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Cytokine-induced prostaglandin E_2 production and cyclooxygenase-2 expression in dental pulp cells: downstream calcium signalling via activation of prostaglandin EP receptor

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Abstract

Chang M-C, Chen Y-J, Tai T-F, Tai M-R, Li M-Y, Tsai Y-L, Lan W-H, Wang Y-L, Jeng J-H. Cytokine-induced prostaglandin E_2 production and cyclooxygenase-2 expression in dental pulp cells: downstream calcium signalling via activation of prostaglandin EP receptor. *International Endodontic Journal*, **39**, 819–826, 2006.

Aim To determine whether (i) proinflammatory cytokines stimulate prostaglandin E_2 (PGE_2) production and cyclooxygenase (COX) gene expression in dental pulp cells, and (ii) pulp cells that express different prostaglandin E_2 receptor (EP) isoforms and their activation by PGE_2 leads to downstream Ca^{2+} signalling.

Methodology Cultured human dental pulp cells were exposed to interleukin (IL)-1β and tumour necrotic factor-alpha (TNF-α). The expression of COX-1 and COX-2 was measured with reverse transcriptase-polymerase chain reaction (RT-PCR). The production of PGE_2 was measured using an enzyme-linked immunosorbent assay. Expression of prostaglandin EP receptor isoforms was studied by RT-PCR, whereas fura-2 fluorescence was used to measure calcium mobilization. The Kruskal–Wallis test and Wilcoxon sum rank test with Bonferroni correction were used for statistical analysis.

Results Interleukin-1β and TNF-α stimulate PGE_2 production of human dental pulp cells ($P < 0.05$). IL-1β stimulated the COX-2 but not COX-1 mRNA expression. Pulp cells express mainly EP2, EP3 and EP1 receptors as analysed by RT-PCR. PGE_2 (0.25–2 μmol L^{-1}) stimulated the Ca^{2+} mobilization as indicated by increase in fura-2 fluorescence.

Conclusions Interleukin-1β and TNF-α may stimulate PGE_2 production in dental pulp cells. Activation of prostaglandin EP receptors in dental pulp cells by PGE_2 may induce Ca^{2+} signalling to regulate cellular biological activity during inflammation.

Keywords: calcium mobilization, inflammation, prostaglandin, prostaglandin receptor, pulp cells.

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Introduction

Mechanical, chemical and microbial irritants to the dental pulp can provoke pain and an inflammatory response (Selzer & Bender 1984). Following injuries, pulp tissue exerts an inherent capacity for repair and regeneration to prevent further environmental insult (Yamamura 1985, Cox & Bergenholtz 1986, Mjor *et al.* 1991). Initially a number of inflammatory cells including polymorphonuclear leucocytes, lymphocytes and macrophages may infiltrate the inflamed dental pulp and affect pulp healing (Cox & Bergenholtz 1986). The final outcome of the pulp status depends on the ability of adjacent pulp cells to proliferate, migrate into the wound surface, differentiate and secrete dentine matrix

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proteins (Yamamura 1985, Mjor *et al.* 1991). Ideally, the wound should be populated by fibroblasts and/or preferentially odontoblasts, which may produce new dentine.

During the infection, healing and inflammatory processes, prostaglandin E_2 (PGE_2) is one of the most important factors that regulate the functions of pulp cells (Okiji *et al.* 1987, 1992, Goodis *et al.* 2000). Fibroblasts and macrophages in inflamed pulpal tissues have been shown to express cyclooxygenase-2 (COX-2) protein, a key enzyme responsible for PGE_2 production (Nakanishi *et al.* 2001). Endotoxin-induced pulp inflammation is accompanied with an increase of PGE_2 production, leading to changes in blood flow and vascular permeability of the pulp (Okiji *et al.* 1987, 1989, Kim *et al.* 1992). Previous studies have shown that thrombin stimulates the proliferation and PGE_2 production of cultured dental pulp cells (Sundqvist *et al.* 1995). In infected dental pulp tissue, bacterial lipopolysaccharide and methyl mercaptan may stimulate the production of various pathogenic cytokines such as interleukin (IL)-1α, IL-β, tumour necrotic factor-alpha (TNF-α) and IL-6 (Yang *et al.* 2003, Coil *et al.* 2004). Some of these proinflammatory cytokines such as IL-1α and TNF-α may stimulate COX-2 mRNA and protein expression in dental pulp cells (Lin *et al.* 2002, Chang *et al.* 2003). Recently, it has been reported that PGE_1 and PGE_2 suppressed protein and DNA synthesis of dental pulp cells (Chang *et al.* 1998a). The stimulatory effects of thrombin (10 U mL^{-1}), a vascular coagulation factor, can be inhibited by PGE_2 (Chang *et al.* 1998a,b). These results imply that PGE_2 is important in modulating the inflammatory and healing processes of the pulp. PGE_2 mediates these effects possibly via its autocrine and paracrine binding to prostaglandin EP receptors over the cell surface and induce downstream signalling. The actions of PGE_2 depend mainly on its binding to four types of PGE receptors, i.e. EP1, EP2, EP3 and EP4, which link to different signal transduction pathways (Abramovitz *et al.* 1995, Narumiya 1995, Negishi *et al.* 1995). PGE_2 stimulates or suppresses the neurotransmitter release and the reabsorption of Na^+ and H_2O from kidneys (Abramovitz *et al.* 1995, Narumiya 1995, Negishi *et al.* 1995). These contradictory actions of PGE_2 further support the presence of diverse PGE_2 receptor isoforms in different organs or tissues. However, no prior studies have reported the expression of EP receptors in dental pulp. The aim of this study was to determine whether (i) proinflammatory cytokines stimulate PGE_2 production and COX gene expression in dental pulp cells, and (ii) dental pulp cells that express different prostaglandin EP receptor isoforms and their activation by PGE_2 leads to Ca^{2+} signalling. Thus specific primers were designed and the polymerase chain reaction (PCR) was used to elucidate the expression of receptor isoforms. Moreover, subsequent calcium mobilization within dental pulp cells in response to PGE_2 activation was evaluated.

Materials and methods

Materials

3-(4,5-Dimethyl-thiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), PGE_2, recombinant IL-1β and TNF-α were obtained from Sigma Chemical Company (St Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), foetal calf serum (FCS), and penicillin/streptomycin were purchased from Gibco Life Technologies (Grand Island, NY, USA). PGE_2 ELISA kits were from Cayman Chemical Company (Ann Arbor, MI, USA). Specific primers of human β-actin, COX-2, COX-1, EP1, EP2, EP3 and EP4 receptors were synthesized by Genemed Biotechnologies Inc. (San Francisco, CA, USA).

Culture of human dental pulp cells

Briefly, human third molars were obtained following appropriate informed consent of the patients. The teeth were immediately split using a hammer and the pulp tissues harvested, cut into small pieces and then cultured by an explant technique in DMEM containing 10% FCS, 1× penicillin/streptomycin. Two strains of human dental pulp cells were established. Cultured dental pulp cells are spindle shape with extended cellular processes but show marked alkaline phosphatase activities, indicating their potential for tissue mineralization (Chang *et al.* 1998a,b, Chan *et al.* 2005). The pulp cells in passage numbers of 3–8 were used for these studies with similar results.

Effects of IL-1β and TNF-α on the PGE_2 production of pulp cells

Pulp cells (1×10^5 cells per well) in 24-well culture plates and 1 mL of DMEM with 10% FCS were exposed to various concentrations of IL-1β and TNF-α for 24 h. Culture medium was collected and the cell number was measured with MTT assay as described previously (Jeng *et al.* 2000, Chan *et al.* 2005).

Effects of IL-1β on the COX-2 and COX-1 mRNA expression of dental pulp cells

Briefly, near confluent pulp cells were incubated in DMEM with 10% FCS and various concentrations of IL-1β for 24 h. Total cellular RNA was isolated using Qiagen RNA isolation kits. Then 2 μg of total RNA was subjected to a reverse transcription (RT) as described previously (Jeng *et al.* 2000). Four microlitres of the cDNA product was used for PCR amplification in a reaction volume of 50 μL comprising 5 μL of 10× Super TAQ buffer, 4 μL of dNTP (2.5 mmol L^{-1}), 1 μL of each specific primers, and 0.2 μL of Super TAQ enzyme (2 U μL^{-1}). Specific primers for human COX-1 are TGCCCAGCTCCTGGCCCGCC GCTT and GTGCATCAA-CACAGGCGCCTCTTC; COX-2 are 5′-TTCA AATG-AGATTGTGGGAAAATTGCT-3′ and 5′-AGATCATCT CTGCC TGAGTATCTT-3′ and primers for β–actin are 5′-AAGAGAGGCA TCCTCACCCT-3′ and 5′-TACATG-GCTGGGGTGTTGAA-3′ (Jeng *et al.* 2000, Chang *et al.* 2004). The amplified DNA products are 304 base pairs (bp), 305 bp and 218 bp for COX-1, COX-2 and β-actin, respectively. The reaction mixture was set at 94 °C for 5 min in the first cycle. Then the reaction was carried out at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min of suitable cycles with a thermal cycler (Perkin Elmer 4800, City of Industry, CA, USA). The quantity of amplified DNA product, which was linear with respect to the input RNA, was used for this report. Lastly, the reaction was complete following an extension of PCR reaction at 72 °C for 10 min. The amplified PCR products were loaded to 1.8% agarose gel electrophoresis and stained with ethidium bromide.

Expression of prostaglandin EP receptor isoforms in pulp cells

Total RNA of human dental pulp cells was isolated as described above (Jeng *et al.* 2000). Expression of prostaglandin EP receptors was detected by reverse transcriptase-polymerase chain reaction (RT-PCR) using specific primers for human EP1, EP2, EP3 and EP4 as used by Ben-Av *et al.* (1995). RT-PCR procedures were completed as described above. The nucleotide sequence for EP1 was sense: TCTACCTCCCTGCAG CGGCCACTG and antisense: GAAGTGGCTG AGGCCGCTGTGCCG GGA with product size of 230 bp. The primers for EP2 were sense: CTTACCTGCAGCTGTACG and antisense: GAT-GGCAAAGACCCAA GG with a product size of 367 bp. For EP3, the sense primer was GAGCACTGCAAGACA-CACACACGGAG and antisense primer was GAT-CTCCATGGGTATTACTGACAA, which generate a PCR product of 398 bp. The primers for EP4 were sense: CTGGCGATCAACCATGCCTATTTC and antisense: TGAGCACCA CCAGGGAGGTGGCAAAAT and an amplified product of 494 bp. RT samples were amplified for 35 cycles of PCR reaction with a denaturing temperature at 94 °C for 30 s, followed by annealing at 55 °C for 30 s and extension at 72 °C for 1 min. The amplified products were subjected to 1.8% agarose gel electrophoresis and detected by ethidium bromide staining.

Measurement of calcium mobilization

Calcium mobilization was measured by detecting fura-2 fluorescence using a temperature-controlled Hitachi F-4500 Spectrofluorometer (Tokyo, Japan) as described previously (Jeng *et al.* 2004). Fura-2 AM can be cleaved by cell membrane esterase and pass into cells. The peak excitation wavelength of fura-2 changes when it binds Ca^{2+}, which released from intracellular store or extracellular influx following activation. Measurement of fluorescence at two excitation-wavelength can be used to estimate intracellular Ca^{2+} concentration (Hayashi & Miyada 1994). Briefly, dental pulp cells were collected in HBSS (145 mmol L^{-1} NaCl, 5.4 mmol L^{-1} KCl, 1 mmol L^{-1} $MgCl_2$, 1.8 mmol L^{-1} $CaCl_2$, 25 mmol L^{-1} glucose, 20 mmol L^{-1} HEPES, pH 7.4) containing 2 μmol L^{-1} of fura-2 AM at 37 °C for 30 min with intermittent shaking. Cells were washed with HBSS and resuspended in HBSS at a concentration of 2.5×10^5 cells mL^{-1}. Pulp cells (0.9 mL) were added into the quartz cuvette with continuous stirring achieved by a magnetic stirrer. Thereafter, various concentrations of PGE_2 (final 0.25–4 μmol L^{-1}) were added. Fluorescence of Ca^{2+}-bound and -unbound fura-2 was measured by rapidly alternating the dual excitation wavelengths of both 340 and 380 nm and electronically measuring the emission fluorescence at wavelength 510 nm. The ratio (R) of the fluorescence at the two wavelengths were computed automatically and used to calculate the changes in $[Ca^{2+}]_i$. The ratio of maximum (R_{max}) and minimum (R_{min}) fluorescence of fura-2 were measured by adding 40 μL of 10% Triton X-100 and by adding a final 5 mmol L^{-1} of EGTA, respectively. Changes in 340/380 ratio were measured with a Hitachi Calcium Measurement Software.

Statistical analysis

Each experiment was repeated at least three times. Non-parametric Kruskal–Wallis tests were used for

multiple comparisons to check whether there was any difference among the groups ($P < 0.05$). Then the Wilcoxon sum rank (Mann–Whitney) test with adjustment by Bonferroni correction was conducted to evaluate whether the differences were significant between two compared groups ($P < 0.0033$).

Results

Effect of IL-1β and TNF-α on PGE_2 production of dental pulp cells

As shown in Fig. 1, basal level of PGE_2 in the culture medium was approximately 99 pg mL^{-1}. After exposure to IL-1β for 24 h, PGE_2 levels increased to 193 pg mL^{-1} (1.95-fold), 328 pg mL^{-1} and 487 pg mL^{-1} (4.9-fold), respectively, by 0.1, 0.5 and 10 ng mL^{-1} of IL-1β. TNF-α also stimulated the PGE_2 production to 613 pg mL^{-1} (6.2-fold) and 893 pg mL^{-1} (9-fold), at concentrations of 0.05 and 5 ng mL^{-1}. No evident change in cell number was noted under these experimental conditions (data not shown).

Effect of IL-1β on COX-1 and COX-2 gene expression of dental pulp cells

Using RT-PCR, IL-1β induced COX-2 mRNA expression of pulp cells in a dose-dependent manner at concentrations ranging from 0.05 to 5 ng mL^{-1}, but IL-1β showed little effect on COX-1 mRNA expression (Fig. 2).

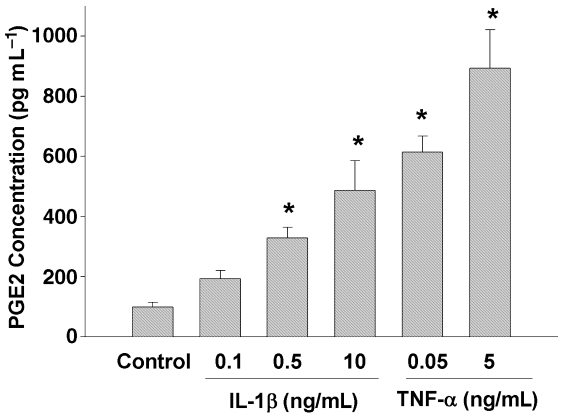

Figure 1 (a) Effect of IL-1β and TNF-α on the PGE_2 production of dental pulp cells. Concentration of PGE_2 in the culture medium after exposure to IL-1β (0.1–10 ng mL^{-1}) and TNF-α (0.05–5 ng mL^{-1}) for 24 h were measured with ELISA. *Marked difference when compared with untreated control ($P < 0.0033$, mean ± SE).

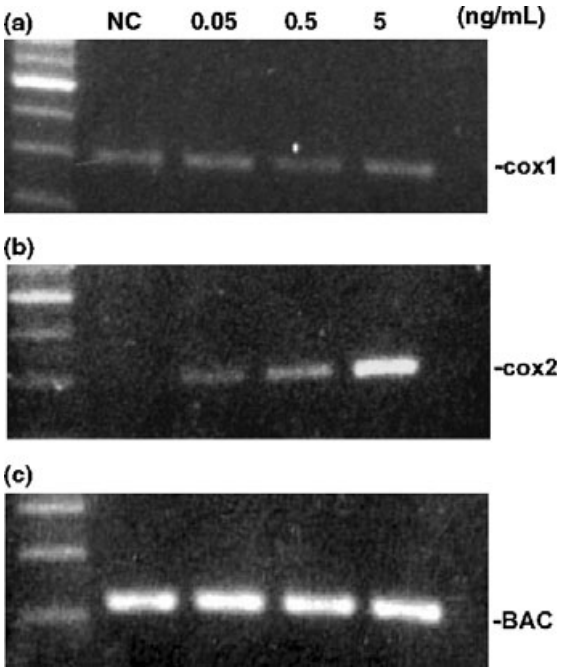

Figure 2 Expression of COX-1, COX-2 and β-actin gene of dental pulp cells following exposure to 0.05–5 ng mL^{-1} of IL-1β for 24 h as analysed by RT-PCR and agarose gel electrophoresis.

Expression of prostaglandin EP receptor isoforms in dental pulp cells

Using specific primers and PCR, we found that pulp cells expressed mainly EP2 receptor (367 bp), lesser amounts of EP3 (398 bp) and EP1 (230 bp) receptors, whereas expression of EP4 (494 bp) was below the detection limit (Fig. 3).

Effect of PGE_2 on calcium mobilization of dental pulp cells

Exposure of pulp cells to PGE_2 also elicited calcium mobilization as indicated by rapid increase of fura-2 fluorescence (as indicated by changes in 340/380 ratio) at concentrations ranging from 0.25 to 2 μmol L^{-1} (data not shown). Quantitatively, PGE_2 elevated the intracellular calcium levels in a dose-dependent manner as indicated by an increase in 340/380 nm fura-2 fluorescence ratio from 0.425 by

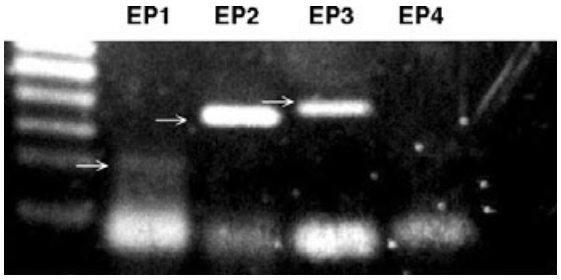

Figure 3 Expression prostaglandin receptor isoform (EP1, EP2, EP3 and EP4) in culture human dental pulp cells. RT-PCR was used to amplify the products using specific primer sets for EP1, EP2, EP3 and EP4. Arrows indicated the expression of EP1, EP2 and EP3 receptor in dental pulp cells, respectively.

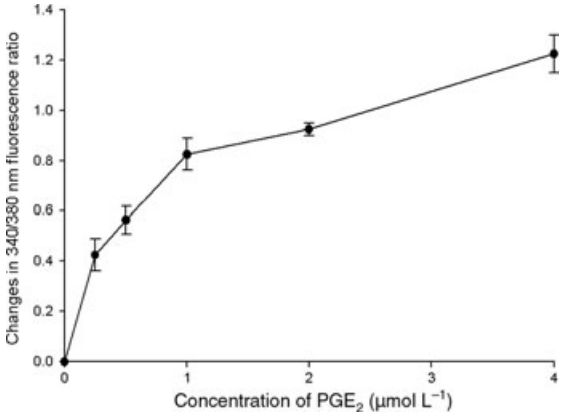

Figure 4 Quantitative value of different concentrations of PGE_2 (0.25–4 μmol L^{-1}) on calcium mobilization in dental pulp cells. Results were expressed as changes in 340/380 nm fluorescence ratio (mean ± SE, $n = 5$).

0.25 μmol L^{-1} PGE_2 to 1.225 by 4 μmol L^{-1} of PGE_2 (Fig. 4).

Discussion

Pulp cells are specialized connective tissue cells, which may differentiate into odontoblasts and are responsible for new dentine formation after pulpal injuries. A number of mediators such as PGE_2, IL-1α and TNF-α are critical in the pathogenesis of inflammatory pulpal diseases. Expression of IL-1α and TNF-α in macrophages and fibroblasts of dental pulp have been detected in surgically exposed rat dental pulp (Tani-Ishii *et al.* 1995). IL-1β and TNF-α stimulate DNA synthesis, hepatocyte growth factor production and matrix metalloproteinase (MMP)-1 mRNA expression in dental pulp cells as well as dermal fibroblasts (Lertchirakarn *et al.* 1998, Ohnishi *et al.* 2000, Lin *et al.* 2001). These events were mediated in a prostaglandin-dependent or -independent manner (Lertchirakarn *et al.* 1998, Ohnishi *et al.* 2000, Lin *et al.* 2001). In the present study, IL-1β and TNF-α induced PGE_2 production of dental pulp cells, indicating these pro-inflammatory cytokines could regulate pulp cell behaviour by inducing PGE_2 production. Consistently, IL-1β and TNF-α are shown to stimulate prostanoid synthesis of pulpal fibroblasts after 1–2 h of exposure (Sundqvist & Lerner 1996). Both COX-1 and COX-2 are critical enzymes regulating PGE_2 production. Previous reports have found the induction of COX-2 mRNA and protein expression as well as IL-6 mRNA expression by IL-1α and TNF-α in dental pulp cells (Lin *et al.* 2002, Chang *et al.* 2003). Exogenous addition of PGE_2 attenuates the IL-1α and TNF-α-induced IL-6 production in dental pulp cells, whereas indomethacin, a COX inhibitor, enhances these events (Lin *et al.* 2002). In the present study, IL-1β also induced COX-2 but not COX-1 mRNA expression, indicating that possibly COX-2 activation by various proinflammatory cytokines is associated with PGE_2 production. Alternatively, cytokine may also activate phospholipase A2 to promote PGE_2 production (Murakami & Kudo 2004). Accordingly, normal pulp tissues express little COX-2 proteins as analysed by immunohistochemical technique, whereas COX-2 protein could be detected in pulpal fibroblasts and macrophages of all inflamed dental pulp tissues (Nakanishi *et al.* 2001). This can be due to the induction of COX-2 gene expression and subsequent PGE_2 production by these cytokines. The activation of COX and PGE_2 production has been shown to suppress DNA and protein synthesis of pulp cells (Chang *et al.* 1998a,b). In addition, PGE_2 inhibits the IL-1β and TNF-α-induced MMP-1 mRNA expression (Chang *et al.* 1998b, Lin *et al.* 2001). PGE_2 is also shown to stimulate glycosaminoglycan (GAG) synthesis and alkaline phosphatase activity of pulp cells (Nagata *et al.* 1991, Hamasaki *et al.* 1992). However, the signalling mechanisms responsible for these PGE_2-mediated events are not well-elucidated.

Prostaglandin GE_2 may regulate the functional activities of cells via its binding to cell surface receptors (e.g. EP1, EP2, EP3 and EP4) (Abramovitz *et al.* 1995, Narumiya 1995, Negishi *et al.* 1995). However, no prior study appears to have addressed the expression of EP receptor isoforms in dental pulp cells. PGE_2 may suppress the IL-1β-induced MMP-3 production via

activation of EP2/EP4 receptors, whereas IL-1β stimulates MMP-3 production via activation of EP1 receptor in gingival fibroblasts (Ruwanpura *et al.* 2004). It was noted that pulp cells expressed mainly EP2 and EP3 receptor and lesser amounts of EP1 receptor, whereas EP4 receptor expression was below the detection limit. This differential expression of EP receptor isoforms may mediate the response of pulp cells to PGE_2 via different signalling. PGE_2 was shown to stimulate cAMP production of rat clonal odontoblast-like RDP4-1 cells and bovine dental pulp cells (Kawase *et al.* 1990, Kido *et al.* 1991). Stimulation of GAG synthesis and alkaline phosphatase activity of pulp cells by PGE_2 and cAMP have also been reported (Nagata *et al.* 1991, Hamasaki *et al.* 1992). Whether activation of which EP receptor isoform is responsible for GAG synthesis and alkaline phosphatase activity of pulp cells should be addressed further. Elevated PGE_2 levels in pulp tissues with carious exposure have been correlated to poor radiological success and clinical outcome (Waterhouse *et al.* 2002). Further studies on the control of COX-2 gene expression, PGE_2 and cAMP production in the dental pulp cells may be helpful for our treatment of inflammatory pulpal and periapical diseases in the future.

Interestingly, an increase of calcium mobilization of pulp cells by PGE_2 was noted. The PGE_2-induced calcium mobilization is possibly associated with activation of EP1/EP3 receptor, which has been reported to mediate calcium mobilization in other cells (Abramovitz *et al.* 1995, Narumiya 1995, Negishi *et al.* 1995) and support of this, the expression of EP1/EP3 receptor in pulp cells was revealed by RT-PCR. L-type calcium channels, which may mediate extracellular calcium influx, have been detected in odontoblasts and pulpal fibroblasts in response to pulpal injury (Westenbroek *et al.* 2004). Elevation of extracellular calcium may stimulate BMP-2 and osteopontin, whereas downregulate BMP-4 and alkaline phosphatase gene expression of pulp cells (Rashid *et al.* 2003). As calcium is important in the repair and dentinogenesis (Yamamura 1985, Couble *et al.* 2000), PGE_2-induced calcium mobilization in dental pulp cells may possibly affect functional activity and dentine mineralization in the pulp.

Conclusion

Proinflammatory cytokines such as IL-1β and TNF-α may stimulate PGE_2 production in dental pulp cells via stimulation of COX-2 gene expression or phospholipase A2 activation. Pulp cells harvest different prostaglandin EP receptors. During pulpal healing and inflammation, activation of prostaglandin EP receptors in dental pulp cells by PGE_2 may induce Ca^{2+} signalling to regulate cellular biological activities, e.g. alterations of cAMP levels, MMP-1 activity, glycosaminoglycan and alkaline phosphatase activities as reported previously (Nagata *et al.* 1991, Hamasaki *et al.* 1992, Lin *et al.* 2001). More studies are needed to determine the effects of differential EP receptor activation by prostaglandins on the functional activity of dental pulp cells.

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