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Cyclooxygenase-2 Induction in *Porphyromonas gingivalis*-Infected THP-1 Monocytic Cells

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Periodontopathogens including *Porphyromonas gingivalis* interact with host periodontal cells and the excessive subsequent host responses contribute a major part to the development of periodontal diseases. Cyclooxygenase (COX)-2-synthesized PGE_2 has detrimental activities in terms of periodontal pathogenesis. The present study investigated induction of COX-2 expression by *P. gingivalis* in human monocytic THP-1 cells. Live *P. gingivalis* increased expression of COX-2, but not that of COX-1, which was demonstrated at both mRNA and protein levels. Elevated levels of PGE_2 were released from *P. gingivalis*-infected THP-1 cells. Pharma-cological inhibition of p38 mitogen-activated protein kinase (MAPK) and extracellular signal-regulated kinase (ERK) substantially attenuated *P. gingivalis*-induced COX-2 mRNA expression. Indeed, activation of p38 MAPK and ERK was observed in *P. gingivalis*-infected THP-1 cells. Also, *P. gingivalis* induced activation of nuclear factor-κB (NF-κB) which is an important transcription factor for COX-2. These results suggest that COX-2 expression is upregulated in *P. gingivalis*-infected monocytic cells, at least in part, via p38 MAPK, ERK, and NF-κB.

Keywords: *Porphyromonas gingivalis*, cyclooxygenase-2, signaling, THP-1

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Introduction

Periodontal diseases are multifactorial infections elicited by a complex of bacterial species that interact with host tissues and cells, inducing a variety of proinflammatory products from various host cells such as interleukin-1 (IL-1), tumor necrosis factor (TNF), and prostaglandins (PGs) (Socransky SS and Haffajee, 1992; Holt and Ebersole, 2005). In particular, PGs are well known as potent soluble mediators in normal biological functions and in various inflammatory lesions. Prostagladin E_2 (PGE_2) has been implicated as a key mediator in the pathogenesis of periodontal diseases and its levels have been shown to be elevated in inflamed gingival tissues. PGE_2 induces vasodilation and increased vascular permeability, resulting in clinical signs of inflammation (Preshaw and Heasman, 2002; Heasman *et al.*, 1998).

Cyclooxygenase (COX), also known as prostaglandin endoperoxide synthase, is a key enzyme in PG 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 stimuli such as lipopolysaccharide (LPS), IL-1, and TNF (Caughey *et al.*, 2001; Shanmugam *et al.*, 2004).

Porphyromonas gingivalis plays a significant role in the progression of chronic periodontitis. It has routinely been reported to be a major member of the pathogenic microbiota in various periodontal diseases characterized by alveolar bone loss. *P. gingivalis* is a gram-negative black pigmented anaerobe that colonizes in periodontal pockets and spreads

into deeper tissues (Socransky and Haffajee, 2005; Holt *et al.*, 1999). In the meantime, the major cell types of periodontal tissues are epithelial cells, fibroblasts, endothelial cells, and macrophages. Moreover, the inflammatory infiltrate which develops in the gingival tissues is dominated by lymphocytes and macrophages (Dixon *et al.*, 2004). According to immunohistochemical studies, PGE_2 is mainly localized in macrophage-like cells in inflamed gingival tissues. Thus, monocytes/macrophages are thought to be one of the major sources of PGE_2 (Garrison and Nichols, 1989).

Previous reports have shown that LPS from *P. gingivalis* induces high levels of COX-2 expression and PGE_2 production in peripheral blood monocytes (Noguchi *et al.*, 2000; Morton and Dongari-Bagtzoglou, 2001). However, the effects of live *P. gingivalis* on COX-2 expression by monocytes/macrophages have never been investigated. The purpose of the present study was to investigate profiling of COX-2 induction by *P. gingivalis* and the involved signaling mechanisms in human monocytic THP-1 cells. The results showed that COX-2 expression is upregulated in *P. gingivalis*-infected THP-1 cells through p38 mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK), and nuclear factor-κB (NF-κB).

Materials and Methods

Reagents

GF109203X, U73122, PD98059, SB203580, and SP 600125 were purchased from Calbiochem (San Diego, CA, USA). Rapamycin was from Cell Signaling Technology (Beverly, MA, USA). Wortmannin and genistein were purchased from Sigma (St. Louis, MO, USA). Anti-COX-2 antibody was from Cayman Chemicals (Ann Arbor, MI, USA). Antibodies to phospho-ERK, phospho-p38, phospho-c-Jun N-terminal kinase (JNK) were from Cell Signaling Technology (Beverly, MA, USA). Anti-β-actin was from Sigma. Unless stated otherwise, all chemicals were 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 and bacteria in logarithmic growth phase were used in all experiments.

Cell culture

THP-1 human monocytic cells were purchased from American Type Culture Collection. The cells were cultured in RPMI 1640 medium (Life Technologies, Grand Island, NY, USA) containing 10% fetal bovine serum and 50 μg/ml gentamicin at 37°C in 5% CO_2.

Infection protocol

Bacteria were washed twice with phosphate-buffered saline (PBS) and once with complete RPMI medium. After resuspension in complete RPMI medium, the optical density of the bacterial suspension was measured at 600 nm, and further diluted to an optical density of 0.5 which corresponded to 5×10^8 CFU/ml. For enzyme-linked immunosorbent assays (ELISA), 1×10^6 THP-1 cells were seeded in 24-well and the cells were incubated with *P. gingivalis* (multiplicity of infection (MOI) of 1:50) in a final volume of 0.5 ml for 12 or 24 h. For Western blots, RT-PCR, and gel shift assays, 2×10^6 THP-1 cells were seeded in 12-well plates and the cells were incubated with *P. gingivalis* (MOI of 1:50) for various times.

Measurement of PGE_2 from culture supernatants

The THP-1 culture supernatants were collected, clarified, and the levels of PGE_2 were quantified using a commercial ELISA kit (R&D Systems) according to the manufacturer's directions.

Measurement of relative mRNA expression of COX-2

Total RNA was isolated and RT-PCR was done as described previously (Kang and Kuramitsu, 2002). Total RNA was prepared with Trizol reagent (Life Technologies) as specified by the manufacturer and quantified spectrophotometrically. Levels of COX-2 mRNA expression were determined by RT-PCR using specific primers. First-strand cDNA was synthesized from 1 μg of RNA using random primers (Promega, Madison, WI, USA) and Molony murine leukemia virus reverse transcriptase (Life Technologies). 2 μl of cDNA products was amplified in 25 μl volumes under a layer of mineral oil using a GeneAmp 9600 thermal cycler (Perkin Elmer Cetus, Norwalk, CT, 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°C (30 s), annealing at 55°C (30 s), and extension at 72°C (60 s). The sequences of primers were 5'-TTCAAATGAGATTGTGGGAAAATTGCT-3', 5'-AGTTCATCTCTGCCTGAGTATCTT-3' for COX-2 (305 bp); 5-GAGTCTTTCTCCAACGTGAGC-3, 5-ACCTGGTACTTGAGTTTCCCA-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 2% agarose gels containing ethidium bromide, visualized by UV transillumination, and photographed.

Western blot

THP-1 cells in 12-well plates were harvested and lysed with 100 μl of Cell Lysis Buffer (Cell Signaling Technology). 30 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 anti-COX-2 polyclonal antibody

(1:500); anti-phospho-ERK, p38, or JNK polyclonal antibody (1:1000, Cell Signaling Technology) and a 1:1500 dilution of 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-β-actin (1:5000).

Gel shift assay

Nuclear extracts were prepared according to the method of Dignam *et al.* (Dignam *et al.*, 1983) with some modifications. Briefly, cells were washed with ice-cold PBS and pelleted. The cell pellet was resuspended in hypotonic buffer (10 mM HEPES (pH 7.9 at 4°C), 0.5 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, 0.2 mM PMSF) and incubated for 10 min on ice, then the cells were lysed by addition of 10% IGEPAL CA-630 (Sigma), followed by vigorous vortex for 10 s. Nuclei were pelleted and resuspended in low-salt buffer (20 mM HEPES (pH 7.9 at 4°C), 25% glycerol, 1.5 mM $MgCl_2$, 20 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, and 0.2 mM PMSF) and added high-salt buffer (20 mM HEPES (pH 7.9 at 4°C), 1.5 mM $MgCl_2$, 0.8 M KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF) in a drop-wise fashion. After 30 min incubation at 4°C, the lysates were centrifuged, and supernatants containing the nuclear proteins were transferred to new vials. Protein concentrations of the nuclear extracts were measured with DC Protein Assay Kit (Bio-Rad). Double-stranded NF-κB-binding DNA probe (5'-AGTTGA GGGGACTTTCCCAGGC-3') was purchased from Promega (Madison, WI, USA), and end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. About 10 μg of nuclear extracts were incubated with 10,000 cpm of probe in 20 μl of reaction buffer containing 10 mM Tris-HCl (pH 7.6), 50 mM KCl, 1 mM EDTA, 5% glycerol, 1 mM DTT, and 200 ng of poly (dI·dC) for 30 min at room temperature. Specific binding of NF-κB was controlled by competition with a 50-fold excess of cold NF-κB probe. The DNA-protein complexes were separated on 4% polyacrylamide gels. The gels were dried and subjected to autoradiography.

Results

COX-2 mRNA expression

Initially, it was determined whether live *P. gingivalis* in a relatively low MOI (1:50) could induce COX-2 and COX-1 mRNAs in THP-1 cells. The cells were infected with *P. gingivalis* for various time periods from 2 to 16 h. Total RNA was isolated and levels of COX-1 and COX-2 mRNA were determined by RT-PCR. Strong COX-2 mRNA induction by *P. gingivalis* was demonstrated at 2-4 h postinfection and the COX-2 message persisted up to 16 h postinfection (Fig. 1). In contrast, COX-1 mRNA was constitutively expressed and not altered during the time course. This suggests that *P. gingivalis* specifically induces the COX-2 isoform in monocytic cells.

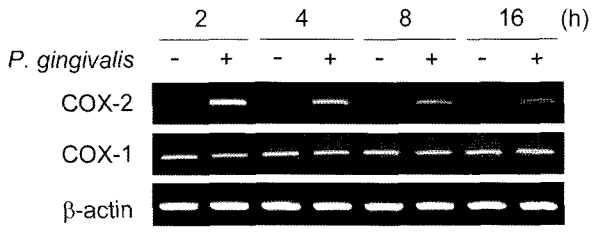

Fig. 1. COX-2 mRNA expression by THP-1 cells in response to *P. gingivalis*. THP-1 cells were infected with *P. gingivalis* (1:50) for the indicated time periods. Total RNA was isolated and levels of COX-1 and COX-2 mRNA were determined by RT-PCR.

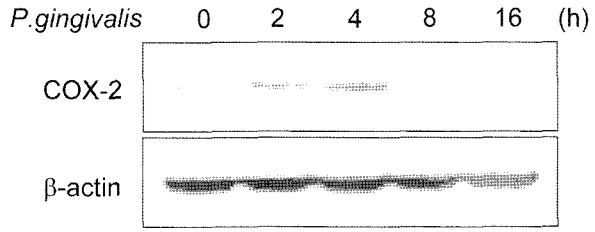

Fig. 2. COX-2 protein expression by THP-1 cells in response to *P. gingivalis*. THP-1 cells were infected with *P. gingivalis* (1:50) for the indicated time periods. Cell lysates were analyzed for COX-2 expression by Western blot analysis.

COX-2 protein expression

The RT-PCR data showed significant induction of COX-2 mRNA by *P. gingivalis*. Next, it was determined whether COX-2 protein levels were also regulated. Western blot analysis with specific COX-2 antibody was carried out using total protein from control and *P. gingivalis*-infected THP-1 cells. Fig. 2 shows that COX-2 protein appeared 2 h after infection, peaking at 4 h, and then gradually declined up to 16 h. This result suggests that *P. gingivalis*-induced COX-2 mRNA is readily translated into COX-2 protein in THP-1 cells.

PGE_2 production

To determine whether the induction of COX-2 mRNA and protein results in increased production of PGE_2, PGE_2 concentrations of the culture supernatants were measured by ELISA. In accordance with the COX-2 expression data, *P. gingivalis*-infected THP-1 cells had significantly elevated PGE_2 levels relative to control cells (Fig. 3). A remarkable increase in PGE_2 production was observed at 24 h postinfection. This suggests that monocytic cells release a considerable amount of PGE_2 in response to *P. gingivalis* infection.

Involvement of ERK and p38 MAPK

The MAPKs are central to many inflammatory signaling pathways (Robinson and Cobb, 1997). In order to evaluate

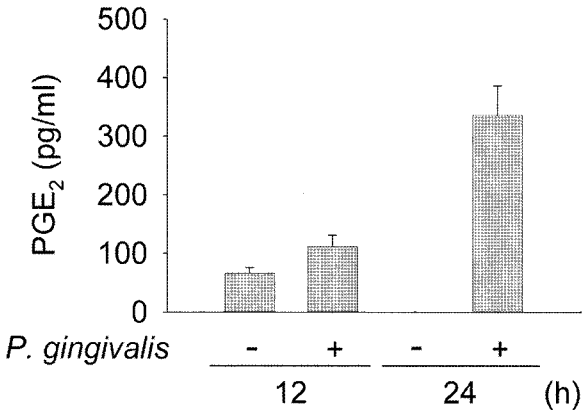

Fig. 3. PGE_2 production by THP-1 cells in response to *P. gingivalis*. THP-1 cells were infected with *P. gingivalis* (1:50) for 12 or 24 h. PGE_2 concentrations of the culture supernatants were measured by ELISA. Data are the means±S.D. of a representative experiment performed in triplicate.

the relative importance of MAPKs and some additional signaling pathways in *P. gingivalis*-induced COX-2 expression in THP-1 cells, specific pharmacological inhibitors were used. THP-1 cells were pretreated with GF109203X (protein kinase 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), or SP600125 (JNK, 10 μM) for 1 h, and then the cells were incubated with *P. gingivalis* for 2 h. Total RNA was isolated and levels of COX-2 mRNA were determined by RT-PCR. Among the inhibitors, SB203580 and PD98059, specific inhibitors of p38 MAPK and ERK, respectively, strongly reduced COX-2 mRNA expression by *P. gingivalis*, with SB203580 more efficient than PD98059 (Fig. 4). This result suggests that p38 MAPK and ERK play important roles for *P. gingivalis*-induced COX-2 expression at the pre-translational level in monocytic cells.

Activation of MAPKs

As the involvement of MAPK pathways in *P. gingivalis*-induced COX-2 expression was demonstrated by the use of inhibitors of MAPKs, it was necessary to determine whether

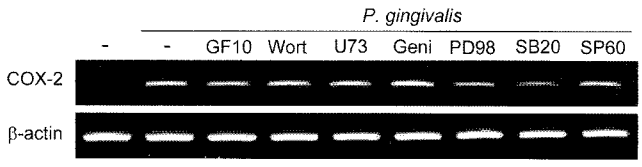

Fig. 4. Effect of various signaling inhibitors on COX-2 expression of THP-1 cells infected with *P. gingivalis*. THP-1 cells were pretreated for 1 h with GF109203X (1 μM), wortmannin (100 nM), U73122 (10 μM), genistein (50 μM), PD98059 (50 μM), SB203580 (10 μM), or SP600125 (10 μM) and then infected with *P. gingivalis*. Total RNA was isolated at 2 h after infection, and levels of COX-2 mRNA were determined by RT-PCR.

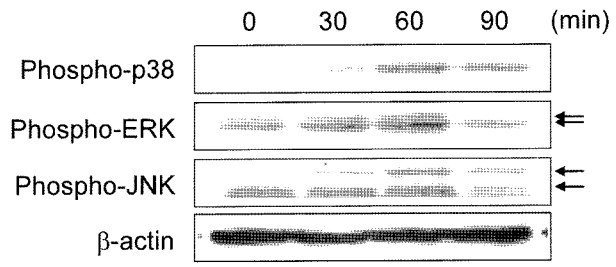

Fig. 5. Activation of MAPKs in *P. gingivalis*-infected THP-1 cells. THP-1 cells were infected with *P. gingivalis* (1:50) for the indicated time periods. Cell lysates were prepared and Western blot analysis was performed for phospho-p38, ERK, or JNK.

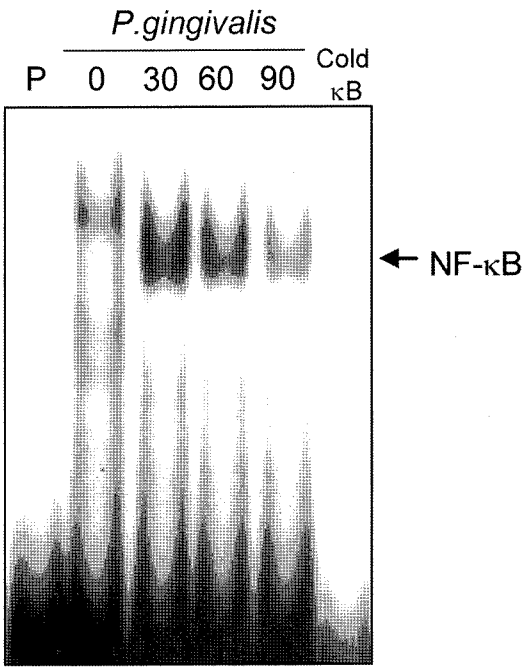

Fig. 6. Activation of NF-κB in *P. gingivalis*-infected THP-1 cells. THP-1 cells were infected with *P. gingivalis* (1:50) for the indicated minutes of time. Nuclear extracts were prepared and activation of NF-κB was determined by gel shift assays. The first lane contained probe incubated without nuclear extract. NF-κB specificity was controlled with a 50-fold excess of cold NF-κB probe.

MAPKs are actually activated by *P. gingivalis* in THP-1 cells. Western blot analysis was performed for phospho-p38, -ERK, or -JNK. Activation of MAPKs occurs through phosphorylation of threonine and tyrosine residues by upstream MAPK kinases (Watanabe *et al.*, 2001). Phosphorylation of p38 and JNK was not detected in control unstimulated THP-1 cells. Exposure of the cells to *P. gingivalis* for 30 min brought about phosphorylation of p38 and JNK, and maximal activation was at 60 min after infection (Fig. 5). Upregulation of ERK phosphorylation was also demonstrated during 30-60 min after infection in THP-1 cells. This result demonstrates that all the three classes of MAPKs are activated in response to *P. gingivalis*.

NF-κB activation

The transcription factors NF-κB, C/EBP, and CREB have been found to be crucial for the regulation of COX-2 transcription (Chun and Surh, 2004). To investigate whether *P. gingivalis* stimulated NF-κB, THP-1 cells were infected with *P. gingivalis* and nuclear extracts were prepared, and activation of NF-κB was determined by gel shift assays. As shown in Fig. 6, strong inducible NF-κB activity was detected after infection with *P. gingivalis*. This result indicates that *P. gingivalis* activates NF-κB in monocytic cells, resulting in enhanced transcription of COX-2 gene.

Discussion

Periodontal diseases are infectious diseases and are associated with the subgingival growth of periodontopathogens. However, much of the damage in the disease is actually the result of the host response to the bacteria, not the result of the bacteria themselves (Baker, 2000). Therefore, interactions of periodontopathogens with host cells and the resultant host cell responses are clearly of fundamental importance. *P. gingivalis* can adhere to and invade various types of host cells and has been observed in deep gingival tissue biopsy specimens (Andrian *et al.*, 2004; Deshpande *et al.*, 1998). Macrophages constitute a substantial proportion of the cells recovered from the gingival tissues, particularly the inflammatory tissues, of patients with periodontitis (Stoufi *et al.*, 1987). Although these macrophages play protective roles in the elimination of periodontopathogens from gingival tissues, they may contribute to periodontal pathogenesis by releasing many inflammatory mediators, including PGE_2.

The present study demonstrated that live *P. gingivalis* stimulates THP-1 monocytic cells to induce COX-2 expression and to produce PGE_2 without affecting COX-1 expression. This is the first report showing that live *P. gingivalis* strongly induces COX-2 in monocytic cells. Purified *P. gingivalis* LPS has been shown to induce COX-2 in monocytes (Noguchi *et al.*, 2000). Further experiments are needed to define whether the cell wall LPS of *P. gingivalis* plays a major role in live *P. gingivalis*-stimulated COX-2 induction by monocytic cells. Although LPS, along with FimA, is generally considered to be a major pathogenic factor of *P. gingivalis*, the host defense system may sense live bacteria differently than individual bacterial components, thus launching a different immune response (Zhou *et al.*, 2005).

This study demonstrated the importance of p38 MAPK, ERK, and NF-κB in *P. gingivalis*-stimulated COX-2 expression by THP-1 cells. Increased phosphorylation of ERK and p38 was observed in *P. gingivalis*-infected THP-1 cells. Pharmacological inhibition of p38 or ERK substantially reduced the COX-2 expression. Overexpression of COX-2 appears to be consequence of both increased transcription and enhanced mRNA stability (Chun and Surh, 2004). 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. As MAPKs regulate these transcription factors, one possible role for upregulated p38 and ERK in *P. gingivalis*-induced COX-2 expression is the positive regulation of the activity of transcription factors. Moreover, p38 has been shown to have a positive effect on COX-2 mRNA stability. The present study showed strong induction of NF-κB activation in *P. gingivalis*-infected THP-1 cells. There has been no previous report on activation of NF-κB and its involvement in COX-2 induction in *P. gingivalis*-infected monocytes/macrophages. Future studies should be aimed to define the activation of C/EBP and CREB and their roles in the COX-2 induction by *P. gingivalis*.

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