Histamine Amplifies Immune Response of Gingival Fibroblasts

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ABSTRACT

Histamine is an important mediator in immune responses, but it is unclear whether periodontal tissues express histamine receptors and are able to respond to histamine. We hypothesized that histamine, inflammatory cytokines, and bacterial components released in inflamed periodontal tissues may be synergistically involved in periodontitis. The present study showed that human gingival fibroblasts mainly express histamine receptor H1R, and responded to histamine to produce interleukin (IL)-8. Stimulation of gingival fibroblasts with tumor necrosis factor-α, IL-1α, and lipopolysaccharide markedly induced IL-8 production, and the IL-8 production was synergistically augmented in the presence of or pre-treatment with histamine. Selective inhibitors of mitogen-activated protein kinases (MAPKs), nuclear factor (NF)-κB, and phospholipase C (PLC) significantly inhibited the synergistic effect. These results indicate that histamine induces IL-8 production from gingival fibroblasts through H1R, and synergistically augments the inflammatory stimuli by amplification of the MAPK and NF-κB through H1R-linked PLC.

Abbreviations used: HDC, histidine decarboxylase; LPS, lipopolysaccharide; IL, interleukin; TNF, tumor necrosis factor; HR, histamine receptor; PLC, phospholipase C; MAPK, mitogen-activated protein kinase; NF, nuclear factor; ERK, extracellular signal-related kinase; JNK, c-Jun N-terminal kinase; R, receptor; TLR, Toll-like receptor; α-MEM, alpha-minimum essential medium; FCS, fetal calf serum; RT-PCR, reverse-transcriptase polymerase chain-reaction; ELISA, enzyme-linked immunosorbent assay; SD, standard deviation; LDH, lactate dehydrogenase.

KEY WORDS: histamine, fibroblasts, inflammation, MAPK, NF-κB.

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INTRODUCTION

Histamine is an important mediator not only in allergic reactions but also in a variety of immune responses, including the production of inflammatory cytokines and the modulation of T-helper cell balance (Dy and Schneider, 2004). Histamine is released from stimulated mast cells or basophils. In addition, histamine is newly synthesized by a histamine-forming enzyme, histidine decarboxylase (HDC), in non-mast cells, and is released without being stored (Kahlson and Rosengren, 1968; Schayer, 1974; Endo, 1982). HDC is induced in various organs or tissues in response to a variety of inflammatory stimuli, including bacterial products, such as lipopolysaccharide (LPS), and inflammatory cytokines, such as interleukin (IL)-1, tumor necrosis factor (TNF)-α, IL-18, and IL-12 (Schayer, 1974; Endo, 1989; Yamaguchi *et al.*, 2000), indicating that histamine might also be induced in the inflamed gingiva of persons with periodontitis.

Histamine receptors (H1R, H2R, H3R, and H4R) belong to the G-protein-coupled receptor superfamily (Dy and Schneider, 2004). H1R and H2R are expressed in various cell types, while the expression of H3R and H4R is restricted to the brain and the hematopoietic cells, respectively. H1R is linked to the activation of phospholipase C (PLC) through the $G\alpha_{q/11}$ protein (Dy and Schneider, 2004). An H1R agonist, 2-[3-(fluoromethyl)phenyl]histamine, induces the activation of nuclear factor (NF)-κB, a ubiquitous transcription factor that is considered to play an important role in inflammatory processes, through $G\alpha_{q/11}$ and Gβγ (Bakker *et al.*, 2001). $G\alpha_{q/11}$ also stimulates mitogen-activated protein kinases (MAPKs), important mediators of signal transduction, through the protein kinase C- and Src family kinase-dependent signaling pathway (Nagao *et al.*, 1998). MAPKs are divided into at least 3 subfamilies: extracellular signal-related kinase (ERK), c-Jun N-terminal kinase (JNK)/stress-activated protein kinase, and p38 MAPK (Davis, 1994). TNF-α, IL-1, and LPS also activate NF-κB and MAPK pathways through respective receptors (Barton and Medzhitov, 2003; Wajant *et al.*, 2003; Subramaniam *et al.*, 2004).

Human gingival fibroblasts, the major constituent of gingival connective tissue, are heterogeneous (Lekic *et al.*, 1997; Sugawara *et al.*, 1998) and express immunological receptors (Rs), such as IL-1R (Kanda-Nakamura *et al.*, 1996), IL-2R (Ozawa *et al.*, 2004), TNFR (Butler *et al.*, 1994), CD14 (Watanabe *et al.*, 1996; Sugawara *et al.*, 1998), and Toll-like receptors (Tamai *et al.*, 2002). Stimulation of gingival fibroblasts with these cytokines and LPS induces the production of various cytokines, such as IL-1, IL-6, and IL-8 (Takada *et al.*, 1991; Watanabe *et al.*, 1996; Sakuta *et al.*, 1998; Sugawara *et al.*, 1998; Tamai *et al.*, 2002; Ozawa *et al.*, 2004). These observations indicate that gingival fibroblasts actively participate in immune responses and inflammatory processes.

It is unclear whether gingival fibroblasts express histamine receptors and are able to respond to histamine, but it is conceivable that, in inflamed periodontal tissues, histamine, inflammatory cytokines, and bacterial components are released together, and may be orchestrating immune

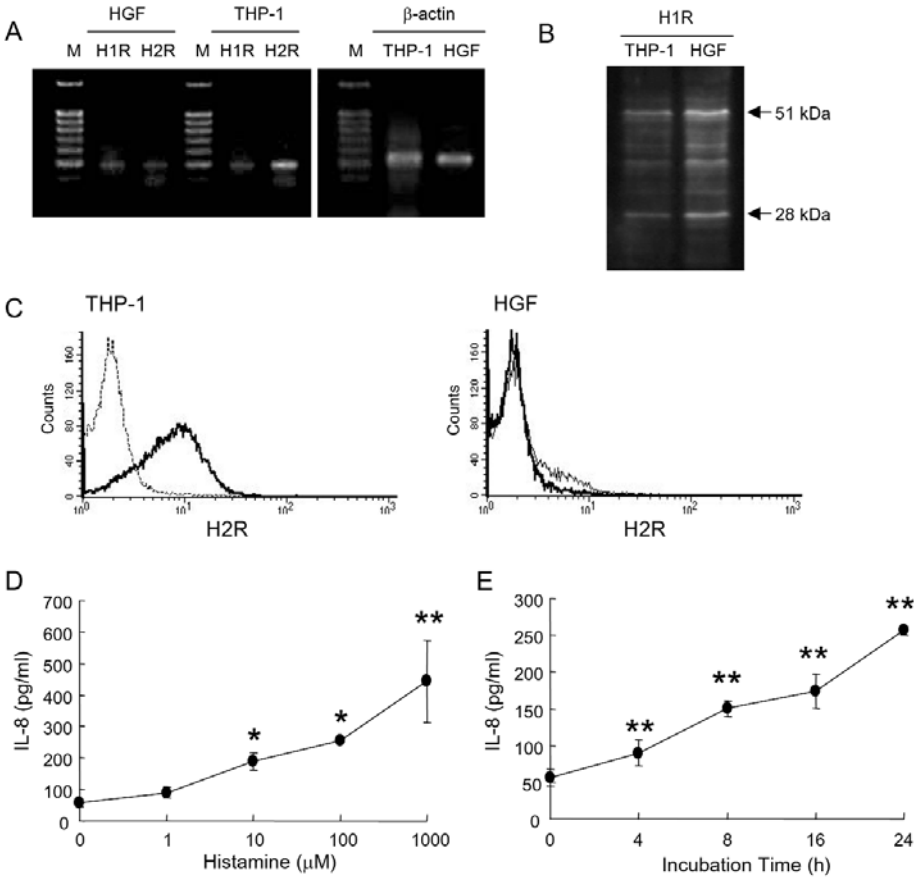

Figure 1. Expression of H1R in human gingival fibroblasts (HGF) and IL-8 secretion from the cells in response to histamine. **(A)** Total RNA was extracted from confluent gingival fibroblasts. THP-1 cells were used as a positive control. cDNA was prepared and analyzed for the mRNA expression of β-actin, H1R, and H2R by RT-PCR. M, molecular-weight marker. **(B)** Cell membrane fraction was separated from gingival fibroblasts and THP-1 cells and mixed with Laemmli sample buffer. Samples (equivalent to 10^6 cells each) were then subjected to Western blotting with rabbit anti-human H1R antibody. **(C)** Gingival fibroblasts and THP-1 cells were stained with 2 different rabbit anti-human H2R antibodies and analyzed by flow cytometry. Results in A, B, and C are representative of those from six donors with similar results. **(D,E)** Gingival fibroblasts were stimulated with the indicated concentrations of histamine for 24 hrs (D) or with 100 μmol/L of histamine for the time indicated (E). Supernatants were then collected, and the concentrations of IL-8 in the supernatants were determined by ELISA. The results are expressed as the mean ± SD for triplicate cultures. $*p < 0.05$, and $**p < 0.01$ compared with the unstimulated control (medium alone).

responses and inflammatory processes. To examine this hypothesis, we used TNF-α, IL-1α, and LPS as major inflammatory stimuli and measured the secretion of IL-8, one of the major inflammatory mediators, from gingival fibroblasts, in response to the inflammatory stimuli with or without histamine. We also used specific signaling inhibitors to elucidate the signaling pathway.

MATERIALS & METHODS

Reagents

Human recombinant IL-1α and TNF-α were supplied by Dainippon Pharmaceutical (Osaka, Japan). Dimaprit was obtained from Wako (Osaka, Japan). Signaling inhibitors were obtained from Calbiochem (San Diego, CA, USA). LPS from *Escherichia coli* O55:B5, 2-pyridylethylamine, and all other reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA), unless otherwise indicated.

Cells and Cell Culture

Human gingival fibroblasts were prepared from explants of normal gingival tissues of persons with adult periodontitis undergoing periodontal surgery after providing informed consent, as previously described (Sugawara *et al.*, 1998). Cells were used at passages 5 through 8. The Ethical Review Board of Tohoku University Graduate School of Dentistry (Sendai, Japan) approved the experimental procedures. Human monocytic THP-1 cells were obtained from the Cell Resource Center for Biomedical Research, Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan) and used as positive controls.

Reverse-transcriptase/Polymerase Chain-reaction (RT-PCR)

Total RNA was isolated from cells (2 x 10^6 cells) by means of a Total RNA Isolation kit (Isogen, Nippon Gene, Tokyo, Japan), according to the manufacturer's instructions. RT-PCR for H1R, H2R, and β-actin was performed as previously described (Gutzmer *et al.*, 2002; Butch *et al.*, 1993). Amplified samples were visualized after electrophoresis on 2% agarose gels and staining with ethidium bromide, and photographed under ultraviolet light.

Western Blotting and Flow Cytometry

Western blotting of cell membrane fractions and flow cytometric analysis with FACSCalibur and CELLQuest software (BD Biosciences, San Diego, CA, USA) were performed as described previously (Sugawara *et al.*, 1998), with the following antibodies: rabbit anti-human H1R (Chemicon International, Temecula, CA, USA), rabbit anti-human H2R (Alphadiagnostic International, San Antonio, TX, USA; GeneTex, San Antonio, TX, USA).

Detection of IL-8

Gingival fibroblasts (10^5 cells/500 μL) were seeded in alpha-minimum essential medium (α-MEM) with 10% fetal calf serum (FCS) in 24-well plates (BD Labware, Lincoln Park, NJ, USA). After incubation for one day, cells were washed with α-MEM 3 times, and test-stimulants were added in 500 μL of α-MEM with 1% FCS for the time indicated. For the inhibition experiments, gingival fibroblasts in 24-well plates were pre-incubated with inhibitors for 30 min to 1 hr at 37°C, and were then stimulated with test-stimulants at 37°C. After the incubation, the levels of IL-8 in the supernatants were determined with an OptEIA human IL-8 enzyme-linked immunosorbent assay (ELISA) kit (BD Biosciences, San Jose, CA, USA).

Statistical Analysis

We replicated all of the experiments in this study at least 3 times to confirm the reproducibility of the results. Experimental values are given as means ± standard deviation (SD) of triplicate assays. The statistical significance of differences between the 2 means was evaluated by a one-way analysis of variance by the Bonferroni or Dunnett method, and values of $p < 0.05$ were considered significant.

RESULTS

Expression of H1R in Gingival Fibroblasts and Secretion of IL-8 in Response to Histamine

We first examined whether human gingival fibroblasts express histamine receptors and are able to respond to histamine. Gingival fibroblasts constitutively expressed H1R and H2R mRNAs, as assessed by RT-PCR (Fig. 1A), and H1R protein, as assessed by Western blotting (Fig. 1B). However, expression of H2R protein was not clearly detected in gingival fibroblasts by Western blotting and flow cytometry (Fig. 1C and data not shown). THP-1 cells were used as a positive control. Stimulation of gingival fibroblasts with histamine at 10 μmol/L significantly induced IL-8 production, and the production was further increased at higher concentrations (Fig. 1D). A time kinetic study showed that the histamine-stimulated IL-8 production from gingival fibroblasts was time-dependent, and the production was highest at 24 hrs (Fig. 1E).

Histamine Synergistically Augments IL-8 Production from Gingival Fibroblasts Induced by TNF-α, IL-1, and LPS

Stimulation of gingival fibroblasts with an inflammatory cytokine TNF-α at 1 and 10 ng/mL for 6 hrs markedly induced IL-8 production from gingival fibroblasts, and the IL-8 levels were high compared with those induced by histamine alone at 100 μmol/L (Med→His *vs.* Med→TNF) (Fig. 2A). Furthermore, the TNF-induced IL-8 production was synergistically augmented in the presence of 100 μmol/L histamine (Med→His *vs.* Med→His + TNF), and the IL-8 levels induced by 1 ng/mL of TNF-α and 100 μmol/L histamine were comparable with those induced by 10 ng/mL of TNF-α alone. Pre-treatment of gingival fibroblasts with histamine for 2 hrs also amplified the IL-8 production by subsequent stimulation with TNF-α (His→TNF).

The synergism was observed with another inflammatory cytokine IL-1α and histamine (Fig. 2B). Stimulation of gingival fibroblasts with LPS for 6 hrs induced low levels of IL-8 (about 50-100 ng/mL), and histamine showed only an additive effect on LPS-induced IL-8 production (data not shown). However, 22-hour stimulation with LPS in the presence of histamine or following pre-treatment with histamine synergistically induced IL-8 production from gingival fibroblasts (Fig. 2C).

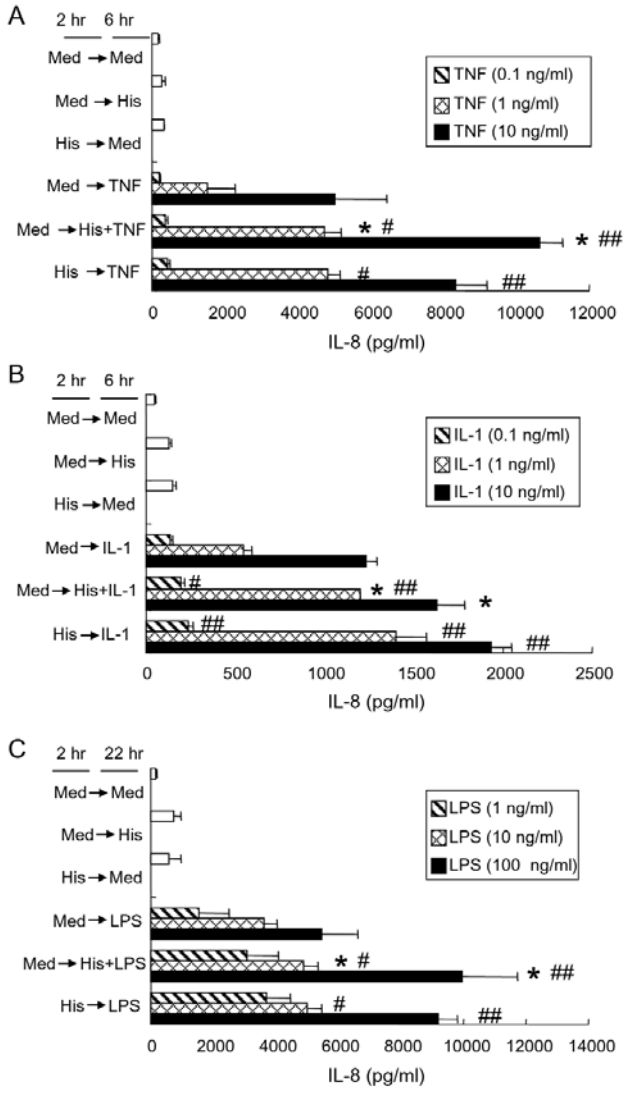

Figure 2. Synergistic effect of histamine for IL-8 production from gingival fibroblasts stimulated with TNF-α, IL-1α, and LPS. Gingival fibroblasts were incubated with 100 μmol/L of histamine (His) or medium (Med) for 2 hrs. The cells were then stimulated with TNF-α **(A)**, IL-1α **(B)**, or LPS **(C)** at the indicated concentrations in the presence or absence of histamine at 100 μmol/L for 6 hrs for TNF-α and IL-1α or 22 hrs for LPS. After the incubation, supernatants were collected, and the concentrations of IL-8 in the supernatants were determined by ELISA. The results are expressed as the mean ± SD for triplicate cultures. *$p < 0.01$ compared with Med→His. #$p < 0.05$ and ##$p < 0.01$ compared with Med→TNF-α, Med→IL-1α, or Med→LPS.

Involvement of Functional H1R in the Synergistic Effect

We next examined whether H1R is functionally involved in the histamine response and the synergistic effect. To examine this, we used H1R agonist 2-pyridylethylamine and H2R agonist dimaprit instead of histamine. The IL-8 production from gingival fibroblasts was significantly augmented in the presence of the H1R agonist at 0.1 and 1 mmol/L (Fig. 3A). In contrast, the H2R agonist at 0.1 mmol/L showed no effect on IL-8 production, and the IL-8 levels were further decreased at 1 mmol/L, due to the toxicity at this concentration, as assessed by lactate dehydrogenase activity. TNF-induced IL-8 production was also markedly augmented by the H1R agonist, which was higher than that of histamine at 100 μmol/L; whereas, the H2R agonist showed no effect (Fig. 3B). Similar results were obtained with IL-1α and LPS as stimulants (data not shown). These results indicate that histamine stimulates gingival fibroblasts through H1R, and that H1R is involved in the inflammatory stimuli-induced synergistic effect.

Involvement of MAPK, NF-κB, and PLC in the Synergistic Effect

Finally, the possible involvement of MAPK, NF-κB, and PLC

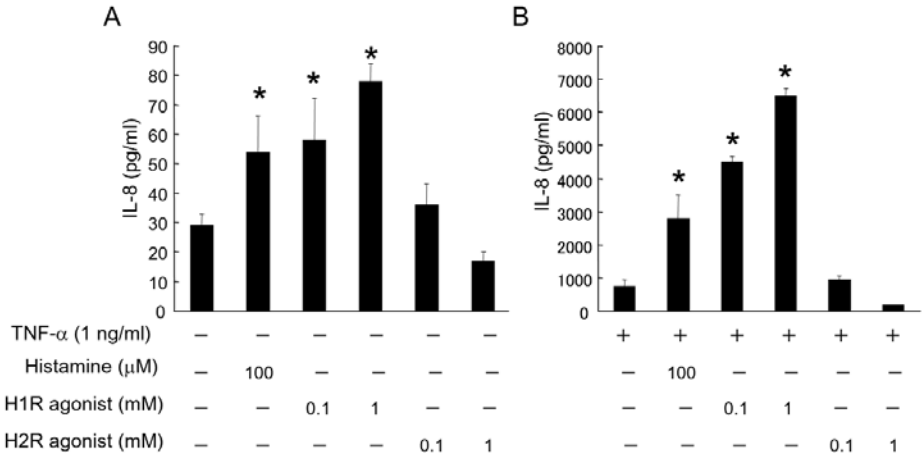

Figure 3. Involvement of functional H1R in the synergistic effects. Gingival fibroblasts were incubated in medium **(A)** or with TNF-α (1 ng/mL) **(B)** in the presence or absence of histamine, the H1R agonist 2-pyridylethylamine, or the H2R agonist dimaprit at the indicated concentrations for 8 hrs. After incubation, the supernatants were collected, and the concentrations of IL-8 in the supernatants were determined by ELISA. The results are expressed as the mean ± SD for triplicate cultures. *$p < 0.01$ compared with medium or TNF-α alone.

in the synergistic effect was examined. Histamine-induced IL-8 production was significantly inhibited by inhibitors of MEK (PD98059), JNK (SP600125), p38 MAPK (SB203580), and NF-κB (pyrrolidine dithiocarbamate, PDTC) (Fig. 4A). The TNF-induced IL-8 production was also significantly suppressed by each of the inhibitors, and was markedly inhibited by a combination of these inhibitors (Fig. 4B). No lactate dehydrogenase activity was detected in all of the supernatants for ELISA (data not shown), indicating that the cell membrane was not damaged by the treatment with inhibitors. The synergistic IL-8 production induced by histamine and TNF-α was also significantly inhibited by these inhibitors (Fig. 4B), indicating that the synergistic effect was mediated by the activation of MAPKs and NF-κB.

H1R is linked to PLC activation through $G\alpha_{q/11}$ (Dy and Schneider, 2004). To examine whether PLC is involved in the synergistic effect, we stimulated gingival fibroblasts with histamine and TNF-α in the presence of PLC inhibitor U73122 or the control compound U73343. The inhibition of PLC suppressed IL-8 production induced by histamine and TNF-α to the level of TNF-α alone, whereas the control compound did not suppress IL-8 production (Fig. 4C). Similar results were obtained with IL-1α and LPS as stimulants (data not shown). These results indicate that $G\alpha_{q/11}$-coupled PLC is involved in the synergism, and that TNF-induced IL-8 production is not mediated by the activation of PLC.

DISCUSSION

Mast cells are detected in healthy gingiva and gingivitis lesions (Gemmell *et al.*, 2004) and have been found in high numbers in chronically inflamed gingival tissue (Steinsvoll *et al.*, 2004), indicating that the source of histamine is gingival mast cells. In addition, histamine is synthesized by histidine decarboxylase and released without being stored from other cell types, such as monocytes/macrophages, neutrophils, and vascular endothelial cells (Kahlson and Rosengren, 1968; Schayer, 1974; Endo, 1982). Therefore, gingival histamine may also be derived from a non-mast-cell origin. The increase in salivary histamine levels is correlated with severity of periodontitis (Venza *et al.*, 2006). These observations suggest that histamine might also be released in the inflamed gingiva of persons with periodontitis. Periodontitis is caused by Gram-negative periodontopathic bacteria, and inflamed gingival epithelial cells, as well as infiltrated lymphocytes, appear to express several inflammatory cytokines, IL-1, IL-6, IL-8, and TNF-α (Lundqvist *et al.*, 1994). Therefore, the histamine synergism shown in this study likely occurs *in vivo*, and histamine in periodontal tissues potentially modulates the initiation and development of periodontitis.

The present study showed that histamine amplifies inflammatory stimuli from TNF-α, IL-1, and LPS, in gingival fibroblasts. The IL-8 levels induced by these inflammatory stimuli with histamine were comparable with those induced by a ten-fold concentration of the inflammatory stimuli alone, indicating that histamine augments the activity of the inflammatory stimuli at the site of inflammation approximately ten-fold as compared with that of the stimuli alone.

Gingival fibroblasts mainly expressed H1R. Gingival fibroblasts also expressed H2R mRNA, but the expression of H2R protein was not clearly detected in the cells (data not shown), indicating that translational and post-transcriptional mechanisms modify or degrade the H2R mRNA transcript. H1R are linked $G\alpha_{q/11}$ proteins (Dy and Schneider, 2004), and $G\alpha_{q/11}$ stimulate NF-κB (Bakker *et al.*, 2001) and MAPKs (Nagao *et al.*, 1998). Signaling through TNFR, IL-1R, and the LPS receptor also activates both cascades (Barton and Medzhitov, 2003; Wajant *et al.*, 2003; Subramaniam *et al.*, 2004), and this study confirmed this evidence. Furthermore, histamine activates MAPK and NF-κB signaling cascades *via* H1R by specific inhibitors and histamine receptor agonists. Synergistic IL-8 production was also significantly suppressed by the inhibition of NF-κB and MAPKs. It has been reported that peptidoglycan induces the activation of NF-κB *via* MAPKs in murine macrophages (Chen *et al.*, 2004). However, the inhibition of MAPKs in stimulated gingival fibroblasts did not result in the inhibition of NF-κB (data not shown). These observations indicate that the amplification of MAPKs and that of NF-κB are equally involved in synergism in gingival fibroblasts.

The principal mechanism of H1R activation is through $G\alpha_{q/11}$, resulting in the activation of PLC (Dy and Schneider, 2004). The inhibition of PLC suppressed the production of IL-8 induced by histamine and TNF-α to the levels of TNF-α alone. The results indicate that histamine activates PLC through H1R, and consequently amplifies the MAPK and NF-κB pathway signaling induced by the inflammatory stimuli.

This study showed that the secretion of IL-8, a major product from gingival fibroblasts, is mediated by the activation of MAPKs and NF-κB, which was consistent with the report from a previous study (Hoffmann *et al.*, 2002). IL-8 mainly

activates neutrophils, promoting their recruitment. IL-8 is also a chemoattractant for other cell types, such as basophils, T-cells, and natural killer cells, and enhances the permeability of endothelial cells (Baggiolini *et al.*, 1997). It has been reported that histamine causes limited inhibition of neutrophil chemotaxis (direct motility) while stimulating chemokinesis (random motility) (Seligmann *et al.*, 1983). Therefore, IL-8 from gingival fibroblasts and histamine in periodontal tissues may control neutrophil locomotion and play an important role in the control of inflammation at the periodontitis site. Since TNF-α, IL-1, and LPS stimulate histamine production through mast-cell-independent HDC induction (Wu *et al.*, 2004), it is also conceivable that histamine is induced by inflammatory cytokines and LPS, and, in turn, augments the biological functions of inflammatory cytokines and LPS.

In conclusion, the present study showed that gingival fibroblasts secrete IL-8 in response to histamine through H1R, and that histamine synergistically augments IL-8 secretion induced by TNF-α, IL-1α, and LPS by the amplification of the MAPK and NF-κB pathway through H1R-linked PLC. Recent studies also reported that an H2R antagonist, cimetidine, prevents periodontitis in a rat model (Hasturk *et al.*, 2006), and that topical cimetidine oral rinse enhances the antibacterial function of human crevicular neutrophils (Van Dyke *et al.*, 2005), although the effect of H1R antagonists on periodontitis was not reported. Therefore, control of histamine receptors at inflammatory sites might be beneficial in the regulation of periodontitis.

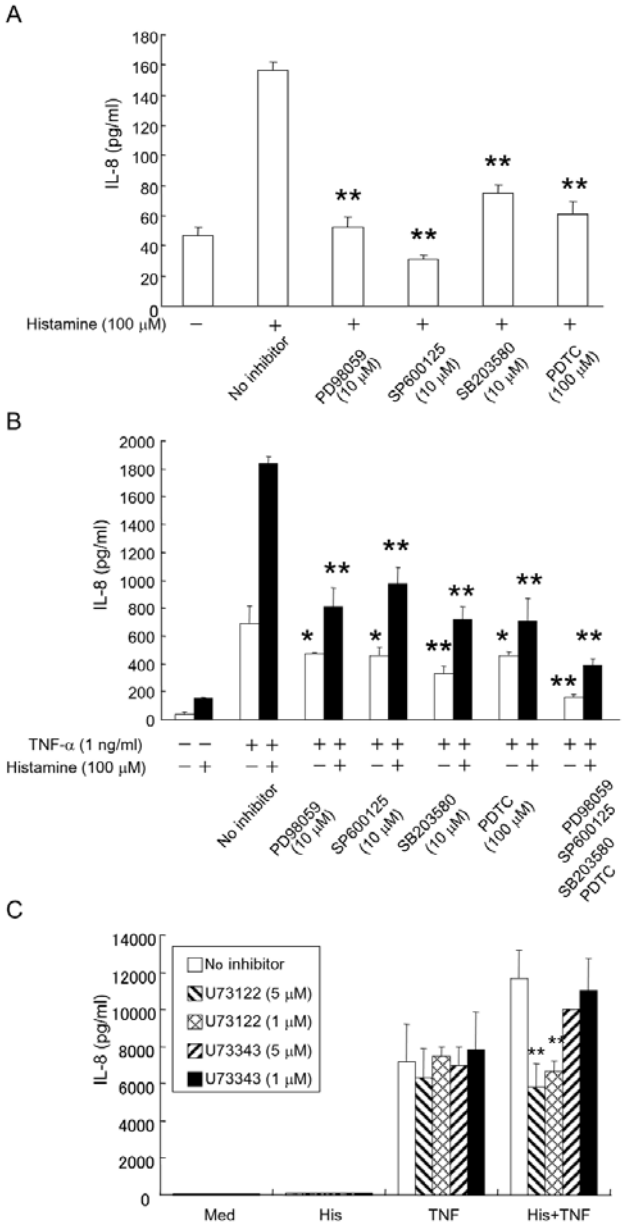

Figure 4. Involvement of MAPK, NF-κB, and PLC on the synergistic effects. **(A,B)** Gingival fibroblasts were pre-treated with or without PD98059, SP600125, SB203580, and/or PDTC at the dose indicated for 1 hr. The cells were then incubated with or without histamine (100 μmol/L) alone (A) or in the presence or absence of histamine (100 μmol/L) and TNF-α (1 ng/mL) (B) for 8 hrs. **(C)** Gingival fibroblasts were pre-treated with or without U73122 or U73343 at the dose indicated for 30 min. The cells were then incubated in the presence or absence of histamine (100 μmol/L) and TNF-α (10 ng/mL) for 8 hrs. After incubation, the supernatants were collected, and the concentrations of IL-8 in the supernatants were determined by ELISA. The results are expressed as the mean ± SD for triplicate cultures. $*p < 0.05$, and $**p < 0.01$ compared with no inhibitor.

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