NFATc Mediates Lipopolysaccharide and Nicotine-Induced Expression of iNOS and COX-2 in Human Periodontal Ligament Cells

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Although nuclear factor of activated T cell (NFAT) plays a key role in inflammation, its anti-inflammatory effects and mechanism of action in periodontitis are still unknown. This study aimed to identify the effects of NFAT on the proinflammatory mediators activated by lipopolysaccharide (LPS) plus nicotine stimulation in human periodontal ligament cells (hPDLCs). The production of nitric oxide (NO) and prostaglandin E2 (PGE2) was evaluated using Griess reagent and an enzyme immunoassay, respectively. The expression of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) and NFAT proteins was evaluated by Western blot analysis. LPS plus nicotine synergistically induced the production of NO and PGE2 and increased the protein expression of iNOS, COX-2 and NFAT. Treatment with an NFAT inhibitor blocked the LPS plus nicotine-stimulated NO and PGE2 release as well as the expression of iNOS and COX-2. Our data suggest that the LPS plus nicotine-induced inflammatory effects on hPDLCs may act through a novel mechanism involving the action of NFAT. Thus, NFAT may provide a potential therapeutic target for the treatment of periodontal disease associated with smoking and dental plaque.

Key Words: Lipopolysaccharides, NFATc transcription factors, Nicotine, Periodontitis

Introduction

Periodontal disease initiation and progression occur as a consequence of the host response to pathogenic bacteria present in the dental biofilm. It is a chronic inflammatory disease that affects the periodontium resulting in the tooth supporting tissue destruction and even in the loss of the alveolar bone. Host immune response against this infection leads to the production of inflammatory mediators. The large amounts of released proinflammatory mediators, in addition to nitric oxide (NO) and prostaglandins (PGs), has been shown to be associated with periodontal disease through the activity of inducible enzymes such as inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2).

The inducible isoform, iNOS, is involved in immune response, which catalyzes the oxidative deamination of L-arginine to produce NO and is thus responsible for
prolonged NO production\(^9\). COX-2 is inducible in inflammatory conditions including periodontitis for the production of large amounts of proinflammatory PGs at the site of inflammation\(^9\). Based on these observations, understanding the regulation of proinflammatory mediators and their effects in periodontal tissues has been the objective of many studies\(^3-7\).

Human periodontal ligament cells produce cytokines and chemokines in response to inflammation promoters, including lipopolysaccharide (LPS)\(^8\). Also, it is reasonable to suggest that periodontal ligament cells play a key role as promoters of periodontal inflammation through these mechanisms\(^9\). Since a recent study suggested that periodontal ligament cells may have important implications for the development of new therapeutic strategies to treat periodontitis\(^10\).

Smoking is a major environmental risk factor in the development and progression of periodontal disease\(^11\), including gingivitis and periodontitis. It is associated with increased pocket depths, a loss of periodontal attachment and alveolar bone and a higher rate of tooth loss\(^12\).

Nicotine, the major toxic component of tobacco smoke, and its metabolites make the early signs of periodontal disease by the inflammatory response. Previous reports demonstrated that nicotine detected on the root surfaces of teeth, in the saliva\(^13\) and in the gingival crevicular fluid of smokers\(^14\). Although, nicotine is a cytotoxic agent to gingival fibroblasts and periodontal ligament cells, inhibiting their viability, attachment, and proliferation\(^11\), little is known about the effect of both nicotine and LPS on inflammatory responses in periodontal ligament cells.

The nuclear factor of activated T cell (NFAT) is composed of a family of transcription factors that includes 5 members, 4 of which are regulated by Ca\(^{2+}\) signaling; NFAT1 (NFATc2 or NFATp), NFAT2 (NFATc or NFATc1), NFAT3 (NFATc4), NFAT4 (NFATx or NFATc3), and calcineurin-independent NFAT5 (TonE-BP or NFATL1). The NFAT family of transcription factors plays a fundamental role in the transcriptional regulation of the immune response and is best known that regulate cytokine production after T-cell activation\(^15\). Furthermore, NFATs are not only restricted to the cells of the immune system, because their expression can be found in other tissues, including the heart\(^16\), muscle\(^17\), brain\(^18\), and endothelium\(^19,20\).

Although, a key function of NFAT in immune cells is to regulate the expression of potent immunomodulatory cytokines, the role of NFAT signaling pathway in periodontal ligament cells remains unclear. Thus, the purpose of the present study was to investigate whether NFATc1 could regulate on LPS and nicotine-induced iNOS and COX-2 expression in hPDLCs.

### Materials and Methods

#### 1. Cell culture

Immortalized hPDLCs\(^21\) transfected with human telomerase catalytic component (hTERT), were kindly provided by Professor Takashi Takata (Hiroshima University, Japan). The cells were cultured in \(\alpha\)-modified Eagle medium (\(\alpha\)-MEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 \(\mu\)g/mL streptomycin in a humidified atmosphere of 5% CO\(_2\) at 37\(^\circ\)C. \(\alpha\)-MEM, FBS, and penicillin/streptomycin were purchased from Gibco BRL Co. (Grand Island, NY, USA).

#### 2. Cell viability assay

The cytotoxicity of LPS (from Porphyromonas gingivalis) plus nicotine was determined by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma-Aldrich Chemical Co., St. Louis, MO, USA) assay. Cells seeded on 96-well microplates at 1 \(\times\) 10\(^4\) cells per well were incubated with LPS plus nicotine for the indicated time period. Medium was removed and then incubated with 100 \(\mu\)L MTT assay solution for 4 hours. Absorbance was measured in an microplate reader (Bio-Rad, Hercules, CA, USA) at 595 nm. The percentage of cell viability was calculated as the ratio of the absorbance of treated media that of the control media \(\times\)100.

#### 3. Quantification of nitric oxide

Thawed 50 \(\mu\)L aliquots of culture supernatant were mixed with 50 \(\mu\)L Griess reagent, comprising: 5% phosphoric acid (Fisher Scientific, Fairlawn, NJ, USA), 1% sulfanilamide and 0.1% N-(1-naphthylethyl)enediamine (Sigma Aldrich). Samples were incubated at room temperature for approximately 10 minutes and then read on an enzyme-
linked immunosorbent assay microplate plate reader (Bio-Rad) at 570 nm.

4. Determination of PGE2 levels

The culture medium of control and treated cells was collected, centrifuged, and stored at −70°C until tested. The level of PGE2 released into culture medium was quantified using a specific enzyme immunoassay according to the manufacturer’s instructions (Amersham, Arlington Heights, IL, USA).

5. Western blotting assay

The treated cells were washed with PBS and cytosolic and nuclear protein extracts were prepared using 1 × Cell Lysis buffer (Santa Cruz Biotechnology, Santa Cruz, CA, USA) supplemented with a protease inhibitor cocktail. Protein concentrations were determined using the Bradford assay (Bio-Rad) as per the manufacturer’s protocol. Proteins (30 μg) were mixed with an equal volume of 2 × sodium dodecyl sulphate (SDS) sample buffer, boiled for 5 minutes, and then resolved by SDS-polyacrylamide gel electrophoresis (10% acrylamide) and transferred to polyvinylidene difluoride membrane, immobilon-P (Millipore Co., Milford, MA, USA). Protein bands were detected using an enhanced chemiluminescence system (Amersham Biosciences, Buckinghamshire, UK, USA) according to the manufacturer’s instructions and exposed to X-ray film.

6. Statistical analysis

Differences among groups were analyzed using one-way analysis of variance with the IBM SPSS Statistics ver. 20.0 (IBM Co., Armonk, NY, USA) computer program. Statistical significance was determined at p < 0.05.

Results

1. Effects of LPS plus nicotine on cytotoxicity

We first assessed the effects of LPS plus nicotine on periodontal ligament cell viability via MTT assay. Periodontal ligament cells exposed to different concentrations of LPS plus nicotine for various lengths of time showed a concentration- and time-dependent reduction in cell viability compared with control cells (Fig. 1).

2. Effect of LPS plus nicotine on the iNOS/COX-2 expression and of NO/PGE2 production

Next, we examined the time course of LPS plus nicotine-induced changes in iNOS/COX-2 levels and NO/PGE2 production in hPDLCs. Co-treatment with LPS (1 μg/mL) plus nicotine (5 mM) resulted in a time-dependent increase of iNOS and COX-2 expression, with maximal induction after 18 or 24 hours of incubation (Fig. 2A). This combination of LPS plus nicotine also increased iNOS-derived NO (Fig. 2B) and COX-2-derived PGE2 (Fig. 2C), with maximal induction after 18 or 24 hours of incubation.

Fig. 1. Effects of lipopolysaccharide (LPS) plus nicotine on cytotoxicity. Cell viability was determined by MTT. Data were obtained from three independent experiments. Values are mean ± standard deviation of three experiments. *Statistically significant difference compared with control (p < 0.05).
Fig. 2. Effects of lipopolysaccharide (LPS) plus nicotine on the iNOS/COX-2 expression and of NO/PGE2 production. Cells were incubated for 24 hours with the indicated concentrations of LPS plus nicotine. The levels of expression were determined by western blotting, ELISA and Griess assay. Data were obtained from three independent experiments. Values are the mean±standard deviation of three experiments. *Statistically significant difference compared with control (p<0.05).

Fig. 3. Effects of lipopolysaccharide (LPS) plus nicotine on the NFATc1 activation and the phosphorylation of MAP kinase (MAPK).

NFATc1 protein activation induced by LPS plus nicotine, because the induction of NFATc1 by various stress stimuli have been implicated in inflammation. As shown in Fig. 3A, the NFATc1 expression induced by nicotine plus LPS was comparable to that in the control cultures in hPDLCs. To elucidate the molecular basis of the responses to NFAT, we examined the effects of LPS plus nicotine on the MAPK signaling pathways in hPDLCs. As shown in Figure 3B, LPS plus nicotine treatment induced the phosphorylation of p38 MAPK but not ERK or JNK.

Fig. 4A. Effects of NFATc1 the inhibitor, cyclosporine A (CsA), on LPS plus nicotine induced iNOS/COX-2 expression and of NO/PGE2 production

Moreover, cyclosporine A (CsA) pretreatment revealed a significant inhibitory effect on LPS plus nicotine-induced iNOS proteins and NO production in hPDLCs (Fig. 4A). In addition, CsA decreased the LPS plus nicotine-induced synthesis of COX-2 proteins and PGE2 production in hPDLCs (Fig. 4B).
Fig. 4. Effects of NFATc1 the inhibitor, cyclosporine A (CsA), on lipopolysaccharide (LPS) plus nicotine induced iNOS/COX-2 expression and of NO/PGE2 production. Cells were pretreated for 4 hours with CsA, and then incubated for 24 hours with the indicated concentrations of LPS plus nicotine. The levels of expression were determined by western blotting, ELISA and Griess assay. Data were obtained from three independent experiments. Values are the mean±standard deviation of three experiments. *Statistically significant difference compared with control (p<0.05). **Statistically significant difference compared with LPS plus nicotine (p<0.05).

Discussion

As the major constituent of the particulate phase of tobacco smoke, nicotine is a major contributor to periodontitis. Although previous studies reported that nicotine causes injury to periodontal ligament cells via multiple mechanisms, including c-Fos, COX-2, heme oxygenase-1 and oxidative stress, the molecular mechanisms of these effects have not been fully elucidated. Elevated *P. gingivalis* levels are present in periodontal lesions and are significantly reduced by successful therapy. LPS is a well-known endotoxin which elicits a variety of inflammatory responses. Furthermore, previous studies have reported that LPS from a periodontal pathogenic bacterium stimulated interleukin (IL) 6 and IL-8 production from hPDLCs, and these inflammatory cytokines play a role in the destruction and disintegration of periodontium. Previous studies have shown that LPS and nicotine is a cytotoxic agent to human fibroblasts derived from periodontium. In this study, we present evidence of LPS plus nicotine-induced cytotoxicity on hPDLCs by inhibiting cell growth and proliferation in a dose-dependent manner. Our results also showed that nicotine enhanced LPS plus nicotine-induced NO, iNOS, COX-2 and PGE2 synthesis in hPDLCs.

NFAT is highly phosphorylated and remain in the cytoplasm in unstimulated cells. When various physiological processes results in an increase in intracellular calcium level, dephosphorylation of NFAT translocate to the nucleus, and induces expression of NFAT target genes. The NFAT family of transcription factors regulates various biological processes such as cell survival, proliferation, migration, invasion, angiogenesis, and neural development and function. NFAT activation was first identified from the extracts of activated T lymphocytes. However, NFAT proteins also play key roles of diverse cellular functions within the cardiovascular system, skeletal muscle, bone, and nervous system. In addition, recent studies have demonstrated that NFATc1 not only a master transcriptional factor for induced in osteoclast precursors but also regulates bone homeostasis. NFATc1 over-expression in osteoblasts stimulates transcriptional...
activity of osterix, which are major osteoblastogenic transcription factors. Furthermore, our previous studies demonstrated that the first report of the expression of NFATc1 mRNA and protein being induced during osteoblastic differentiation in hPDLCs. However, the induction of NFAT, COX-2 and iNOS by LPS plus nicotine has not been verified in hPDLCs. Thus, we hypothesized that the induction of NFATc1 by LPS plus nicotine in hPDLCs may be responsible for the COX-2- and iNOS-inducing effects of treatment with LPS plus nicotine. Our first finding was that treatment of LPS plus nicotine synergistically induced NFATc1 activation in hPDLCs.

Moreover, MAP kinases play a critical role in the regulation of cell growth and differentiation and in the control of cellular responses to cytokines and other stresses. In the present study, LPS plus nicotine induced MAPK phosphorylation (Fig. 3B). In the present study, p38 MAPK was slightly activated in LPS plus nicotine-stimulated cells, while the level of phosphorylated ERK and JNK was basal level, indicating a constitutive activation of these pathways in hPDLCs. This suggests that, even though LPS plus nicotine-activated p38 MAPK play differential roles in iNOS and COX-2 induction.

In addition, the NFATc1 inhibitor CsA blocked the LPS plus nicotine-induced increases in iNOS/COX-2 expression and NO/PGE2 production. CsA, widely used pharmacological inhibitor, are known to block the calcineurin/NFATc1 signaling pathway. Our results are in agreement with Mena et al., who reported that COX-2 upregulation was inhibited by the NFAT antagonist CsA in endothelial cells.

These results suggest that LPS plus nicotine induced iNOS and COX-2 as well as NO and PGE2 production via the NFATc1-dependent pathway in hPDLCs. Based on these findings, we propose that NFATc1 as an inflammatory mediator represents a novel preventive or therapeutic target in periodontitis.

Summary

Host immune response and immune system are the main causes of individual susceptibility to periodontal diseases. Bacterial infection and smoking is an important environmental risk factor involved in the causation and progression of periodontal tissue destruction. Therefore, the aim of this study was to identify the effects of NFAT signaling pathway on LPS plus nicotine stimulated iNOS and COX-2 expression, a therapeutic target in periodontal disease, in hPDLCs. We also examined NO and PGE2 synthesis in LPS plus nicotine-stimulated hPDLCs. This study is the first to demonstrate that the inhibition of NFATc1 in LPS plus nicotine-stimulated hPDLCs results in the suppression of iNOS and COX-2 expression, as well as the reduction of NO and PGE2. Therefore, targeted NFATc1 regulation during inflammation, by modulating NO and PGE2 levels in PDL cells, may represent a novel pharmacological approach in the prevention or treatment of periodontal diseases caused by dental plaque and smoking.

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