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Induction of Prostaglandin E_2 by *Porphyromonas gingivalis* in Human Dental Pulp Cells

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Cyclooxygenase-2 (COX-2)-mediated prostaglandin E_2 (PGE_2) plays a key role in development and progression of inflammatory responses and *Porphyromonas gingivalis* is a common endodontic pathogen. In this study, we investigated induction of COX-2 and PGE_2 by *P. gingivalis* in human dental pulp cells (HDPCs). *P. gingivalis* increased expression of COX-2, but not that of COX-1. Increased levels of PGE_2 were released from *P. gingivalis*-infected HDPCs and this PGE_2 increase was blocked by celecoxib, a selective COX-2 inhibitor. *P. gingivalis* activated all three types of mitogen-activated protein kinases (MAPKs). *P. gingivalis*-induced activation of nuclear factor-κB (NF-κB) was demonstrated by the results of phosphorylation of NF-κB p65 and degradation of inhibitor of κB-α (IκB-α). Pharmacological inhibition of each of the three types of MAPKs and NF-κB substantially attenuated *P. gingivalis*-induced PGE_2 production. These results suggest that *P. gingivalis* should promote endodontic inflammation by stimulating dental pulp cells to produce PGE_2.

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Key words: Human dental pulp cells, prostaglandin E_2, cyclooxygenase-2, *Porphyromonas gingivalis*

Introduction

Endodontic infections refer to those that occur within the tooth pulp, root canal system or at the root apex. Normally the pulp and root canal system are sterile. However, bacteria may enter through cracks around restorations, areas of exposed dentin and possibly microfracture, or through trauma to the tooth. Endodontic infections have a polymicrobial nature, with obligate anaerobic bacteria dominating the microbiota in primary infections. The most prevalent cultivable bacteria from root canals are *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, *Pseudoramibacter alactolyticus*, *Parvimonas micra*, *Streptococcus mitis*, *S. intermedius*, and other streptococci [1-4]. It is noteworthy that the well-known periodontal pathogen, *P. gingivalis*, is one of the commonest bacteria detected in endodontic infections and it can participate in the pathogenesis of apical periodontitis [5].

The main cellular components of the pulp are peripherally located odontoblasts and stromal fibroblasts. There are also undifferentiated mesenchymal and immune cells. Cells in human dental pulp that express Toll-like receptors contribute to trigger immune responses to bacteria [6,7]. Increased expression of pro-inflammatory mediators are found in inflamed pulp, including cytokines, chemokines, adhesion molecules, and

prostaglandin E_2 (PGE_2) [8].

PGE_2 has been implicated in most of the inflammatory and destructive changes that occur in periapical lesions, such as vasodilation, increased vascular permeability, and bone resorption [9]. Cyclooxygenase (COX) is a key enzyme in prostaglandin biosynthesis. COX-1 and COX-2 catalyze the conversion of arachidonic acid to prostaglandins and related eicosanoids. COX-1 is constitutively expressed in most cells and plays a role in basal physiological functions in several cells and tissues. COX-2, on the other hand, is usually expressed at low or undetectable levels in most tissues and cells, but is significantly induced by inflammatory stimuli [10].

In spite of the importance of *P. gingivalis* and PGE_2 in endodontic pathogenesis, there have been no reports that determined PGE_2 production by *P. gingivlais* in pulp cells. Therefore, the aim of this study was to investigate the production of PGE_2 by *P. gingivalis* and the involved mechanisms in human dental pulp cells (HDPCs).

Materials and Methods

Reagents

PD98059, SB203580, SP600125, GF109203X, and U73122 were purchased from Calbiochem (San Diego, CA, USA). Wortmannin, genistein, and SC-514 were purchased from Sigma (St. Louis, MO,USA). Antibodies to phospho-extracellular signal-regulated kinase (ERK), phospho-p38, phospho-c-Jun N-terminal kinase (JNK) were from Cell Signaling Technology (Beverly, MA, USA). Antibodies to phospho-NF-κB p65 and inhibitor of κB-α (IκB-α) were also from Cell Signaling Technology. Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was from Sigma.

Bacterial culture

P. gingivalis 381 was grown in Trypticase soy broth supplemented with yeast extract (1 mg/ml), hemin (5 μg/ml), and menadione (1 μg/ml). The bacteria were incubated anaerobically (85% N_2, 10% H_2, and 5% CO_2) at 37°C.

HDPCs culture

HDPCs were kindly provided by professor Ji-Yeon Jung (Department of Oral Physiology, Chonnam National University Dental School). HDPCs were grown in minimum essential medium α (Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in 5% CO_2.

RT−PCR

HDPC cells were plated onto 3-cm dishes (5×10^5cells/dish). The next day, the cells were stimulated with *P. gingivalis* for various times. Total RNA was prepared with Trizol reagent (Invitrogen, Carlsbad, CA, USA) as specified by the manufacturer and was quantified spectctrophotometrically. First-strand cDNA was synthesized from 1 μg of RNA using random primers (Promega, Madison, WI, USA) and Molony murine leukemia virus reverse transcriptase (Promega). 2 μl of cDNA products were amplified in 25 μl volumes under a layer of mineral oil using a GeneAmp 2700 thermal cycler (Applied Biosystems, Foster City, CA, USA). Each PCR reaction mixture contained 50 mM KCl, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 0.2 μM each dNTP, 1 U *Taq* DNA polymerase, and 0.5 μM of each primer. Each cycle consisted of denaturation at 94℃ (30 s), annealing at 57℃ (30 s), and extension at 72℃ (60 s). The sequences of primers were 5′-TTCAAATGAGATTGTGGGAAAATTGCT-3′, 5′-AGTTCATCTCTGCCTGAGTATCTT-3′ for COX-2 (305 bp); 5′-GAGTCTTTCTCCAACGTGAGC-3′, 5′-ACCGGTAC TTGAGTTTCCCA-3′ for COX-1 (350 bp); and 5′-AGCGGGAAA TCGTGCGTG-3′, 5′-CAGGGTACATGGTGGTGCC-3′ for β -actin (300 bp). The PCR products of 10 μl were fractionated on 1.2% (w/v) agarose gels containing RedSafe (Intron Biotechnology, Korea), visualized by UV transillumination, and photographed.

ELISA

The HDPCs were seeded in 12-well plates (3×10^5 cells/well). The next day, the cells were stimulated with *P. gingivalis* for various times. Cell culture supernatants were sampled and centrifuged at 100 ×g for 10 min for clarification of debris. The levels of PGE_2 was quantified using commercial ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s directions.

Western blot

HDPC cells were plated onto 10-cm dishes (2×10^6 cells/dish). The next day, the cells were stimulated with *P. gingivalis* for various times. The cells were harvested and lysed with 300 μl of Cell Lysis Buffer (Cell Signaling Technology). 30-50 μg of each boiled sample was resolved by SDS-PAGE (10%) and transferred to a polyvinylidene difluoride membrane (Bio-Rad,

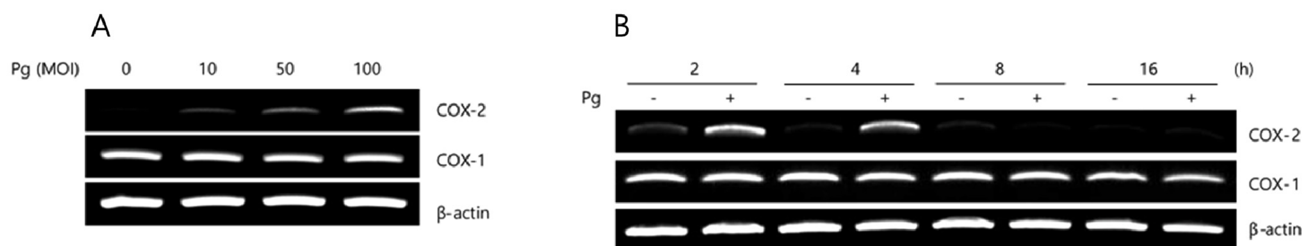

Fig. 1. COX-2 mRNA expression by HDPCs in response to *P. gingivalis*. (A) HDPCs were infected with increasing MOIs of *P. gingivalis* for 3 h. (B) HDPCs were infected with *P. gingivalis* (1:100) for various times. Total RNA was isolated and levels of COX-1 and COX-2 mRNA were determined by RT-PCR.

Hercules, CA, USA). The membrane was probed with rabbit polyclonal antibodies against phospho-ERK, phosphor-p38, phosphor-JNK, phosphor-p65, or IκBα (1:1000, Cell Signaling Technology) and a 1:1500 dilution or horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Cell Signaling Technology). Immunoreactive proteins were detected by enhanced chemiluminescence (LumiGLO, Cell Signaling Technology). The same membrane was stripped and reprobed with anti-GAPDH (1:2000)

Statistical analysis

Our experiments were conducted in three independent experiments to confirm the reproducibility of the results. The data are presented as means with standard deviations (SD). Statistical analysis of one-way analysis of variance (ANOVA) with Tukey-Kramer multiple comparisons test was performed using GraphPad InStat (GraphPad Software, La Jolla, CA, USA). A p-value < 0.05 was considered statistically significant.

Results

Induction of COX−2 mRNA expression

First, we determined whether *P. gingivalis* could induce COX-2 and COX-1 mRNAs in HDPCs. HDPCs were infected with increasing MOIs of *P. gingivalis* for 3 h. Total RNA was isolated and levels of COX-1 and COX-2 mRNA were determined by RT-PCR. Expression of COX-2 mRNA was induced by *P. gingivalis* in a multiplicity of infection (MOI)-dependent manner. A relatively low MOI of 1:10 could induce COX-2 mRNA. In contrast, COX-1 mRNA was constitutively expressed and not altered by *P. gingivalis* infection (Fig. 1 A). In order to determine the time course of COX-2 mRNA expression, HDPCs were infected with *P. gingivalis* (MOI=1:100) for various time periods from 2 h to 16 h. Strong COX-2 expression was demonstrated at 2-4 h postinfection but the COX-2 message did not appear at 8-h and 16-h time points (Fig. 1 B).

Production of PGE_2

To determine whether the induction of COX-2 mRNA leads to increased production of PGE_2, PGE_2 concentrations of the culture supernatants were measured by ELISA. *P. gingivalis* stimulated HDPCs to produce PGE_2. Three to four-fold increase of PGE_2 production was demonstrated in *P. gingivalis*-infected cells at both time points of 12 h and 24 h. Moreover, this PGE_2 increase was completely blocked by celecoxib, a selective COX-2 inhibitor (Fig.2).

Activation of MAPKs and NF−κB pathways

As activation of mitogen-activated kinases (MAPKs)and NF-κB plays important roles in the induction of COX-2, we determined whether MAPKs and NF-κB pathways are activated by *P. gingivalis* in HDPCs. Western blot analysis demonstrated that *P. gingivalis* induced phosphorylation of all three types

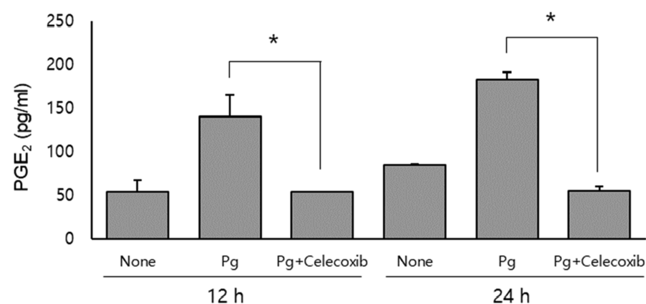

Fig. 2. PGE_2 production by HDPCs in response to *P. gingivalis*. HDPCs were pretreated with celecoxib (10 μM) for 1 h and then infected with *P. gingivalis* (1:100) for 12 or 24 h. PGE_2 concentrations of the culture supernatants were measured by ELISA. Data are the means±S.D. of a representative experiment performed in triplicate. The asterisks indicate significant differences .

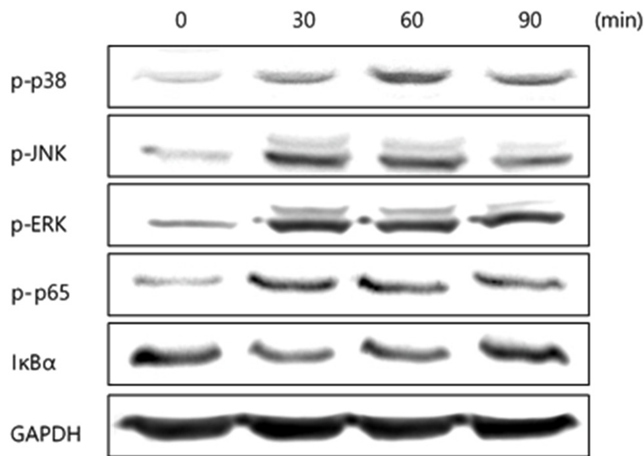

Fig. 3. Activation of MAPKs and NF-κB in *P. gingivalis*-infected HDPCs. HDPCs were infected with *P. gingivalis* (1:100) for the indicated time periods. Cell lysates were prepared and Western blot analysis was performed for phospho-p38, phospho-ERK, phospho-JNK, phospho-p65, or IκB-α.

of MAPKs (ERK, p38, and JNK) through the time course of 30-90 min. Phosphorylation of p65 NF-κB and degradation of IκB-α were also demonstrated (Fig. 3).

Effects of various signaling inhibitors

In order to evaluate the relative importance of various signaling pathways in *P. gingivalis*-induced PGE_2 production in HDPCs, specific pharmacological inhibitors were used. HDPCs were pretreated with GF109203X (proteinkinase C, 1 μM), wortmannin (phosphatidylinositol 3-kinase, 100 nM), U73122 (phospholipase C, 10 μM), genistein (protein tyrosine kinase, 50 μM), PD98059 (ERK, 50 μM), SB203580 (p38 MAPK, 10 μM), SP600125 (JNK, 10 μM), or SC-514 (NF-κB, 30 μM) for 1 h, and then the cells were incubated with *P. gingivalis* for 12 h. PGE_2 concentrations of the culture supernatants were measured by ELISA. Among the inhibitors, SB203580, PD98059, SP600125, genistein, and SC-514 blocked PGE_2 production stimulated by *P. gingivalis*. In contrast, PGE_2 production was significantly elevated in the presence of U73122 and wortmannin.

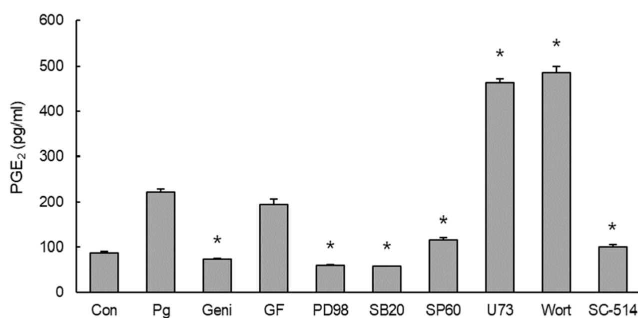

Fig. 4. Effect of various signaling inhibitors on *P. gingivalis*-stimulated PGE_2 in HDPCs. HDPCs were pretreated for 1 h with GF109203X (1 μM), wortmannin (100 nM), U73122 (10 μM), genistein (50 μM), PD98059 (50 μM), SB203580 (10 μM), SP600125 (10 μM), or SC-514 (30 μM) and then infected with *P. gingivalis* (1:100) for 12 h. PGE_2 concentrations of the culture supernatants were measured by ELISA. Data are the means±S.D. of a representative experiment performed in triplicate. The asterisks indicate significant differences compared to *P. gingivalis* stimulation without inhibitors.

Discussion

The present study demonstrated that *P. gingivalis* stimulates HDPCs to express COX-2 and to produce PGE_2 without affecting COX-1 expression. The induction of PGE_2 production was COX-2-mediated, as it was completely blocked by celecoxib, a selective COX-2 inhibitor. The use of selective COX-2 inhibitors has resulted in pain relief after endodontic treatment [11].

This study demonstrated the importance of MAPKs and NF-κB in *P. gingivalis*-stimulated PGE_2 production by HDPCs. Increased phosphorylation of ERK, p38, and JNK was observed in *P. gingivalis*-infected HDPCs and pharmacological inhibition of MAPKs blocked the PGE_2 production. COX-2 is the primary COX controlling PGE_2 synthesis in response to inflammatory stimuli. Transcription of COX-2 gene requires binding of transcription factors, including NF-κB, C/EBP, and CREB, to the promoter region of COX-2 gene [12]. As MAPKs regulate these transcription factors, activated MAPKs may positively regulate the activity of the transcription factors in *P. gingivalis*-stimulated HDPCs [13]. The present study showed involvement of NF-κB activation in *P. gingivalis*-induced PGE_2 production. The NF-κB activation was demonstrated by the results of p65 phosphorylation and IκB-α degradation [14]. Activation of NF-κB mainly occurs via phosphorylation and subsequent degradation of IκB-α. Optimal induction of NF-κB target genes also requires phosphorylation of NF-κB proteins, such as p65 [15].

The present study showed that genistein strongly inhibited the PGE_2 induction, implying involvement of protein tyrosine kinases. A previous report showed that genistein treatment exerted a significant inhibitory effect on NF-κB activation, leading to downregulation of COX-2 in gastric cancer cells [16].

Therefore, the inhibitory action of genistein in our study may also be mediated by inhibition of NF-κB activation. Interestingly, PGE_2 production by *P. gingivalis* was significantly increased by inhibition of phospholipase C or phosphatidylinositol 3-kinase. Further studies are needed to define the role of these signaling pathways in PGE_2 production by HDPCs.

The present study showed for the first time that *P. gingivalis* stimulated HDPCs to express COX-2, leading to induced production of PGE_2. These results suggest that *P. gingivalis* should promote endodontic inflammation, at least in part, by stimulating dental pulp cells to produce PGE_2.

Acknowledgments

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Conflict of interest

The authors declare no conflict of interest.

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