Regulation of German cockroach extract-induced interleukin-8 expression in human airway epithelial cells

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Regulation of German cockroach extract-induced interleukin-8 expression in human airway epithelial cells

Directed by Professor Tai Soon Yong

The Doctoral Dissertation submitted to the Department of Medical Science, the Graduate School of Yonsei University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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June 2007

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Yonsei University

June 2007

ACKNOWLEDGEMENTS

I would like to express my deep appreciation for Prof. Tai Soon Yong, thesis supervisor, who gave me the wonderful opportunity to be a Ph.D. And I would also like to thank Prof. Kyu Earn Kim and Myung Hyun Sohn for their willingness to take me into their research group and support me to concentrate on my study throughout. I am indebted to Prof. Se Kyu Kim and Jung Won Park for their kind help and enthusiasm.

Thanks are due to Prof. Jae Woo Kim for his passionate guidance to my study, Dr. Kyoung Yong Jeong for continuous offer for cockroach extract and Dr. Hye Yung Yum for her sincere encouragement.

I am very grateful to all my colleagues and friends, Soo Young Choi, Jung Ho Sohn, Kyung-Hwa, Hyun-Jung, Ji-Eun, Hea-Sun, Jung-Yeon, Kyung-A, who gave me pleasant memories and power to endure difficulties in studying.

Finally, with all my heart, I would like to thank my families for taking care of me patiently and helping me with everything for the completion of this work.

Through the last 4 years, I could have learned not only scientific techniques but also more importantly, the method of scientific thinking, how to identify a research problem, how to form and carry out a research plan and how to get results with even attitude to my life. I am sure what I have learned here will greatly benefit my future careers and I am willing to be the one not to be contrary to your expectations.

June, 2007

Kyung Eun Lee

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ABSTRACT

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(Directed by Professor Tai-Soon Yong)

Cockroaches have been implicated as a cause of respiratory allergies such as asthma. Interleukin (IL)-8 plays an integral role in the coordination and persistence of the inflammatory process in the chronic inflammation of the airways in asthma. We investigated the mechanism by which German cockroach extract (GCE) triggers IL-8 release from human airway epithelial cells.

Chemical inhibitors were pretreated before addition of GCE for promoter activity and protein synthesis of IL-8. Transcriptional activity of IL-8 promoter was analyzed by mutational, deletional anaylsis and

electrophoretic mobility shift assay (EMSA).

Stimulation of H292 cells with GCE resulted in a time- and concentration-dependent induction of IL-8 transcription and protein synthesis. IL-8 promoter deletion analysis indicated that position -132 to +41 was essential for GCE-induced IL-8 transcription. And mutants with substitutions in activator protein (AP)-1, nuclear factor (NF)-IL6 and NF-κB binding sites revealed a requirement for NF-κB and NF-IL6, but not AP-1, in GCE-induced activation of the IL-8 promoter. The DNA binding activities of NF-κB and NF-IL6 were induced by GCE, as determined by EMSA. The chemical inhibition of extracellular signal-regulated kinase (ERK) attenuated GCE-induced transcriptional activity and protein synthesis. In addition, through aprotinin treatment and PAR2 small interfering (si) RNA transfection, it was proven that protease of GCE is consistent with the regulation of GCE-induced IL-8.

We conclude that GCE with protease activity induced IL-8 expression is regulated by transcriptional activation of NF-κB and NF-IL6 coordinating with ERK pathway in human airway epithelial cells.

Key words: German cockroach extract, airway epithelial cell, IL-8, ERK, NF-IL6, NF-κB

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I. INTRODUCTION

Cockroach allergy has been recognized as an important cause of asthma and allergic rhinitis. Cockroach asthma has been described as a severe disease, associated with perennial symptoms and high levels of total IgE. Cockroaches produce several allergens that induce sensitization, and exposure to high levels of cockroach allergens in the home is a major risk factor for symptoms in sensitized individuals [1]. The most common domestic species of cockroach are *Blattella germanica* (German cockroach) and *Periplaneta americana* (American cockroach) [2]. It appears that cockroach allergens derive from several sources, including cockroach saliva, fecal materials, secretions, cast skins, debris, and dead bodies. The allergenicity of cockroach

extract has been demonstrated in human subject by means of skin test, bronchial provocation tests, and RASTs [3]. So far, identified allergens of German and American cockroach include Bla g 2 (inactive aspartic protease), Bla g 4 (lipocalin or calycin), Bla g 5 (glutathione-S-transferase), Bla g 6 (troponin C), the Group 1 cross-reactive allergens Bla g 1 and Per a 3 (arylphorin), and Per a 7 or Bla g 7 (tropomyosin) [1, 4]. Many aeroallergens have been shown to possess enzymatic activity, including house dust mite (*Dermatophagoides pteronyssinus*) cysteine protease Der p 1 and serine proteases Der p 3 and Der p 9; fungal extracts, including *Alternaria alternata* and *Cladosporium herbarum*; and the cat allergen *Felis domesticus* (Fel d 1) [5]. Previously, we reported that GCE with protease activity has a direct effect on human airway epithelial cells in particular generating Ca2+ release [6]. Although the precise pathophysiological role of the proteolytic activity of these cockroach allergens has not been fully elucidated in vivo, recent data provide evidence that the proteolytic activity amplifies allergen-induced bronchial asthma. In addition, this proteolytic activity has been implicated in the regulation of epithelial cell release of GM-CSF, IL-8 and IL-6 in vitro [7, 8].

Among these cytokines, IL-8 belongs to the C-X-C subfamily of chemokines and is a potent chemoattractant and stimulus of neutrophils. IL-8

has been shown to affect other inflammatory and immunocompetent cells and is therefore believed to play a pivotal role in inflammatory disease. It is also found in bronchoalveolar lavage fluid and serum from patients with asthma[8]. Patients with status asthmaticus also exhibit dramatic increases of this cytokine in their airways [9]. IL-8 gene is regulated transcriptionally, post-transcriptionally, and translationally, but major part of this regulation occurs at the transcriptional level [10]. In a number of studies, it was found that a sequence spanning nucleotides -1 to -133 within the 5' flanking region of the IL-8 gene is essential and sufficient for transcriptional regulation of the gene [11]. This promoter element contains a NF-κB element that is required for activation in all cell types as well as AP-1 and CCAAT enhancer-binding factor (C/EBP or NF-IL6) sites. The transcription factor NF-κB appears to play a key role in the regulation of lung epithelial cell cytokine expression [12]. Unlike the NF-κB site, the AP-1 and C/EBP sites are not essential for induction but are required for maximal gene expression [11, 13]. Different signal transduction pathways, such as protein kinase C (PKC), cyclic AMP-dependent protein kinase (PKA), protein tyrosine kinase, phophatidylcoline-specific phospholipase, or mitogen-activated protein kinase, can regulate IL-8 gene expression in various cell types [14]. To date, numerous lines of functional evidence indicated that the activity of transcription factors that can

bind to NF-κB, AP-1, and CCAAT/enhancer-binding protein (NF-IL6) binding sites in the IL-8 promoter are regulated by the coordinated activation of the MAP kinases ERK, c-Jun NH2-terminal kinase (JNK), and p38 MAP kinase (p38 MAPK) in response to stimuli that induce IL-8 production [15]. It is therefore likely that the protease activity of GCE-induced responses activate similar pathways to regulate the release of pro-inflammatory cytokines and trigger the immune response seen in GCE-related asthma. Thus, in this study we examined whether GCE induces IL-8 in epithelial cells and investigated the intracellular mechanisms which regulate IL-8 gene expression.

II. MATERIALS AND METHODS

1. Cell culture

The human epithelial carcinoma cell line H292 was obtained from American Type Culture Collection (ATCC, MD, USA). Cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 100 U/mL Penicillin and streptomycin (GibcoBRL, NY, USA). H292 cells were grown in sterile T-75 tissue culture flasks and maintained at 37℃ in an incubator with 5% CO_2.

2. German cockroach extract

Thirty grams of live or frozen German cockroaches were pulverized in liquid nitrogen. The defatted sample was then placed in 200 mL of 1:1 ethyl ether/ethyl acetate and extracted with slow stirring at 4℃ overnight in PBS (pH 7.4) containing 6 mM/L 2-mercaptoethanol and 1 mg/mL 1-phenyl-3-(2-thiazolyl)-2-thiourea to prevent melanization. The extract was then centrifuged at 10,000 rpm for 30 minutes at 4℃, and the supernatant was filtered through a 0.22 μm filter. The supernatant of cockroach crude extract was shown to be negative for endotoxin by E-toxate assay (Sigma Chemical, MO, USA) and was measured as 0.00926 EU/mL by the QCL-1000 chromogenic Limulus amebocyte lysate kit (Cambrex, MD, USA). Total protein in the extract was 3.60 mg/mL, as evaluated with a Bradford assay

reagent (Bio-Rad, CA, USA).

3. Reagents and antibodies

Specific antibodies (Abs) against phophorylated (p)-p44/42, p-p38, p-JNK and control Abs were purchased from Cell Signaling Technology (New England Biolabs, MA, USA) and p65, p50, c/EBPα and c/EBPβ were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). E-64 and aprotinin were from Sigma-Aldrich (MO, USA). PD98059, U0126, SB202190, JNK Inhibitor II, Curcumin and CAPE were purchased from Calbiochem (CA, USA).

4. Measurement of human IL-8 protein

Cells grown on 12-well plates were stimulated with GCE at 37℃ in RPMI 1640 (GibcoBRL, NY, USA) by a series of doses and induction times, as specified. Supernatants were aspirated and stored at -70℃ until assayed. IL-8 levels in the culture supernatants were determined by specific ELISA (enzyme-linked immunosorbent assay) against human IL-8 according to the manufacturer's guideline (R&D system, MN, USA).

5. RT-PCR

Total RNA was isolated from H292 cells cultured with GCE over specified induction times using RNeasy Mini Kit (Qiagen, CA, USA). For synthesis of first strand cDNA, total RNA (2 μg) was resuspended in 12 μl diethyl-pyrocarbonate-treated water with random hexamers. The mix was incubated at 65℃ for 10 min and cooled on ice. First strand buffer (100 mM Tris-HCl, pH 8.4, 250 mM KCl), 10 mM dNTP mix, 0.1M DTT, and 25 U Superscript II RT (GibcoBRL, NY, USA) were then added to samples. The samples were incubated at 42℃ for 60 min, and the reverse transcriptase was heat-inactivated at 70℃ for 10 min. PCR conditions for human IL-8 PCR assay included an initial denaturation step at 94℃ for 5 min; 30 cycles of 94℃ (30 sec), 56℃ (30 sec), 72℃ (30 sec); and, a final extension step of 72℃ for 5 min. Primer sequences were as follows: human IL-8: 5'-AGA TAT TGC ACG GGA GAA-3' (sense) and 5'-GAA ATA AAG GAG AAA CCA-3' (antisense); GAPDH: 5'-ACC ACA GTC CAT GCC ATC AC-3' (sense) and 5'-TCC ACC ACC CTG TTG CTG TA-3' (antisense). The final PCR products were run on ethidium bromide-stained 1.5% agarose gels in 0.5 X TAE buffer.

6. Western immunoblot analysis

Cells grown on dishes were stimulated with GCE at 37℃ in serum-free RPMI 1640 for specified induction times. Briefly, after treatment, cells were

washed with cold 1X PBS, and lysed for 40 min on ice in lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1 mM EDTA, 1mM EGTA, 10% glycerol, and 0.5% NP-40) containing 1 mM dithiotreitol, 0.1 mM sodium vanadate. Samples were mixed with SDS-PAGE buffer containing β-mercaptoethanol, heated at 95℃ for 3 min and ultracentrifuged before loading onto polyacrylamide gels (5% stacking, 10% resolving gel). After electrophoresis, separated proteins were transferred to a nitrocellulose membrane for 2 hrs at 200 mAmp. Membranes were blocked with TBS with 5% dry milk for 1 hr and incubated with specific Abs in TBS/0.1% Tween and 5% dry milk overnight at 4℃. After washing and incubating with secondary Abs, an ECL detection system was used for protein detection (New England Biolabs, MA, USA).

7. Construction of IL-8 promoter deletion fragments

The -1322/+42 fragment of full length human IL-8 promoter was subcloned into a luciferase reporter plasmid (-1322/+42 hIL-8/Luc) (Promega, WI, USA). Six deletional constructs were made to identify important regulatory elements on the hIL-8 promoter related to GCE-induced gene expression.

Site-directed mutagenesis of the AP-1, NF-IL6 or NF-κB binding site in the -132/+42 hIL-8 promoter was introduced by PCR with mutagenic primers to

obtain mutAP-1, mutNF-IL6 and mutNF-κB luciferase plasmids using the GeneTailor site-directed mutagenesis system (Invitrogen, CA, USA). Primer sequences were as follows: mutAP-1: 5'-GAA TTC GCC CTT GTG TGA TAG ATC TGG TTT GCC C-3' (sense) and 5'-TCA CAC AAG GGC GAA TTC CAG CAC ACT GGC-3' (antisense); mutNF-IL6: 5'-CTG AGG GGA TGG GCC ATC GAG TGA AAA TCG TGG A-3' (sense) and 5'-ATG GCC CAT CCC CTC AGG GCA AAC CTG AGT-3' (antisense); mutNF-κB:5'-GGC CAT CAG TTG CAA ATC GTT AAC TTT CCT CTG A-3' (sense) and 5'-ACG ATT TGC AAC TGA TGG CCC ATC CCC TCA-3' (antisense).

To measure transcription activity from the IL-8 promoter, cells were transfected with the relevant reporter plasmids by a liposome-mediated technique (Invitrogen, CA, USA). Transfection efficiency was assessed by co-transfection with pSV-β-galactosidase. After treatment with GCE, cells were harvested and analyzed for luciferase and β-galactosidase activity (luciferase and β-galactosidase enzyme assay systems, Promega, WI, USA) by a luminometer.

8. Electrophoretic mobility shift assays (EMSA)

Nuclear extracts were prepared by NE-PER nuclear and cytoplasmic extraction reagents (Pierce, IL, USA). EMSAs were performed using nuclear

extract binding buffer (100 mM Tris, 500 mM KCl, 10 mM DTT, pH 7.5), and biotin-labeled probes incubated at room temperature for 20 min. The sequences of the sense strands of the oligonucleotides used for EMSA were as follows: NF-IL6: 5'-TGC AGA TTG CGC AAT CTG CA-3' (Santa Cruz, CA, USA) and NF-κB: 5'-AGT TGA GGG GAC TTT CCC AGG C-3' (Promega, WI, USA). For supershift experiments, binding reactions were conducted in the presence of specific antibodies to individual p65, p50, c/EBPα and c/EBPβ proteins (overnight at 4℃) before the addition of biotin-labeled probes. The protein-DNA complexes were electrophoresed on 6% polyacrylamide gels at 4℃ in 0.5x TBE and transferred to a nylon membrane. The UV coss-linked membrane was then exposed to X-ray film for detection by Lightshift chemiluminescent EMSA kit (Pierce, IL, USA).

9. Small interfering RNA (siRNA)

Human PAR2 siRNA was purchased from Santa Cruz Biotechnology, Inc (CA, USA). Cells were transiently transfected with human PAR2 siRNA by Lipofectamine 2000 (Invitrogen, CA, USA), according to the manufacturer's protocol. Control siRNA-A (Santa Cruz, CA, USA) served as a scrambled siRNA control. PAR2 knockdown was assessed by RT-PCR at 44 h after transfection. Total RNA, cDNA and specific primers (IL-8 & GAPDH) used

were described in above RT-PCR.

10. Statistical analysis

Data were expressed as mean ± SEM from three to six independent experiments. Statistical analysis comparing treatment and control groups was assessed by Student's *t* test, and $p < 0.05$ was considered significant.

III. RESULTS

1. German cockroach extract (GCE) induces IL-8 expression in H292 cells

GCE was analyzed by SDS-PAGE, and proteins were detected by Coomassie Blue staining (Figure 1). GCE significantly enhanced IL-8 production in a time- and dose-dependent manner in H292 cells (Figure 2. A). Based on 100 μg/mL GCE treatment, IL-8 production was increased by 23.9 ± 0.6, 72.9 ± 14.7, 692.1 ± 78.3, 1165.2 ± 36.2, 1495.3 ± 57.0 pg/mL according to 1, 2, 6, 12, 24 hrs, respectively. Levels of IL-8 mRNA transcripts were also stimulated by GCE (50 μg/mL) in a time-dependent manner (Figure 2. B).

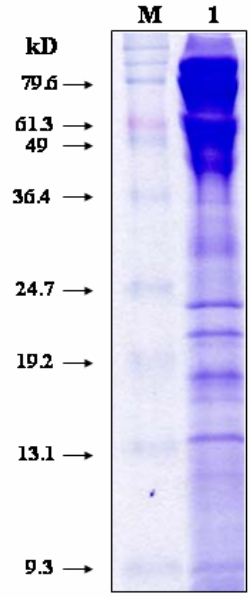
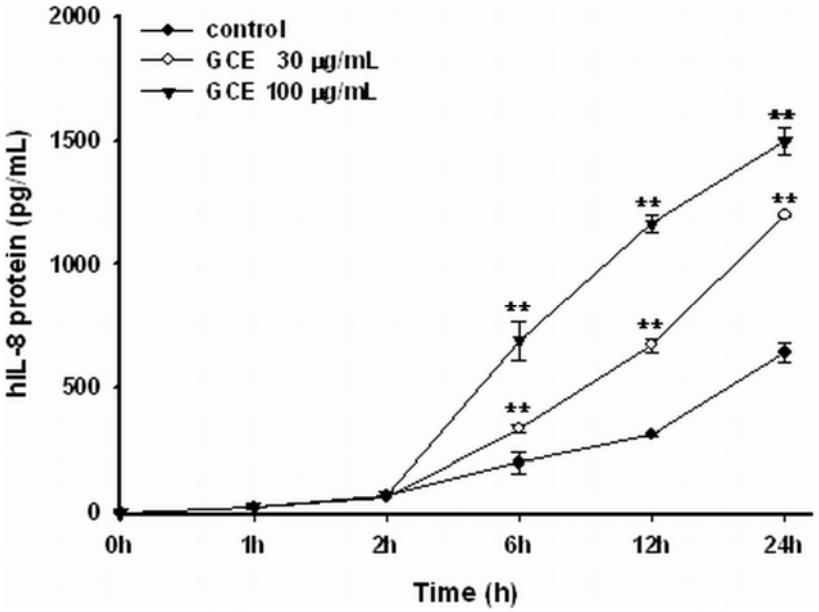
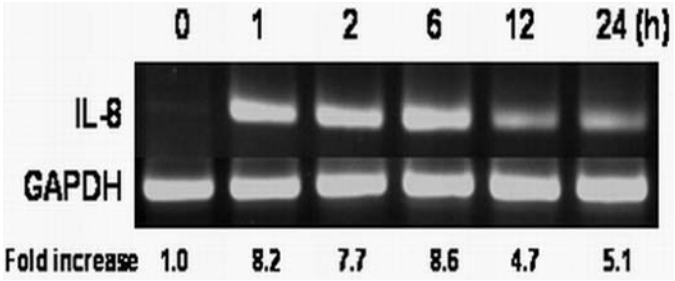


Figure 1. German cockroach extract (GCE) was analyzed by SDS-PAGE, and proteins were detected by Coomassie Blue staining.



A



B

Figure 2. Effect of GCE on IL-8 protein abundance and mRNA. A: IL-8 concentrations of supernatants collected from control cultures and those treated with GCE were measured by ELISA, B: The RNA from H292 cells treated with GCE for the indicated times was harvested and used for RT-PCR. The data represent the mean ± SEM from six separate experiments.

2. Endotoxin dose not affect to GCE-induced IL-8

Cockroach crude extract was shown to be negative for endotoxin by E-toxate assay (Sigma Chemical, MO, USA) and was measured as 0.00926 EU/mL by the QCL-1000 chromogenic LAL kit (Cambrex, MD, USA). And, after boiling GCE for 5 min to remove endotoxin, we incubated H292 cells with GCE and the boiled GCE (100 μg/mL) depending on times. As shown Figure 3, both GCE and the boiled GCE-induced IL-8 proteins were similarly increased depending on times.

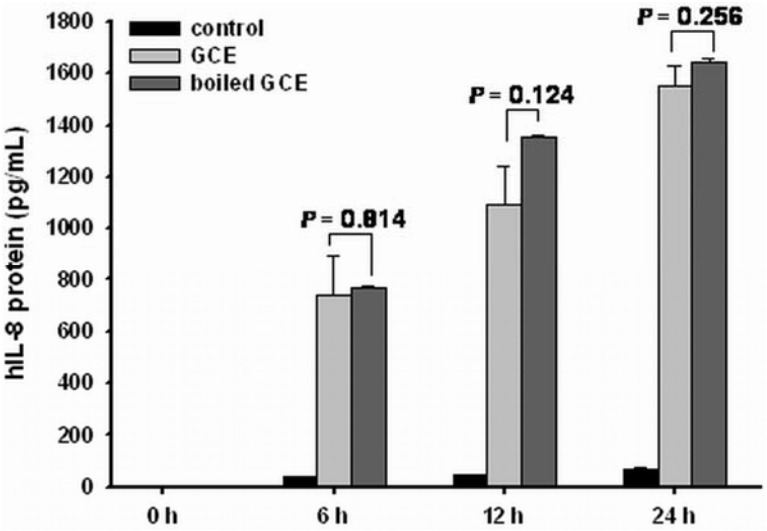
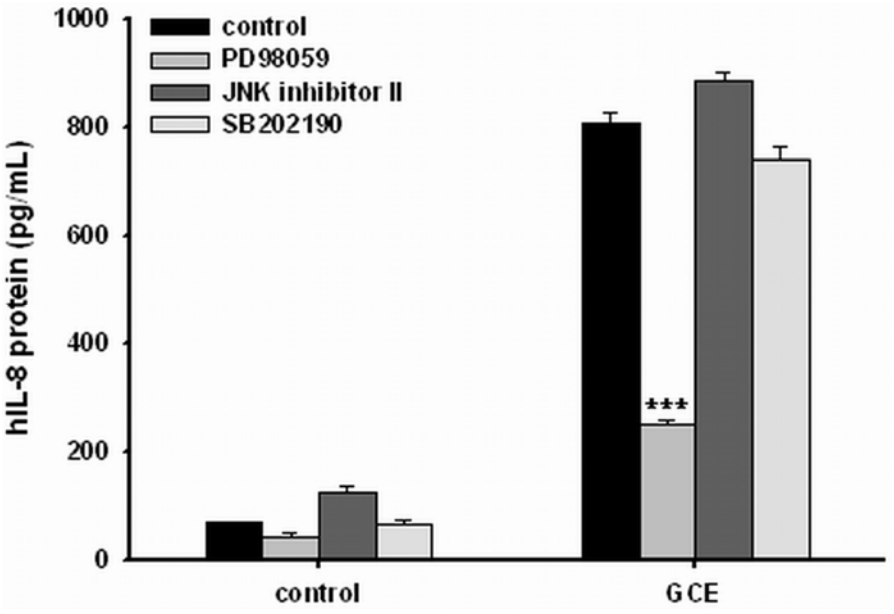


Figure 3. Effect of endotoxin of GCE on IL-8 protein. H292 cells were treated with GCE and the boiled GCE for 6, 12, 24 hrs. The IL-8 concentrations of supernatants from cultures were measured by ELISA. The data represents the mean ± SEM of at least three independent experiments performed in duplicate.

3. Effect of ERK on GCE-induced IL-8

To determine whether MAP kinases could regulate IL-8 expression, we measured activation of ERK, p38 and JNK MAP kinases by chemical inhibitor treatment and anti-phosphorylated ERK (p44/42), p38 and JNK protein Abs. First, chemical inhibition of ERK activation by PD98059 significantly decreased GCE-induced IL-8 protein synthesis. However, inhibitions of p38 and JNK by SB202190 and JNK inhibitor II failed to effectively attenuate IL-8 protein abundance in H292 cells (Figure 4. A). Second, in the result of western blot analysis, resting cells before exposure exhibited extremely low levels of phosphorylated p44/42 MAP kinase. Exposure to GCE resulted in a distinct increase in phosphorylated p44/42 MAP kinase levels in H292 cells. After a 5 min exposure to GCE, phosphorylation of p44/42 MAP kinase was detected and continued for a period of 40 min post-exposure. This was followed by a decrease to basal levels after 60 min (Figure 4. B). On the other hand, phosphorylations of p38 and JNK were not activated by GCE, as seen in Figure 4. B. Third, based on the hypothesis that EKR have central role in GCE-induced IL-8, we pretreated ERK inhibitors (U0126 and PD98059) with various concentrations before GCE treatment (100 μg/mL). In case of U0126, IL-8 protein was decreased to over 76% (Figure 4. C) and PD98059 also inhibited over 55% compared to

GCE alone (Figure 4. D).



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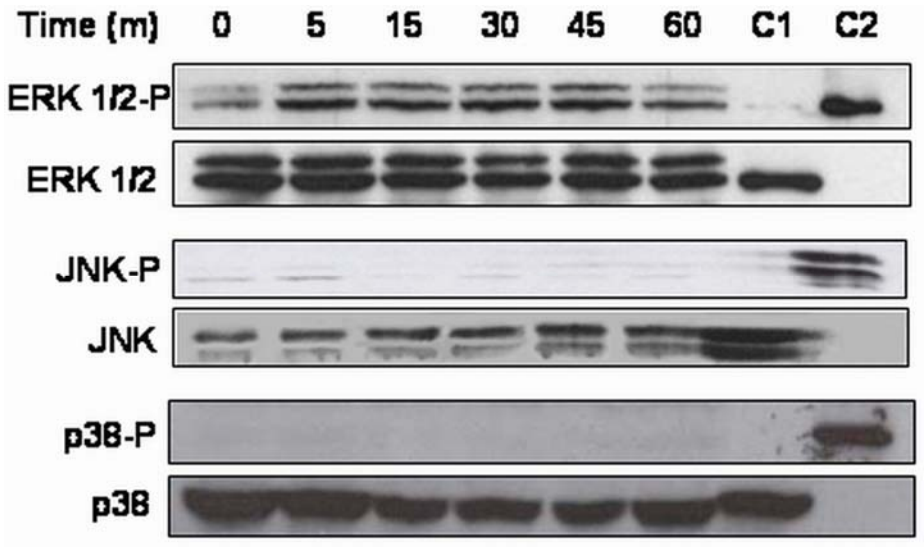

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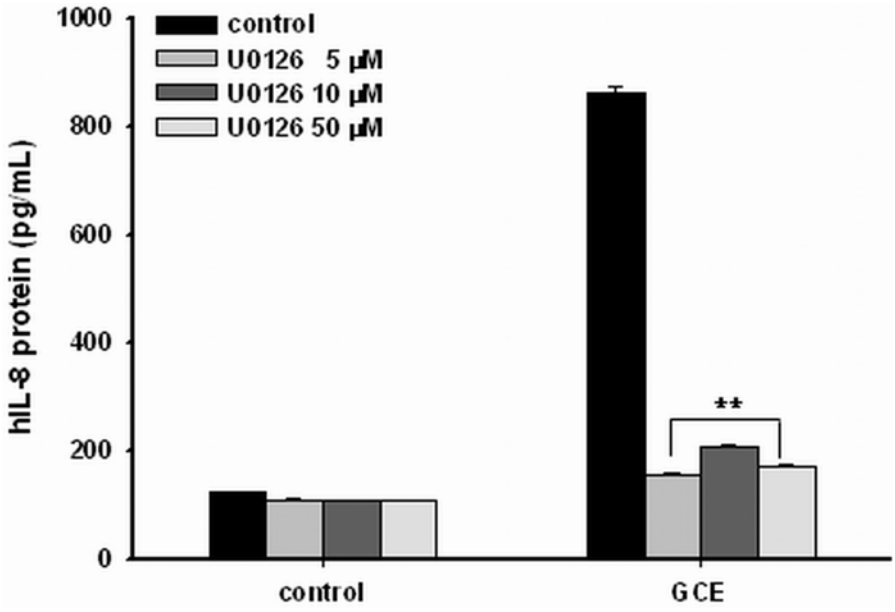
1000
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hIL-8 protein (pg/mL)
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U0126 5 μM
U0126 10 μM
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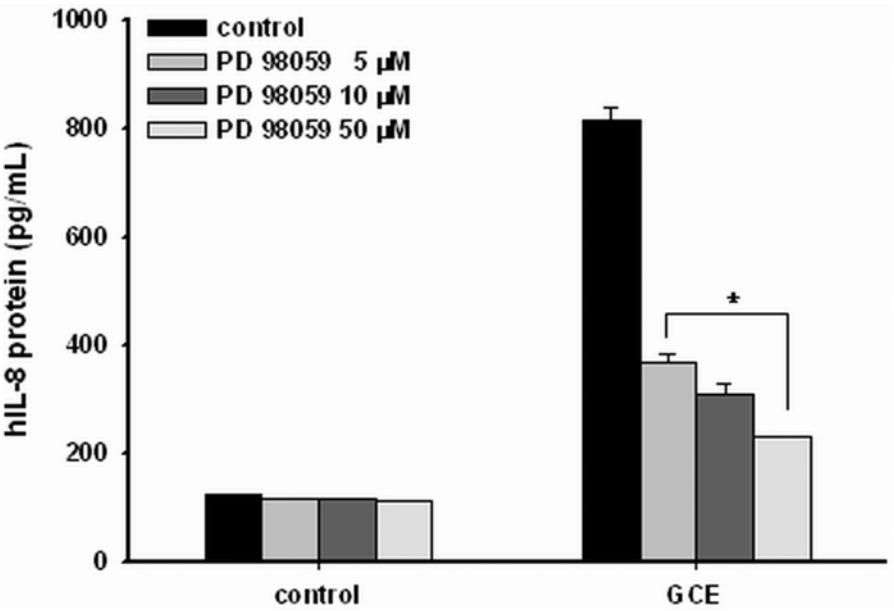
1000
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PD 98059 10 μM
PD 98059 50 μM
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control
GCE

D

Figure 4. Requirement of MAP kinases for GCE-induced IL-8 expression. (A) Effect of pretreatment with inhibitors of MAP kinases on GCE-induced IL-8 expression. Protein of cell supernatants was measured by ELISA. ***$p<0.001$ vs. GCE alone. (B) Phosphorylation assays of MAP kinase activities. Cells treated with GCE were harvested at the indicated time points, lysed, and equal amounts of cell extracts were resolved on 10% acrylamide gels and then subjected to Western blot analysis. C1: non-phosphorylated control, C2: phosphorylated control. (C) Effect of pretreatment with MEK 1/2 inhibitor U0126 on GCE-induced IL8 expression. **$p < 0.01$ vs. GCE alone. (D) Effect of pretreatment with MEK 1 inhibitor PD98059 on GCE-induced IL8 expression. *$p < 0.05$ vs. GCE alone. All data represent the mean ± SEM of at least three independent experiments performed in duplicate.

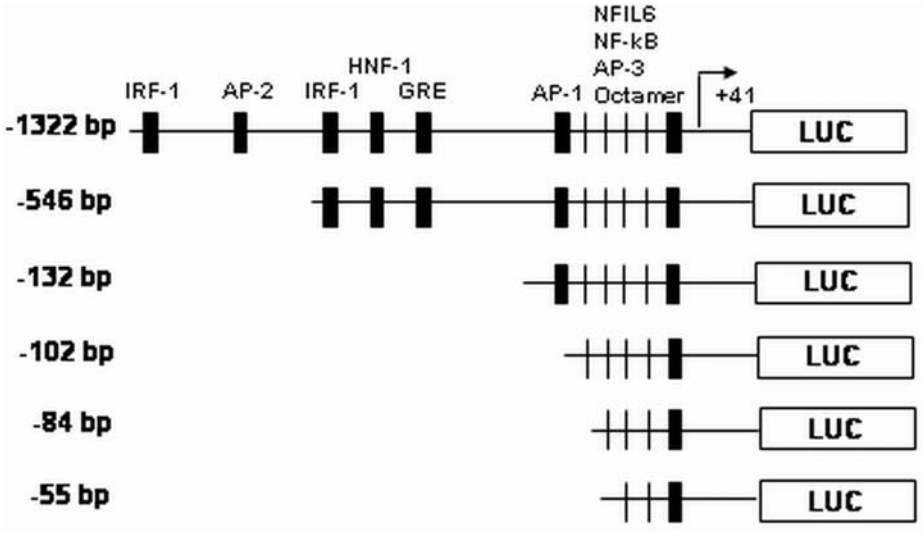
4. Production of IL-8 promoter fragments and promoter activity in a concentration-dependent manner

H292 cells were transiently transfected with IL-8 promoter constructs, -1322, -546, -132, -102, -84, -55 (Figure 5. A) and incubated with GCE (100 μg/mL) for 24 hrs. Luciferase activities (normalized by β-galactosidase expression) of them are 1.1, 1.9, 4.5 and 1.75 (Figure 5. B). However, the GCE-induced luciferase expression from promoter sequences which included a deletion under -102 was substantially reduced and no longer significant. In summary, the construct -132 was more expressive than -1322, -546 and -102 on GCE-stimulated luciferase production. Through the treatment with GCE in a concentration-dependent manner, the capacity of GCE to activate the transcription of a luciferase reporter gene driven by the IL-8 promoter (-132/+41) was measured. As shown in Figure 5. C, IL-8 promoter activities were significantly induced by GCE compared to control.

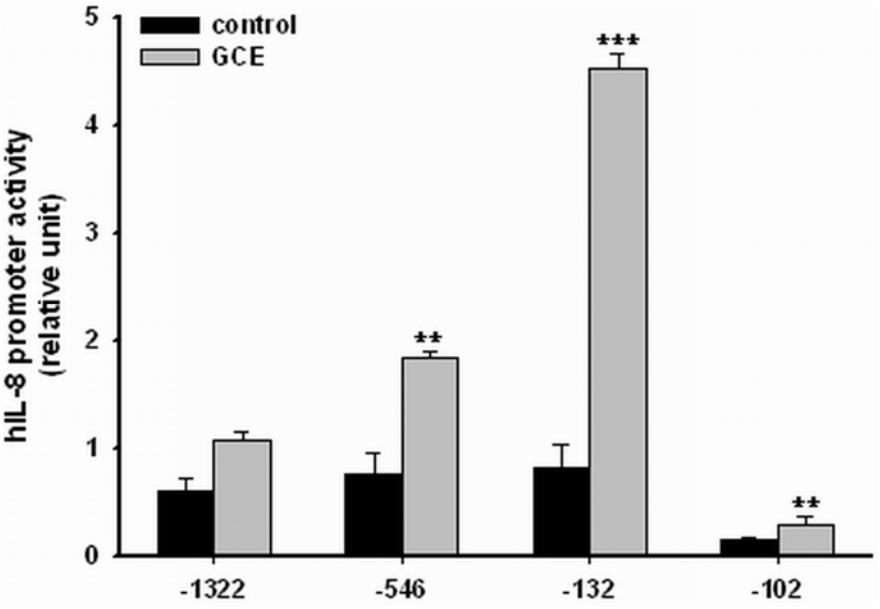
5. ERK regulates IL-8 promoter activity

To test the requirement of ERK, p38 or JNK MAP kinase activation on IL-8 promoter activity, we transiently transfected cells with -132 construct and then pretreated with PD98059, SB202190 and JNK inhibitor II before GCE treatment. Only inhibition of ERK with PD98059 (50μM) totally abolished

GCE-induced IL-8 promoter activation (Figure 5. D).



A



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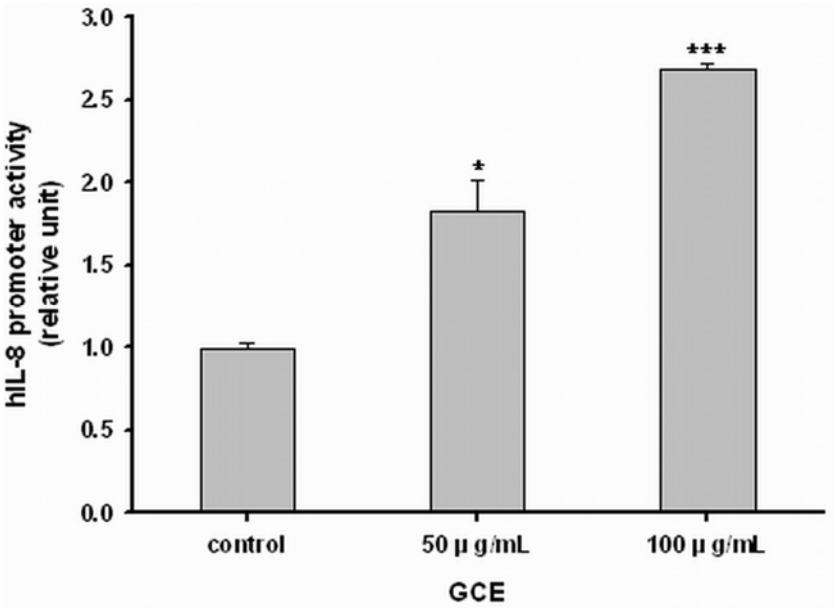
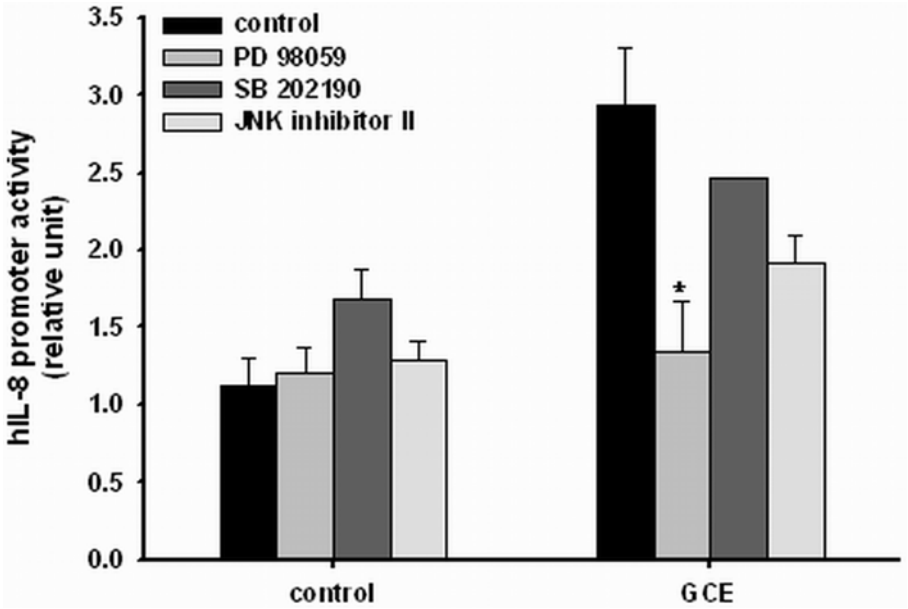

hIL-8 promoter activity
(relative unit)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
***
control
50 μ g/mL
100 μ g/mL
GCE

C

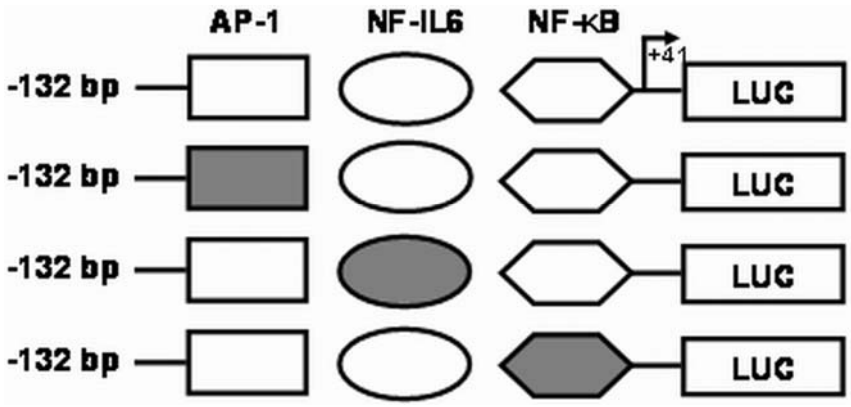

control
PD 98059
SB 202190
JNK inhibitor II
hIL-8 promoter activity
(relative unit)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
control
GCE

D

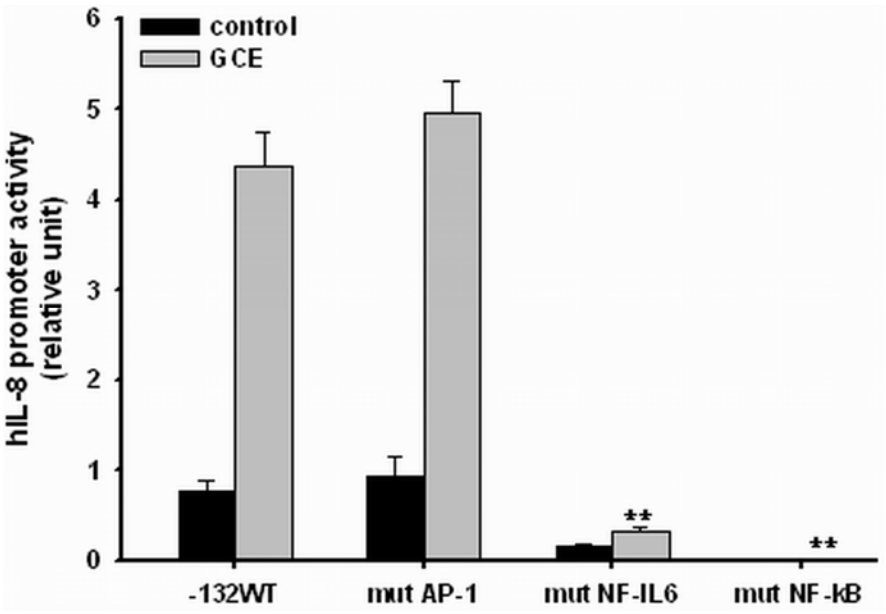
Figure 5. Deletional analysis and transcriptional activity of IL-8 promoter. (A) Schematic diagram of IL-8 promoter fragments. (B) Effect of GCE on transcriptional activities of 5'-upstream deletion fragments of IL-8 promoter. Cells transfected with fragmens of IL-8 promoter subcloned into a luciferase reporter gene were stimulated with GCE, and luciferase activity was measured by a luminometer. **$p < 0.01$, ***$p < 0.001$ vs. -1322/+41 IL-8. (C) Cells transfected with -132/+41 IL-8 were stimulated with different GCE concentrations. *$p<0.05$, ***$p<0.001$ vs. control. (D) Cells transfected with -132/+41 IL-8 were pretreated with inhibitors of MAP kinases and then stimulated with GCE. *$p<0.05$ vs. GCE alone. All data represent the mean ± SEM of at least three independent experiments performed in duplicate.

6. Implication of the NF-IL6 and NF-κB sites in IL-8 promoter activity induced by GCE

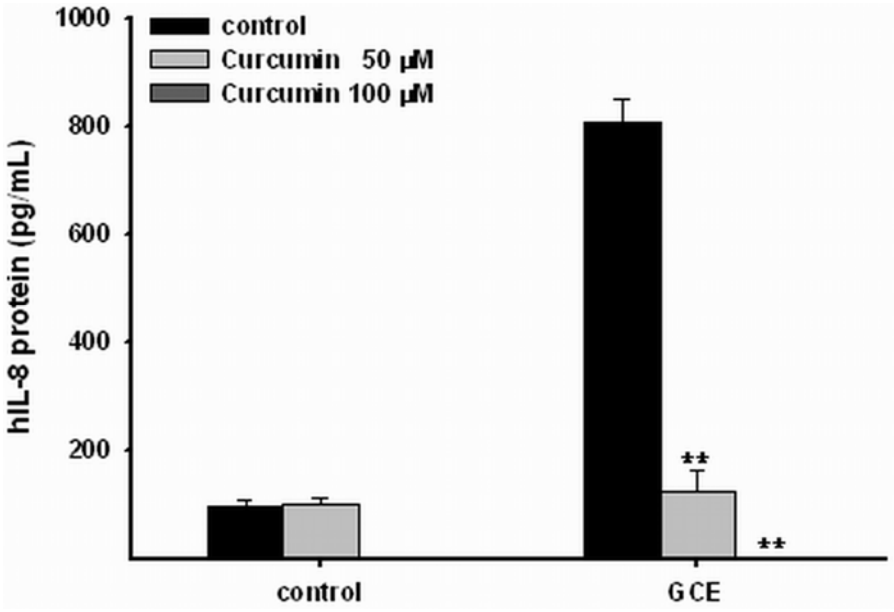
Based on our above results, the construct -132/+41 was determined as wild type (WT) IL-8 promoter for identification of GCE-activated transcription factors. The IL-8 promoter is widely characterized and involved several binding sites for transcription factors, including the AP-1 (-126/-120), NF-IL6 (-94/-81) and NF-κB (-80/-70) sites [16]. To test the implication of each site related with GCE stimulation, we used IL-8 promoter constructs containing site specific mutations. As shown in Figure 6. A and B, the NF-IL6 site mutation reduced GCE-induced IL-8 promoter activity by 91%. In case of the NF-κB site mutation, IL-8 promoter activity by GCE was completely abolished. In contrast, construct with a mutated AP-1 site produced more IL-8 promoter activation compared to the -132WT (Figure 6. B). To confirm the role of NF-κB on protein level, we pretreated NF-κB inhibitor, curcumin (known as a NF-κB and AP-1 inhibitor) [17] and CAPE (known as a specific inhibitor of NF- κB) [18] dependent on concentrations before GCE treatment (100 μg/mL). Both curcumin and CAPE significantly inhibited IL-8 protein compared to GCE alone (Figure 6. C, D).


AP-1
NF-IL6
NF-κB
+41
-132 bp
LUC
-132 bp
LUC
-132 bp
LUC
-132 bp
LUC

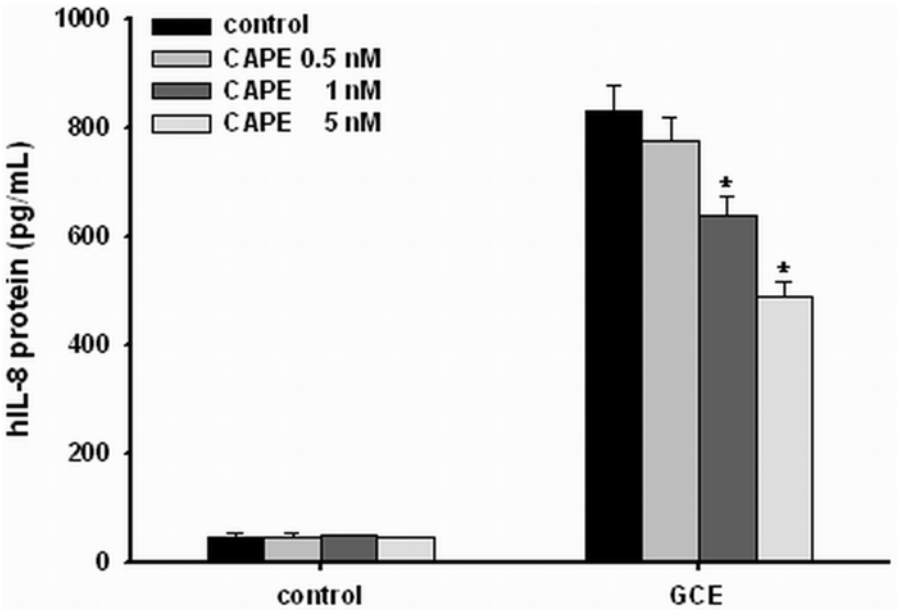
A


control
GCE
hIL-8 promoter activity
(relative unit)
0
1
2
3
4
5
6
**
**
-132WT
mut AP-1
mut NF-IL6
mut NF-kB

B


1000
800
600
400
200
hIL-8 protein (pg/mL)
control
Curcumin 50 μM
Curcumin 100 μM
**
**
control
GCE

C

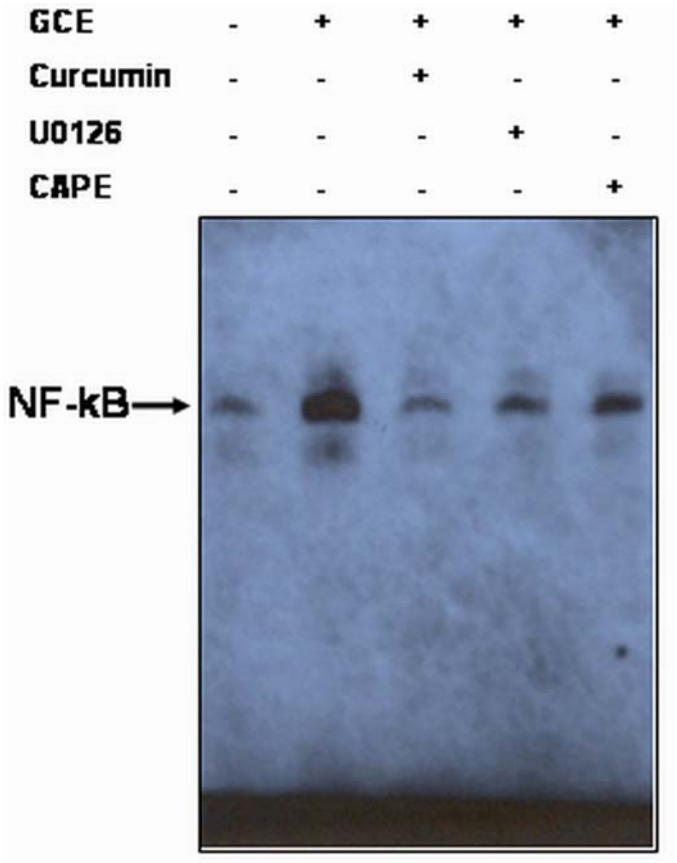

1000
800
600
400
200
0
hIL-8 protein (pg/mL)
control
CAPE 0.5 nM
CAPE 1 nM
CAPE 5 nM
*
*
control
GCE

D

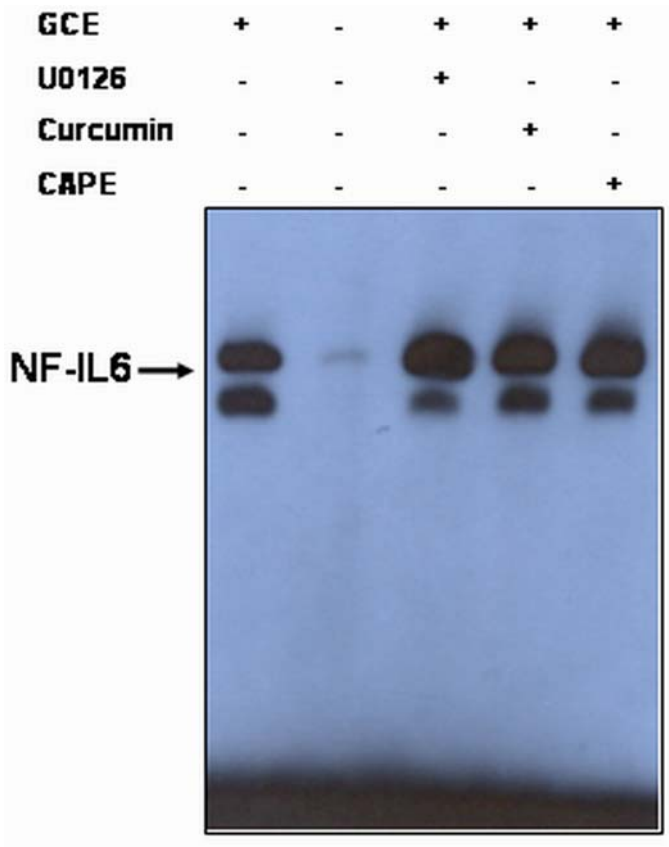
Figure 6. Implication of AP-1, NF-IL6 and NF- kB binding sites in IL-8 promoter activity stimulated by GCE. (A) Schematic diagram of -132/+41 IL-8 promoter fragments having various mutations in either AP-1, NF-IL6 or NF-kB transcription factor binding site. (B) Contributions of each site in GCE-induced IL-8 promoter activation. Cells transfected with -132/+41 WT, mutated (mut) AP-1, mut NF-IL6 and mut NF-κB promoter constructs were stimulated with GCE before measurement of luciferase activity. **$p < 0.01$ vs. -132 WT. (C) Effect of pretreatment with NF-kB (or AP-1) inhibitor, Curcumin on GCE-induced IL-8 expression. Protein from cell supernatants was measured by ELISA. **$p < 0.01$ vs. GCE alone. (D) Effect of pretreatment with a specific NF-κB inhibitor, CAPE on GCE-induced IL-8 protein. *$p < 0.05$ vs. GCE alone. All data represent the mean ± SEM of at least three independent experiments performed in duplicate.

7. Effect of GCE on NF-IL6 and NF-κB binding activity

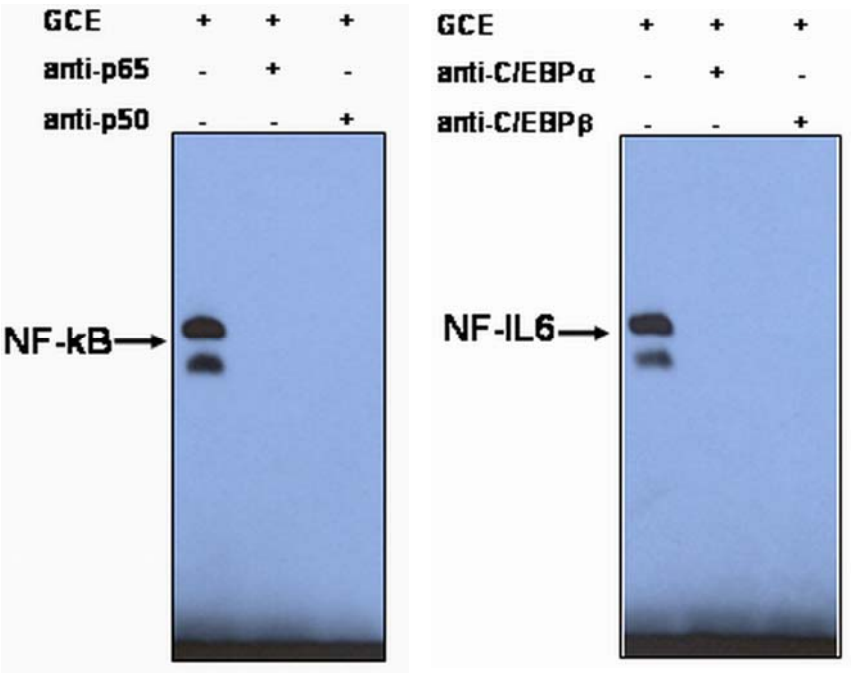
Besides our previous result of involvement of the NF-IL6 and the NF-κB by chemical inhibitor treatments or their specific mutations, we studied whether NF-IL6 and NF-κB DNA binding was induced by GCE. As presented in Figure 7. A, exposure of H292 cells to GCE (100 μg/mL) for 12 hrs resulted in NF-IL6 DNA binding activity and pretreatment with U0126 (50 μM) partially inhibited its binding activity. Figure 7. B illustrates that GCE induced NF-κB DNA binding activity and pretreatment with each curcumin (50 μM), CAPE (10 nM) or U0126 (50 μM) interfered its binding activity. To study the nature of the complex induced by GCE, supershift assays were performed by specific antibody against p65, p50, C/EBPα or C/EBPβ. As shown in Figure 7. C and D, the GCE inducible complex contained p65, p50, C/EBPα and C/EBPβ supershifted using antibodies that recognize specifically these proteins.



A



B



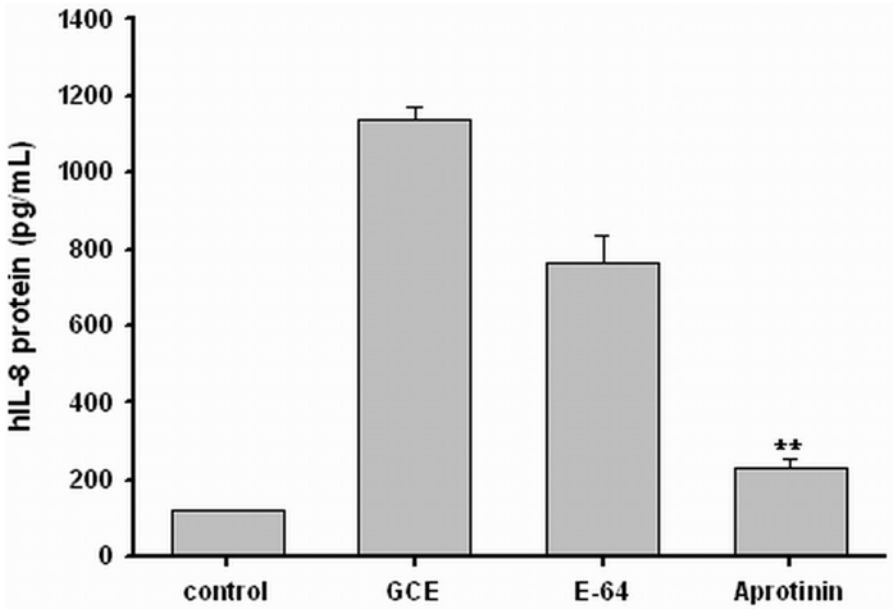
C **D**

Figure 7. Effect of GCE on the activation of NF-IL6 and NF-κB DNA binding activities. Electrophoretic mobility shift assay (EMSA) demonstrates GCE-induced binding of nuclear proteins to an oligonucleotide encoding the NF-κB (A) or NF-IL6 (B) consensus binding sequence. (C), (D) The specificity of complex formation was tested by the inclusion of specific anti-p50, anti-p65, anti-C/EBPα or anti-C/EBPβ Abs. Similar results were obtained in two other independent experiments.

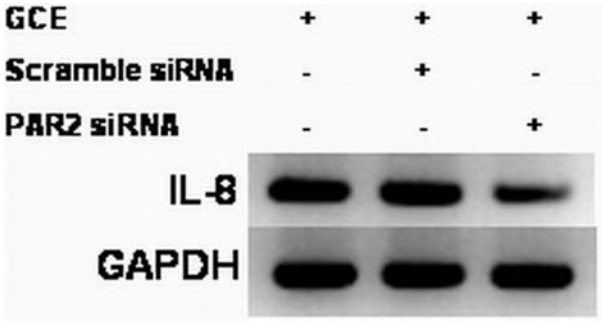
8. Effect of Cockroach proteases on IL-8 induction

Cockroaches have been known to have protease activity and this activity has been implicated in the regulation of epithelial cell release of GM-CSF, IL-8 and IL-6 in vitro [7, 8]. In our study, to confirm whether the protease activity of GCE affect IL-8 production, we incubated GCE with each protease inhibitor, E-64 or aprotinin for 1 hr before addition to cells. As shown in Figure 8. A, aprotinin known as a serine protease inhibitor significantly decreased IL-8 induction by GCE.

Another experiment for protease activity of GCE is that H292 cells were transiently transfected with either scrambled siRNA (control siRNA) or PAR2 siRNA. At 44 hrs after transfection, PAR2 siRNA knockdown was determined by RT-PCR. Figure 8. B illustrates that cells transfected with PAR2 siRNA were partially blocked on GCE-induced IL-8 expression.



A



B

Figure 8. Effect of cockroach protease on IL-8 expression. (A) Cells were treated with GCE incubated with protease inhibitor, E-64 or aprotinin for 1h. Protein from cell supernatants was measured by ELISA. **$p < 0.01$ vs. GCE alone. The data represents the mean ± SEM of at least three independent

experiments performed in duplicate. (B) Cells were transiently transfected with human PAR2 siRNA and scramble siRNA control and then treated with GCE for 12h. PAR2 knockdown was assessed by RT-PCR at 44 h after transfection. Similar results were obtained in two other independent experiments.

IV. DISCUSSION

Cockroaches produce several allergens. Exposure and sensitization to these allergens are associated with the development of acute asthma morbidity [19, 20]. Airborne allergens including cockroach, fungus or house dust mite can induce the release of proinflammatory mediators, such as IL-8, IL-6, eotaxin, GM-CSF, or RANTES by airway epithelial cells [7, 21]. These mediators trigger the accumulation of inflammatory cells, such as eosinophils and neutrophils, to perpetuate the chronic allergic inflammation of the airways. Especially, IL-8 is a potent chemoattractant for neutrophils and plays a major role in promoting inflammation, as found in severe asthma [22]. Here we report a direct action of GCE on the transcriptional regulation of IL-8 expression coordinating with ERK pathway in epithelial cells.

First, our results demonstrate that GCE can synergistically increase IL-8 protein depending on times and concentrations in human epithelial cells. Also, we studied that GCE induced a time-dependent expression of IL-8 mRNA. IL-8 gene regulation in bronchial epithelial cells is predominantly modulated at the transcriptional level [23] and significant IL-8 expression requires at least one or two MAPK pathways [10]. Because MAP kinases have been shown to regulate cytokine expression in a number of cell types, including lung epithelial cells [24-28], we examined the requirements of ERK, p38 and JNK

for transcription from IL-8 promoter and IL-8 protein expression. In our findings, we show that inhibition of ERK by pretreatment with chemical inhibitor PD98059 and U0126 abolished completely GCE-induced transcription and protein abundance of IL-8. On the other hand, inhibitions of both p38 and JNK with each respective chemical inhibitor failed to reduce the transcription and protein expression of IL-8. Furthermore, only ERK signaling intermediates (with the exception of p38 and JNK) are phosphorylated and detected by anti-phosphorylated 44/42. These results suggest that ERK pathway more strongly contributes to IL-8 expression by GCE than p38, JNK.

Second, based on a well characterized IL-8 promoter sequence, analysis of promoter deletions indicated that the region from -132 to +41 is minimally essential part for IL-8 expression and the region under -102 did not affect promoter activity of IL-8. In addition, this region was more expressive than -1322/+41 and -546/+41 in reporter gene assay. This promoter element -1 to -132 contains AP-1, NF-IL6, NF-κB [10] and IL-8 gene expression is mainly regulated by these elements [29]. For mutation of different binding sites for transcription factors, we used the fragment from -132 to +41 of IL-8 promoter as wild type. The results of site-directed mutational analysis show that NF-IL6 and NF-κB but not AP-1 binding sites are essential for GCE-induced activation of IL-8 promoter. Specifically, mutation of the IL-8 promoter AP-1

site did not reduce the transcriptional activity, whereas mutation of the IL-8 promoter NF-kB and NF-IL6 site blocked the response to GCE by 100% and 91%. Therefore, our deletional and mutataional analysis demonstrate that NF-kB and NF-IL6 were necessary for induction of IL-8 transactivation in response to GCE. Maximal activation of IL-8 has been shown to be regulated by the binding of both NF-κB and NF-IL6 transcription factors [30]. NF-IL6 has shown to bind weakly to the IL-8 promoter, but when NF-κB is bound to the adjacent site, NF-IL6 shows strong cooperative binding [31]. NF-κB plays a critical role in immune and inflammatory responses and many of the stimuli that increased inflammation in asthmatic airways resulted in the activation of NF-κB, particularly proinflammatory cytokines. However, considering the result of deletional analysis that -121/+41 (deleted AP-1 binding site) totally lost IL-8 promoter activity, the function of AP-1 could not be excluded in GCE-induced IL-8 promoter activation. In 16HBe cells and airway epithelial cells, AP-1 appears to act as a basal level enhancer element of IL-8 transcription, which can be increased by powerful stimuli such as TNF-α via NF-κB [31, 32].

Third, we examined whether chemical inhibitors of NF-κB blocked IL-8 protein synthesis by GCE. Pretreatment with curcumin completely abrogated protein abundance of GCE-induced IL-8. Because curcumin has known to be

an inhibitor for NF-κB and AP-1, we confirmed that IL-8 production was attenuated by dose-dependent use of CAPE (which is a potent and specific inhibitor of NF-κB) [17]. Additionally, our data showed that GCE stimulation led to activation of both NF-IL6 and NF-κB DNA binding, as shown by EMSA. NF-IL6 binding to the IL-8 promoter is not affected by inhibitor treatments of NF-κB, instead is slightly disturbed by U0126. Although we were unable to do inhibition test of IL-6 with the few existing inhibitors commercially available, we believe that NF-IL6 also have key role to mediate GCE-stimulated IL-8 expression by gel shift assay and mutational analysis. In contrast, the binding activity of NF-κB to DNA was totally abolished by U0126, CAPE and curcumin. Our data demonstrates that the activation of NF-κB and NF-IL6 induces IL-8 promoter activation.

Interestingly, in our studies, we examined whether cockroach proteases regulate IL-8 in human epithelial cells. Recent studies have explored the importance of PAR2 in airway inflammation and our previous study proved that whole-body cockroach extract elicited calcium flux in human airway epithelial cells via PAR-2 [33]. Through the pretreatment with protease inhibitors and GCE, aprotinin known as a serine protease inhibitor significantly reduced IL-8 production. In addition, PAR2 siRNA transiently tansfected cells were blocked to synthesize GCE-induced IL-8 mRNA,

compared to reagent and scramble siRNA control.

In conclusion, we demonstrated that the mechanism by which German cockroach extract induces IL-8 expression is regulated by the transcriptional activation of NF-κB and NF-IL6. Also, our data have shown that GCE-induced transcriptional regulations of them are mediated by ERK MAP kinase pathway. Although protease of GCE was partially blocked in IL-8 induction by protease inhibitors and PAR2 siRNA, it is thought that cockroach proteases are consistent with IL-8 expression (Figure 9). Further studies are underway to investigate the signaling from PAR2 activation to IL-8 synthesis.

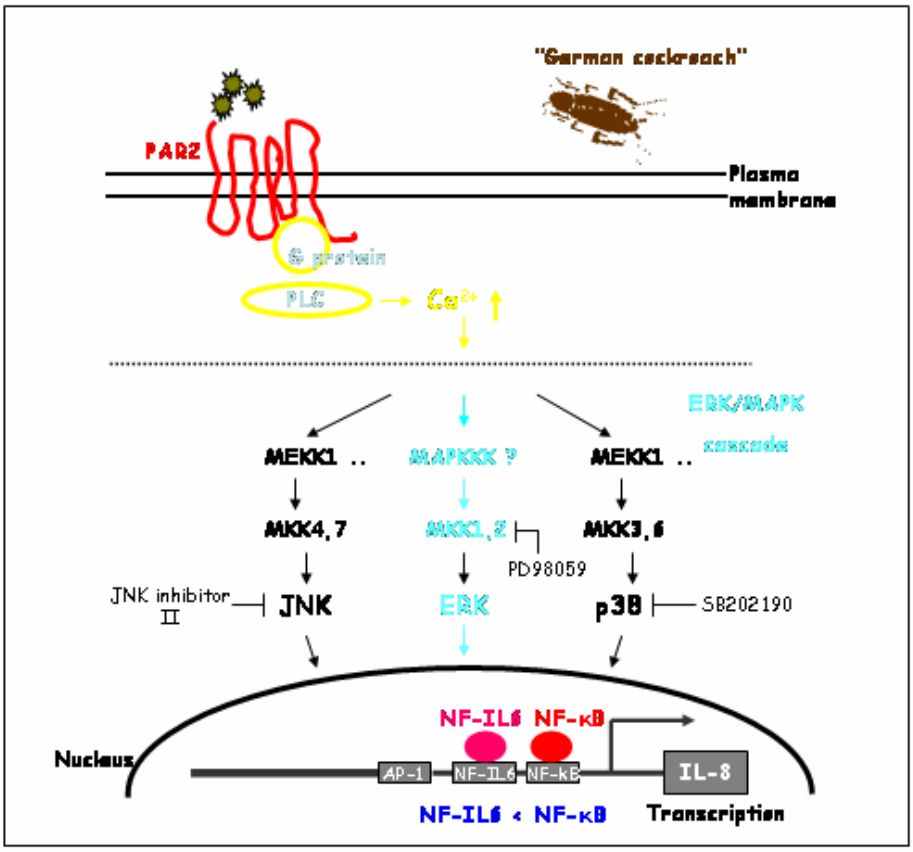


Figure 9. Model of NF-IL6 and NF-κB transactivation in GCE-induced IL-8 expression. The activity of transcriptional factors, AP-1 and NF-IL6 is dependent on ERK of MAP kinases.

CONCLUSIONS

1. German cockroach extract with protease activity synergistically increases IL-8 in human airway epithelial cells.

2. Activation of EKR increased German cockroach extract-induced IL-8 transcription.

3. ERK increases NF-IL6 and NF-κB activity to regulate IL-8 promoter activity in human airway epithelial cells.

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Abstract (in Korean)

인체 기관지 상피세포 내 독일바퀴 항원의 Interleukin-8 유전자 발현조절기작

<지도교수 용 태 순>

연세대학교 대학원 의과학과

이 경 은

목적: 바퀴는 알레르기 질환을 유발하는 중요한 기인항원으로 가옥 내에 널리 퍼져 분포하며 잔류물이 집먼지 속에 남아 호흡기를 통하여 흡입되어 알레르기항원으로 작용한다고 알려지고 있다. 본 연구에서는 독일바퀴항원이 기관지 상피세포 내에서 염증작용을 일으키는 사이토카인인 Interleukin-8 (IL-8)을 유도하는지와 바퀴항원이 사이토카인 발현에 관여하는 조절기작을 밝히고자 하였다.

방법: 배양한 호흡기상피세포에 바퀴항원농도와 반응시간들을 달리하여 처리하였다. IL-8 발현을 위한 transcriptional activity 는 IL-8 promoter constructs 를 제작하고 delectional analysis 와 mutational

analysis 를 통해 각각 조사하였다. Mitogen-activated protein (MAP) kinase 의 연관성여부와 활성화되는 전사인자들을 확인하기 위해 chemical inhibitor 를 처리하거나 electrophoretic mobility shift assay (EMSA) 방법을 이용하였다.

결과: H292 세포에 독일바퀴 항원을 노출시켰을 때 시간과 농도가 증가함에 따라 IL-8 의 생성이 증가되었으며, ERK inhibitor 인 PD98059 를 전처치했을 때 IL-8 생성이 감소하였으나 p38 과 JNK inhibitor 를 각각 전처치 하였을 때는 의미있는 감소를 보이지 않았다. 또한 luciferase assay 를 이용하여 deletional analysis 를 실시한 결과 IL-8 promoter 의 -132/+41 에 해당하는 부위가 IL-8 발현에 밀접한 연관이 있음을 알 수 있었으며, mutational analysis 와 chemical inhibitor 전처치를 통해 NF-IL6 와 NF-κB 전사인자가 독일바퀴에 의한 IL-8 발현에 관여하고 있음을 확인할 수 있었다. EMSA 결과를 통해 NF-IL6 와 NF-κB 의 translocation 을 확인하였으며 ERK inhibitor 전처치를 한 경우 이들의 translocation 이 감소함을 알 수 있었다.

결론: 독일바퀴 항원은 기관지 상피 세포에서 ERK pathway 를 통해 IL-8 promoter 내 NF-IL6 와 NF-κB 를 활성화시키고 이들을 통해 IL-8 유전자 발현을 조절하는 것으로 사료된다.

핵심되는 말: 독일바퀴항원, 호흡기 상피세포, IL-8, ERK, 전사인자