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

Ga-Yeon Son[a,b,1], Aran Son[a,1], Yu-Mi Yang[a], Wonse Park[c], Inik Chang[a], Jae-Ho Lee[d], Dong Min Shin[a,b,*]

[a] Department of Oral Biology, Yonsei University College of Dentistry, Seoul, Republic of Korea
[b] BK21 PLUS Project, Yonsei University College of Dentistry, Seoul, Republic of Korea
[c] Department of Advanced General Dentistry, Yonsei University College of Dentistry, Seoul, Republic of Korea
[d] Department of Pediatric Dentistry, Yonsei University College of Dentistry, Seoul, Republic of Korea

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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.

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;

* Corresponding author at: Department of Oral Biology, Yonsei University College of Dentistry, 50 Yonsei-ro, Seodaemon-gu, Seoul 120-752, Republic of Korea. Fax: +82 2 364 1085.
E-mail address: dmshin@yuhs.ac (D.M. Shin).
[1] These authors equally contributed to this work.

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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