dimethyl sulphoxide (DMSO) (Sigma, Mo, USA) and diluted to the desired concentration in IMDM (final DMSO concentration 0.02% v/v). An equal amount of DMSO was added in control samples (medium only).

II-2. Cell viability

Cell viability was measure by MTT assay. Cells (2×10^4 cells/well) were seeded in 96-well u-bottom culture plates with IMDM at 37°C, 5% CO_2. Cells were treated with various concentration of genistein (0-50 μM) and incubated in 37°C for 24 h. After treatment, MTT (0.5 mg/mL) in medium was added to each wells and incubated at 37°C for 4 h. After incubation, MTT solution was removed and formazan product was dissolved in a solvent (DMSO:ethanol = 1:1) into colored solution. Absorbance was measured by ELISA microplate reader at 570 nm wavelength (Biotech, CA, USA).

II-3. Polymerase chain reaction and reverse transcription (RT)-PCR

Total RNA was isolated from genistein-treated cells using TRI reagent

(Sigma) according to manufacturer's protocol. To synthesize cDNA, 0.5 μg of total RNA was primed with oligo dT and reacted with mixture of M-MLV RTase, dNTP, and reaction buffer (Promega, WI, USA). To measure the mRNA level of inflammatory cytokines, PCR was performed using synthetic cDNA and selective primers : IL-6 forward, GAG GCA CTG GCA GAA AAC AA ; IL-6 reverse, TTG GGT CAG GGG TGG TTA TT ; IL-1β forward, GTA CCT GAG CTC GCC AGT GA ; IL-1β reverse, TGA AGC CCT TGC TGT AGT GG ; TNF-α forward, CCA TCA GAG GGC CTG TAC CT ; TNF-α, reverse; CAG ACT CGG CAA AGT CGA GA ; GAPDH forward, AAG GGT CAT CAT CTC TGC CC ; GAPDH reverse, GTG ATG GCA TGG ACT GTG GT. The PCR products stained with Loading star (Dain Biotechnology, Seoul, Korea) were electrophoresed on 1% agarose gel and bands were detected by UV transilluminator (CoreBio, CA, USA).

II-4. Western blot analysis

HMC-1 (2×10^6 cells/well) were seeded in 60Φ cell culture dish and starved with serum free IMDM for 6 h. After starvation, cells were pretreated with genistein for 30 min, and then stimulated with 20 nM / PMA and 1 μM calcium ionophore A23187 for 15, 30, and 60 min. Treated cells were washed by cold-PBS and lysed by modified RIPA buffer (50 mM Tris-HCl, 0.1% sodium dodecyl sulphate, 0.5% sodium deoxycholate, 1% NP-40, and 150 mM sodium chloride, pH 8.0) at 4°C for 30 min. The lysates were centrifuged at 13,000 ×g for 15 min and the supernatant was used for protein samples. Protein concentration was measured according to the manufacturers' instructions by colorimetric BCA kit (Thermo scientific, PA, USA). Equivalent amounts

of protein were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and electrophoretically transferred to polyvinylidene fluoride (PVDF) membrane (Millipore, MA, USA). The membranes were incubated with blocking (5% skim milk in TBS) for 1 h. After blocking, membranes probed with anti-ERK, anti-p-ERK, anti-p-38, and anti-p-p38 primary antibodies (Santa Cruz Biotechnology, CA, USA). And then, the membranes were incubated with horseradish peroxidase-conjugated anti-rabbits or anti-mouse secondary antibodies (Santa Cruz Biotechnology) for 2 h. Band detection were visualized using chemiluminescence (ECL) (Biorad, CA, USA) detection system and exposed to radiographic film.

II-5. Enzyme-linked immunosorbent assay

HMC-1 cells (1×10^6 cells/well) were seeded in 96-well u-bottom culture plates. Cells were pretreated with various concentration of genistein (0-50 μM) for 30 min, and then stimulated by treatment of PMA/A23187 for 48 h. Cultured cells were cleared separated by microcentrifugation and the supernatant was used for samples. The quantification of IL-6 release was measured by Human IL-6 ELISA MAX™ Deluxe Sets (BioLegend, CA, USA), according to manufacturer's protocol. Briefly, standards and samples were incubated on capture antibody coated plate at 4°C, overnight. Detection antibody was incubated for 1 h and Avidin-HRP bind to detection antibody. To visualization, substrate solution was added to each well, and then the reaction was stopped by stop solution (2N H_2SO_4). Absorbance was measured by ELISA microplate reader at 405 nm wavelength.

II-6. Statistical anaylsis

All results were expressed as the means ± SD of the indicated number of experiments. Statistical significance was estimated using a Student's t-test for unpaired observations, and the differences were compared with regard to statistical significance by one-way ANOVA, followed by LSD *post hoc* test. A P of < 0.05 was considered significant.

III. Results

III-1. Genistein had no effect on cytotoxicity in a human mast cell line (HMC-1)

In an initial series of experiments, we investigated whether genistein would influence the cytotoxicity of a human mast cell line-1 (HMC-1). HMC-1 were treated with various concentrations of genistein, ranging from 0 to 50 μM, for 24 h and subjected to a MTT assay. As shown in Fig. 1, addition of 50 μM genistein did not alter HMC-1 numbers when compared to the control or cells treated with only medium (DMSO [0.02%] in IMDM). Therefore, we used this range of genistein concentrations to determine the anti-inflammatory effects of genistein during mast cell activation.

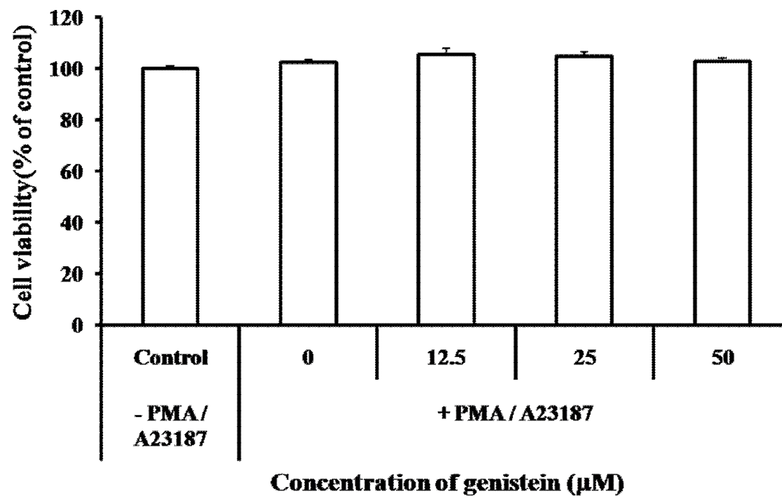

Fig. 1. Cytotoxicity of genistein on HMC-1. HMC-1 (2×10^4 cells/well) were pretreated with genistein (0-50 μM) and then incubated for 24 h. Cell viability was determined by MTT assay. Data are representative of three independent experiments. Each datum represents the mean ± SEM of three independent experiments.

III-2. Genistein suppresses expression levels of pro-inflammatory cytokines in PMA/A23187-induced HMC-1

Pro-inflammatory cytokines are important mediators of inflammation, cell recruitment, and allergenic responses (Woolley and Tetlow, 2000). To evaluate the effect of genistein on the gene expression of pro-inflammatory cytokines, we first treated HMC-1 with genistein, then stimulated the cells with PMA (20 nM) and A23187 (1 μM), and analyzed the gene expression of the pro-inflammatory cytokines using RT-PCR. As shown in Fig. 2A, a high level of genistein (50 μM) significantly suppressed the gene expression of IL-1β and IL-6. However, TNF-α gene expression remained unaltered (Fig. 2B).

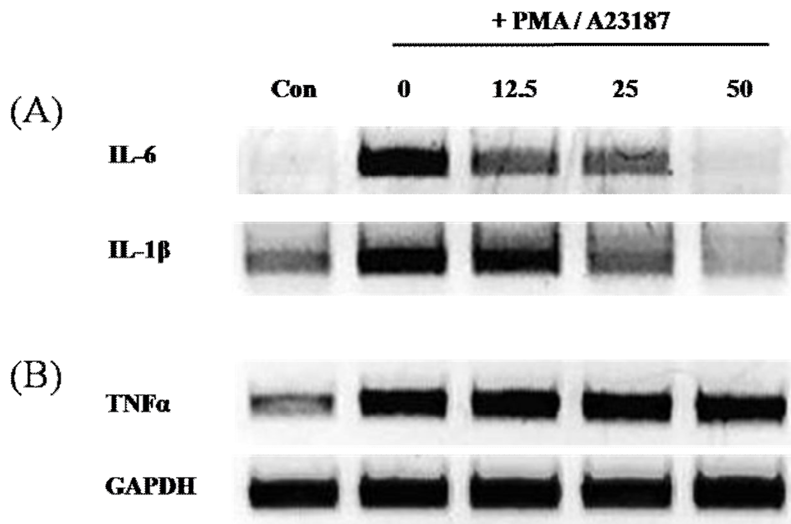

Fig. 2. Effect of genistein on gene expression of inflammatory cytokines in activated HMC-1. HMC-1 (1×10^5 cells/well) were pretreated with various concentrations of genistein (0-50 μM) for 30 min and stimulated by PMA (20 nM) and A23187 (1 μM) for 2 h. Total RNA was extracted and gene expression of IL-1β, IL-6, and TNF-α was quantified by RT-PCR. Products were electrophoresed on a 1% agarose gel, stained with Loading star, and captured using a Kodak DC 290 digital camera. Data are representative of three independent experiments.

III-3. Genistein significantly inhibits IL-6 production in PMA/A23187-induced HMC-1

IL-6 is crucial for mast cell maturation; activated mast cells increase IL-6 mRNA associated with protein kinase C (PKC) activity and also up-regulate histamine production (Conti et al., 2002). Previously, we found that genistein suppressed gene expression of pro-inflammatory cytokines IL-1β and IL-6. To confirm the effect of genistein on the gene expression of pro-inflammatory cytokines, culture supernatants were assayed for cytokine levels by ELISA. HMC-1 were pretreated with genistein (0-50 μM) for 30 min, and then stimulated with PMA and A23187 for 48 h. As shown in Fig. 3, genistein strongly decreased the production of IL-6 in PMA/A23187-induced HMC-1. These results indicate that genistein inhibits pro-inflammatory cytokines such as IL-1β and IL-6 in PMA/A23187-activated HMC-1.

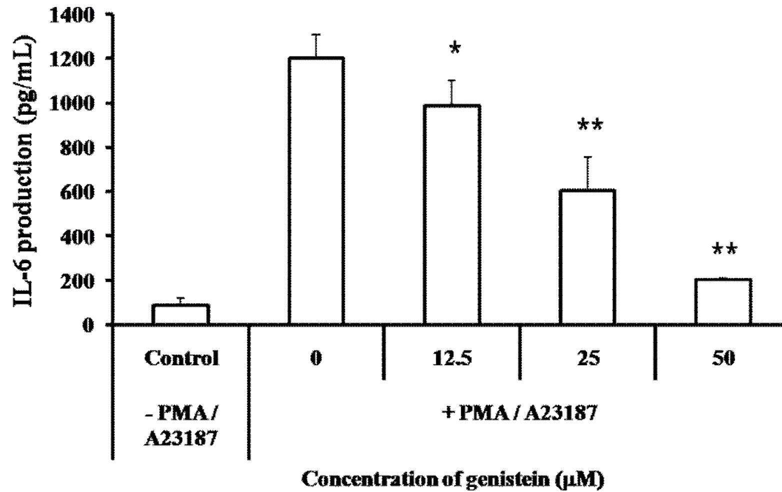

Fig. 3. Effect of genistein on production of IL-6 in activated HMC-1. HMC-1 (1×10^6 cells/well) were pretreated with genistein (0 to 50 μM) for 30 min, then stimulated with PMA (20 nM) and A23187 (1 μM) for 48 h. IL-6 levels in the supernatant were measured using ELISA and represented as the mean ± SEM of three independent experiments. *$p<0.05$ and **$p<0.01$ compared with PMA/A23187 only groups.

III-4. Genistein regulates phosphorylation of ERK

To evaluate the mechanism of the effect of genistein on gene expression of pro-inflammatory cytokines, we investigated the influence of genistein on MAPK phosphorylation. IL-1β and IL-6 expression is regulated by a transcription factor, NF-κB, and activated by MAPK pathways (de Bittencourt Pasquali et al., 2013). In order to investigate the effects of genistein on the MAPK signaling pathways, HMC-1 were pretreated with 50 μM genistein and kinase activation was assessed. Phosphorylation of p38 MAPK and ERK1/2 were measured by phosphor-specific western blotting. As shown in Fig. 4, genistein inhibited the phosphorylation of ERK1/2 in PMA/A23187-induced mast cells. These results indicate that genistein inhibits pro-inflammatory cytokine production *via* regulation of the ERK pathway.

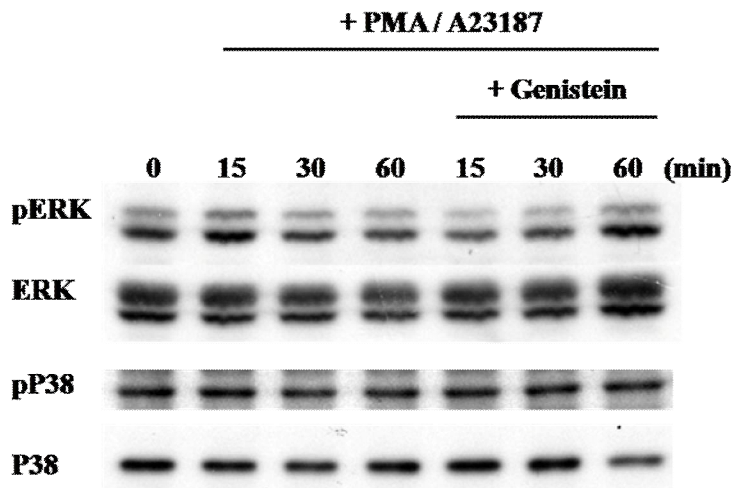

Fig. 4. Effect of genistein on MAPK phosphorylation in activated HMC-1. Cells were treated with 50 μM genistein for 15, 30, and 60 min. Cell lysates were prepared and blotted with anti-phospho-ERK1/2, anti-ERK1/2, anti-phospho-p38, and anti-p38 antibodies. A signal was detected with biotinylated goat anti-rabbit IgG and visualized using enhanced chemiluminescence. The results are representative of four experiments.

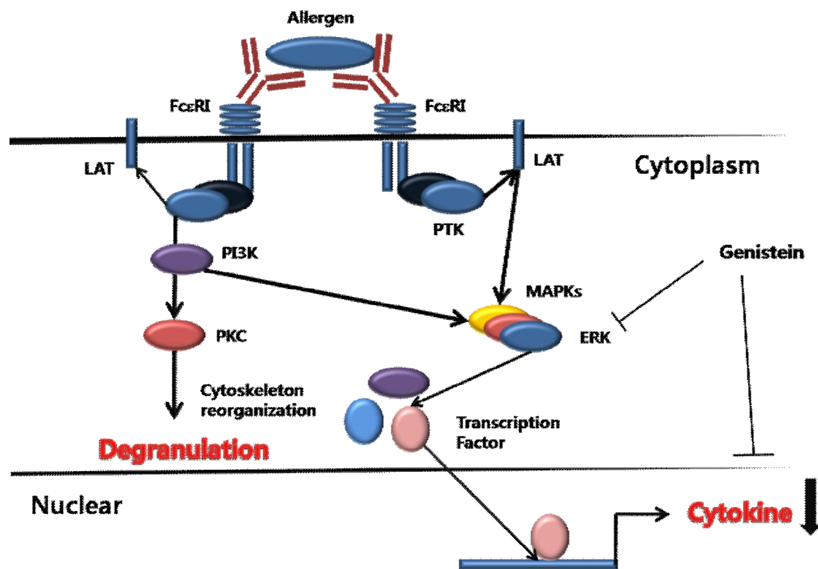

Fig. 5. The simplified depiction of the anti-allergic mechanism of genistein on PMA/A23187-induced human mast cells activation. Treatment of genistein inhibited the mRNA expression of IL-1β and IL-6 genes. Moreover, the production of IL-6 was inhibited by genistein treatment on PMA/A23187-induced human mast cells activation. Genistein induced down-regulation of ERK 1/2 in activated human mast cells.

IV. Discussion

Allergic diseases including asthma and anaphylaxis are a severe health burden for many nations. In the United States, an estimated 20 million patients are treated for allergic diseases, at a cost exceeding $15 billion annually (Pullen et al., 2012). Anaphylaxis is a Type I hypersensitivity reaction mediated by IgE-activated mast cells and occurs locally and systemically; it is caused by various inflammatory mediators such as histamine, tryptase, and several cytokines from activated mast cells. These inflammatory mediators affect leukocyte recruitment, and cause vasodilation, increased vascular permeability, and bronchial-constriction (Galli et al., 2008). Therefore, many researchers have sought to develop new therapeutic adjuvants for allergenic inflammation *via* regulation of mast cells. To address this, we investigated the anti-inflammatory effects of genistein, a phytoestrogen, on mast cell activation.

Generally, PMA activates PKC, resulting in activation of mast cells. In addition, the calcium ionophore, A23187, increases the permeability of the cell membrane to Ca^{2+} and can selectively activate gene expression of calcium-regulated genes (Resendez et al., 1986). In addition, A23187 alone has been shown to induce granule release in mast cells (Zhang et al., 2011). In this study, we used HMC-1, an immature human mast cell line derived from the peripheral blood of a patient suffering from mast cell leukemia. HMC-1 lacks FcεRI, making it difficult to activate the mast cells through IgE-mediated responses. In addition, we also used co-treatment with PMA and A23187 to activate mast cells.

Genistein is a phytoestrogen isolated from *Genista tinctoria*; the chemical name is derived from the generic name. Kim et al. reported that genistein-4′-O-α-L-rhamnopyranosyl-(1→2)-β-D-glucopyranoside from *Sophora japonica* (Leguminosae) ameliorates mast cell-mediated allergic inflammation in vivo and in vitro. Moreover, this report by Kim et al. suggests that the anti-inflammatory effect of the genistein compound is involved in the regulation of inflammatory cytokines, including IL-8 and TNFα (Kim et al., 2011). Recent studies found that genistein inhibited transcription factors, including GATA-binding protein (GATA)-3 and signal transducer and activator of transcription (STAT)-6, which control the Th1/Th2 response in an asthma mouse model. Specifically, genistein decreased Th2-type cytokine levels and attenuated ovalbumin (OVA)-induced airway inflammation (Gao et al., 2012). However, the underlying mechanisms by which genistein inhibits inflammatory mediators in mast cell activation remain unknown.

To our knowledge, this is the first report showing that genistein inhibits pro-inflammatory cytokines in activated mast cells. We found that genistein significantly suppressed expression of IL-1β and IL-6 in PMA/A23187-induced mast cells (Fig. 2A). In general, mast cells release an array of mediators with potential to cause allergic inflammation, such as the pro-inflammatory cytokines IL-1β, IL-6, and TNF-α. Specifically, IL-1β plays an important role in allergic response, the local accumulation of IL-6 is associated with a local allergic reaction, and both IL-1β and IL-6 promote inflammation and a mast cell-mediated immune response (Yu and Li, 2000; Ganeshan and Bryce, 2012; Ganeshan et al., 2013). These reports indicate that reduction of pro-inflammatory cytokines from mast cells

is one of the keys to reducing inflammatory symptoms. Our results showing inhibition of IL-1β and IL-6 expression by genistein support the idea that genistein has an anti-inflammatory effect resulting from the reduction of these mediators in mast cells. Moreover, the induction of these cytokines was involved in the activation of the MAPK and NF-κB pathway. Blackwell et al. reported on the role of NF-κB activation in the regulation of cytokine production in allergic inflammation (Blackwell et al., 1997). NF-κB activation is also associated with MAPK activation. To address this, we investigated the inhibitory effect of genistein on pro-inflammatory cytokines *via* regulation of MAPK. We found that genistein inhibits the phosphorylation of ERK. However, p38 MAPK levels were not altered by pretreatment with genistein in activated mast cells (Fig. 4). These results suggest that the inhibitory effect of genistein on pro-inflammatory cytokine production is associated with ERK signaling pathways.

In conclusion, genistein inhibits the gene expression and production of pro-inflammatory cytokines IL-1β and IL-6, but did not alter TNF-α levels. Moreover, genistein attenuates activation of the ERK signaling pathways (Fig. 5). Our study also suggests that genistein has potential for use as a treatment for allergic inflammation and anaphylactic shock.

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