ORIGINAL ARTICLE

Serine Protease Inhibitor Gabexate Mesilate Attenuates American Cockroach–Induced Bronchial Damage and Inflammatory Cytokine Release

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Abstract

Background and objective: Allergic airway diseases are not only a T_H2-mediated chronic airway inflammation, but also a condition of epithelial barrier defects and dysfunction. Allergens with protease activities are known factors that initiate respiratory epithelial damage. Cockroach allergy is the second leading cause of allergic respiratory airway diseases in Taiwan, and cockroach allergens have strong serine protease activity. This study aimed to determine the protective effect of the direct local administration of gabexate mesilate (GM) on American cockroach allergen (CraA)–induced human bronchial epithelial cell inflammation.
Methods: BEAS-2B cells, from the human bronchial epithelial cell line, were stimulated with CraA or co-cultured with different doses of GM. Cellular morphologic changes were observed by microscopy and changes in chemokine mRNA expression and protein levels were determined by semi-quantitative reverse transcription–polymerase chain reaction (RT-PCR) and ELISA. Effects of specific inhibitors of ERK1/2 (U0126), JNK (SP600125), and p38 MAPK (SB203580) on CraA-induced chemokine mRNA expression were also tested by RT-PCR.
Results: GM prevented CraA-induced bronchial epithelial cell detachment and morphological changes. It had superior and more extensive suppression effects than specific target MAPK inhibitors in CraA-induced mRNA expression of IL-8, monocyte chemotactic protein (MCP) 1, chemokine (C-C motif) ligand 20, and granulocyte-macrophage colony-stimulating factor from the cells in a dose-dependent manner. CraA-induced IL-8 and MCP-1 protein production from BEAS-2B cells was also attenuated by GM.
Conclusions: The serine protease inhibitor GM has local protective effects against CraA-induced bronchial epithelial inflammation. The development of an inhaled or intranasal protease inhibitor may be a potential strategy for the treatment of allergic airway diseases induced by allergens with protease activities.

Key words: Cockroach allergy. Bronchial epithelial cells. Serine protease inhibitor. Gabexate mesilate.

Resumen

Antecedentes y objetivo: Las enfermedades alérgicas de las vías respiratorias no son sólo fruto de una inflamación crónica de las vías respiratorias mediada por Th2, sino que también se encuentran defectos físicos y funcionales de la barrera epitelial. En este sentido, es conocido el papel de los alérgenos con actividad proteasa que son los factores conocidos de inicio del daño epitelial respiratorio. La alergia a las cucarachas es la segunda causa principal de enfermedades alérgicas de las vías respiratorias en Taiwan y estos alérgenos poseen una potente actividad serín-proteasa. Este estudio tuvo como objetivo determinar el efecto protector del mesilato de gabexate (GM) contra la inflamación inducida por la administración local directa de alérgenos de la cucaracha americana (CRAA), sobre las células epiteliales bronquiales humanas.
Métodos: Se empleó la línea de células epiteliales bronquiales, células BEAS-2B, las cuales se estimularon con CRAA o fueron co-cultivadas con diferentes dosis de GM. Se analizaron los cambios morfológicos celulares por microscopía y los cambios en la expresión de ARNm de diferentes quimiocinas, así como los niveles de proteína. Se utilizaron métodos semi-cuantitativos de RT-PCR y ELISA. Los efectos de inhibidores específicos de ERK1/2 (U0126), JNK (SP600125), y p38 MAPK (SB203580) en las expresión de ARNm de quimiocinas inducidas por CRAA, también se ensayaron por RT-PCR.

Resultados: El mesilato de gabexate impidió el desprendimiento de las células epiteliales y los cambios morfológicos inducidos con CRAA a nivel bronquial. Sus efectos supresores fueron más potentes y prolongados que los obtenidos con inhibidores específicos de MAPK sobre la expresión del ARNm de IL-8, MCP-1, CCL20 y GMSF en una forma dosis-dependiente. La producción proteica de IL-8 y MCP-1 de las células BEAS-2B también fue menor cuando se añadió GM.
Conclusiones: El mesilato de gabexate, inhibidor serín-proteasa , tiene efectos protectores locales contra la inflamación bronquial epitelial inducida por la cucaracha americana. El desarrollo de un inhibidor de la proteasa, por vía inhalada o intranasal, puede ser una estrategia potencial para el tratamiento de enfermedades de las vías respiratorias alérgicas inducidas por alérgenos con actividad proteasa.
Palabras clave: Alergia a cucaracha. Células epiteliales bronquiales. Inhibidor de la serín-proteasa. Mesilato de gabexate.

Introduction

There is an increasing body of evidence that allergic respiratory diseases not only are an unwanted T_H2-mediated immune overresponse to allergens, but also involve defects and dysfunction of the epithelial barrier [1-4]. These defects may further facilitate the passage of allergens and other agents into the airway tissue, leading to chronic inflammation of the airways [2]. Upon exposure to allergens with protease activities, such as house dust mites [5,6], cockroaches [7,8], and molds [9], respiratory epithelial cells are directly activated via protease-activated receptors (PARs), triggering cascades of innate immune responses and causing further airway damage. One of the major American cockroach allergens is serine protease [10]. A previous study by our group showed that the effect of American cockroach allergen (CraA)-induced IL-8 production from pulmonary epithelial cells is dependent on serine protease activity [11].

Cockroach allergy is the second leading type of allergic respiratory disease in Taiwan, accounting for more than 50% of all asthma cases [11,12]. Studies show that asthmatic children with cockroach allergy have more severe symptoms and more frequent hospitalizations [13], yet the effects cannot be entirely explained by IgE-mediated inflammation [14]. Although stringent environmental control of cockroach allergens has proven to be effective, only some 50% of families follow appropriate cleaning instructions [15,16].

Gabexate mesilate (GM) is a synthetic serine protease inhibitor. In the clinical setting, it has been used intravenously to treat acute pancreatitis and disseminated intravascular coagulation [17-21]. Intraperitoneal injection of GM has also been shown to attenuate allergen-induced airway inflammation and eosinophilia in a murine asthma model [22].

The aim of this study was to investigate whether direct local administration of GM protects against CraA-induced human bronchial epithelial cell inflammation.

Materials and Methods

Extract and Chemicals

CraA was prepared from *Periplaneta americana* with Coca's solution and further purified by Endotoxin Detoxi-Gel (Pierce), as described previously [11]. GM was purchased from the Ono Pharmaceutical Company and dissolved in phosphate buffered solution (PBS) before use. Other reagents were purchased from Sigma unless otherwise stated.

As none of the experiments in this study involved humans, institutional review board approval was not required.

BEAB- 2B Cell Cultures and Stimulation

The BEAS-2B cells, a cell line derived from adenovirus 12-SV40 hybrid virus–transformed human bronchial epithelial cells, were purchased from Taiwan Bioresource Collection and Research Center. The cells were cultured in serum-free LHC-9 medium (GIBCO) with penicillin (100 U/mL) and streptomycin (100 µg/mL) in 100-mm culture dishes precoated with a solution of 10 µg/mL fibronectin, 30 µg/mL rat collagen, and 10 µg/mL bovine serum albumin (BSA) in LHC basal medium (GIBCO). Upon confluence, the cells were detached by enzyme-free cell dissociation solution (Invitrogen) to minimize the proteolytic activation of PARs. Cells were used at passages 10-20.

For the experiments, the cells were seeded at a density of 3×10^5 in a 35-mm dish and grown to confluence. These were then rinsed with d-PBS and incubated with various concentrations of CraA at different times to extract RNA and to observe, using light microscopy (Carl Zeiss), progressive morphologic changes when exposed to various additives. At the end of each experiment, cell viability and cell number were determined by trypan blue exclusion for analyzing potential cytotoxic reactions.

RNA Extraction and Complementary DNA Synthesis

At specified points, BEAS-2B cells were homogenized in Trizol reagent (Invitrogen) according to the manufacturer's instructions. The concentration and purity of the RNA were determined by measuring the absorbance at 260 and 280 nm in a NanoDrop ND-2000 spectrophotometer (Thermo). Extracted RNA was reverse transcribed into first-strand complementary DNA (cDNA) from 1 µg of total RNA using oligo$(dT)_{20}$ and random hexamers and SuperScript III reverse transcriptase (Invitrogen). The reaction was performed at 50°C for 60 minutes, followed by a heat denaturation step at 85°C for 5 minutes.

Gene Expression of Chemokines by Reverse Transcription–Polymerase Chain Reaction

Expression of IL-8, monocyte chemotactic protein-1 (MCP-1), chemokine (C-C motif) ligand 20 (CCL20), and granulocyte-macrophage colony-stimulating factor

Table 1. Oligonucleotide Primers Used For Reverse Transcript-Polymerase Chain Reaction (PCR)

PCR Product (Size)	Forward (5' to 3')	Reverse (5' to 3')
IL-8 (248 bp)	TTGGCAGCCTTCCTGATT	AACTTCTCCACAACCCTC
MCP-1 (199 bp)	CCCCAGACACCCTGTTTT	TCAAAACATCCCAGGGGTAGA
CCL20 (318 bp)	TACTCCACCTCTGCGGCGAATCAGAA	GTGAAACCTCCAACCCCAGCAAGGTT
GM-CSF (265 bp)	GCTGCTGAGATGAATGAAAC	AGTCAAAGGGGATGACAAG
β-Actin (227 bp)	AAAGACCTGTACGCCAACACAGTGC	CCGGACTCGTCGTCATACTCCTGCTTGC

Abbreviations: bp, base pair; GM-CSF, granulocyte-macrophage colony-stimulating factor.

(GM-CSF) mRNA were analyzed by reverse transcription–polymerase chain reaction (RT-PCR). The reaction mixture contained 2 mM of $MgCl_2$, 200 μM of dNTP, 1.25 U of hot-start SuperTherm Gold DNA polymerase (Hoffman-La-Roche), 10 pmole of specific sense and antisense primers for each cytokine gene, and 10 ng of first-strand cDNA in a total volume of 50 μL. After beginning with a single preincubation step at 95°C for 10 minutes, PCR was performed under the following conditions: denaturation at 94°C for 1 minute, annealing at 60°C for 1 minute, and extension at 72°C for 1 minute in a thermocycler (Perkin Elmer). The number of cycles used was dependent on the transcript amplified. β-Actin cDNA was used as an internal control. The primer sequences used are listed in Table 1.

The PCR products were analyzed by 2% agarose gel electrophoresis and ethidium bromide staining and captured by a Kodak molecular imaging system. Results were expressed as the ratio of mRNAs of chemokines to β-actin after densitometric determination of the bands in agarose gel.

Detection of Phosphorylation of ERK1/2, JNK and p38 by Western Blot

BEAS-2B cells grown in 35-mm dishes were stimulated with 5 μg/mL of CraA for 0, 5, 10, 20, 30, and 40 minutes. After washing with ice-cold PBS, the cells were lysed in 200 μL PRO-PREP protein extraction solution (iNtRON biotechnology) and harvested with a cell scraper. Cell suspensions were placed on ice for 20 minutes and centrifuged at 14 000 g for 5 minutes at 4ºC. Protein concentration was determined by the Bradford method (Bio-Rad) using BSA as standard. Protein samples (30 μg/lane) were separated on 4% to 12% SDS-PAGE and transferred to nitrocellulose membranes (Bio-Rad). The membrane was blocked with 5% skim milk for 1 hour at room temperature. After washing 3 times with PBST, the membrane was incubated with the primary antibodies at an appropriate dilution with gentle agitation overnight at 4ºC, followed by horseradish preoxidase–coupled secondary antibody (Sigma).

We used rabbit antihuman phospho-ERK1/2 antibody (1:2000, R&D), rabbit antihuman phospho-JNK antibody (1:2000, R&D), and rabbit antihuman phospho-p38 MAPK antibody (1:1000, cell signaling) as primary antibodies. Bands were visualized by enhanced chemiluminescence (Super-signal West Pico, Pierce) and Hyperfilm ECL (Amersham Biosciences). To calculate relative intensity, the membranes were stripped and reprobed with nonphosphorylated anti-ERK1/2 (1:5000, R&D), JNK (1:5000, R&D), and p38 MAPK (1:1000, cell signaling) antibodies.

IL-8 and MCP-1 Measurements by Sandwich ELISA

The levels of IL-8 and MCP-1 in culture supernatants were detected using ELISA kits (R&D) according to the manufacturer's instructions.

Statistical Analysis

Data were presented as means (SEM). Statistical significance between means was analyzed with the Mann-Whitney U test or Dunnett 1-way analysis of variance as appropriate. All statistical analyses were performed using the SPSS statistic software version 10.0. *P* values of less than .05 were considered to be statistically significant.

Results

GM Prevented CraA-Induced Bronchial Epithelial Cell Detachment and Morphological Changes

GM at 4 different doses (36, 75, 150 and 300 μg/mL) was cotreated with 5 μg/mL of CraA for 24 hours on confluent BEAS-2B cells. We observed the morphological changes of the cells under light microscopy. As shown in Figure 1, GM prevented CraA-induced cell detachment and morphological changes in a dose-dependent manner (Figure 1, C to F).

GM Had a Superior Effect to MAPK Inhibitors in Suppressing CraA-Induced Chemokine mRNA Expression

We studied the kinetics of chemokine mRNA expression in response to 5 μg/mL of CraA at 1, 2, 3, 4, 5, 16, and 24 hours by semi-quantitative RT-PCR. Levels of IL-8, MCP-1, CCL20, and GM-CSF were significantly upregulated as early as 2 hours after stimulation (Figure 2A). Treatment with GM also significantly reduced CraA-induced IL-8, MCP-1, GM-CSF, and CCL20 expressions in a dose-dependent manner. Maximal inhibition for all 4 chemokines was reached at 300 μg/mL (Figure 2B).

Using Western blotting, we assessed whether activation of MAPK was associated with CraA-induced inflammation in BEAS-2B cells. We found that after a 5-minute incubation

period with CraA, phosphorylation of ERK1/2 and JNK increased markedly and continued for at least 40 minutes (Figure 3A). Phosphorylation of p38 MAPK was also detected after CraA stimulation for 5 minutes but it decreased to baseline levels after 10 minutes.

Subsequently, specific target MAPK inhibitors, including U0126 (an ERK1/2 inhibitor), SP600125 (a JNK inhibitor), and SB203580 (a p38 MAPK inhibitor) were used to check their effects on CraA-induced chemokine expression. At a concentration of 6.25 μM, U0126 significantly attenuated CraA-induced IL-8, MCP-1, and GM-CSF gene expressions, but not CCL20 expression (Figure 3B). Similarly, SB203580 (6.25 μM) significantly suppressed CraA-induced IL-8, MCP-1, GM-CSF, and CCL20 gene expressions, while SP600125 inhibited only MCP-1 and GM-CSF.

GM Attenuated CraA-Induced IL-8 and MCP-1 Protein Production of BEAS-2B Cells

The BEAS-2B cells released IL-8 and MCP-1 proteins when stimulated with CraA (5 μg/mL). The protein levels started to increase 3 hours after stimulation and continued to do so up to 24 hours (Figure 3, black bars). However, when CraA was co-cultured with 300 μg/mL, GM, IL-8 and MCP-1 levels in BEAS-2B cells decreased significantly at all of the indicated time points (Figure 4, gray bars).

Discussion

In this study, our data show that the serine protease inhibitor, GM, exerts local protective effects against airway inflammation induced by cockroach allergen, a common allergen with known strong protease activity. Our results reveal that GM not only reduces CraA-induced detachment and morphologic changes to the bronchial epithelial cells, but also attenuates CraA-induced chemokine mRNA expression and protein production. The protective effects against CraA-induced chemokine release were even more extensive in GM than in the specific target MAPK inhibitors, U0126, SP600125, and SB203580. In addition to previous reports that GM inhibits human mast cell tryptase activity [23] and PAR-2, thus reducing tissue damage in the lungs [24], our data further support the protective effects of GM on bronchial cells.

In previous reports, we showed that cockroach-allergic asthmatic patients have significantly higher serum IL-8, MCP-1, CCL20, and GM-CSF levels than nonallergic individuals [25], and that CraA-induced IL-8 from human airway epithelial cells signaled via extracellular signal regulatory kinase and jun N-terminal kinase but not via p38 mitogen-activated protein kinase [11]. Using a cell model, the current study demonstrates similar results to those previously observed in patients. Moreover, the data suggest that CraA-induced IL-8,

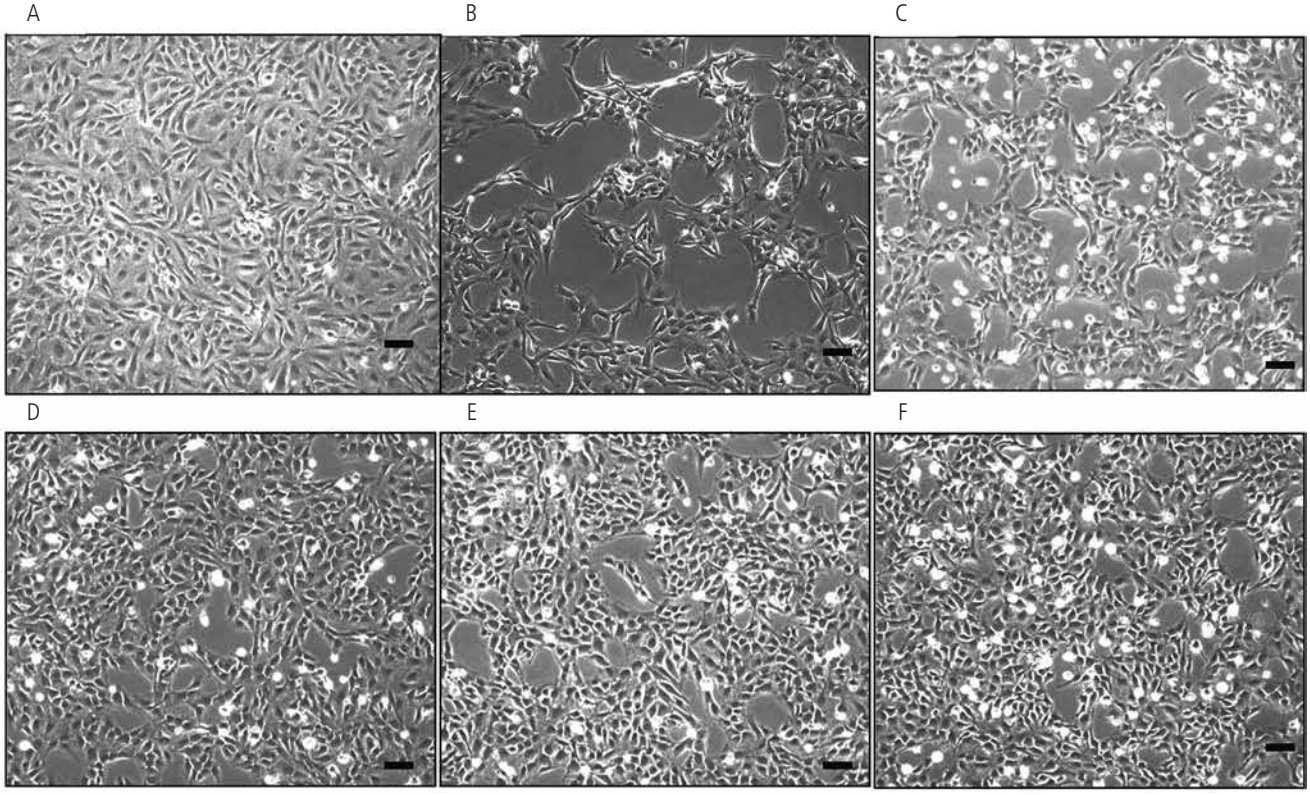

Figure 1. Effects of gabexate mesilate (GM) on CraA-induced cellular morphologic changes. The BEAS-2B cells were incubated for 24 hours with (A) medium alone, (B) 5 μg/mL CraA alone, (C) CraA plus 36 μg/mL, (D) 75 μg/mL, (E) 150 μg/mL, and (F) 300 μg/mL of GM. Original magnification ×100; scale bar, 100 μm. CraA indicates American cockroach allergen.

MCP-1, CCL20, and GM-CSF may signal through different MAPK pathways. Activation of p38, but not ERK1/2 or JNK, is critical in inducing CCL20 from CraA-stimulated BEAS-2B cells. In contrast, blocking ERK1/2, but not JNK or p38, inhibited GM-CSF production of CraA-stimulated BEAS-2B cells. We therefore propose a possible mechanism of GM in CraA-induced bronchial epithelial inflammation as shown in Figure 5. However, it should be noted that our data are based on an in vitro cell model. More experiments involving in vivo models are required to further explore the proposed hypothesis.

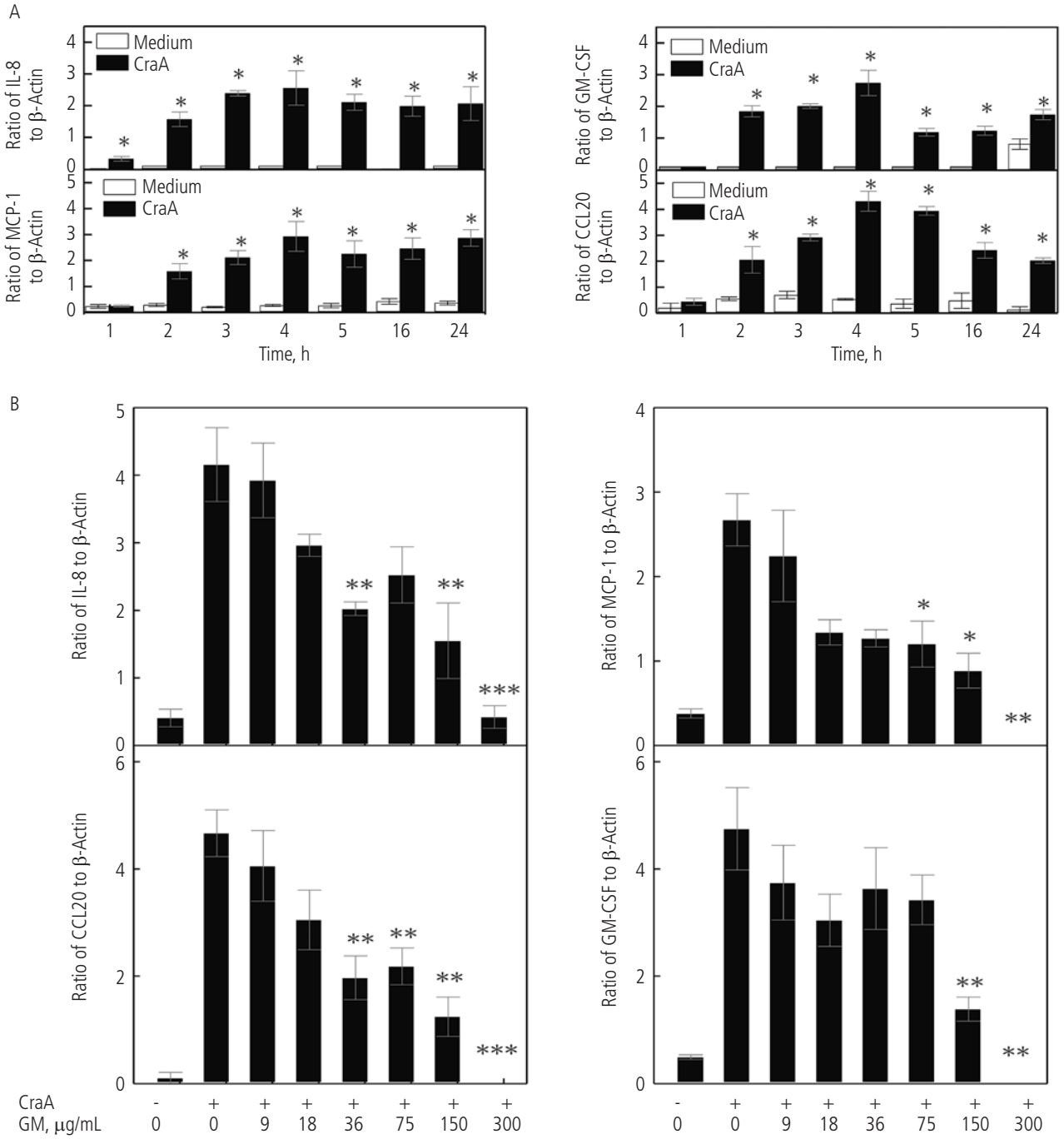

Figure 2. The effects of gabexate mesilate (GM) on CraA-induced chemokine mRNA expression from BEAS-2B cells detected by semi-quantitative reverse transcription–polymerase chain reaction. A, Stimulation with CraA (5 μg/mL) induced significant IL-8, MCP-1, GM-CSF, and chemokine (C-C motif) ligand 20 (CCL20) mRNA expression. * $P<.05$, by Mann-Whitney test, compared to response to medium alone. B, GM significantly downregulated CraA-induced IL-8, MCP-1, GM-CSF and CCL20 mRNA expression in a dose-dependent manner. * $P<.05$; ** $P<.01$; *** $P<.001$, by 1-way analysis of variance with the Dunnett *t* test. CraA indicates American cockroach allergen; MCP-1, monocyte chemotactic protein 1; GM-CSF, granulocyte-macrophage colony-stimulating factor; CCL20, chemokine (C-C motif) ligand 20.

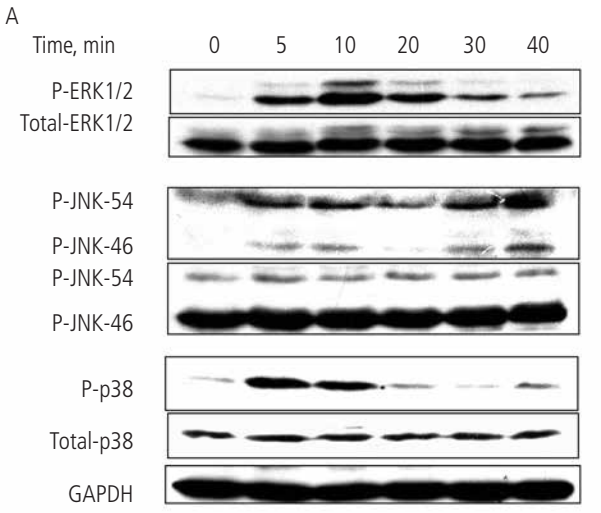

Figure 3. A, Western blot analysis of CraA on MAPK phosphorylation in BEAS-2B cells.

Animal studies using inhaled GM in CraA-allergic asthmatic mice are underway in our laboratory.

The airway epithelium is the first protective barrier against external stimuli, including allergens. It plays a sentinel role in the pathogenesis of asthma [26,27]. Further recruitment of inflammatory cells into the airway by chemokines is a critical process in the development of chronic inflammatory airway disease [28-30]. Inhibition of protease activity or members of MAPK families may provide novel therapeutic targets for patients allergic to allergens with protease activities [31-33]. However, there is also concern since intrinsic proteases and MAPKs are expressed ubiquitously and systemic inhibition may result in unwanted adverse effects. Accordingly, local delivery may be a preferential option.

In conclusion, the serine protease inhibitor GM has local protective effects against CraA–induced bronchial epithelial inflammation. The development of inhaled or intranasal protease inhibitors may be a potential strategy in the treatment of allergic airway diseases induced by allergens with protease activities.

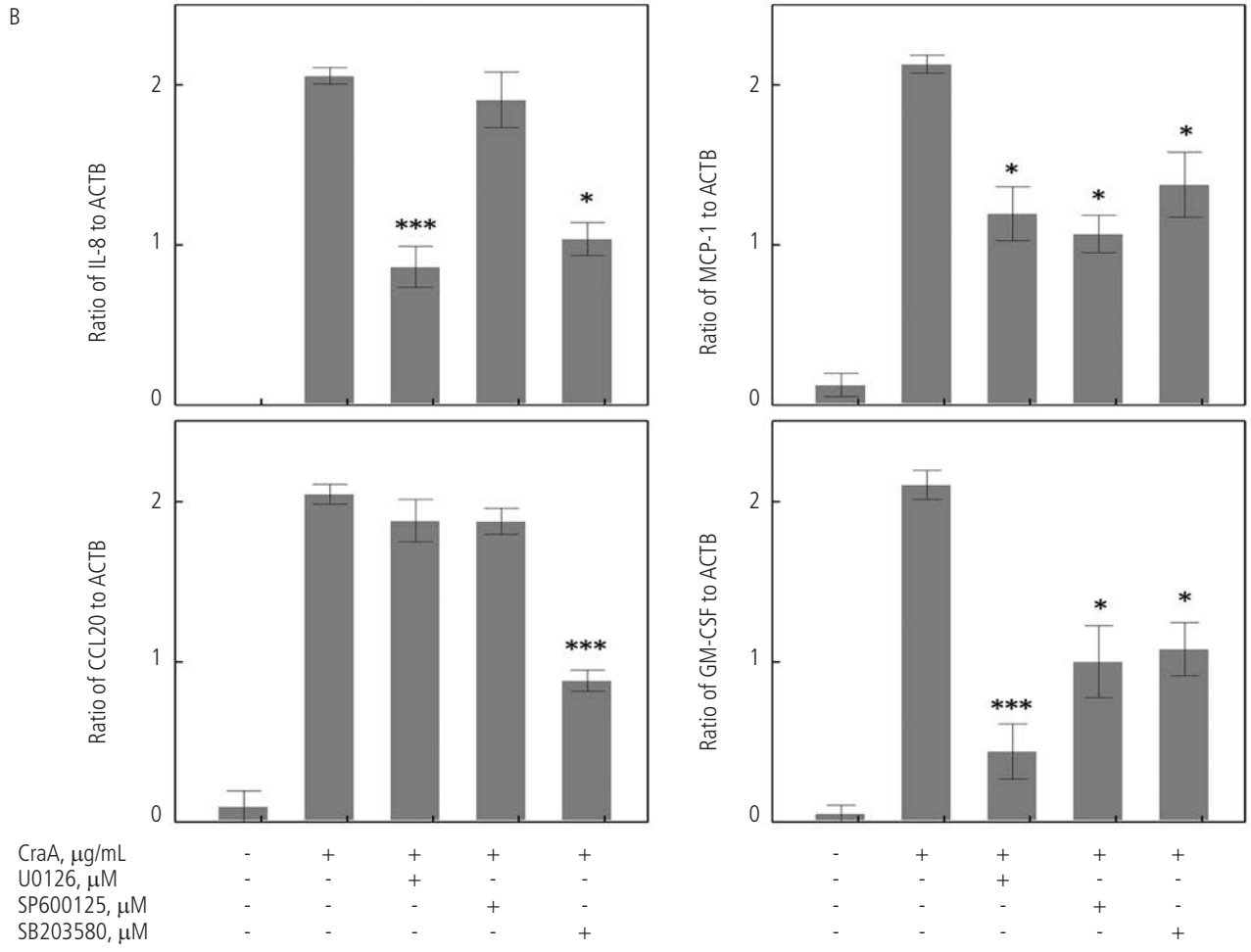

Figure 3. B, Effects of MAPK inhibitors on chemokine transcription by semi-quantitative reverse transcription–polymerase chain reaction. The relative levels of the genes were expressed as the ratio to β-actin. The values shown are the mean (SEM) of 3 independent experiments. *P<.05; **P<.01; ***P<.001, by 1-way analysis of variance with the Dunnett t test. CraA indicates American cockroach allergen.

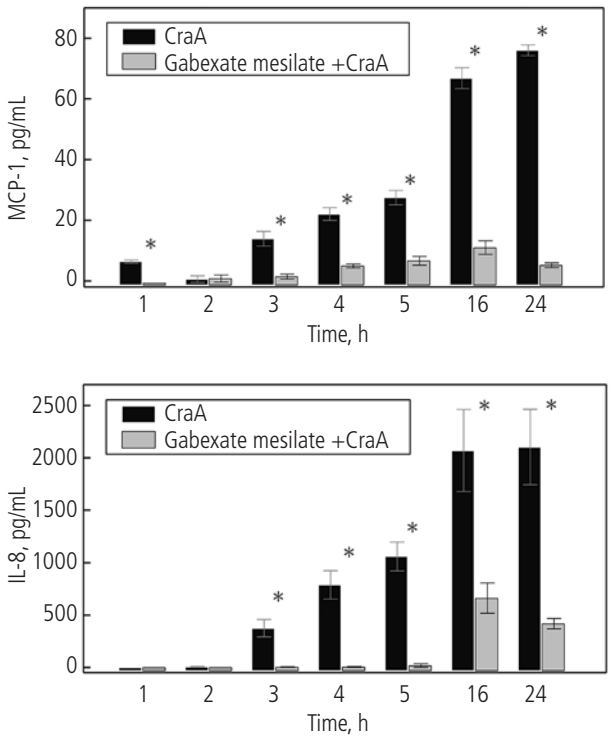

Figure 4. Time effects of gabexate mesilate on CraA-induced IL-8 and MCP-1 protein production from BEAS-2B cells by ELISA. Data are presented as the mean (SEM) of 3 independent experiments. *$P<.05$, by the Mann-Whitney test, compared to response to medium alone. CraA indicates American cockroach allergen; MCP-1, monocyte chemotactive protein 1.

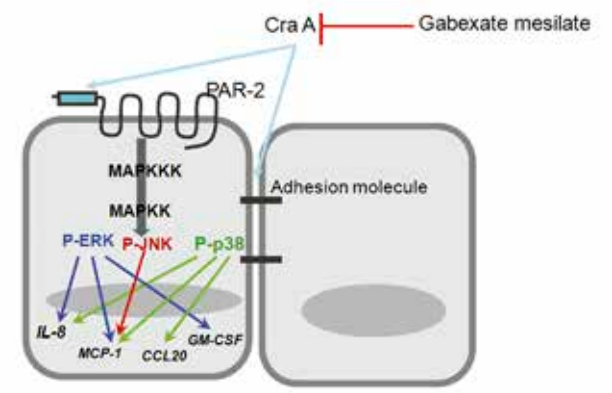

Figure 5. Proposed signaling mechanisms of CraA-induced chemokine gene expression from bronchial epithelial cells. CraA indicates American cockroach allergen.

Funding

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

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