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Original Article

Fusobacterium nucleatum stimulates dental pulp cells to produce prostaglandin E_2 via mitogen-activated protein kinases activation

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Fusobacterium nucleatum is an important endodontic pathogen, and prostaglandin E_2 (PGE_2) has important roles in the development and progression of inflammatory responses. In this study, we investigated the effects of *F. nucleatum* on PGE_2 production in human dental pulp cells (HDPCs). Cells were stimulated with *F. nucleatum* and PGE_2 protein and cyclooxygenase (COX)-1/2 mRNA levels determined by enzyme-linked immunosorbent assay and reverse transcription-polymerase chain reaction, respectively. The activation of mitogen-activated protein (MAP) kinases was examined by Western blotting. *F. nucleatum* strains increased PGE_2 production by HDPCs in a dose-dependent manner. Moreover, strain 10953 made a stronger response than strain 49256. *F. nucleatum* dose-dependently increased COX-2 mRNA levels without affecting COX-1 expression. Furthermore, celecoxib, a selective COX-2 inhibitor, blocked PGE_2 production by *F. nucleatum*. Finally, *F. nucleatum* activated all three MAP kinases. The pharmacological inhibition of each MAP kinase significantly attenuated *F. nucleatum*-induced PGE_2 production. These results suggested *F. nucleatum* induced COX-2-mediated PGE_2 production by HDPCs by activating MAP kinases, thereby promoting endodontic inflammation.

Key Words: Cyclooxygenase-2; Dental pulp; Fusobacterium nucleatum; Prostaglandin E_2

Introduction

Endodontic infections are infections of the tooth pulp and root canal system. Under normal healthy conditions, intact enamel and dentin protect the pulp, acting as a physical barrier to injury and microbial intrusion and the enclosed vital dental pulp is sterile. However, bacteria may enter through cracks around restorations, areas of exposed dentin and possibly microfracture, or through trauma to the tooth [1]. Endodontic infections are polymicrobial and are made up of predominantly anaerobic bacteria and some facultative bacteria. A tooth with an infected nonvital pulp is a reservoir of infection that is isolated from the patient's immune response and will eventually produce a periradicular inflammatory response. The most prevalent cultivable bacteria from root canals are *Fusobacterium nucleatum*, *Porphyromonas gingivalis*, *Pseudoramibacter alactolyticus*, *Parvimonas micra*, *Streptococcus mitis*, *S. intermedius*, and other streptococci [2,3].

F. nucleatum, a Gram-negative anaerobe, is one of the most abundant species in the oral cavity. While it is a well-known periodontal pathogen, it is also frequently associ-

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ated with endodontic infections such as pulp necrosis and apical periodontitis [4]. *F. nucleatum* also mediates important biofilm-organizing behavior and interactions with host cells through the expression of numerous adhesins [5].

The dental pulp is a soft connective tissue which has a support role for the dentin. The main cellular components of the pulp are peripherally located odontoblasts and stromal fibroblasts. The dental pulp cells are known to synthesize various mediators of inflammation in response to bacterial components [6,7]. In fact, increased expression of pro-inflammatory mediators is found in inflamed pulp, including cytokines, chemokines, adhesion molecules, and prostaglandins (PGs) [8].

The presence and elevated levels of PGE_2 in inflamed pulp and periradicular tissues have been reported [9,10]. 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 [11]. Human dental pulp from painful teeth had higher levels of PGE_2 than that from asymptomatic teeth [12]. Cyclooxygenase (COX) is an enzyme responsible for converting arachidonic acid to PGs. Two types of COX have been elucidated. 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 an inducible enzyme, which is responsible for the production of PGs involved in inflammation [13,14].

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

Materials and Methods

Reagents

PD98059, SB203580, SP600125, and celecoxib were purchased from Merck (Burlington, MA, USA). Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody was also from Merck. 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).

Bacterial culture

F. nucleatum ATCC 10953 and *F. nucleatum* ATCC 49256 were grown in Trypticase soy broth supplemented with yeast extract (5 mg/mL), hemin (10 μg/mL), cysteine (0.5 mg/mL) and menadione (5 μg/mL). The bacteria were incubated anaerobically (85% N_2, 10% H_2, and 5% CO_2) at 37°C. The number of bacteria for experiments was measured spectrophotometrically. The optical density of the bacterial suspension was measured at 600 nm, and the concentrations of bacteria were calculated using the preset standard curve.

HDPCs culture

HDPCs were kindly provided by Professor Ji-Yeon Jung (Department of Oral Physiology, Chonnam National University Dental School, Gwangju, Korea). 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 mg/mL streptomycin at 37°C in 5% CO_2.

RT-PCR

HDPCs were plated onto 3-cm dishes (5×10^5 cells/dish). The next day, the cells were stimulated with *F. nucleatum* for various times. Total RNA was prepared with Trizol reagent (Invitrogen, Carlsbad, CA, USA) as specified by the manufacturer and was quantified spectrophotometrically. 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). Two 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 mM each dNTP, 1 U *Taq* DNA polymerase, and 0.5 μM of each primer. Each cycle consisted of denaturation at 94℃ (30 seconds), annealing at 57℃ (30

seconds), and extension at 72℃ (60 seconds). The sequences of primers were 5′-TTCAAATGAGATTGTGGGAAAATTGCT-3′, 5′-AGTTCATCTCTGCCTGAGTATCTT-3 for COX-2 (305 bp); 5′-GAGTCTTTCTCCAACGTGAGC-3′, 5′-ACCGGTACTTGAGTTTCCCA-3′ for COX-1 (350 bp); and 5′-AGCGGGAAATCGTGCGTG-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, Seongnam, 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 *F. nucleatum* for various times. Cell culture supernatants were sampled and centrifuged at 100 ×g for 10 minutes 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

HDPCs were plated onto 10-cm dishes (2×10^6 cells/dish). The next day, the cells were stimulated with *F. nucleatum* for various times. The cells were harvested and lysed with 300 μL of Cell Lysis Buffer (Cell Signaling Technology). Thirty μg of each boiled sample was resolved by SDS-PAGE (10%) and transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA, USA). The membrane was probed with rabbit polyclonal antibodies against phospho-ERK, phosphor-p38, or phosphor-JNK (1:1,000; Cell Signaling Technology) and a 1:1,500 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 (1:2,000).

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. Statistical analysis of one-way analysis of variance with Tukey-Kramer multiple comparisons test was performed using GraphPad InStat (GraphPad Software, La Jolla, CA, USA). Differences were considered significant at the level $p<0.05$.

Results

Two strains of *F. nucleatum* induced production of PGE_2 with different potency

We firstly determined whether *F. nucleatum* stimulates HDPCs to produce PGE_2. Two different strains of *F. nucleatum* (ATCC 10953 and ATCC 49256) were added to HDPCs with varying multiplicity of infections (MOIs) and PGE_2 concentrations of the culture supernatants were measured by ELISA. Both strains robustly stimulated HDPCs to produce PGE_2 ($p<0.05$). However, there was strain difference in that 10953 was much stronger than 49256 in the ability to induce the production of PGE_2. An MOI of 1:10 of 10953 was comparable to an MOI of 1:100 of 49256 in stimulating HDPCs to produce PGE_2 (Fig. 1). We used strain 10953 for later experiments.

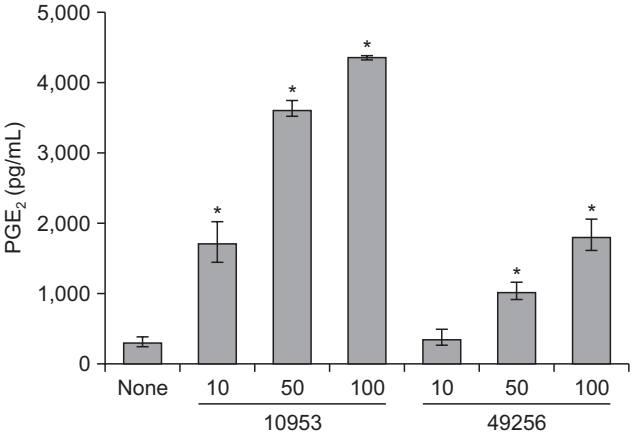

Fig. 1. Production of prostaglandin E_2 (PGE_2) in *Fusobacterim nucleatum*-infected human dental pulp cells (HDPCs). HDPCs were infected with increasing MOIs of *F. nucleatum* 10953 or 49256 for 18 h. PGE_2 concentrations of the culture supernatants were measured by ELISA. Data are the means±standard deviations of a representative experiment performed in triplicate. MOIs, multiplicity of infections. The asterisks indicate significant differences ($p<0.05$).

F. nucleatum induced mRNA expression of COX-2 and an inhibitor of COX-2 blocked the production of PGE_2

Next, we determined whether *F. nucleatum* induces COX-2 and COX-1 mRNAs in HDPCs. 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 *F. nucleatum* in an 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 *F. nucleatum* infection (Fig. 2A). We then examined the effect of celecoxib, a selective COX-2 inhibitor, on the production of PGE_2 by *F. nucleatum*. HDPCs were pretreated

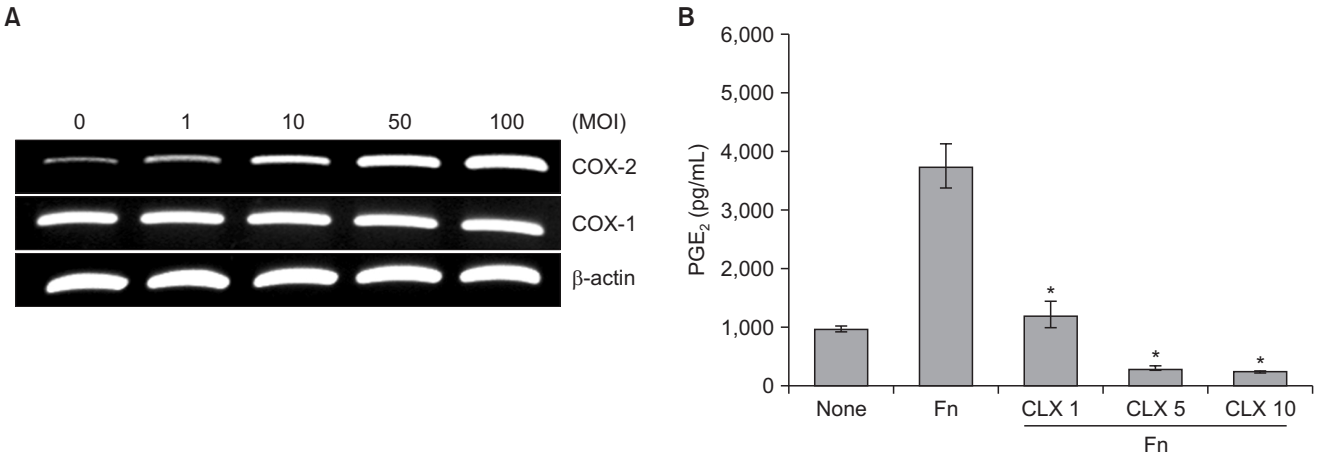

Fig. 2. (A) Expression of cyclooxygenase (COX)-2 mRNA in *Fusobacterim nucleatum*-infected human dental pulp cells (HDPCs). HDPCs were infected with increasing MOIs of *F. nucleatum* 10953 for 3 h. Total RNA was isolated and levels of COX-1 and COX-2 mRNA were determined by RT-PCR. (B) Blockade of prostaglandin E_2 (PGE_2) production by celecoxib. HDPCs were pretreated with celecoxib (CLX, 1–10 μM) for 1 h and then infected with *F. nucleatum* 10953 (1:50) for 18 h. PGE_2 concentrations of the culture supernatants were measured by ELISA. Data are the means±standard deviations of a representative experiment performed in triplicate. MOIs, multiplicity of infections; Fn, *F. nucleatum*. The asterisks indicate significant differences (p<0.05) compared to *F. nucleatum* stimulation without celecoxib.

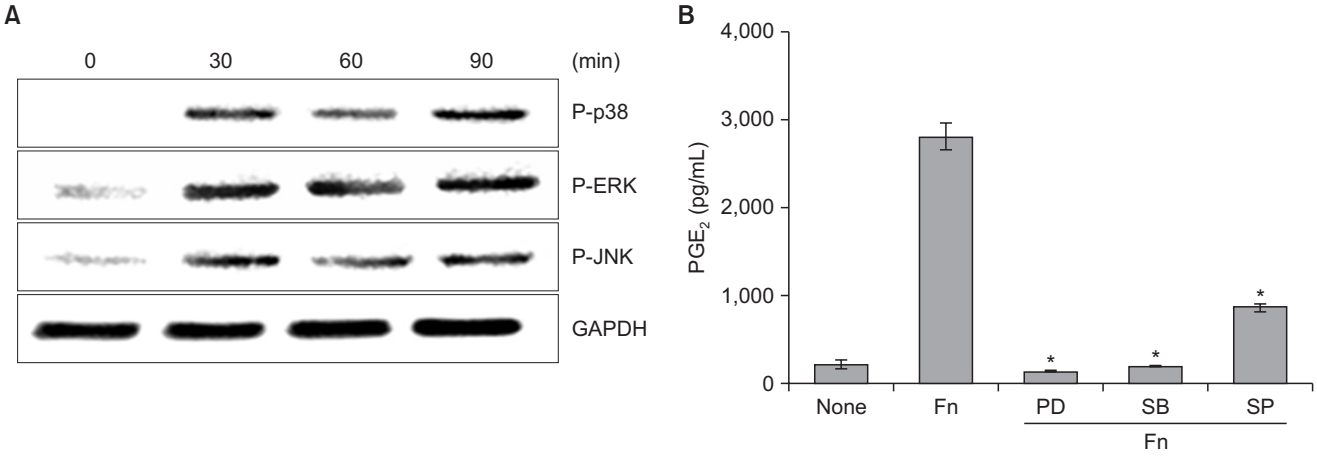

Fig. 3. (A) Activation of mitogen-activated protein (MAP) kinases in *Fusobacterim nucleatum*-infected human dental pulp cells (HDPCs). HDPCs were infected with *F. nucleatum* 10953 (1:50) for the indicated time periods. Cell lysates were prepared and Western blot analysis was performed for phospho-p38, phospho-extracellular signal-regulated kinase (ERK), and phospho-c-Jun N-terminal kinase (JNK). (B) Effect of selective inhibitors of MAP kinases on *F. nucleatum*-stimulated prostaglandin E_2 (PGE_2) production in HDPCs. HDPCs were pretreated for 1 h with PD98059 (PD, 50 μM), SB203580 (SB, 10 μM), or SP600125 (SP, 10 μM) and then infected with *F. nucleatum* 10953 (1:50) for 18 h. PGE_2 concentrations of the culture supernatants were measured by ELISA. Data are the means±standard deviations of a representative experiment performed in triplicate. Fn, *F. nucleatum*. The asterisks indicate significant differences (p<0.05) compared to *F. nucleatum* stimulation without inhibitors.

with increasing doses of celecoxib and stimulated with *F. nucleatum*. As shown in Fig. 2B, the PGE_2 production by *F. nucleatum* was completely blocked by celecoxib ($p<0.05$).

F. nucleatum activates all the three members of mitogen-activated protein (MAP) kinases and pharmacological inhibition of MAP kinases suppresses the production of PGE_2

As activation of MAP kinases plays important roles in the induction of various inflammatory responses, we determined whether the pathways of MAP kinases are activated by *F. nucleatum* in HDPCs. Western blot analysis demonstrated induced phosphorylation of all three types of MAP kinases (ERK, p38, and JNK) through the time course of 30-90 minutes (Fig. 3A). To evaluate the importance of the activation of MAP kinases in *F. nucleatum*-induced production of PGE_2, specific pharmacological inhibitors were used. HDPCs were pretreated with working concentrations of PD98059 (ERK inhibitor), SB203580 (p38 inhibitor), or SP600125 (JNK inhibitor), and *F. nucleatum*-stimulated PGE_2 production were measured by ELISA. Each inhibitor of three members of MAP kinases greatly attenuated the production of PGE_2 stimulated by *F. nucleatum* ($p<0.05$, Fig. 3B).

Discussion

Our study demonstrated that *F. nucleatum* stimulates HDPCs to produce PGE_2. Although two different strains of *F. nucleatum*, ATCC 10953 and ATCC 49256, firmly increased the production of PGE_2 by HDPCs, strain 10953 achieved a much more increased production of PGE_2 than strain 49256. Several subspecies of *F. nucleatum* have been recognized including ssp. *nucleatum*, ssp. *polymorphum*, and ssp. *vincentii* [15]. Strain 10953 belongs to *F. nucleatum* ssp. *polymorphum* and 49256 belongs to *F. nucleatum* ssp. *vincentii* [16]. We do not know at present the reason behind the strain difference of the potency in stimulating HDPCs. Strain-specific impact of *F. nucleatum* on neutrophil function was reported [17]. Moreover, *F. nucleatum* was reported to invade oral fibroblasts in a strain-specific manner, in which 10953 resulted in a greater invasion than 49256 [16].

COX-2 is the key enzyme regulating the production of PGs. Our study showed that *F. nucleatum* induced COX-2 expression without affecting COX-1 expression in HDPCs. A relatively low MOI of 1:10 could robustly increase the expression of COX-2 mRNA. This is in accordance with the production of PGE_2, which was also firmly increased by an MOI of 1:10. To formally demonstrate that the *F. nucleatum*-increased production of PGE_2 is mediated via COX-2, we examined the effect of celecoxib, a selective COX-2 inhibitor, on the PGE_2 production by *F. nucleatum*. As the PGE_2 production was blocked by celecoxib, we conclude that *F. nucleatum* stimulates HDPCs to induce COX-2 enzyme that leads to increased release of PGE_2. The increased production of PGE_2 within pulpal tissues should play a pathogenic role during pulpal disease progression.

Expression of COX-2 is induced by various extracellular signals and all signals converge to the activation of MAP kinases that regulate COX-2 mRNA levels [18]. Transcription of COX-2 gene is regulated by several transcription factors including NF-κB, C/EBP, and CREB and MAP kinases regulate these transcription factors [19]. Our study demonstrated the importance of MAP kinases in *F. nucleatum*-stimulated PGE_2 production by HDPCs. Increased phosphorylation of ERK, p38, and JNK each was evident following *F. nucleatum* infection through the time course of 30-90 minutes. *F. nucleatum* has been reported to activate all three types of MAP kinases in gingival fibroblasts and macrophages [20,21]. These results suggest that *F. nucleatum* stimulation is strong enough to activate all three members of MAP kinases in various cell types. Moreover, pharmacological inhibition of MAP kinases greatly inhibited the PGE_2 production by *F. nucleatum*. Taken together, it is thought that *F. nucleatum* robustly activates MAP kinases that positively regulates the transcription factors responsible for COX-2 gene expression, leading to PGE_2 production.

The present study showed for the first time that *F. nucleatum* stimulated HDPCs to induce COX-2, leading to production of PGE_2. Our study suggests that *F. nucleatum* should promote endodontic inflammation after the penetration into the pulp, at least in part, by stimulating the production of PGE_2.

Conflicts of Interest

The authors declare that they have no competing interests.

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