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Acidification induces OGR1/Ca^{2+}/calpain signaling in gingival fibroblasts

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ABSTRACT

Gingivitis, the mildest form of periodontitis, is generally considered a consequence of prolonged exposure of the gingiva to periodontal pathogens. On the other hand, several epidemiologic reports have suggested that other etiologic factors such as oral acidification may also increase the susceptibility of the periodontium to destruction. However, the pathologic mechanism underlying the effects of oral acidification on the gingiva is still largely unknown. In this study, we analyzed molecular pathways mediating the influence of the acidic environment on human gingival fibroblasts (HGFs). Acidic extracellular pH caused biphasic increase of intracellular Ca^{2+} level ($[Ca^{2+}]_i$) through activation of ovarian cancer G protein-coupled receptor 1, phospholipase C, and Ca^{2+} release from the endoplasmic reticulum, but not through voltage-gated Ca^{2+} channels or extracellular Ca^{2+} influx via transient receptor potential cation channel subfamily V member 1. The acidic environment was also transiently cytotoxic for HGFs; however, the activation of pro-apoptotic proteins poly (ADP-ribose) polymerase-1 and BAX was not observed. Furthermore, we found that intracellular matrix metalloproteinase 1 was consistently upregulated in HGFs grown in regular medium, but significantly reduced in the acidic medium, which depended on $[Ca^{2+}]_i$ increase, lysosomal pH homeostasis, and Ca^{2+}-dependent protease calpain. Considering that HGFs, essential for oral wound healing, in the *in vitro* culture system are placed in wound repair-like conditions, our findings provide important insights into molecular mechanisms underlying HGF functional impairment and chronic damage to the gingiva caused by the acidic intraoral environment.

1. Introduction

The gingiva is a part of the periodontium, serving not only as a supportive and connective tissue but also as an effective immune barrier to infiltration of pathogens into deeper periodontal layers. Prolonged exposure to periodontal bacteria such as *Porphyromonas gingivalis* triggers inflammatory responses in the gingiva, known as gingivitis [1], which can ultimately lead to a loss of supporting connective tissue, alveolar bone, and teeth. However, several epidemiologic studies suggest that genetic disorders and/or environmental factors such as cigarette smoke contribute to the development of gingivitis regardless of infection [2]. Thus, people suffering from xerostomia and Sjögren's syndrome characterized by progressive deterioration of salivary functions show increased susceptibility to dental caries, changes in soft tissues, and oral infections [3]. Saliva constantly bathing teeth and oral mucosa has a significant buffering capacity and plays an essential role in the maintenance of intraoral pH which is highly acidic in patients with pathological xerostomia, Sjögren's syndrome, and gastroesophageal reflux disease [3,4]. These data suggest that acidification of the intraoral environment is a major factor in promoting gingivitis; however, the underlying molecular mechanisms are largely unknown.

Gq-coupled ovarian cancer G protein-coupled receptor 1 (OGR1) is a proton-sensing receptor fully activated at extracellular pH (pH_e) $\leq$ 6.8 [5]. OGR1 activation induces the formation of inositol triphosphate (IP_3) and increase of intracellular Ca^{2+} level ($[Ca^{2+}]_i$), and is involved in several pathological conditions [6–9]; thus, OGR1 overexpression was observed in human medulloblastoma, a cancer of neuronal precursor cells [8]. OGR1 was shown to suppress metastasis of prostate and breast cancer cells [6,7], and its activation in endplate chondrocytes promoted apoptosis [9]. Regarding

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OGR1 functions in the oral cavity, it was observed that the exposure of periodontal ligament cells to bacterial pathogens and allergens induced OGR1-mediated increase of $[Ca^{2+}]_i$, promoting the release of pro-inflammatory cytokines [10]. Considering the diverse activities of OGR1, we hypothesized that OGR1/Ca^{2+} signaling might mediate the pathophysiological responses induced by acidification in the gingiva, which ultimately cause chronic tissue damage.

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases involved in wound healing and tissue remodeling, and in processing bioactive proteins, including growth factors, cytokines, and cell-surface receptors [11]. MMP1 is capable of degrading fibrillar collagens, and has been implicated in diseases characterized by exaggerated extracellular matrix (ECM) degradation, such as periodontitis [12,13]. MMP expression in fibroblasts is normally low, but is upregulated in pathological conditions such as wound healing and tissue repair [14]. In this study, we examined whether increased concentration of extracellular H^+ induced intracellular OGR1/Ca^{2+} signaling and changes in MMP expression in human gingiva fibroblasts (HGFs). Our results indicate that acidic pH_e elicits $[Ca^{2+}]_i$ increase through OGR1, resulting in lysosomal degradation of MMP1.

2. Materials and methods

2.1. Materials

Capsazepine, nicardipine, U73122, U73343, and $CuCl_2$ were from Sigma Aldrich, and the Vybrant cytotoxicity assay kit was obtained from Invitrogen. For human (h)OGR1 silencing, we used the Trifecta Dicer-substrate RNAi kit (Integrated DNA Technologies). Primers for standard PCR assay were synthesized by Genotech, and antibodies against poly (ADP-ribose) polymerase-1 (PARP-1), BAX, β-actin, and MMP1 were obtained from Cell Signaling Technology, Inc., Santa Cruz Biotechnology, Inc., and R&D Systems, respectively.

2.2. Cell culture and transfection

HGFs (CRL-2014) obtained from American Type Culture Collection were cultured in DMEM supplemented with 10% FBS and 1% antibiotics at 37 °C in a 5%-CO_2 incubator. To examine the effects of extracellular acidification, sodium bicarbonate-free DMEM supplemented with HEPES (25 mM) was used. To silence hOGR1, HGFs were seeded on 35-mm culture dishes at 80% confluence and transfected the next day with double-stranded hOGR1-specific siRNAs using Lipofectamine (Invitrogen) according to the manufacturer's protocol. OGR1 siRNA sequences were as follows:

#1: 5′-GCAAAUAAAGCAAAGUAACUGGAAA-3′,
3′-GACGUUUAUUUCGUUUCAUUGACCUUU-5′;
#2: 5′-GGAGAAUUGAGGGAGUUAUAGGCAG-3′,
3′-ACCCUCUUAACUCCCUCAAUAUCCGUC-5′;
#3: 5′-ACUCUGUAAUGGAUUUAUGUAGCCC-3′,
3′-ACUGAGACAUUACCUAAAUACAUCGGG-5′.

After transfection, cells were incubated for additional 72 h and used for experiments.

2.3. $[Ca^{2+}]_i$ measurement

$[Ca^{2+}]_i$ was measured using Ca^{2+}-sensitive fluorescence dye, Fura-2/AM (Sigma Aldrich) as described [15]. Briefly, cells were seeded on coverslips (22 × 22 mm) at 80% confluence the day before experiment, and loaded with Fura-2 (5 μM final concentration) and incubated for 50 min at 37 °C. Cells attached to coverslips were placed at the bottom of a perfusion chamber and perfused with pre-warmed (37 °C) HEPES buffer containing (mmol/L): 140 NaCl, 5 KCl, 1 $MgCl_2$, 10 HEPES, 1 $CaCl_2$, 10 glucose (adjusted to 310 mOsm, pH 7.4 or 6.5). Ca^{2+}-free HEPES containing 1 mmol/L EGTA instead of $CaCl_2$ was used. $[Ca^{2+}]_i$ was measured at excitation wavelengths of 340 nm and 380 nm, and emitted fluorescence was detected at 510 nm by a CCD camera. Collected images were analyzed using the MetaFluor Software (Molecualr Devices) and presented as the F_{340}/F_{380} ratio.

2.4. Western blotting

Cells incubated in different conditions were lysed with RIPA buffer (Pierce Biotechnology) containing protease inhibitors. After protein quantification, total proteins (10 μg) were separated by SDS-PAGE and transferred onto a PVDF membrane, which was incubated with primary antibodies (all antibodies were diluted at 1:1000) for O/N at 4 °C. HRP-conjugated IgG (1:2000) was used as secondary antibody. Enhanced chemiluminescence (ECL) detection system (Thermo Scientific) was used for detecting the immunoreactive proteins, according to the manufacturer's protocols.

2.5. Reverse transcription-PCR

Total mRNA was extracted by Trizol (Invitrogen) and cDNA was synthesized using the PrimeScript RT reagent kit (Takara, Shiga) and used as a template for PCR. The amplified products were separated on agarose gels and analyzed in a FluorChem imager (ProteinSimple). The following primers were used:

OGR1 forward 5′-ATAAACAGGATGGCGGTG G-3′,
reverse 5′-AGGAGTTGTCTGCAGTGATG-3′;
GAPDH forward 5′-AGGGCTGCTTTTAACTCTGGT-3′,
reverse 5′-CCCCACTTGATTTTGGAGGGA-3′;
MMP1 forward 5′-TTGTGGCCAGAAAACAGAAA-3′,
reverse 5′-TTCGGGGAGAAGTGATGTTC-3′.

2.6. Cytotoxicity assay

Cells were seeded into 96-well plates at > 80% confluence, and then were incubated under indicated conditions and culture medium was analyzed for glucose-6-phosphate dehydrogenase (G6PD) release using the Vybrant Cytotoxicity kit according to manufacturer's protocol. Fluorescence (excitation, 544 nm; emission, 590 nm) was measured every 5 min for 30 min at 37 °C, and the data were expressed as the percentage of total G6PD in lysates of cells grown in parallel wells.

2.7. Cell migration assay

Cells seeded in two-well Culture-Insert μ-Dishes (35 mm) at > 80% confluence were transfected with siOGR1 and scrambled RNA (control), incubated for additional 72 h, and silicone inserts were removed after treatment with acidic medium. Cell images were taken every 12 h, and the data were presented as relative values compared to control (pH 7.4, scrambled).

2.8. Statistical analysis

The data were expressed as the mean ± SEM of at least three independent experiments. Statistical analysis was conducted with SPSS version 14.0 (SPSS Inc.). Differences were evaluated using one-way ANOVA followed by Tukey's post hoc test, and $P < 0.05$ was considered statistically significant.