Prostaglandin F<sub>2α</sub>-Induced Interleukin-8 Production in Human Dental Pulp Cells Is Associated With MEK/ERK Signaling

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Abstract
Prostaglandin F<sub>2α</sub> (PGF<sub>2α</sub>) and interleukin-1beta (IL-1β) levels are elevated in inflamed dental pulp. The roles of IL-1β and PGF<sub>2α</sub> in the pathogenesis of pulpal inflammation await investigation. We found that IL-1β stimulated PGF<sub>2α</sub> production of human dental pulp cells. IL-1β and PGF<sub>2α</sub> (0.5–10 μmol/L) also induced IL-8 production and mRNA expression in pulp cells. Aspirin inhibited IL-1β-induced PGF<sub>2α</sub>, but not IL-8 production. PGF<sub>2α</sub>-induced IL-8 production and mRNA expression were inhibited by U0126 (an inhibitor of mitogen-activated protein kinase kinase [MEK1/2]) inhibitor), whereas SQ22536 (an adenylate cyclase inhibitor) enhanced this event. These results indicate that IL-1β–induced IL-8 production in pulp cells is not mainly via direct activation of cyclooxygenase and PGF<sub>2α</sub> generation. PGF<sub>2α</sub>-induced IL-8 production is possibly via activation of MEK/extracellular signal-regulated kinase signaling, but not by activation of adenylate cyclase. IL-1β and PGF<sub>2α</sub> might involve the pathogenesis of pulpal inflammation via induction of IL-8 production. (J Endod 2009;35:508–512)

Key Words
Dental pulp, interleukin-8, mitogen-activated protein kinase, PGF<sub>2α</sub>, signaling

Dental pulp is a loose connective tissue characterized by its particular location and morphology and is completely enclosed by mineralized dentin (1). It consists of an odontoblast layer adjacent to the dentin and an immunocompetent tissue containing pulp cells, extracellular matrix, nerves, and vascular elements (2). When exposed to mechanical injury or direct invasion by microorganisms and their metabolic products, pulpal inflammation and even necrosis are often noted (3, 4). A number of chemical factors such as cytokines, chemokines, substance P, and prostaglandins (PGs) are suggested to mediate pulpal inflammation (5–7). Interleukins and PGs have been implicated in many aspects of the inflammatory processes including vasodilation, increased vascular permeability, chemotaxis of inflammatory cells, and cartilage destruction (8). The increased release of PGs plays a pathogenic role during progression of pulpal diseases. Cyclooxygenase-2 (COX-2) is an inducible and rate-limiting enzyme believed to be responsible for PG synthesis from arachidonic acid at the site of inflammation (9).

Prostaglandin F<sub>2α</sub> (PGF<sub>2α</sub>) is one of the PG family members that has diverse functions such as mitogenic to fibroblasts and osteoblasts as well as the induction of bone resorption (10, 11). PGF<sub>2α</sub> might also affect the pathobiologic processes of tissue inflammation and cardiovascular and rheumatic diseases (12). Recently, it has become clear that this type of prostanoid might stimulate mitogenic signals and regulate gene transcription. PGF<sub>2α</sub> binds to a G-protein–coupled receptor (FP) that consists of 2 known isoforms, FP<sub>a</sub> and FP<sub>b</sub>. These isoforms are generated by alternative mRNA splicing and differ in their carboxy-terminal domains (13, 14).

It has been reported that cultured rat pulp cells generate mainly PGs and thromboxane B<sub>2</sub> (TXB<sub>2</sub>) (5). The amounts of interleukin-8 (IL-8) and PGF<sub>2α</sub> are significantly elevated during inflammation of human and rat dental pulp (15–17), suggesting a positive relationship between PGF<sub>2α</sub> levels and dental pulp inflammation. However, the roles and effects of PGF<sub>2α</sub> in human dental pulp are not fully clear. The aim of this research was to evaluate whether IL-1β and PGF<sub>2α</sub> might stimulate IL-8 production in dental pulp cells and their signal transduction pathways.

Materials and Methods
We obtained 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), and IL-1β from Sigma Chemical Company (St Louis, MO). Dulbecco modified Eagle medium (DMEM), fetal bovine serum (FBS), and penicillin/streptomycin from Gibco (Life Technologies, Grand Island, NY). PGF<sub>2α</sub> enzyme-linked immunosorbent assay (ELISA) kits and PGF<sub>2α</sub> were from Cayman Chemical Company (Ann Arbor, MI). IL-8 ELISA kits were from Biosource (Biosource International, Inc, Camarillo, CA). Ethidium bromide, agarose, and kits for reverse transcription (RT) and polymerase chain reaction (PCR) were obtained from HT Inc (Cambridge, UK). Total RNA isolation kits were from Qiagen Inc (Santa Clarita, CA). Specific PCR primers for β-actin (BAC) and IL-8 were synthesized by Genemed Biotechnologies, Inc (San Francisco, CA).
Culture of Human Dental Pulp Cells

By the approval of Ethics Committee, National Taiwan University Hospital, primary human dental pulp cells were cultured in DMEM containing 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin as described previously (18–20). The dental pulp cells in passage numbers from 3–8 were used for these experiments. Generally, dental pulp cells were exposed to IL-1β for 24 hours; IL-8 level was determined (n = 5). (B) Pulp cells were exposed to IL-1β for 24 hours; PGF$_{2\alpha}$ level was determined (n = 7). (C) Pulp cells were incubated with PGF$_{2\alpha}$ for 3 days; IL-8 production was measured by ELISA (n = 5) (D) Effect of aspirin on IL-1β-induced PGF$_{2\alpha}$ production in pulp cells (n = 8). (E) Effect of aspirin on IL-1β–induced IL-8 production in pulp cells (n = 4). Results were expressed as mean ± standard error of the mean. *Significant differences when compared with control group. **Difference when compared with IL-1β–treated group (P < .05).

**Figure 1.** Stimulation of IL-8 and PGF$_{2\alpha}$ production by IL-1β in dental pulp cells and its modulation by aspirin. (A) Pulp cells were exposed to IL-1β for 24 hours; IL-8 level was determined (n = 5). (B) Pulp cells were exposed to IL-1β for 24 hours; PGF$_{2\alpha}$ level was determined (n = 7). (C) Pulp cells were incubated with PGF$_{2\alpha}$ for 3 days; IL-8 production was measured by ELISA (n = 5) (D) Effect of aspirin on IL-1β–induced PGF$_{2\alpha}$ production in pulp cells (n = 8). (E) Effect of aspirin on IL-1β–induced IL-8 production in pulp cells (n = 4). Results were expressed as mean ± standard error of the mean. *Significant differences when compared with control group. **Difference when compared with IL-1β–treated group (P < .05).
pulp cells are spindle-shaped in appearance with extended cellular processes and express alkaline phosphatase activity (data not shown).

**Stimulation of IL-8 Production by IL-1β and PGF$_{2α}$**

Briefly, $1 \times 10^5$ pulp cells were seeded in 24-well culture plates in DMEM with 10% FBS. After 24 hours, the medium was decanted and changed with fresh medium containing various concentrations of IL-1β and PGF$_{2α}$. Culture medium was collected at indicated time point, and then viable cell number was estimated by MTT assay as described previously (21). IL-8 and PGF$_{2α}$ levels in the culture medium were determined by ELISA (21, 22). In some experiments, various inhibitors (aspirin, U0126, or SQ22536) were added 30 minutes before the addition of IL-1β or PGF$_{2α}$ and then coincubated for indicated time points. Culture medium was collected for analysis.

**Stimulation of IL-8 mRNA Expression by IL-1β and PGF$_{2α}$**

Briefly, $3 \times 10^6$ pulp cells were inoculated into 10-cm culture dishes in DMEM with 10% FBS. After 24 hours, medium was decanted and changed with fresh medium with various concentrations of IL-1β and PGF$_{2α}$. After 24 hours of exposure, total RNA was isolated by using RNA isolation kits. The RNA was used for RT-PCR as described previously (19, 21). About 3 µg of denatured RNA was reverse transcribed in a total reaction mixture of 45 µL containing 4 µL of random primer (500 µg/mL), 8 µL of deoxyribonucleoside triphosphate (dNTP) (2.5 mmol/L), 4.5 µL of 10 RT buffer, 1 µL of RNase inhibitor (40 U/µL), and 0.5 µL of RT (21 µL/µL) and double distilled water at 42°C for 90 minutes in a thermal cycler. The generated cDNA was thereafter used for PCR amplification in a reaction mixture comprising 5 µL of 10× Super Taq buffer, 4 µL of dNTP (2.5 mmol/L), 1 µL of each specific primer, 0.2 µL of Super Taq enzyme (2 U/µL) and double distilled water. The specific primers for IL-8 are sense, 5'-CACAAGAGCCAGGAAGAAAC-3' and the anti-sense, 5'-CTACAACA GACCCACACAATAC-3' (product size of 459 base pair [bp]) (23). The PCR amplification for BAC primers (218 bp) (19, 21) was used for control. The PCR condition was 25–35 cycles for denaturing at 94°C for 30 seconds, annealing at 55°C for 1 minute, and extension at 72°C for 30 seconds followed by 72°C for additional 10 minutes. In some experiments, U0126 or SQ22536 was added 30 minutes before the addition of PGF$_{2α}$ and then

**Figure 2.** Stimulation of IL-8 mRNA expression by IL-1β and PGF$_{2α}$ in dental pulp cells. (A) Pulp cells were exposed to IL-1β for 24 hours; IL-8 gene expression was determined by RT-PCR (n = 3). (B) Pulp cells were exposed to PGF$_{2α}$ for 3 days; IL-8 mRNA expression was evaluated by RT-PCR (n = 3). One representative picture was shown.

**Figure 3.** Effect of U0126 (MEK1/2 inhibitor) and SQ22536 (adenylate cyclase inhibitor) on PGF$_{2α}$-induced IL-8 production and mRNA expression in dental pulp cells. Pulp cells were pretreated with U0126 (A) (n = 10) and (C) (n = 3) or SQ22536 (B) (n = 4) and (D) (n = 3) for 30 minutes and then coincubated with PGF$_{2α}$ for 3 days. Medium was collected for analysis of IL-8 level by ELISA. RNA was isolated for PCR amplification of BAC and IL-8 gene expression. Results were expressed as mean ± standard error of the mean in (A) and (B). In (C) and (D), one representative experiment was shown. *Significant differences when compared with control. **Significant difference when compared with PGF$_{2α}$-treated group (P < .05).
Aspirin — 0.5 ng/mL (Fig. 1). Cells concentrations ranging from 0.5–20 ng/mL. Moreover, PGF2α exposure to IL-1α further stimulated the IL-8 production by 7.7-fold at concentrations of 1–5 ng/mL (Fig. 1B). On the other hand, PGF2α induced IL-8 production of pulp cells by 4.6-fold to 8.9-fold at concentrations ranging from 0.5–20 μmol/L (Fig. 1C). Interestingly, the induction of PGF2α production in dental pulp cells by IL-1β was suppressed by aspirin (100 and 200 μmol/L), with 79%–89% of inhibition (Fig. 1D). On the contrary, the IL-1β-stimulated IL-8 production in the dental pulp cells was not significantly inhibited by aspirin at similar concentrations (Fig. 1E). At these experimental conditions, IL-1α, PGF2α, and aspirin showed little cytotoxicity to pulp cells as analyzed by MTT assay (data not shown).

**IL-1β and PGF2α Stimulated IL-8 mRNA Expression in Dental Pulp Cells**

As shown in Fig. 2A, IL-8 mRNA expression was stimulated after exposure to IL-1β for 24 hours at concentrations ranging from 0.05–5 ng/mL. Moreover, PGF2α also stimulated IL-8 gene expression of dental pulp cells at concentrations of 1–20 μmol/L (Fig. 2B).

**Figure 4.** Possible signaling pathways responsible for stimulation of IL-8 production in dental pulp cells by IL-1β and PGF2α. One is the MEK/ERK/RSK-p-CREB signaling pathway. The other is adenylate cyclase/PKA-p-CREB signaling pathway. RSK or PKA might phosphorylate and activate CREB. The activated CREB (p-CREB) might translocate into nucleus and regulate gene transcription, including IL-8.

**Statistical Analysis**

Three or more separate experiments were performed. Differences between control and experimental groups were analyzed by one-way analysis of variance and post hoc Bonferroni test. A P value <.05 was considered to be statistically significantly different between groups.

**Results**

**IL-1β Induced IL-8 and PGF2α Production in Dental Pulp Cells**

Untreated dental pulp cells generated only a small amount of IL-8. IL-1β stimulated IL-8 production in a dose-dependent manner. IL-8 levels in the culture medium dramatically increased by 39-fold even after exposure to 0.025 ng/mL of IL-1β. IL-1β further stimulated the IL-8 production by 336-fold to 390-fold at concentrations of 0.25–0.5 ng/mL (Fig. 1A). IL-1β also stimulated PGF2α production by 7.7-fold at concentrations of 1–5 ng/mL (Fig. 1B). On the other hand, PGF2α induced IL-8 production of pulp cells by 4.6-fold to 8.9-fold at concentrations ranging from 0.5–20 μmol/L (Fig. 1C). Interestingly, the induction of PGF2α production in dental pulp cells by IL-1β was suppressed by aspirin (100 and 200 μmol/L), with 79%–89% of inhibition (Fig. 1D). On the contrary, the IL-1β-stimulated IL-8 production in the dental pulp cells was not significantly inhibited by aspirin at similar concentrations (Fig. 1E). At these experimental conditions, IL-1α, PGF2α, and aspirin showed little cytotoxicity to pulp cells as analyzed by MTT assay (data not shown).

**Effect of U0126 and SQ22536 on PGF2α-Induced IL-8 Production and mRNA Expression**

U0126 (1 and 10 μmol/L), a mitogen-activated protein kinase kinase (MEK1/2) inhibitor, effectively attenuated the PGF2α-induced IL-8 production in pulp cells, with 59% and 95% of inhibition, respectively (Fig. 3A). Interestingly, SQ22536 (100 and 250 μmol/L), an adenylate cyclase inhibitor, could not prevent the PGF2α-induced IL-8 production and even enhanced this event (Fig. 3B). Accordingly, U0126 also inhibited the PGF2α-induced IL-8 mRNA expression in dental pulp cells (Fig. 3C). Similar to the results of ELISA, SQ22536 (100 and 250 μmol/L) enhanced the PGF2α-induced IL-8 mRNA expression in dental pulp cells (Fig. 3D).

**Discussion**

During pulpal and periapical inflammation, various mediators such as cytokines (IL-1α, IL-1β, tumor necrosis factor–α, IL-8, etc), PGs (PGE2, PGF2α, etc), and TXB2 are excessively generated (5, 6, 24, 25). IL-1α and IL-1β, as proinflammatory cytokines, have been shown to stimulate COX-2 expression and PGE2 production in dental pulp cells (19, 26). Similarly, IL-8 and PGF2α production of pulp cells is also induced by IL-1β in this study. Because our cultured human dental pulp cells have undergone several passages in vitro, these effects are most likely on pulp fibroblasts, the major stroma cells of dental pulp. This is further supported by in vitro observation of an elevated IL-1β and IL-8 expression in infiltrated inflammatory cells such as lymphocytes, macrophages, and endothelial cells but not in fibroblasts in inflamed dental pulp (6, 17, 27), as well as increased prostanoid (eg, PGE2 and PGF2α) production in inflamed dental pulp (5, 15). Generation of IL-1β, PGF2α, and IL-8 might have effects on immune cells, vasculature, and neurons in vitro to promote neutrophil chemotaxis and increase the vascular permeability of the dental pulp, all contributing to pulpal inflammation. Whereas presence of these inflammatory mediators in dental pulp is intended to remove exogenous insult and promote pulpal healing, persistent and severe pulpal inflammation will eventually lead to necrosis of dental pulp.

In this study, we also found that IL-1β stimulates both PGF2α, and IL-8 production in dental pulp cells. Intriguingly, PGF2α also induced IL-8 production, but markedly less than that induced by IL-1β. Moreover, aspirin, a COX-1 and COX-2 inhibitor, suppressed the IL-1β—induced PGF2α production, but not IL-8 production. This indicates that IL-1β—stimulated IL-8 production is not mediated mainly via COX activation and prostanoid production. IL-1β has been shown to suppress pulp cell proliferation and promotes collagen degradation in dental pulp by alteration of metalloproteinases (28, 29). IL-8 also recruits the neutrophils in response to bacterial by-products (6, 30). This suggests that IL-1β as well as IL-8 and PGF2α are important in the pathogenic processes of pulp diseases.

Whereas PGF2α level is elevated in inflamed dental pulp (5, 15), little is known about the effects of PGF2α on the dental pulp. PGF2α has been shown to induce the differentiation of cementoblasts and stimulate the matrix metalloproteinase activity as well as IL-6 production of gingival fibroblasts (10, 11, 31). In this study, exposure to PGF2α stimulates IL-8 production of pulp cells. Binding of PGF2α to receptors might potentially stimulate signal transduction and regulate the biologic activity of pulp cells. We found the induction of cyclic adenosine monophosphate responsive element-binding protein/activating transcription factor 1 (CREB/ATF-1) phosphorylation soon after exposure of pulp cells to PGF2α (unpublished observations). Activation of CREB/ATF-1 might be mediated by both upstream activation of MEK/extracellular signal-regulated kinase (ERK)/ribosomal protein S6 kinase (RSK) and adenylate kinase.
cyclophosphamide/cyclic adenosine monophosphate (cAMP) dependent protein kinase (PKA) (Fig. 4) (32, 33). PGF₂α and PGE₂ have been shown to stimulate calcium mobilization and cAMP production in rat clonal RDP-4-1 dental pulp cells (34). We therefore further explored whether signaling by MEK/ERK/CREB–ATF-1 or adenylate cyclase/PKA/CREB–ATF-1 is responsible for the PGF₂α-induced IL-8 production in pulp cells (Fig. 4). Interestingly, the MEK1/2 inhibitor, U0126, at popularly used concentrations (1 and 10 μmol/L) (35) effectively attenuated the PGF₂α-induced IL-8 production and mRNA expression with a 50% effective concentration of about 1 μmol/L. Accordingly, PGE₂ has been found to stimulate protein synthesis and basic fibroblast growth factor expression in vascular smooth muscle cells via activation of ERK signaling (36).

Inducing the ERK signaling by 2-hydroxyethyl methacrylate promotes pulp cell survival (37), suggesting that ERK signaling might play regulatory roles on pulp cell functions. On the contrary, SQ22536, an adenylate cyclase inhibitor, enhanced PGE₂-induced IL-8 production in pulp cells. SQ22536 has been popularly used to suppress adenylate cyclase with a 50% inhibitory concentration of about 200 μmol/L (38, 39), comparable to our study. These results indicate the presence of crosstalk between these 2 signaling pathways of the dental pulp, and the adenylate cyclase negatively regulates COX activity.

In conclusion, IL-1β might stimulate IL-8 and PGE₂ production in dental pulp cells and contribute to the pathogenesis of pulpal inflammation. IL-1β-induced IL-8 production via mainly COX-independent manner. The induction of IL-8 production of dental pulp cells by PGF₂α is mediated by MEK/ERK/CREB–ATF-1 signaling. These results are important for future development of pharmacological agents to control pulpal inflammation.

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References