Nuclear factor-κB contributes to the anti-inflammatory effects of resveratrol

Stimulation with heat-killed *P. gingivalis* slightly increased the nuclear level of the p65 subunit of NF-κB, whereas live *P. gingivalis* had no effect (Fig. 6). Resveratrol treatment inhibited the heat-killed *P. gingivalis*-induced increase in the nuclear level of the p65 subunit of NF-κB. Consistent with this, the cytoplasmic level of the p65 subunit was slightly decreased by heat-killed *P. gingivalis*, and this decrease was inhibited by treatment with resveratrol.

Discussion

In this study, a relatively low dose of resveratrol (8–15 μM) suppressed the expression of *MCP-1* and *IL-1β* by epi 4 cells stimulated with live or heat-killed *P. gingivalis*, and that of *IL-8* by epi 4 cells stimulated with live *P. gingivalis*. IL-8 induces chemotaxis and phagocytosis primarily in neutrophils, whereas MCP-1 facilitates chemotactic activity in monocytes and macrophages, and gingival epithelial cells are the important source of both chemokines. IL-1β is a proinflammatory cytokine secreted by a variety of cells, mainly by hematopoietic cells. After the IL-1β precursor is cleaved by caspase-1 as a result of inflammasome activation, it is released into the extracellular space (20). The expression of these cytokines increases in periodontitis lesions and decreases after periodontal treatment, and they are central to the pathogenesis of periodontitis (21). In Fig. 3, IL-1β in the supernatants was undetectable and did not reflect the changes in gene expression, which may be due to insufficient activation of inflammasome.

The mechanisms by which resveratrol exerts its anti-inflammatory effects are unknown. It has been proposed that resveratrol directly activates SIRT1 through an allosteric mechanism (22,23). SIRT1 activation can lead to NF-κB inactivation via the deacetylation of NF-κB-p65 at the lysine 310 residue (13,24). The reduction of intracellular ROS level by the antioxidative properties of resveratrol may constitute another mechanism of resveratrol-induced inactivation of NF-κB (15,25). These representative studies on the mechanism of effect of resveratrol were mainly performed using cells or organs involved in metabolism.

However, the mechanisms by which resveratrol acts may differ between organs and cells with distinct functions. Only a few studies have investigated its anti-inflammatory effects in epithelial cells other than HGECs (7,26). Zaidi *et al.* (26) showed the inhibitory effects of resveratrol on the production of IL-8 and ROS by *Helicobacter pylori*-stimulated gastric epithelial cells. Donnelly *et al.* (7) demonstrated that resveratrol inhibited granulocyte-macrophage colony-stimulating factor, IL-8 and cyclooxygenase-2, and had antioxidative effects, and decreased the activation levels of NF-κB and activator protein 1 in airway epithelial cells. These reports did not analyze the association between SIRT1 or AMPK and the action of resveratrol.

In this study, a relatively low dose of resveratrol slightly increased the expression of *SIRT1* by HGECs. *SIRT1* knockdown was well performed by using specific siRNA, and the knockdown increased the expression of the *IL-8*, *MCP-1* and *IL-1β* genes, likely due to anti-inflammatory properties of SIRT1 (13). However, neither inhibition of SIRT1 nor knockdown of *SIRT1* counteracted the anti-inflammatory effects of resveratrol, indicating that these effects occur independently of SIRT1. In addition, the effects were not mediated by activation of the AMPK pathway. Unlike the study by Zaidi *et al.* (26), our data do not support an antioxidative role for resveratrol, which may be explained by differences in the cell type or methodologies used. Therefore, although the suppression of NF-κB signaling may mediate the effects of resveratrol on heat-killed *P. gingivalis*-stimulated HGECs, the detailed mechanism remains unclear. Interestingly, our data suggest that the effects of resveratrol on live *P. gingivalis*-stimulated HGECs may not be mediated via NF-κB signaling. However, our method may have been unable to detect marginal increases in NF-κB induced by live *P. gingivalis* because of its weak stimulatory

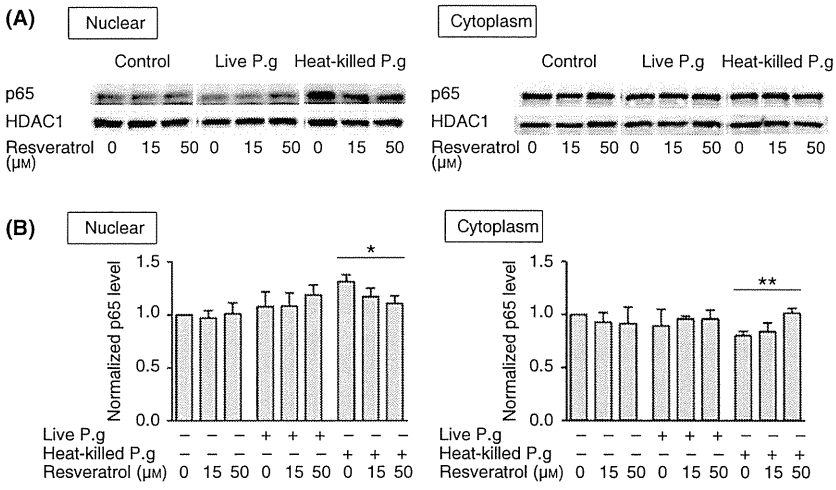

Fig. 6. The effect of resveratrol on the translocation of the p65 subunit of nuclear factor-κB. Epi 4 cells were cultured for 3.5 h in the presence of 15 or 50 μM resveratrol. In the last 1.5 h of culture, cells were stimulated with P.g. Nuclear and cytoplasmic proteins were extracted, and the p65 subunit of nuclear factor-κB present in each compartment was detected by western blotting analysis. (A) Representative figures. (B) The band intensity of nuclear p65 was normalized to that of HDAC1, whereas that of cytoplasmic p65 was normalized to that of GAPDH. Data are expressed as the mean ± SD of three independent experiments. $*p < 0.05$; $**p < 0.01$ vs. no treatment with resveratrol. HDAC1, histone deacetylase 1; P.g, *P. gingivalis*.

potential. Another possibility could be the involvement of other signaling pathways in the anti-inflammatory mechanisms. Specifically, mitogen-activated protein kinase, phosphatidylinositol 3-kinase/Akt and mammalian target of rapamycin signaling pathways are likely candidates (27–29).

Few studies have addressed the potential of resveratrol as a therapeutic supplement for periodontitis. Resveratrol decreased *P. gingivalis* lipopolysaccharide (LPS)-induced production of IL-1β, IL-6, IL-8, IL-12, tumor necrosis factor-α and nitric oxide in periodontal ligament cells, although the signaling pathways involved were not analyzed (8). Conversely, resveratrol inhibited the *P. gingivalis* LPS-induced adhesion of leukocytes to endothelial cells by reducing the expression of cell adhesion molecules, such as intercellular adhesion molecule 1 and vascular cell adhesion molecule 1, mainly via NF-κB inhibition (30). More recently, oral administration of resveratrol into a rat model of periodontitis suppressed alveolar bone loss and decreased IL-17 levels in the gingiva (31). In addition to anti-inflammatory effects, resveratrol has bone-protective properties, which also seem beneficial to periodontal health: it facilitated osteogenesis in mesenchymal stem cells, a process mediated via SIRT1 activation (32), whereas the compound suppressed RANKL-induced osteoclastogenesis via inactivation of NF-κB and inhibition of ROS production (33,34). Moreover, oral administration of resveratrol has demonstrated bone-protective effects in rodent models (35–37).

To conclude, a relatively low dose of resveratrol has anti-inflammatory effects on periodontopathogen-stimulated HGECs, which may be partly mediated by the suppression of NF-κB signaling. The mechanism may be independent of both SIRT1 and AMPK pathways, suggesting that resveratrol exhibits different activities in different cell types.

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RESEARCH REPORTS
Biological

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Epithelial TRPV1 Signaling Accelerates Gingival Epithelial Cell Proliferation

ABSTRACT

Transient receptor potential cation channel subfamily V member 1 (TRPV1), a member of the calcium-permeable thermosensitive transient receptor potential superfamily, is a sensor of thermal and chemical stimuli. TRPV1 is activated by noxious heat (> 43°C), acidic conditions (pH < 6.6), capsaicin, and endovanilloids. This pain receptor was discovered on nociceptive fibers in the peripheral nervous system. TRPV1 was recently found to be expressed by non-neuronal cells, such as epithelial cells. The oral gingival epithelium is exposed to multiple noxious stimuli, including heat and acids derived from endogenous and exogenous substances; however, whether gingival epithelial cells (GECs) express TRPV1 is unknown. We show that both TRPV1 mRNA and protein are expressed by GECs. Capsaicin, a TRPV1 agonist, elevated intracellular Ca^{2+} levels in the gingival epithelial cell line, epi 4. Moreover, TRPV1 activation in epi 4 cells accelerated proliferation. These responses to capsaicin were inhibited by a specific TRPV1 antagonist, SB-366791. We also observed GEC proliferation in capsaicin-treated mice *in vivo*. No effects were observed on GEC apoptosis by epithelial TRPV1 signaling. To examine the molecular mechanisms underlying this proliferative effect, we performed complementary (c) DNA microarray analysis of capsaicin-stimulated epi 4 cells. Compared with control conditions, 227 genes were up-regulated and 232 genes were down-regulated following capsaicin stimulation. Several proliferation-related genes were validated by independent experiments. Among them, *fibroblast growth factor-17* and *neuregulin 2* were significantly up-regulated in capsaicin-treated epi 4 cells. Our results suggest that functional TRPV1 is expressed by GECs and contributes to the regulation of cell proliferation.

KEY WORDS: gingiva, epithelium, transient receptor potential cation channels, capsaicin, periodontal diseases, microarray analysis.

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INTRODUCTION

Transient receptor potential cation channel subfamily V member 1 (TRPV1), also known as the capsaicin receptor or vanilloid receptor 1, is a non-selective ligand-gated cation channel activated by a range of exogenous and endogenous physical and chemical stimuli (Caterina *et al.*, 1997). Noxious heat (> 43°C), acidic conditions (pH < 6.6), and capsaicin, the pungent compound present in red chilies, are prototypical activators of TRPV1 (Dhaka *et al.*, 2006). TRPV1 was originally identified on peripheral nociceptive fibers, where its activation evokes pain or discomfort and initiates reflexes to protect the host (Lin *et al.*, 2007). Recently, TRPV1 channels were also found to play a sensory role in non-neuronal cells, such as keratinocytes (Peier *et al.*, 2002), gastric epithelial cells (Lee *et al.*, 2007), and urinary bladder epithelial cells (Birder, 2005). Other studies revealed that the activation of epithelial TRPV1 affects numerous biological processes, including proliferation, differentiation, and apoptosis (Ip *et al.*, 2012; Liu *et al.*, 2012).

Gingival epithelial cells (GECs) contribute to homeostasis in periodontal tissues by forming a physical barrier protecting against exogenous noxious agents. In addition, GECs sense and respond to bacterial stimuli by activating pathogen recognition receptors, including Toll-like and nucleotide-binding oligomerization domain (NOD)–like receptors (Ji *et al.*, 2009). GECs are also exposed to physical and chemical stimuli, including high temperatures, mechanical pressure, and acids derived from foods, microbes, and inhaled antigens. However, the expression of TRPV1 as an environmental sensor and its physiological functions in GECs are poorly understood.

In this study, we hypothesized that TRPV1 is expressed by GECs and is involved in cellular functions in the gingival epithelium. We examined the expression of TRPV1 mRNA and protein in both human and mouse GECs. We investigated the cellular functions mediated by TRPV1 in GECs *in vitro* and *in vivo*. Finally, we explored the downstream effectors of TRPV1 signaling by cDNA microarray analysis.

MATERIALS & METHODS

Reagents and Antibodies

Capsaicin (≥ 95% purity, from *Capsicum* sp.) was purchased from Sigma-Aldrich Corporation (St. Louis, MO, USA). SB-366791 was purchased from

Focus Biomolecules (Plymouth Meeting, PA, USA). Anti-TRPV1 antibody for immunostaining was obtained from Alomone Labs (Jerusalem, Israel). Rabbit anti-mouse glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and peroxidase-labeled anti-rabbit antibody (GE Healthcare, Little Chalfont, Buckinghamshire, UK) were used for Western blotting experiments.

Cell Preparation and Culture

Prior to inclusion in this study, all human participants provided written informed consent according to a protocol that was reviewed and approved by the Institutional Review Board of the Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan. Human GECs were prepared from clinically normal gingival tissue obtained following the extraction of an uninfected third molar, as previously described (Takahashi *et al.*, 2010). The simian virus 40 (SV40)-immortalized human gingival epithelial cell line, epi 4, was established and maintained as previously described (Murakami *et al.*, 2002; Takahashi *et al.*, 2011). Human embryonic kidney (HEK)-293 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin.

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and Wound-healing Assays *in vitro*

The MTT assay was performed according to the manufacturer's (Sigma-Aldrich Corporation) instructions for analyzing proliferation. For the wound-healing assay, epi 4 cells were grown in 12-well plates, and a small linear scratch was created in the confluent monolayer by gentle scraping with sterile pipette tips. Photographs of wound closure were taken at the end of the experiment and analyzed with ImageJ software (National Institutes of Health, Bethesda, MD, USA), and the relative gap closure was measured.

Mice

All experiments were performed in accordance with the Regulations and Guidelines on Scientific and Ethical Care and Use of Laboratory Animals of the Science Council of Japan and were approved by the Institutional Animal Care and Use Committee at Niigata University, Niigata, Japan. Six- to eight-week-old male C57BL/6 mice were obtained from Japan SLC, Inc. (Shizuoka, Japan), maintained under specific pathogen-free conditions, and fed regular chow and sterile water.

Immunohistochemistry

Fixed mandibles were dissected, decalcified, embedded, and sectioned as previously described (Sulniute *et al.*, 2011), with minor modifications. Paraffin sections were incubated with anti-TRPV1 antibody overnight. Immunoreactivity was detected with biotinylated chicken anti-rabbit immunoglobulin (IgG) (Abcam, Cambridge, UK) in an avidin-biotin-immunoperoxidase system (Vector Laboratories, Inc., Burlingame, CA, USA). Counterstaining was performed with hematoxylin (Polysciences, Inc., Warrington, PA, USA). For the immunohistochemistry of cultured cells, epi 4 cells were seeded in a Lab-Tek™ Chamber Slide (Nunc, Rochester, NY, USA) at a density of 5×10^4 cells/well. The attached cells were partially fixed with chloroform/acetone, washed in phosphate-buffered saline (PBS), and stained with anti-TRPV1 antibody. The sections were then imaged by microscopy (Biozero BZ-8000; Keyence Corporation, Osaka, Japan).

Transfection of a TRPV1 Overexpression Vector

A TRPV1 overexpression plasmid was kindly provided by Dr. Ardem Patapoutian (Scripps Research Institute, La Jolla, CA, USA). The HEK-293 cells were transfected with Lipofectamine® 2000 (Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions. TRPV1 expression was confirmed by conventional PCR and Western blotting at 24 hr post-transfection.

Polymerase Chain-reaction (PCR) and Gel Electrophoresis

Total RNA was isolated from gingival tissues and cells with TRI Reagent® (Molecular Research Center, Inc., Cincinnati, OH, USA). cDNA was synthesized with Transcriptor Universal cDNA Master (Roche Molecular Systems, Inc., Branchburg, NJ, USA). Conventional PCR was performed with a GeneAmp® PCR System 7700 (Applied Biosystems, Carlsbad, CA, USA), and PCR products were run on 1.5% agarose gels and visualized with SYBR® Safe DNA (Invitrogen Corporation, Carlsbad, CA, USA). Quantitative PCR was performed on a LightCycler® 480 (Roche Molecular Systems) with EagleTaq Master Mix (Roche Molecular Systems). The relative expression level of each mRNA was normalized to that of *GAPDH* mRNA by the *delta delta* Ct method (Livak and Schmittgen, 2001). The custom-designed oligonucleotide sequences (Invitrogen Corporation) used for both conventional and quantitative PCR are summarized in the Appendix Table.

Western Blotting

Total protein was extracted with M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, Inc., Rockford, IL, USA). Protein concentration was determined with a Pierce Bicinchoninic Acid Protein Assay Kit (Pierce Biotechnology, Inc., Rockford, IL, USA). Each sample was solubilized in sodium dodecyl sulfate (SDS) sample buffer, separated by SDS-polyacrylamide gel electrophoresis, and transferred to polyvinylidene fluoride membranes (EMD Millipore Corporation, Billerica, MA, USA). After incubation with each antibody, target proteins were detected with ECL Plus Western blotting detection reagents (GE Healthcare) and a LumiVision PRO 400EX system (Aisin Seiki Co., Ltd., Aichi, Japan).

Ca^{2+} Influx Assay

Intracellular Ca^{2+} changes were examined with the Calcium Kit II-Fluo 4 (Dojindo Laboratories, Tokyo, Japan), according to the manufacturer's instructions. In brief, cells seeded in 96-well

plates were treated with loading buffer containing Fluo4-AM at 37°C for 1 hr. Following replacement of the buffer with the recording medium, fluorescent intensity was measured, 1 min after stimulation, with a microplate fluorometer (TriStar LB 941; Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany).

Bromodeoxyuridine (BrdU) Labeling *in vivo*

The BrdU labeling was used to examine GEC proliferation *in vivo*. Mice were injected intraperitoneally with 100 mg/kg of BrdU (BD Pharmingen, San Jose, CA, USA) in PBS. Maxillary jawbones from euthanized mice were dissected 72 hr after BrdU injection, and fixed, decalcified, then embedded in paraffin. BrdU staining was performed with the BrdU *in situ* detection kit (BD Pharmingen), according to the supplier's recommendations.

TdT-dUTP Nick-end-labeling Assay

Epi 4 cells were grown in a Lab-Tek™ Chamber Slide at a density of 5×10^4 cells/well. Apoptotic cells were detected by the terminal deoxynucleotidyl transferase (TdT)-mediated biotin-dUTP nick-end-labeling (TUNEL) method with the In situ Apoptosis Detection Kit (Takara Bio, Inc., Shiga, Japan). Briefly, the cells were fixed with 4% paraformaldehyde for 15 min, permeabilized for 5 min, incubated with TdT end-labeling cocktail for 60 min, and then incubated with anti-fluorescein isothiocyanate (FITC) conjugate for 30 min. To quantitate apoptotic cell death, we calculated the percentage of TUNEL-positive cells relative to total cells after counting the numbers of TUNEL-positive cells and total cells in three random fields using a fluorescent microscope.

cDNA Microarray Analysis

RNA samples were amplified and labeled with Cy3 and a Quick Amp Labeling Kit (Agilent Technologies, Inc., Santa Clara, CA, USA), according to the manufacturer's protocol. Following labeling and purification, Cy3-labeled cRNAs were competitively hybridized onto an Agilent 4 × 44 K whole human genome oligo microarray slide (Agilent Technologies, Inc.), then scanned in an Agilent GeneArray Scanner (Agilent Technologies, Inc.). The detailed protocol and microarray data used in our study were deposited in the National Center for Biotechnology Information Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo; Accession Numbers: GSE57759).

Statistical Analysis

All experiments were performed in triplicate for each set of conditions and repeated at least twice. All data are expressed as the mean ± standard error of the mean. Statistical analyses were performed with GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA). The Student's *t* test was applied to compare the differences between the groups. The means of multiple groups were compared by analysis of variance followed by Tukey's test. A p value of $< .05$ was considered statistically significant.

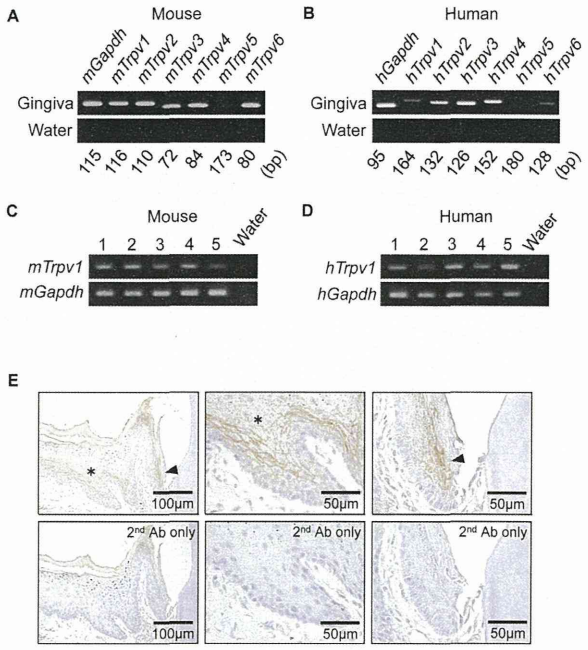

Figure 1. TRPV1 expression in the gingival epithelium. **(A, B)** PCR products of TRPV 1–6 after 33 cycles of amplification from mouse and human gingival tissues. Values show their predicted length in base pairs. *Glyceraldehyde 3-phosphate dehydrogenase* (*Gapdh*) was used as an internal control. Water samples were used as a negative control. **(C, D)** Expression of *Trpv1* mRNA in five different individual samples from mouse and human gingival tissues, respectively. **(E)** Representative immunohistochemical staining of murine gingival sections with anti-TRPV1 antibody at low (left panel) and high (right 2 panels) magnification. TRPV1 immunoreactivity was detected in the basal and suprabasal layers (asterisk) and junctional epithelium (arrowhead). Nuclei were counterstained with hematoxylin. The lower panels are sections without primary antibody, which served as negative controls.

RESULTS

TRPV1 Expression and Localization in Gingival Tissue

First, we examined the mRNA expression profiles of TRPV family members by conventional PCR. All TRPV family members were detected in mouse and human gingival tissues, with the exception of *Trpv5* (Figs. 1A, 1B). Of the TRPV channels, the physiological and biological roles of TRPV1 have been studied most extensively; therefore, we focused on identifying and characterizing TRPV1 in GECs in subsequent experiments. *Trpv1* mRNA expression was detected by conventional PCR in the gingival tissues of five different individuals for both mice and humans (Figs. 1C, 1D). We further confirmed TRPV1 localization in gingival tissues by immunohistochemistry. TRPV1 immunoreactivity was predominantly found within the gingival epithelial layer (Fig. 1E). The suprabasal layers showed more intense immunostaining compared with the basal layers and the junctional epithelium (Fig. 1E).

Expression of Functional TRPV1 in GECs

To confirm TRPV1 expression in GECs, we performed conventional PCR using homogeneous populations of GECs cultured

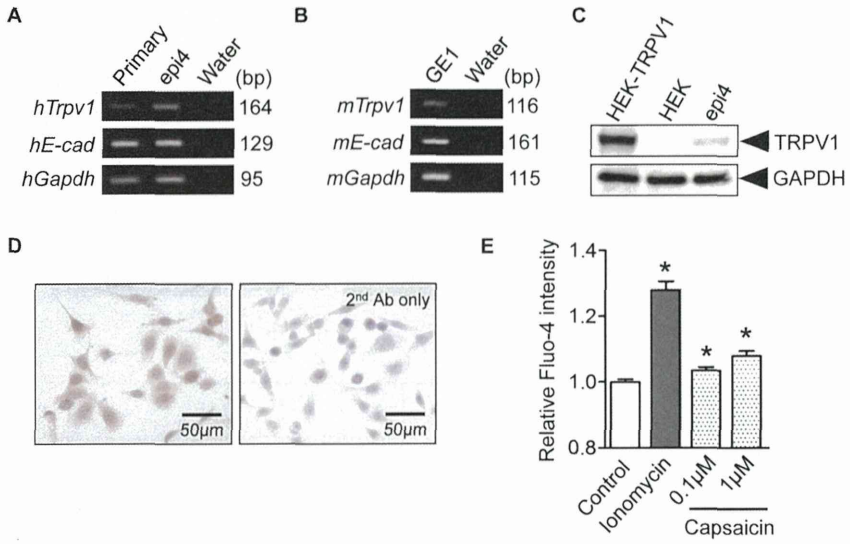

Figure 2. Cultured gingival epithelial cells (GECs) express functional TRPV1. **(A)** Expression of *Trpv1* mRNA was confirmed in primary GECs, in the human gingival epithelial cell line, epi 4, and **(B)** in the murine gingival epithelial cell line, GE1. E-cadherin (*E-Cad*) was used as an epithelial cell marker. *Gapdh* was used as an internal control. **(C)** Endogenous expression of TRPV1 protein in GECs, shown by Western blotting. Human embryonic kidney (HEK)-293 cells, transfected with the TRPV1 expression vector (HEK-TRPV1), served as a positive control. Untransfected cells (HEK) served as a negative control. Equal loading was assessed by GAPDH immunoblotting. **(D)** Epi 4 cells were grown in wells of Lab-Tek™ Chamber Slides to subconfluence (70%). After fixation, cells were incubated with anti-TRPV1 overnight at 4°C. Cells were counterstained with hematoxylin, and mounted for microscopy. The right panel is a section without primary antibody, which served as a negative control. **(E)** TRPV1-mediated intracellular Ca^{2+} influx in epi 4 cells. Intracellular Ca^{2+} changes were analyzed as fluorescence intensity changes of the green-fluorescent calcium indicator, *Fluo-4*. Capsaicin (0.1, 1 μM) treatment induced the elevation of intracellular [Ca^{2+}] in a dose-dependent manner. A calcium ionophore (Ionomycin, 10 ng/mL) was used as a positive control. $*p < .05$ *vs.* unstimulated cells.

in vitro. TRPV1 expression was evident in primary cultured human GECs, in the SV40-immortalized human gingival epithelial cell line, epi 4 (Fig. 2A), and in the murine gingival epithelial cell line, GE1 (Fig. 2B). To analyze TRPV1 protein expression, we first validated the specificity of the anti-TRPV1 antibody for HEK-293 cells overexpressing TRPV1, then confirmed TRPV1 expression in epi 4 cells by Western blotting (Fig. 2C). TRPV1 expression by GECs was also demonstrated by immunostaining with epi 4 cells (Fig. 2D). Functional TRPV1 expression was demonstrated by Ca^{2+} influx assay in GECs; epi 4 cells were stimulated with the TRPV1 agonist capsaicin (1 μM), causing significant intracellular Ca^{2+} increase (Fig. 2E). Analysis of these data, taken together, suggests that GECs express functional TRPV1 channels.

Epithelial TRPV1 Signaling Accelerates GEC Proliferation

Disruption of the epithelial barrier and the subsequent invasion of exogenous substances into the gingiva facilitate the progression of periodontal breakdown. Preservation of epithelial proliferation is crucial for maintenance of the epithelial barrier and protection of the host. We performed MTT and wound-healing assays to evaluate the role of TRPV1 signaling in GECs. A significant increase in the number of epi 4 cells was evident after treatment with capsaicin (1 μM) for 72 hr (Fig. 3A). Pretreatment of epi 4 cells with the TRPV1 antagonist (SB-366791) prevented this pro-proliferative effect, suggesting a TRPV1-dependent mechanism (Fig. 3B). Consistent with these findings, wound-healing assays showed TRPV1-dependent, pro-proliferative effects of capsaicin on GECs (Figs. 3C, 3D). To assess the proliferative activity of capsaicin *in vivo*, we performed BrdU pulse-chase experiments. The mice received BrdU intraperitoneally and were euthanized 72 hr later, and their gingival tissues were processed for histologic analysis. As expected, the number of BrdU-positive cells was higher in mice fed with capsaicin compared with those fed the normal diet (Fig. 3E). In addition, transcript levels of proliferating cell nuclear antigen (PCNA) were significantly higher in the capsaicin-treated group than in the normal-diet group (Fig. 3F). No effects were observed on GEC apoptosis by epithelial TRPV1 signaling (Appendix Fig. A-D). Collectively, these results demonstrate the pro-proliferative effects of capsaicin on GECs *in vitro* and *in vivo*.

Identification of Candidate Genes Associated with TRPV1-dependent GEC Proliferation

To elucidate the molecular mechanisms of capsaicin-induced GEC proliferation, we performed cDNA microarray analysis. Gene expression profiles of the epi 4 cells stimulated with capsaicin (1 μM) for 4 hr were compared with those of untreated cells (four samples/group). The data were normalized and filtered by a fold change of > 1.5 and < 1.5 with an adjusted p value $< .05$, respectively. The 227 up-regulated and 232 down-regulated genes were sorted according to Gene Ontology "growth factor activity" (GO: 0008083). In total, four genes were identified and validated in independent experiments by quantitative PCR. Both *fibroblast growth factor* (*FGF*)-*17* and *neuregulin* (*NRG*) *2* were significantly up-regulated in capsaicin-treated epi 4 cells relative to untreated cells (Fig. 4). Analysis of these data suggests that TRPV1 signaling in GECs may induce transcriptional up-regulation of growth factors, resulting in increased proliferation.

DISCUSSION

In this study, we demonstrated that TRPV1 channels are robustly expressed by human and mouse GECs. Furthermore, we found that epithelial TRPV1 signaling promotes GEC proliferation. Previous studies have identified functional TRPV1 expression in other epithelial cells, including bronchial (Yang *et al.*, 2013),

corneal (Pan *et al.*, 2011), and mammary (Kang *et al.*, 2003) epithelial cells. In the oral cavity, Wang *et al.* (2011) reported TRPV1 mRNA expression in three oral epithelia (buccal, palatal, and lingual) and also found that TRPV5 mRNA was not expressed in these regions. These expression profiles are consistent with our results. To the best of our knowledge, our study provides the first evidence of TRPV1 expression in human and mouse GECs. Analysis of our data also suggests that GECs act as sensors of changes in the physical and chemical environment and may function in protecting against noxious stimuli from exogenous substances by promoting GEC proliferation.

Although we have demonstrated that TRPV1 signaling increases GEC proliferation *in vitro*, previous studies on the role of TRPV1 in proliferation are conflicting. Involvement of TRPV1 in pro-proliferative effects was reported in prostate epithelial cells (Malagarie-Cazenave *et al.*, 2009), colonic epithelial cells (Liu *et al.*, 2012), and human keratinocytes (Denda *et al.*, 2010). In contrast, anti-proliferative effects of TRPV1 were found in lung (Brown *et al.*, 2010) and prostate stromal (Venier *et al.*, 2012) cells. The effects elicited by capsaicin may depend on the cell or tissue types studied. In oral epithelia, a proliferation marker, PCNA, expression was predominantly expressed in the suprabasal layers in the oral gingival epithelium (Celenligil-Nazliel *et al.*, 2003), suggesting that the proliferating cells of the gingival epithelium are mainly localized in the suprabasal rather than in the basal layers. Our immunohistochemical staining of periodontal tissues showed that TRPV1 immunoreactivity was most intense in the suprabasal layer, indicating that TRPV1 expressed in the gingival epithelium acts as an inducer of cell proliferation rather than an anti-proliferative effector.

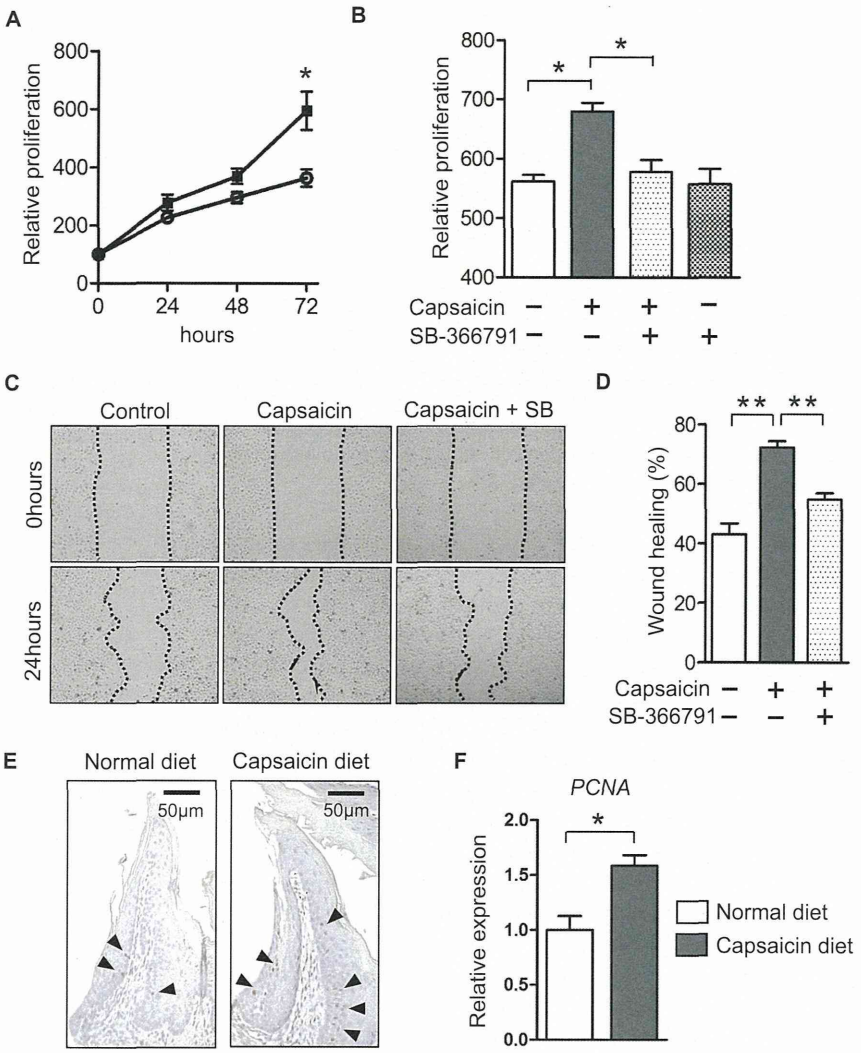

Figure 3. Epithelial TRPV1 signaling accelerates GEC proliferation. **(A)** The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to examine the proliferative activity of epi 4 cells after treatment with the TRPV1 agonist, capsaicin, for 24, 48, and 72 hr. The relative cell proliferation of epi 4 treated with 1 μM capsaicin (solid square) for 72 hr was significantly increased compared with that of untreated cells (open circle). **(B)** Pretreatment with the TRPV1 antagonist, SB-366791 (1 μM), for 30 min inhibited capsaicin-induced cell proliferation at 72 hr. SB-366791 treatment alone had no effects on the proliferation of GECs. **(C)** To further confirm the effect of TRPV1 on proliferation *in vitro*, we performed wound-healing assays with epi 4 cells. Representative images after capsaicin treatment (1 μM) with or without SB-366791 (1 μM) for 24 hr are shown. Dotted lines indicate the margins of cells. **(D)** The level of cell migration into the wound scratch was quantified as the percentage of wound healing in the untreated group after 24-hour treatment. **(E)** Representative bromodeoxyuridine (BrdU) staining of murine gingival sections. Mice were treated with dietary capsaicin (100 parts *per* million in chow) for 7 days before BrdU injection. Increased numbers of BrdU-positive cells (arrowheads) were found in the gingiva of the capsaicin-treated group compared with the normal-diet group. **(F)** Expression of a proliferation marker, proliferating cell nuclear antigen, was significantly higher in the capsaicin-treated group. $*p < .05$; $**p < .01$ *vs.* unstimulated cells or the normal-diet group.

To determine the molecular mechanism of TRPV1-mediated GEC proliferation, we performed cDNA microarray analysis. Up-regulation of two proliferation-related genes, *FGF-17* and *NRG2*, in capsaicin-stimulated GECs was validated by quantitative PCR. Both FGF-17 and NGR2 are growth factors involved in a variety of biological processes, including cell growth (Falls, 2003; Polnaszek *et al.*, 2004). In addition, various FGF family members have been demonstrated to contribute to the autocrine regulation of epithelial cell proliferation (Maheshwari *et al.*, 2001). Takayama *et al.* (2002) reported the expression of cognate receptors for FGF on

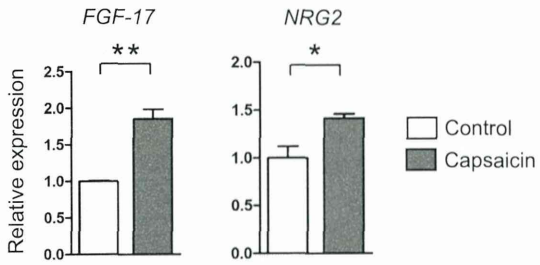

Figure 4. Expression of candidate genes related to TRPV1-mediated cell proliferation. Epi 4 cells were stimulated with 1 μM capsaicin for 4 hr, and the gene expression profiles were analyzed by microarray. Among the up-regulated genes found to be related to cell proliferation by microarray analysis, statistical validation for *fibroblast growth factor (FGF)-17* and *neuregulin (NRG) 2* was discovered by independent experiments using quantitative polymerase chain-reaction. $*p < .05$; $**p < .01$ *vs.* unstimulated cells.

GECs. This suggests that TRPV1 activation contributes to periodontal tissue homeostasis by triggering a positive feedback loop involving the production of multiple growth factors.

Disruption of periodontal tissue homeostasis has the potential to cause the initiation and progression of periodontitis (Hajishengallis, 2014). One limitation of our study is its lack of information on the possible involvement of TRPV1 in the pathogenesis of periodontal diseases. A recent study using immunohistochemistry showed that TRPV1 expression and distribution differed in subgingival specimens from patients with periodontitis and healthy individuals (Öztürk and Yildiz, 2011). However, further studies using both immunohistochemical and other biological methods are required. A more thorough understanding of the mechanism of action of capsaicin in GEC biology could potentially lead to the development of new therapeutic approaches for periodontal diseases.

In conclusion, we confirmed the expression of TRPV1 by GECs isolated from humans and mice. Our results suggest that TRPV1 channels function in the detection of a variety of chemical stimuli and in modulating epithelial wound-healing by affecting proliferation. Further studies are required to elucidate the contribution of TRPV1 to periodontal diseases.

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