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Airborne allergens induce protease activated receptor-2-mediated production of inflammatory cytokines in human gingival epithelium

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ABSTRACT

Objective: In reaching the airways inhaled allergens pass through and contact with the oral mucosa. Although they are often responsible for initiating asthmatic attacks, it is unknown whether airborne allergens can also trigger chronic inflammation of gingival epithelial cells leading to chronic periodontitis. In this study, we investigated the inflammatory responses of human gingival epithelial cells (HGECs) to airborne allergens, particularly German cockroach extract (GCE) with a focus on calcium signaling.
Design: HGECs isolated from healthy donors were stimulated with GCE. Intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured with Fura-2-acetoxymethyl ester (Fura-2/AM) staining. Expression of inflammatory cytokines interleukin (IL)-8, IL-1β, IL-6, and NOD-like receptor family, pyridine domain-containing (NLRP) 3 was analyzed using reverse transcription-polymerase chain reaction (RT-PCR).
Results: GCE promoted increase in the $[Ca^{2+}]_i$ in a dose-dependent manner. Depletion of endoplasmic reticulum (ER) Ca^{2+} by the ER Ca^{2+} ATPase inhibitor thapsigargin (Tg) but not the depletion of extracellular Ca^{2+} abolished the GCE-induced increase in $[Ca^{2+}]_i$. Treatment of phospholipase C (PLC) inhibitor (U73122) or 1,4,5-trisinositolphosphate (IP_3) receptor inhibitor (2-APB) also prevented GCE-induced increase in $[Ca^{2+}]_i$. Protease activated receptor (PAR)-2 activation mainly mediated the GCE-induced increase in $[Ca^{2+}]_i$ and enhanced the expression of IL-8, NLRP3, IL-1β, and IL-6 in HGECs.
Conclusions: GCE activates PAR-2, which can induce PLC/IP_3-dependent Ca^{2+} signaling pathway, ultimately triggering inflammation via the production of pro-inflammatory cytokines such as IL-1β, IL-6, IL-8, and NLRP 3 in HGECs.

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1. Introduction

Asthma is known for a chronic inflammatory disease of the conducting airways caused by a combination of complex and incompletely understood environmental and genetic factors. Among the many environmental factors, indoor allergens (e.g., dust mites, cockroaches, cat dander and fungi) are associated with the incidence of asthma (Gregory & Lloyd, 2011). As a primary barrier against various exogenous pathogens, oral epithelial cells can be directly exposed to airborne allergens when people breathe through their mouth (Desrosiers, Nguyen, Ghezzo, Leblanc, & Malo, 1998). Additionally, several studies have identified a close relationship between asthmatics and their oral health. Patients with asthma had a higher incidence of caries, a higher level of gingivitis, and a lower stimulated salivary secretion rate than those of healthy individuals (Mehta, Sequeira, Sahoo, & Kaur, 2009; Stensson et al., 2011). Despite these reports, it is unknown whether airborne allergens have a direct effect on the inflammation of gingival epithelial cells.

The German cockroach (*Blattella germanica*) is a small cockroach that one of the main domestic cockroach species in the houses (Arruda et al., 2001). Several allergens (Bla g 1, Bla g 2, Bla g 4, Bla g 5, and Bla g 6) have been identified from German cockroach (Arruda, Vailes, Benjamin, & Chapman, 1995; Arruda, Vailes, Hayden, Benjamin, & Chapman, 1995; Arruda, Vailes, Mann et al., 1995; Arruda, Vailes, Platts-Mills, Hayden, & Chapman, 1997; Bhat, Page, Tan, & Hershenson, 2003; Pomes et al., 1998). Although none of these are active proteases, protease activity has been recognized in German cockroach extract (GCE) (Hong et al., 2004;

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Pomes et al., 1998). Protease stimulatory signals induce inflammation that is characteristic of allergic reactions by opening tight junctions, causing desquamation, and producing cytokines, chemokines, and growth factors (Reed & Kita, 2004).

Protease-activated receptors (PARs), a family of four subtypes (PAR-1, -2, -3, and -4), are highly expressed in the cells of blood vessels, connective tissue, leukocytes, epithelium, and airway cells. They are 7-transmembrane-spanning G protein-coupled receptors (GPCRs) that generate G_q, $G_{12/13}$ and G_i protein-mediated signal transduction. Among them, activated G_q protein activates phospholipase C (PLC) to generate inositol 1,4,5-trisphosphate (IP_3) and stimulate IP_3-receptor (IP_3R)-mediated Ca^{2+} release from the endoplasmic reticulum (ER) into the cytosol. Gene transcription affected by increased intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) produces integrins, chemokines, and cytokines, as well as cyclooxygenase 2. Proteases cleave PARs irreversibly at a specific site in the extracellular N-terminus, which binds to the second extracellular loop of each PAR to trigger receptor activation. Extracellular endogenous proteases, as well as exogenous proteases, activate PARs; thrombin activates PAR-1, PAR-3, and PAR-4, and trypsin activates PAR-2 (Reed & Kita, 2004). Moreover, one of German cockroach allergens, Bla g 2, has serine protease which has been shown to activate PAR-2 (Hong et al., 2004; Page, Hughes, Bennett, & Wong, 2006).

Human gingival epithelial cells (HGECs) express PAR-1, -2, and -3 but not -4. Arginine-specific protease from *Porphyromonas gingivalis* activates PARs and induces interleukin (IL)-6 secretion (Lourbakos et al., 2001). Proteases secreted by *P. gingivalis* induce the expression of beta-defensin 2 and CC chemokine ligand 20 through a PAR-2-dependent mechanism (Dommisch et al., 2007). In addition, PAR-2 activation is known to be involved in periodontal disease (Lamont et al., 1995). However, the effect of allergens, particularly the proteases from GCE, on HGECs has not been reported. In the present study, we investigated the mechanism of GCE-induced production of pro-inflammatory cytokines mainly focused on the Ca^{2+} signaling.

2. Materials and methods

2.1. Reagents

Keratinocyte Basal Medium-2 (KBM-2) was purchased from Lonza (Walkersville, MD, USA). Collagenase A and Dispase II were obtained from Roche (Mannhein, Germany) and U73122, U73433, thrombin, trypsin, soybean trypsin inhibitor (SBTI), and histamine were the products of Sigma Chemical Co., Ltd. (St. Louis, MO, USA). PAR-2 agonist peptide (PAR-2 AP; SLIGRL-NH_2) was purchased from Tocris (Ellisville, MO). Thapsigargin (Tg) was obtained from Alexis Biochemical (San Diego, CA, USA). Fura-2-acetoxymethyl ester (Fura-2/AM) and Pluonic F-127 were purchased from Molecular Probe (Eugene, OR, USA).

2.2. Study subjects

The study population consisted of 40 subjects (11 men and 29 women; age range 18–29 years) attending the outpatient clinic of the Department of Advanced General Dentistry, Yonsei University College of Dentistry. These patients had no systemic disorders or complications. Written informed consent was obtained from those who agreed to participate voluntarily and ethical clearance was obtained from the Institutional Review Board. All experimental protocol were reviewed and approved by the Research Ethics Committee of Yonsei University College of Dentistry and Dental Hospital.

2.3. Primary HGEC culture

Human gingival epithelium was resected during the extraction of healthy donor's wisdom teeth as described previously (Lamont et al., 1995). In brief, human gingival epithelium was separated from connective tissues after treatment with Collagenase A and Dispase II for 45 min and placed in Trypsin/EDTA for 20 min. The isolated single-cell suspensions was maintained in KBM-2 at 37 °C in a humidified atmosphere composed of 5% CO_2/95% air. When a cell density of 80% confluence was reached, cells were harvested and subcultured. Primary HGECs within 3–4 passages were used for all experiments.

2.4. Preparation of endotoxin-free GCE

GCE (*Blattella germanica*) was kindly provided by the Arthropods of Medical Importance Resource Bank (Yonsei University College of Medicine, Seoul, Korea). The amount of total protein in the extract was measured by BCA™ protein assay (Pierce Biotechnology, Rockford, IL). For endotoxin-free GCE, endotoxin was removed using Detoxi-Gel™ Endotoxin Removing Gel (Pierce Biotechnology). The amount of endotoxin in GCE was measured with the Endpoint Chromogenic Limulus Amebocyte Lysate Test Kit (Lonza) and confirmed to be below 0.01 EU/ml.

2.5. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from HGECs using the Trizol reagent (Invitrogen Corp., Carlsbad, CA, USA) and then cDNA synthesis was performed using AccuPower® RT PreMix (BIONEER, Daejeon, Korea) according to the manufacture's protocol. cDNAs were amplified by PCR with HiPi™ Thermostable DNA polymerase

Table 1
List of primers used for polymerase chain reaction (PCR).

Gene	Primer sequence (5′ → 3′)	Product size (bp)	Annealing temperature (°C)
GAPDH	Forward: CGGAGTCAACGGATTTGGTCGTAT Reverse: AGCCTTCTCCATGGTGGTGAAGAC	307	58
IL-8	Forward: ATGACTTCCAAGCTGGCCGTGGCT Reverse: TCTCAGCCCTCTTCAAAAACTTCT	292	58
NLRP3	Forward: CATTCGGAGATTGTGGTT Reverse: GTTGCCTCGCAGGTAAAG	454	54
IL-1β	Forward: CAGTGAAATGATGGCTTATTAC Reverse: CTTTCAACACGCAGGACAGGT	548	56
IL-6	Forward: AGCCACTCACCTCTTCAGAACGAA Reverse: TACTCATCTGCACAGCTCTGGCTT	306	54

(Elpis, Pusan, Korea) and primers (Table 1). PCR products were electrophoresed on 1.2% agarose gels.

2.6. Measurement of intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$)

HGECs were cultured on collagen-coated cover glasses. Cells were loaded with 5 μM of Fura-2/AM in the presence of 0.05% Pluronic F-127 for 30 min in physiological saline solution (PSS). The composition of PSS was as follows (in mM): NaCl, 140; KCl, 5; $MgCl_2$, 1; HEPES, 10; $CaCl_2$, 1; glucose, 10 titrated to pH 7.4 with NaOH and the osmolarity was 310 mOsm. Changes in $[Ca^{2+}]_i$ were measured by means of fura-2 fluorescence with excitation wavelengths of 340 and 380 nm, respectively, and an emission wavelength of 510 nm. The emitted fluorescence was monitored with a CCD camera and analyzed with a MetaFluor system (Molecular Devices, PA, USA). Fluorescence images were obtained at 2 s intervals. The fluorescence ratio was calculated as follows: Ratio = F_{340}/F_{380}

3. Results

3.1. GCE induces Ca^{2+} release from the ER via the PLC/IP_3-dependent pathway in HGECs

To investigate whether GCE evoked Ca^{2+} signaling in HGECs, $[Ca^{2+}]_i$ was measured for GCE concentrations ranging from 1 to 100 μg/ml. Although 1 μg/ml of GCE has no effect on the $[Ca^{2+}]_i$, higher concentrations of GCE treatment induced increase in the $[Ca^{2+}]_i$ within 2–5 min (Fig. 1A). The peak value was also dependent on the concentration of GCE (EC_{50}: 23.21 μg/ml) (Fig. 1B).

The $[Ca^{2+}]_i$ increase may be derived from an influx of extracellular Ca^{2+} or the release of Ca^{2+} from intracellular stores such as an ER. To determine the Ca^{2+} source, we first treated HGECs with GCE (50 μg/ml) in the absence of extracellular Ca^{2+}. The GCE-induced increase in $[Ca^{2+}]_i$ was maintained in a Ca^{2+}-free medium, suggesting that these $[Ca^{2+}]_i$ changes are dependent on the release of Ca^{2+} from intracellular Ca^{2+} stores (Fig. 1C). Tg, a specific inhibitor of the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) pump, selectively depletes Ca^{2+} stores in the ER. Therefore, we treated the cells with Tg (1 μM) in Ca^{2+}-free medium. Tg treatment induced an increase in the $[Ca^{2+}]_i$, reflecting inhibition of the SERCA pump and leakage of Ca^{2+} from the ER into the cytosol. After depletion of the ER Ca^{2+} with Tg, GCE failed to induce an increase in the $[Ca^{2+}]_i$ (Fig. 1D, *left*). In a reciprocal experiment, GCE treatment reduced the increase in $[Ca^{2+}]_i$ by Tg (Fig. 1D, *right*).

To determine the involvement of the PLC-dependent IP_3 pathway in GCE-induced increase in $[Ca^{2+}]_i$, we first pretreated HGECs with U73122 (10 μM), a specific blocker of PLC, or its inactive analog U73343 (10 μM) for 5 min. U73122 prevented GCE-induced increase in $[Ca^{2+}]_i$; whereas, U73343 had no effect (Fig. 1E). The cells were also treated with the IP_3R blocker 2-aminoethoxydiphenyl borate (2-APB; 75 μM) to determine whether GCE-induced increase in $[Ca^{2+}]_i$ was mediated by IP_3R. As shown in Fig. 1F, treatment with 2-APB inhibited the increase in

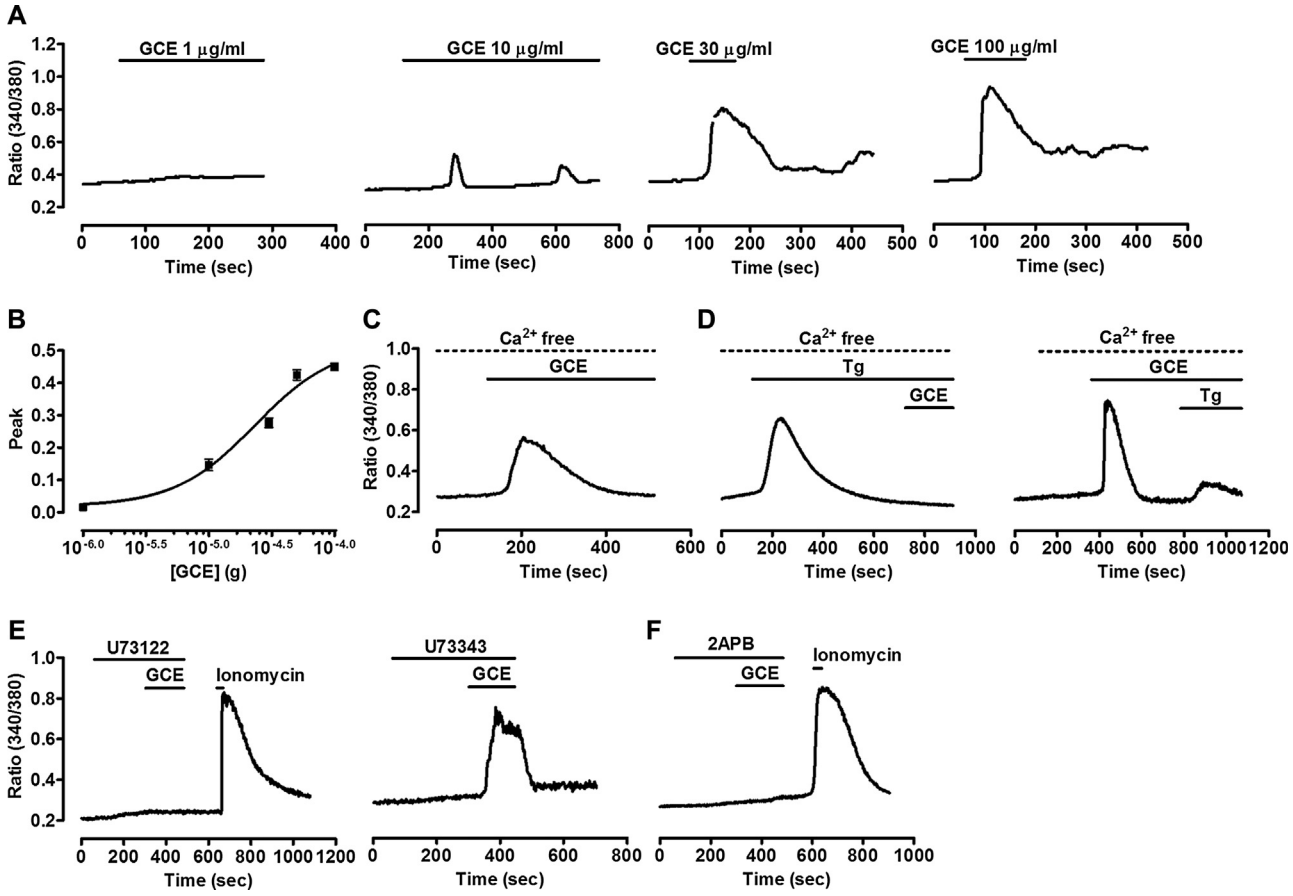

Fig. 1. German cockroach extract (GCE)-induced Ca^{2+} responses in human gingival epithelial cells (HGECs).
(A) Change in $[Ca^{2+}]_i$ induced by various concentrations of GCE ranging from 1 to 100 μg/ml ($n = 3$). (B) Analysis of dose-dependent changes in the peak of fluorescence at the 340/380 ratio. (C) Effect of extracellular Ca^{2+} on GCE (50 μg/ml)-induced increase in $[Ca^{2+}]_i$ ($n = 4$). (D) Effect of thapsigargin (Tg; 1 μM) on GCE (50 μg/ml)-induced increase in $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+} ($n = 3$). (E) Effect of U73122 (10 μM) and U73343 (10 μM) on GCE-induced increase in $[Ca^{2+}]_i$ ($n = 4$). Ionomycin was used as a positive control. (F) Effect of 2-APB (75 μM) on GCE-induced increase in $[Ca^{2+}]_i$ ($n = 4$).

$[Ca^{2+}]_i$, suggesting that GCE increases the $[Ca^{2+}]_i$ by inducing Ca^{2+} release from the ER through the PLC/IP_3-dependent pathway.

3.2. *PAR-2 mediates GCE-induced $[Ca^{2+}]_i$ increases*

GCE contains not only allergens with protease activity but also endotoxins (Bhat et al., 2003; Page et al., 2006). To rule out the possibility that endotoxin plays a role in GCE-induced increase in $[Ca^{2+}]_i$, we measured the effect of endotoxin-free (<0.01 EU/ml) GCE on the change in the $[Ca^{2+}]_i$. As shown in Fig. 2A, both endotoxin-free and whole GCE induced increase in $[Ca^{2+}]_i$, suggesting the effect of GCE was not because of endotoxin.

The serine protease activity of GCE may increase $[Ca^{2+}]_i$ through activation of PARs. To identify which type of PAR is cleaved by GCE, we used a desensitization protocol previously developed by our laboratory (Hong et al., 2004). GCE-induced increase in $[Ca^{2+}]_i$ was abolished by SBTI (10 μM), and pretreatment with SBTI also reduced the ability of GCE to increase $[Ca^{2+}]_i$ (Fig. 2B). Although the initial stimulation of cells with thrombin (1 U), which activates PAR-1, -3, and -4 but not -2, resulted in a transient increase in the $[Ca^{2+}]_i$, subsequent and repeated stimulation was unable to increase the $[Ca^{2+}]_i$. After desensitization of the receptors, treatment with both histamine (100 μM), which is used as a positive control by activating the G_q-coupled H1 histamine receptor, and endotoxin-free GCE still evoked an increase in $[Ca^{2+}]_i$, indicating that the endotoxin-free GCE-induced increase in $[Ca^{2+}]_i$ is not mediated by PAR-1, -3, or -4 (Fig. 2C). By contrast, after desensitization with trypsin (1 μM), which activates PAR-2, histamine induced the $[Ca^{2+}]_i$ rise, but endotoxin-free GCE failed to increase the $[Ca^{2+}]_i$, suggesting that PAR-2 is the receptor primarily activated by proteases in GCE (Fig. 2D). It has been known that GCE caused oscillations of the $[Ca^{2+}]_i$ via activation of PAR-2 (Hong et al., 2004). Additionally, chitinase-activated PAR-2 induced PLC/IP_3-dependent Ca^{2+} signaling in human airway epithelial cells (Hong et al., 2008). To confirm the involvement of PAR-2, we used PAR-2 AP and SBTI, a reversible competitive inhibitor of trypsin, and other trypsin-like proteases such as chymotrypsin, plasmin, and plasma kallikrein. Treatment with PAR-2 AP (100 μM) induced an increase in the $[Ca^{2+}]_i$ (Fig. 2E).

3.3. *PAR-2 induces the expression of pro-inflammatory cytokines*

PAR-2 induces production of pro-inflammatory cytokines such as interleukin (IL)-6 and IL-8 in epithelial cells (Asokananthan et al., 2002; Eckmann, Kagnoff, & Fierer, 1993). To determine whether PAR-2 activated by GCE has a direct effect on inflammation in HGECs, we examined cytokine expression. Both GCE (100 μg/ml) and trypsin (1 μM) significantly increased IL-8 levels (Fig. 3A). In addition, SBTI prevented GCE-induced IL-8 expression (Fig. 3B). NOD-like receptor family, pyridine domain-containing (NLRP) 3 was also induced by both GCE and trypsin, and this induction was reduced by SBTI (Fig. 3C and D). Finally, we also observed that both GCE and trypsin stimulated the production of IL-1β and IL-6 (Fig. 3E and F).

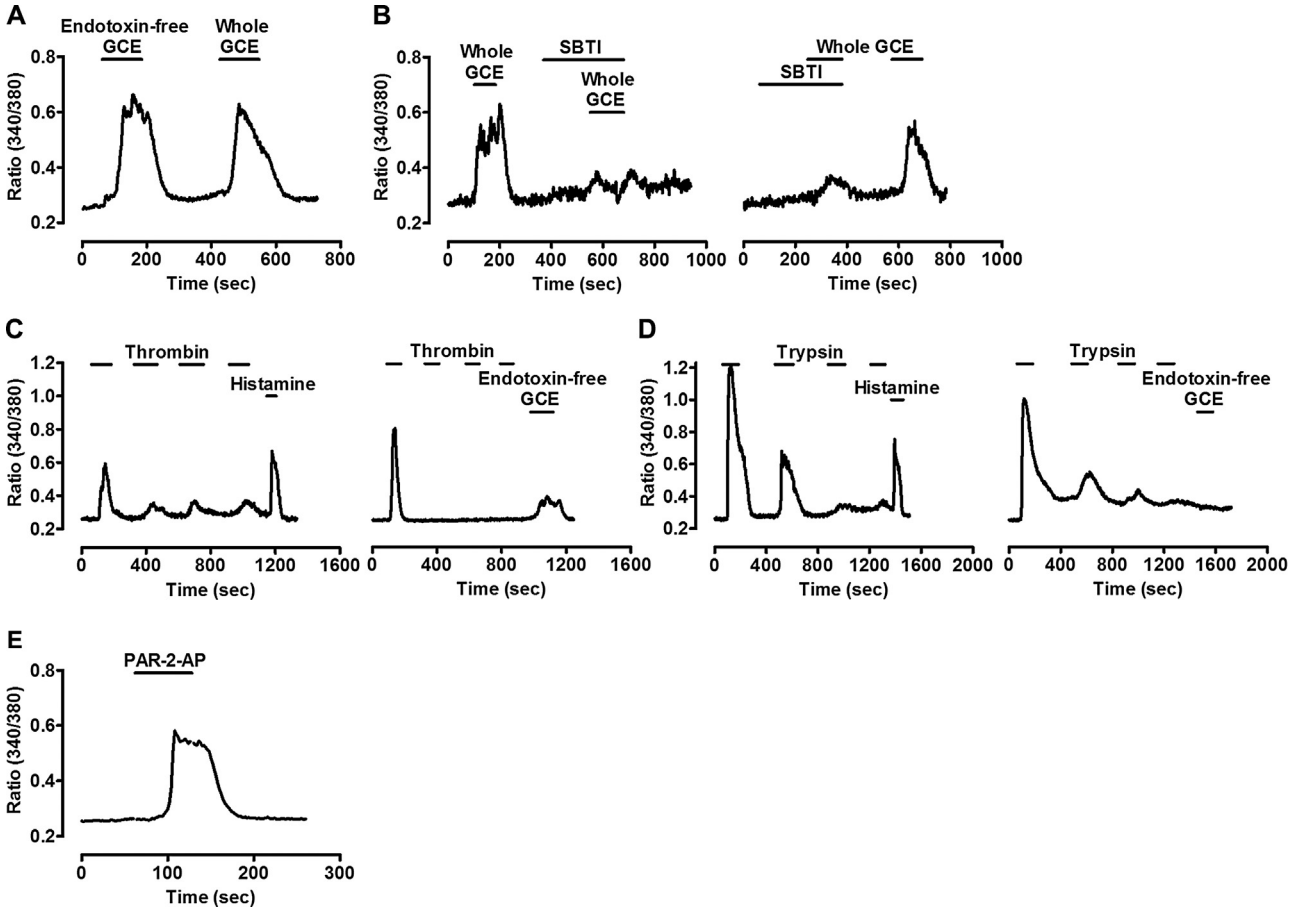

Fig. 2. Effect of protease-activated receptor (PAR) desensitization on GCE-induced $[Ca^{2+}]_i$ increases.
(A) Change in the $[Ca^{2+}]_i$ by endotoxin-free (<0.01 EU/ml) GCE (50 μg/ml) and whole GCE (50 μg/ml) ($n = 4$). (B) Effect of soybean trypsin inhibitor (SBTI; 100 μM) on the GCE-induced increase in $[Ca^{2+}]_i$ ($n = 3$). (C) After desensitization by repeated stimulation with thrombin (1 U), cells were treated with histamine (100 μM) or endotoxin-free GCE (50 μg/ml) and then the $[Ca^{2+}]_i$ was measured ($n = 3$). (D) After desensitization by repeated stimulation with trypsin (1 μM), cells were treated with histamine (100 μM) or endotoxin-free GCE (50 μg/ml), and the $[Ca^{2+}]_i$ was measured ($n = 3$). (E) Effect of PAR-2 agonist peptide (PAR-2 AP; 100 μM) on GCE-induced increase in $[Ca^{2+}]_i$ ($n = 4$).

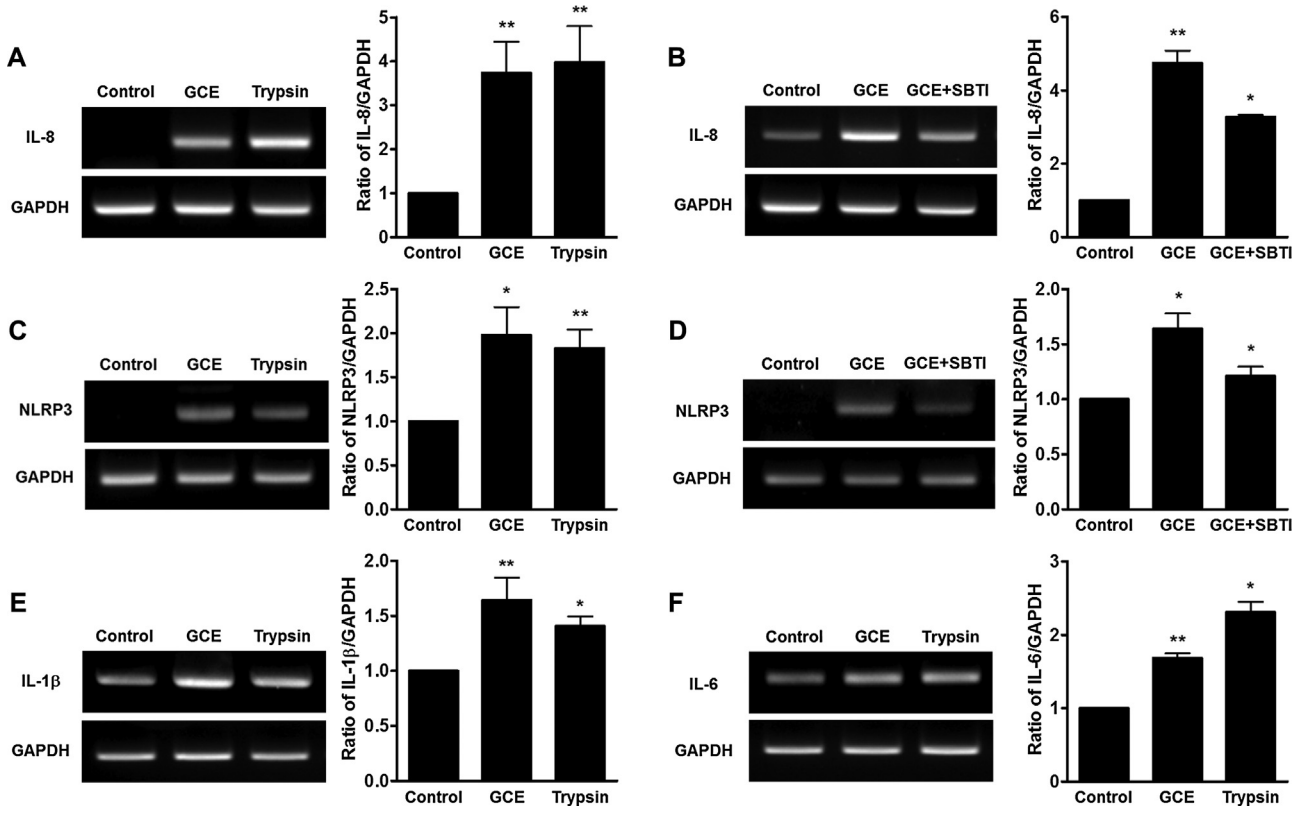

Fig. 3. Expression of inflammatory cytokines by GCE in HGECs.
HGECs were stimulated with GCE (100 μg/ml) alone or with SBTI (100 μM) for 3 h. Total mRNA was extracted from the cells, and the mRNA levels of interleukin (IL)-8 (A; $n = 13$ and B; $n = 3$), NOD-like receptor family, pyridine domain-containing (NLRP) 3 (C; $n = 7$ and D; $n = 3$), IL-1β (E; $n = 6$), and IL-6 (F; $n = 3$) were measured by reverse transcription-polymerase chain reaction (RT-PCR). Trypsin (1 μM) was used as a positive control. The mRNA levels of inflammatory cytokines were quantified after the value was normalized to GAPDH. The asterisks denote statistically significant differences between the compared values: $^{*}p < 0.05$, $^{**}p < 0.01$.

4. Discussion

Both asthma and periodontal disease have an inflammatory nature, and moreover, an association between poor oral health and chronic lung disease has been reported (Hyyppa, 1984; Ostergaard, 1977). The gingival epithelium can be directly exposed to the inhaled airborne allergens causing asthma before the allergens reach to airway epithelial cells (Desrosiers et al., 1998). Thus, it is highly possible that asthmatic allergens trigger inflammatory responses in gingival epithelial cells as it does in respiratory epithelium. The purpose of our study is whether the endotoxin and proteases from GCE known to initiate asthma attack also activates inflammatory responses in oral cavity. Unlike other numerous studies regarding the effects of GCE on airway epithelial cells, few studies have been conducted with oral epithelial cells. Here, we found that GCE activates the PAR-2-mediated PLC/IP_3-dependent pathway released Ca^{2+} from Tg-sensitive ER stores in HGECs. We also showed that GCE stimulated expression of inflammatory cytokines such as IL-8, NLRP3, IL-1β, and IL-6. With our knowledge, this is the first study determining the GCE-induced inflammatory responses and underlying molecular mechanism in the oral epithelial cells.

GCE protease activity induced IL-8 expression, which was dependent on transcriptional activation of NF-κB and NF-IL6 (Lee et al., 2007). Changes in Ca^{2+} levels activated by bacterial products are implicated in activation of NF-κB-dependent gene expression in airway cells (Chun & Prince, 2006). In addition, Ca^{2+} mediates maturation of the IL-1β through activation of caspase-1 via the NLRP3 inflammasome (Lee et al., 2012). Therefore, as seen in GCE-induced asthma, it is likely that the GCE-induced increase in $[Ca^{2+}]_i$ activates similar pathways to modulate the release of inflammatory cytokines and generate an immune response in gingival epithelial cells.

A previous report suggests that high dose of cockroach proteins may cause the chronic inflammation without any conclusive evidence (Sohn et al., 2004). Although most of our results indicate Ca^{2+} release occurred within 2–5 min with a high dose of GCE (50 or 100 μg/ml), it is difficult to conclude whether the response is acute or chronic. We used this as an ideal *in vitro* experimental condition for examining intracellular Ca^{2+} signaling in human gingival epithelial cells. In our study, we particularly focused on the effect of allergens with serine protease activity. It is well known that one of the main allergens from German cockroach, Blag2, contains serine protease and activates PAR-2 (Hong et al., 2004; Page et al., 2006). Moreover, two major allergens derived from dust mite, Der p3 and Der p9 also contain serine proteases, and activates PAR-2 and cytokine release (Sun, Stacey, Schmidt, Mori, & Mattoli, 2001). Thus, our study could be expanded to apply to the role of seine protease from various allergens in the inflammatory responses.

In addition to proteases, GCE contains bacterial endotoxins, which may be responsible for the increased $[Ca^{2+}]_i$. PAR-2 is up-regulated in endothelial cells *in vitro* after exposure to lipopolysaccharide, IL-1β, and tumor necrosis factor-α (Nystedt, Ramakrishnan, & Sundelin, 1996). We measured the $[Ca^{2+}]_i$ after treatment of the cells with endotoxin-free GCE to exclude the possible interference of endotoxin. As shown in Fig. 2, endotoxin-free GCE induced increase in $[Ca^{2+}]_i$ as much as whole GCE increased $[Ca^{2+}]_i$, suggesting that the increase in $[Ca^{2+}]_i$ was mediated through the PAR signaling pathway exclusively via PAR-2 in HGECs. It is highly likely that endotoxin in GCE may induce inflammatory effects; therefore endotoxin-free GCE should have

used to measure the levels of inflammatory cytokines. However, previous reports have shown that endotoxin does not affect GCE-induced cytokine expression (Bhat et al., 2003; Lee et al., 2007; Page et al., 2006). Based on these results, it would be reasonable to consider that endotoxin in whole GCE was not responsible for the induction of cytokine expression in gingival epithelial cells.

In the previous studies, PAR-2 activation mainly increases the expression of IL-8 compared to that of IL-1 and IL-6 (Arruda, Vailes, Benjamin et al., 1995; Arruda, Vailes, Mann et al., 1995; Arruda et al., 2001; Mehta et al., 2009; Pomes et al., 1998). In case of NLRP3, few studies regarding its role in the GCE-mediated PAR-2 activation are available. Therefore, we conducted the experiments to study the effect of SBTI on the only expression of IL-8 and NLRP3.

Our study showed that, similar to airway epithelial cells, GCE triggers PAR-2-mediated PLC/IP_3-dependent pathway activation in gingival epithelial cells, which releases Ca^{2+} from ER stores, and the activation of PAR-2 eventually, activates a pro-inflammatory reaction. This result supports the idea that the association between asthma and periodontal disease may be explained by either pathological activation of the inflammatory process or interactions between the two diseases. Many studies have examined periodontal health parameters in asthmatics. If the two diseases are closely associated, it would be of interest to determine whether oral health could be a diagnostic marker for asthma. In addition, our and others' studies strongly suggest that PAR-2 may be a novel target for both asthma and gingivitis treatment. Therefore, development of a PAR-2 inhibitor is urgently needed to test its effects on GCE-related immune responses.

Conflict of interest

The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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Competing interest

None declared.

Ethical approval

All experimental protocol were reviewed and approved by the Research Ethics Committee of Yonsei University College of Dentistry and Dental Hospital. Informed consent was obtained from all volunteers according to the requirements of the Institutional Review Board.

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