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Nicotine and lipopolysaccharide stimulate the production of MMPs and prostaglandin E_2 by hypoxia-inducible factor-1α up-regulation in human periodontal ligament cells

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Background and Objective: Although hypoxia-inducible factor 1α (HIF-1α) is up-regulated in the periodontal pockets of periodontitis patients, the expression and precise molecular mechanisms of HIF-1α remain unknown in human periodontal ligament cells (PDLCs). The aim of this study was to explore the effects, as well as the signaling pathway, of nicotine and lipopolysaccharide (LPS) on the expression of HIF-1α and on the production of its target genes, including cyclooxygenase-2 (COX-2)-derived prostaglandin E_2 (PGE_2), MMP-2 and MMP-9 in PDLCs.

Material and Methods: The expression of COX-2 and HIF-1α proteins was evaluated using western blotting. The production of PGE_2 and MMPs was evaluated using enzyme immunoassays and zymography, respectively.

Results: LPS and nicotine synergistically induced the production of PGE_2, MMP-2 and MMP-9, and increased the expression of MMP-2, MMP-9, COX-2 and HIF-1α proteins. Inhibition of HIF-1α activity by chetomin or knockdown of *HIF1α* gene expression by small interfering RNA markedly attenuated the production of LPS- and nicotine-stimulated PGE_2 and MMPs, as well as the expression of COX-2 and HIF-1α. Furthermore, pretreatment with inhibitors of COX-2, p38, extracellular signal-regulated kinase, Jun N-terminal kinase, protein kinase C, phosphatidylinositol 3-kinase and nuclear factor-kappaB decreased the expression of nicotine- and LPS-induced HIF-1α and COX-2, as well as the activity of PGE_2 and MMPs.

Conclusion: These data demonstrate novel mechanisms by which nicotine and LPS promote periodontal tissue destruction, and provide further evidence that HIF-1α is a potential target in periodontal disease associated with smoking and dental plaque.

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Periodontal disease initiation and progression occur as a consequence of the host response to microorganisms in the dental biofilm. Periodontal bacteria and virulence factors, such as lipopolysaccharide (LPS), induce the release of various inflammatory molecules, including cytokines and prostaglandin E_2 (PGE_2), in inflamed periodontal tissues (1,2). Among inflammatory mediators involved in periodontitis, PGE_2 has been associated with periodontitis as it is a potent stimulator of bone resorption, and increased PGE_2 levels have been reported in gingival tissue and gingival fluid from patients with periodontitis (3,4). Moreover, administration of nonsteroidal anti-inflammatory drugs – inhibitors of PGE_2 production – have been shown to reduce the progression of alveolar bone resorption in patients with periodontitis, implying that PGE_2 is a key mediator in the pathogenesis of periodontal disease (5).

MMPs are a group of zinc-dependent endopeptidases that include collagenases, gelatinases, stromelysins, membrane-associated MMPs and other MMPs. Several members of the MMP family are involved in periodontal tissue destruction, including MMP-1, MMP-2, MMP-3, MMP-8, MMP-9 and MMP-13 (6). For example, significant concentrations of MMP-2 and MMP-9 have been demonstrated in both the gingival crevicular fluid and the periodontal tissues of patients and animals with periodontal disease (7,8), and MMP-9 levels in gingival crevicular fluid have been used to determine the stage of periodontitis (9). Indeed, MMP-2 and MMP-9 are involved in the progression, course and treatment of periodontitis (10). However, the role and the function of MMPs in periodontal tissue destruction is not completely understood.

Tobacco smoking might be an important risk factor and a prognostic factor for the development of periodontal disease (11). Nicotine, a major component of cigarette smoke, influences host–bacteria interactions and has been detected in the saliva and gingival crevicular fluid of smokers (12). The levels of expression of MMP-2 and MMP-9 are increased in nicotine-treated gingival fibroblasts and osteoblasts (13,14). We previously reported that the induction of cellular antioxidants and phase II enzymes contributes to the cellular defense mechanisms against nicotine-induced cytotoxicity and to RANKL expression in periodontal ligament cells (PDLCs) (15).

The combination of nicotine and LPS increases the expression of MMP-1, MMP-2, MMP-3 and tissue-type plasminogen activators in osteoblasts (16), stimulates the formation of osteoclast-like cells by increasing macrophage colony-stimulating factor and PGE_2 production in osteoblasts (17), and increases PGE_2 and cyclooxygenase-2 (COX-2) expression in osteoblasts (18). Furthermore, nicotine and LPS synergistically induce the production of nitric oxide and PGE_2, and increase the expression of inducible nitric oxide synthase (iNOS) and COX-2 via heme oxygenase-1 in PDLCs (19).

The transcriptional activator hypoxia-inducible factor 1 (HIF-1) is a master regulator of O_2 homeostasis that controls multiple physiological processes by regulating the expression of hundreds of genes (20). HIF-1 is a heterodimeric protein composed of a constitutively expressed HIF-1β subunit and an O_2-regulated HIF-1α subunit. Under hypoxic conditions, HIF-1α escapes ubiquitin degradation, forms a heterodimer with HIF-1β, binds to the cis-acting element (hypoxia-responsive elements) and activates downstream hypoxia-responsive genes (20,21). In addition to hypoxia, HIF-1α activity is up-regulated by a variety of nonhypoxic signals, including inflammatory cytokines, growth factors and bacterial products in normoxic conditions (22,23). Moreover, nicotine not only induces HIF-1α expression, but also stimulates the phosphatidylinositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways in human lung cancer cells (24). Taken together, these data suggest that the response to nicotine and LPS could involve HIF-1α activation and hypoxia signaling in human PDLCs. This assumption was further supported by reports of up-regulation of HIF-1α in the periodontal pockets of patients (25) and of up-regulation of interleukin (IL)-6 in gingival and synovial fibroblasts (26). However, the expression of HIF-1α has not been well elucidated in human periodontal ligament (PDL) tissue.

As PDLCs are the major constituents of the periodontium and are highly affected during periodontitis, the purpose of this study was to investigate the effects of nicotine and LPS on HIF-1α expression and on its major trans-regulating factors (its downstream target genes), including COX-2, MMP-2 and MMP-9, which are involved in periodontal tissue destruction, as well as on their signaling pathways in human PDLCs.

Material and methods

Reagents

LPS from *Porphyromonas gingivalis*, nicotine and anti-MKP-1 serum were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO, USA). Mouse anti-human HIF-1α serum was purchased from BD Biosciences Pharmingen (San Diego, CA, USA). Mouse anti-human COX-2 serum was from Cayman Chemical (Ann Arbor, MI, USA). The human PGE_2 ELISA Kit was from R&D Systems, Inc. (Minneapolis, MN, USA). Antibodies against phospho-Akt, Akt, PI3K, phospho-PDK-1, MMP-2 and MMP-9 were from Cell Signaling (Beverly, MA, USA). The inhibitors SB203580, PD98059, SP600125, LY294002, Ro318220, NS-398, chetomin and PDTC were from Calbiochem (La Jolla, CA, USA).

Cell culture

We used the immortalized human PDL cell lines by transfection with telomerase catalytic subunit hTERTgene (27), which was kindly provided by professor Takashi Takata (Department of Oral and Maxillofacial Pathobiology, Hiroshima Uniiversity, Japan). Cells were cultured in Dulbecco's modified Eagle's minimal essential medium supplemented with 10% fetal bovine serum, 100 U/mL of penicillin and 100 μg/mL of streptomycin in a humidified atmosphere of 5% CO_2 at 37°C.

Treatments

For stimulation experiments, the cells were seeded into culture dishes and

cultured in Dulbecco's modified Eagle's minimal essential medium containing 10% fetal bovine serum for 3 d until 70% confluent, after which the medium was replaced with serum-free medium in order to minimize any serum-induced effects on PDLCs. Subsequently, the cells were exposed to LPS plus nicotine for 24 h. All treatments were performed in duplicate.

Western blot analysis

Cells (1×10^6) from each set of experiments were harvested and washed twice in cold Tris-buffered saline then solubilized in ice-cold 1% Triton X-100 lysis buffer. After 30 min on ice, the lysates were clarified by centrifugation. Proteins (20 μg) were resolved by sodium dodecyl sulfate–polyacryamide gel electrophoresis (SDS-PAGE; 10% acrylamide), transferred to nitrocellulose membranes and probed with specific antibodies (diluted 1/1000), then incubated with secondary horseradish peroxidase-conjugated antibody (diluted 1/5000). Proteins were detected by the enhanced chemiluminescence system, according to the manufacturer's instructions (Amersham, Buckinghamshire, UK), and exposed to X-ray film.

Measurement by ELISA

The concentrations of PGE_2 in the culture supernatants were determined by an ELISA kit, according to the manufacturer's recommended procedure (R & D Systems, Minneapolis, MN, USA). The plates were read at 450 nm on a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Zymography

PAGE was carried out using a mini protein system (Bio-Rad, Richmond, CA, USA). A 10% SDS-PAGE running gel containing 0.1% (weight by volume) gelatin was overlaid with a 4% acrylamide stacking gel. A 10-μL sample was mixed with an equal volume of Laemmli sample buffer (10% SDS; 125 mM Tris–HCl, pH 6.8, 10% glycerol and 0.002% Bromophenol Blue) and incubated for 2 h at 37°C; the sample was then applied to the gel, which was run at 100 V for 4 h at 4°C. After electrophoresis, the gels were soaked and washed twice in 2.5% Triton X-100 for 30 min at 37°C and then incubated in 50 mM Tris buffer, pH 7.5, containing 5 mM $CaCl_2$, 1 μM $ZnCl_2$ and 0.2% Brij 35 (volume by volume) with gentle shaking at 37°C for 18 h. Following incubation, the gels were stained for 2 h with 0.5% Coomassie Brilliant Blue G-250 in a solution of 20% acetic acid and 20% methanol, and were then destained for 30 min in the same solution without Coomassie Blue R-250. MMP activity was visualized as clearance zones in the stained gels. Clear bands representing areas of gelatinolytic activity appeared over the blue background. After rinsing in distilled water for 30 min, the Coomassie-stained gel was scanned and the digital images were obtained from the scanner.

HIF-1α small interfering RNA transfection

Small interfering RNA (siRNA)-annealed oligonucleotide duplexes for HIF-1α (Sequence $5' \rightarrow 3'$ sense: AGAGGUGGAUAUGUGUGGGdtdt; antisense: CCCACACAUAUCCACCUCUdtdt) and a negative control (Catalog No. SN-1003) were purchased from Bioneer Corporation (Daejeon, South Korea) and human PDLCs were transfected with siRNA using Lipofectamine2000 (Gibco; Invitrogen Ltd, Paisley, UK) following the manufacturer's instructions.

Statistical analysis

Differences among the groups were analyzed using one-way analysis of variance combined with Duncan's multiple range tests.

Results

Nicotine and LPS induce cytotoxicity, HIF-1α and COX-2 expression, and MMP and PGE_2 production

We first assessed the effects of nicotine alone on the expression levels of HIF-1α and COX-2, and on PGE_2 production. PDLCs exposed to different concentrations of nicotine for 24 h showed a concentration-dependent increase in HIF-1α and COX-2 expression, and in PGE_2 production, compared with control cells (Fig. 1A and 1D).

To explore the effects of nicotine and LPS on the expression levels of HIF-1α and COX-2 and on PGE_2 production, PDLCs were treated with various concentrations of nicotine and 1 μg/mL of LPS for 24 h. As shown in Fig. 1B, low basal expression levels of HIF-1α and COX-2 proteins were present in the control, unstimulated cells. LPS significantly increased the levels of expression of HIF-1α and COX-2 proteins in PDL cells. Furthermore, expression of HIF-1α and COX-2 were enhanced by LPS and nicotine. However, HIF-1β protein was not induced. The combination of LPS and nicotine also increased COX-2-derived PGE_2, with maximal induction at 1 μg/mL of LPS and 5 mM nicotine (Fig. 1E). In addition, nicotine and LPS induced HIF-1α and COX-2 expression, and PGE_2 production increased in a time-dependent manner, with maximal induction occurring after 24 h of incubation (Fig. 1C, 1D and 1F).

In order to evaluate the effects of nicotine and LPS on cytotoxicity, we evaluated cell viability using the MTT assay. Figure 2A demonstrates that the cell viability decreased in a dose-dependent manner following exposure to nicotine. Treatment with the combination of nicotine and LPS resulted in significant cytotoxicity in a concentration- and time-dependent manner (Fig. 2B and 2C). Maximal cytotoxicity was achieved at 1 μg/mL of LPS and 10 mM nicotine in PDLCs.

Next, the effects of nicotine- and LPS-induced MMP-2 and MMP-9 enzyme activity and gene expression were examined, because MMP-2 and MMP-9 activation may be associated with tissue destruction related to periodontal inflammation (10). As shown in Fig. 2D and 2G, nicotine significantly increased the levels of *MMP2* and *MMP9* mRNA and MMP-2 and MMP-9 protein in PDLCs. Furthermore, MMP-2 and MMP-9 protein expression and enzyme activity were enhanced by the combination of

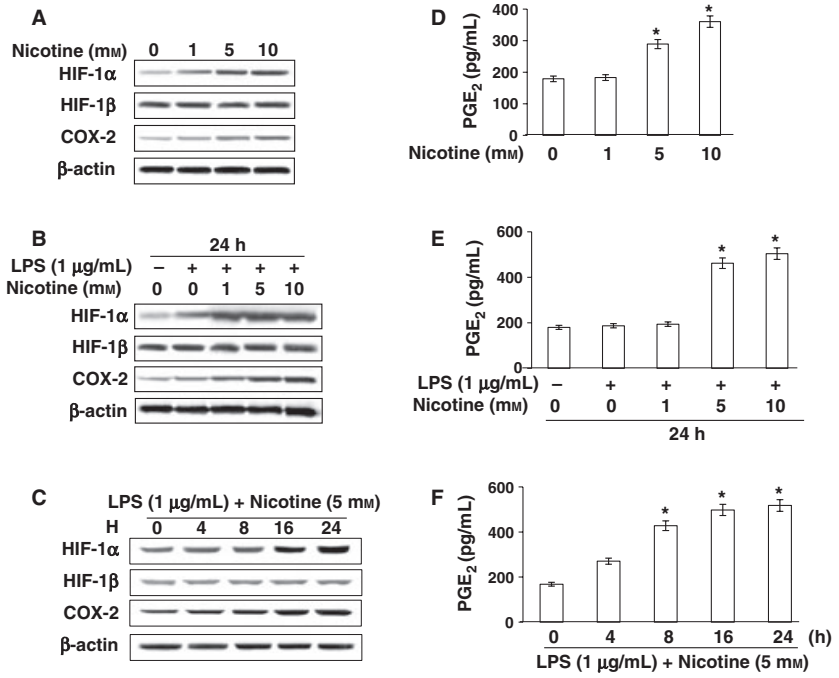

Fig. 1. Effects of lipopolysaccharide (LPS) and nicotine on the expression of hypoxia-inducible factor (HIF)-1α and HIF-1β proteins (A–C) and on the production of prostaglandin E_2 (PGE_2) (D–F). Cells were incubated for 24 h with the indicated concentrations of nicotine. The levels of expression were determined by western blotting, ELISA and zymograms. Data were obtained from three independent experiments. Values are the mean ± standard deviation of three experiments. *Statistically significant difference compared with control, $p < 0.05$.

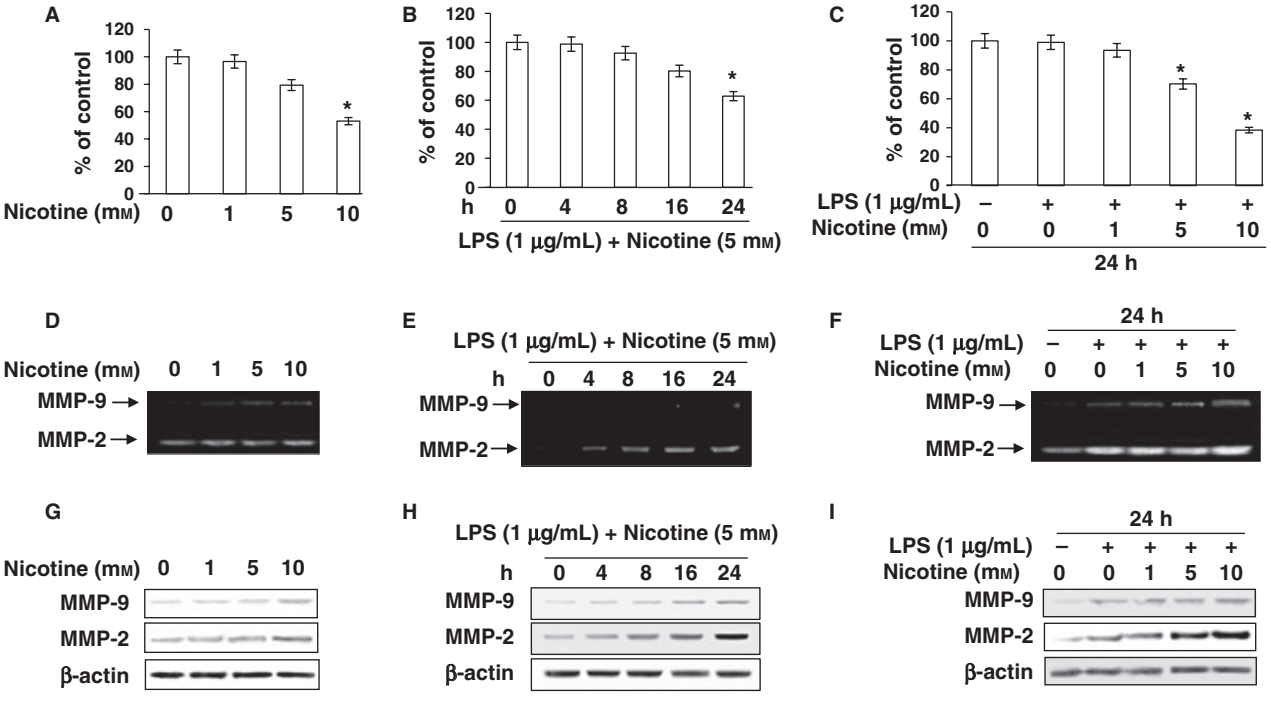

Fig. 2. Effects of lipopolysaccharide (LPS) and nicotine on cytotoxicity (A–C) and on the expression and activity of MMP-2 and MMP-9 protein (D–I). Cells were incubated for 24 h with the indicated concentrations of nicotine. Cell viability was measured using the MTT assay. Expression levels were determined by western blotting and by zymograms. Data were obtained from three independent experiments. Values are mean ± standard deviation of three experiments. *Statistically significant difference compared with control, $p < 0.05$.

nicotine and LPS, in a dose- and time-dependent manner (Fig. 2E, 2F, 2H and 2I).

Effects of HIF-1α inhibition on nicotine- and LPS-induced COX-2α expression and MMP production

To test whether nicotine and LPS are involved in COX-2 expression and MMP production regulated by HIF-1α, we used inhibitors of HIF-1α (chetomin) and HIF-1α siRNA. The siRNA and chetomin successfully inhibited HIF-1α protein expression, but the scrambled siRNA used as a control did not (Fig. 3A). HIF-1α siRNA and the HIF-1α inhibitor abolished the induction effects of nicotine alone, LPS alone and the combination of nicotine and LPS, on PGE_2 production and COX-2 expression (Fig. 3A and 3B). However, chetomin and HIF-1α siRNA did not affect nicotine alone, LPS alone or the combination of nicotine and LPS-induced cytotoxicity (Fig. 3C). Moreover, HIF-1α siRNA and chetomin blocked the stimulatory effects of LPS alone, nicotine alone and the combination of LPS and nicotine on MMP-2 and MMP-9 production and protein expression (Fig. 3D and 3E).

Effects of COX-2 inhibition on nicotine- and LPS-induced HIF-1α expression and MMP production

Because COX-2 is up-regulated in nicotine- and LPS-treated PDLCs, we investigated the effect of the COX-2 inhibitor on nicotine- and LPS-mediated HIF-1α expression and MMP production. As shown in Fig. 4A, treatment of PDLCs with the COX-2 inhibitor, NS-398, significantly suppressed the stimulatory effects of nicotine alone, LPS alone and the combination of nicotine and LPS on COX-2 and HIF-1a protein expression. The zymography, western blot and ELISA analyses showed that PGE_2, MMP-2 and MMP-9 protein expression and enzyme activity increased as a result of treatment with nicotine alone, LPS alone and the combination of nicotine and LPS and were blocked by the addition of NS-398 (Fig. 4B–E).

Effects of nicotine and LPS on activation of signal pathways

The PI3K/Akt signal transduction cascade regulates MMPs and HIF-1α (24). To examine whether nicotine and LPS signal via this pathway in PDLCs, we examined the expression of PI3K and the phosphorylation state of Akt and 3-phosphoinositide-dependent protein

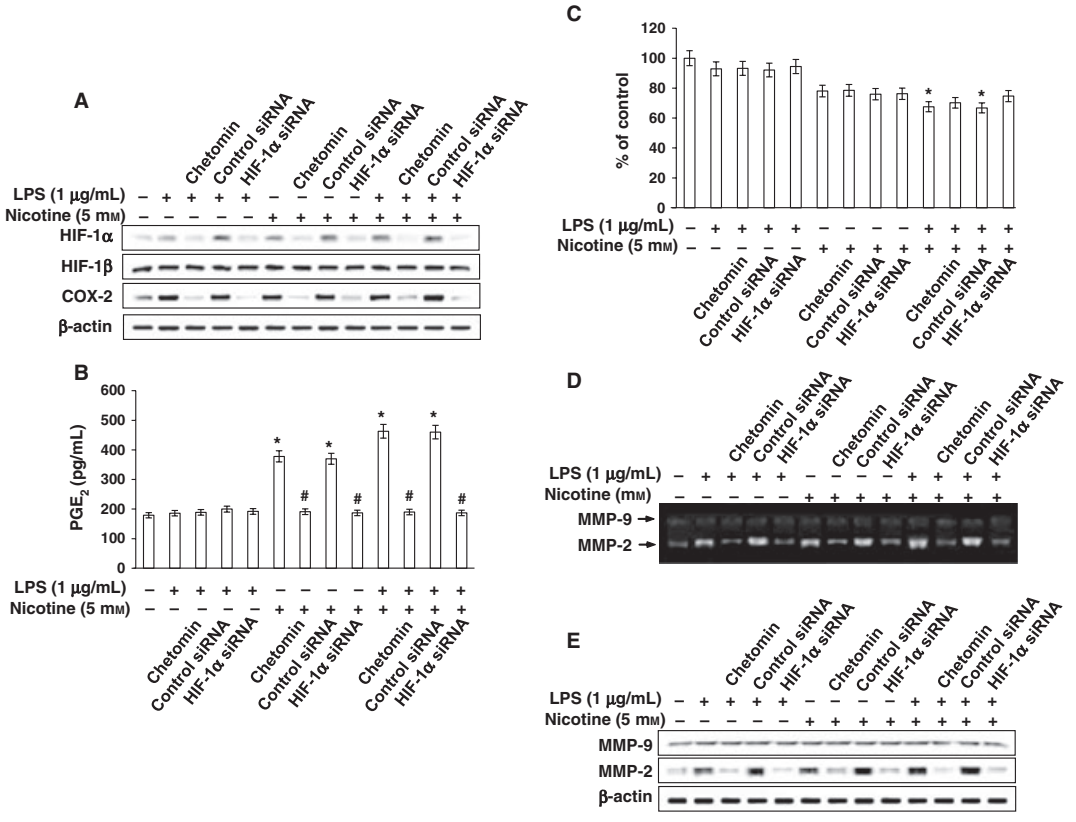

Fig. 3. Effects of hypoxia-inducible factor 1α (HIF-1α) inhibition on lipopolysaccharide (LPS) plus nicotine-induced HIF-1α (A), cytotoxicity (B), prostaglandin E_2 (PGE_2) (C) and MMP-2 and MMP-9 (D, E) production by periodontal ligament cells (PDLCs). Cells were pretreated for 1 h with 1 nM chetomin (an inhibitor of HIF-1α) or were transiently transfected with control small interfering RNA (siRNA) and HIF-1α siRNA, following 24 h of treatment with LPS plus nicotine. Expression levels were determined by western blotting, ELISA and zymograms. Cell viability was measured using the MTT assay. The mean ± standard deviation of three experiments is shown. *$p < 0.05$ with respect to the untreated control group. $p < 0.05$ with respect to the group treated with LPS alone, with nicotine alone and with the combination of 1 μg/mL of LPS plus 5 mM nicotine.

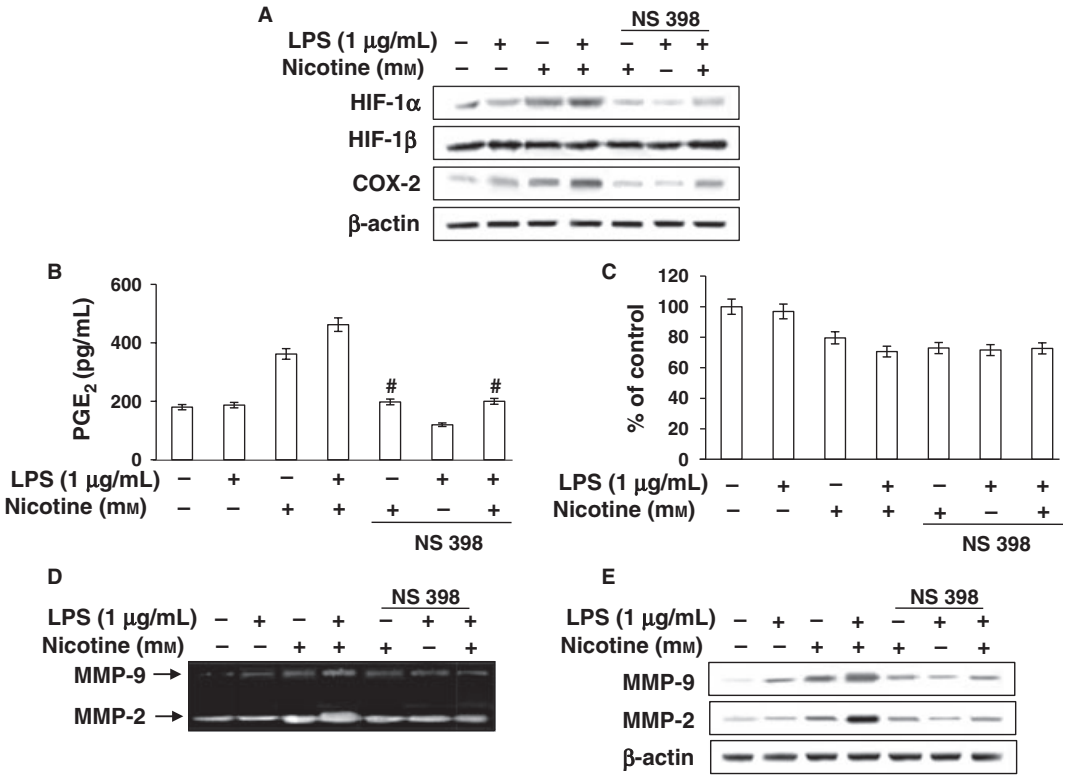

Fig. 4. Effects of the cyclooxygenase-2 (COX-2) inhibitor on lipopolysaccharide (LPS) plus nicotine-induced production of hypoxia-inducible factor 1α (HIF-1α) protein (A) and prostaglandin E_2 (PGE_2) production (B), on cytotoxicity (C) and on MMP-2 and MMP-9 protein expression and activity (D, E). Cells were pretreated for 1 h with 5 μM NS-398 (COX-2 inhibitor) and then incubated with 1 mg/mL of LPS plus 5 mM nicotine for 24 h (A–D). *$p < 0.05$ with respect to the untreated control group. $p < 0.05$ with respect to the group treated with LPS alone, nicotine alone and the combination of 1 μg/mL of LPS plus 5 mM nicotine.

kinase-1 (PDK-1). Western blotting revealed that nicotine and LPS increased the expression of PI3K, with a maximal response at 60 min. In addition, nicotine and LPS significantly phosphorylated both PI3K downstream targets, PDK-1 and Akt (Fig. 5A).

Moreover, to investigate the downstream PI3K signaling pathway, we probed nicotine- and LPS-treated cell lysates for protein kinase C (PKC). As shown in Fig. 5B, nicotine and LPS significantly increased the expression of PKC, with maximal expression detected at 60 min. Given that MAPK and the nuclear factor-kappa B (NF-κB) signaling pathway are involved in nicotine- and LPS-treated PDLCs (19), we examined the expression of MAPK phosphatase-1 (MKP-1), a family related to MAPK. As shown in Fig. 5B, nicotine and LPS markedly induced MKP-1 expression in a time-dependent manner.

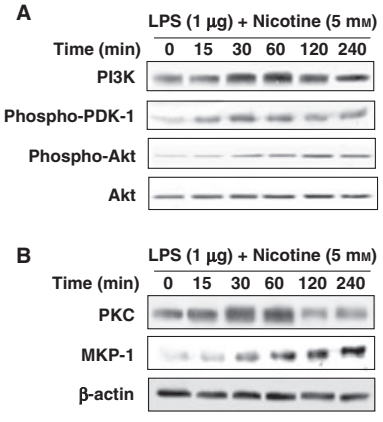

Fig. 5. Effects of lipopolysaccharide (LPS) plus nicotine-induced phosphatidylinositol 3-kinase (PI3K) activation (A), and protein kinase C (PKC) and MAPK phosphatase-1 (MKP-1) activation (B) in human periodontal ligament cells (PDLCs). Results are from three independent representative experiments.

Effects of PI3K, PKC, MAKP and NF-κB inhibitors on nicotine- and LPS-induced COX-2 expression and MMP production

To further investigate whether nicotine and LPS induce HIF-1α expression through the PI3K/Akt, PKC, MAPK and nuclear factor-kappaB (NF-κB) pathways, cells were pre-incubated with various pharmacological inhibitors of signaling intermediates before treatment with nicotine and LPS. Treatment with the p38 inhibitor (SB203580), the extracellular signal-regulated kinase (ERK) inhibitor (PD98059), the Jun N-terminal kinase (JNK) inhibitor (SP600125), the pan-PKC inhibitor (RO318220), the PI3K

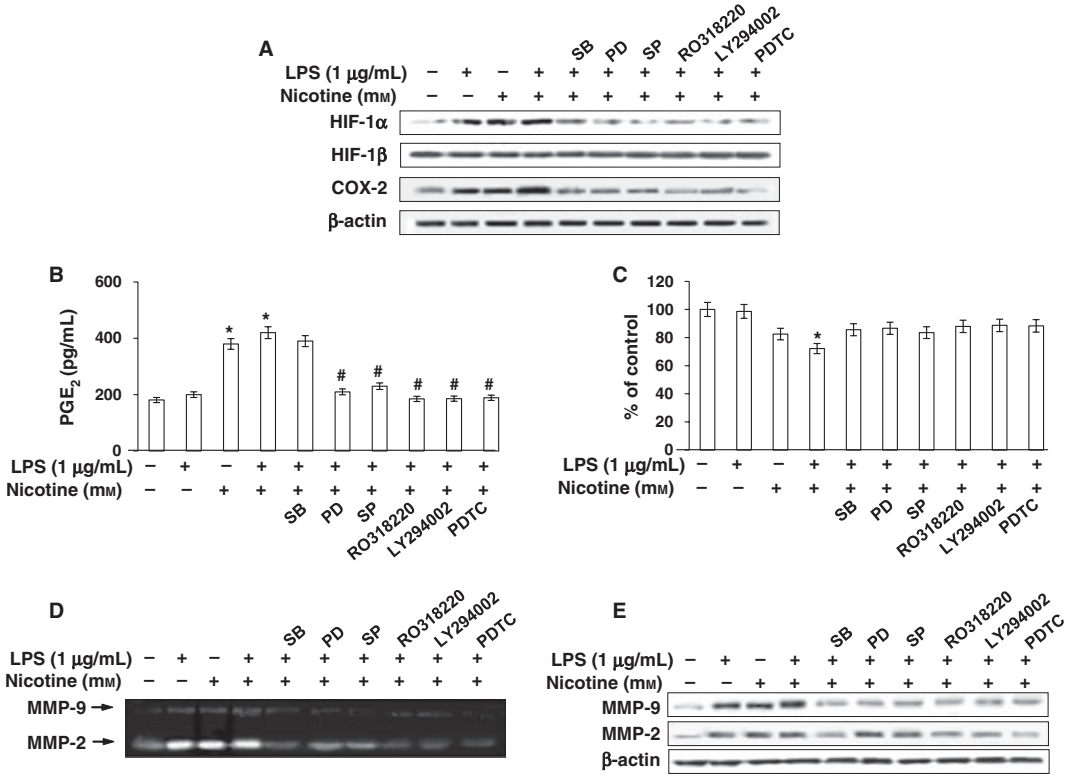

Fig. 6. Effects of various signal pathway inhibitors on lipopolysaccharide (LPS) plus nicotine-induced expression of hypoxia-inducible factor 1α (HIF-1α) protein (A), prostaglandin E_2 (PGE_2) production (B), cytotoxicity (C) and MMP-2 and MMP-9 protein expression and activity (D, E) in human periodontal ligament cells (PDLCs). Cells were pretreated for 1 h with various key signal pathway inhibitors [20 μM SB203580, the p38 mitogen-activated protein kinase (p38 MAPK) inhibitor; 20 μM PD98059, the extracellular signal-regulated kinase 1/2 (ERK1/2) inhibitor; 10 μM SP600125, the Jun N-terminal kinase (JNK) inhibitor; 20 μM RO318220, the protein kinase C (PKC) inhibitor; 10 μM LY294002, the phosphatidylinositol 3-kinase (PI3K) inhibitor; and 1 mM PDTC, the nuclear factor-kappaB (NF-κB) inhibitor] then incubated with 1 μg/mL of LPS plus 5 mM nicotine for 24 h. Data are the mean ± standard deviation values of three experiments. *$p < 0.05$ with respect to the untreated control group. $p < 0.05$ with respect to the group treated with 1 μg/mL of LPS plus 5 mM nicotine. PD, PD98059; SB, SB203580; SP, SP600125.

inhibitor (LY294002) and the NF-κB inhibitor (PDTC) blocked the effects of LPS plus nicotine on the expression of HIF-1α and COX-2 protein (Fig. 6A), but did not affect cytoxicity (Fig. 6C). Similar down-regulation was observed with PGE_2 production, MMP-2 and MMP-9 gelatinolytic activities, and MMP-2 and MMP-9 protein expression (Fig. 6B, 6D and 6E).

Discussion

The host inflammatory response to smoking and periodontopathogens is considered a major causative factor in the local tissue destruction observed in periodontitis (1,2,11). Although our previous studies demonstrated that nicotine and LPS cause degradation of PDLCs via multiple mechanisms, including Nrf-2, heme oxygenase-1 and endoplasmic reticulum stress (15,19,28), the molecular mechanisms of periodontal tissue destruction have not been fully elucidated.

To select the experimental concentration of nicotine, we assumed that nicotine infiltrates the gingival epithelium and influences the function of PDLCs. Cigarettes can deliver up to 1.9 mg of nicotine per cigarette (29). If one smokes a pack of cigarettes each day, one can be exposed to 38 mg of nicotine. Smokeless tobacco contains 0.59–3.35% (weight by weight) nicotine (30), and the saliva of a user of smokeless tobacco can contain up to 1560 μg/mL (9.6 mM) of nicotine (31). The levels of nicotine used are those concentrations of nicotine observed in the blood circulation of habitual cigarette smokers (0.06–1.2 mM) (32) and the saliva levels of long-term snuff users (0.6–9.6 mM) (31). Nicotine is able to rapidly penetrate the epithelial barrier of skin or oral mucosa where it may be partially retained and metabolized. As the epithelial barrier of the inflamed gingiva may be more disorganized and may have widened intercellular spaces (33), the fibroblasts of the inflamed periodontium could be reached almost directly by nicotine. Existing periodontal disease may facilitate the penetration of nicotine through the epithelial barrier. Thus, fibroblasts of the inflamed periodontium may be directly exposed to high concentrations of nicotine (34).

Many studies have shown the effects of continued smoking on persistent gingival bleeding, vertical bone loss

and poor treatment outcomes (11,35). Similarly, *in vitro* studies have demonstrated that nicotine inhibits the attachment and growth of human gingival fibroblasts (HGFs) and PDLCs (36–38). Nicotine concentrations higher than 3.9 mM resulted in changes in the morphology of the cytoskeletal elements, microtubules and vimentin (34). Moreover, HGFs and PDLCs exposed to nicotine have an increased number of vacuoles and decreased fibroblast growth at nicotine concentrations higher than 7.8 mM (34). Nicotine doses between 10.5 and 15.5 mM in HGFs and PDLCs cause irreversible toxicity compared with lower concentrations (34). In addition, altered the response to transforming growth factor-beta1 by decreasing the morphologic change from HGFs to myofibroblast cells (39), supporting the theory that nicotine interferes with wound closure by changing the ability of the cells to contract wounds. In this study, treatment of PDLCs with nicotine concentrations of 1–10 mM induced cytotoxicity in dose-dependent manner, which is consistent with the results obtained for nicotine concentrations of 2.5–25 mM in human PDLCs (36) and of 1–20 mM in HGFs (37). This cytotoxic effect of nicotine may partly explain the role of smoking as an important risk factor regarding the susceptibility to destruction of the periodontium and the low regenerative response during periodontal therapy. Five millimolar nicotine was mainly used in the present study because this concentration has been previously shown to modulate nicotine-induced COX-2 and iNOS expression in PDLCs (19). However, in our preliminary study, treatment with < 1 mM nicotine did not significantly up-regulate COX-2 and iNOS expression, or HIF-1α expression (data not shown), and therefore we did not use such nicotine concentrations in the HIF-1α-related experiments in this study.

The LPS of *P. gingivalis* is a potent stimulator of inflammatory cytokine production and tissue destruction in periodontitis, resulting in tooth loss (40). Previously we reported that 1 μg/mL of *P. gingivalis* LPS up-regulated COX-2 and iNOS expression in PDLCs (19). Therefore, 1 μg/mL of LPS was used in the present study. Given that LPS from *P. gingivalis* and nicotine are indices of mouth cleaning and smoking, LPS and nicotine were used to study the modulation of inflammatory molecules. The present study was designed to clarify the role of HIF-1α in regulating COX-2 expression and MMP production as a therapeutic target in periodontal disease.

In addition to tumor promotion, HIF-1α appears to play a role in inflammatory processes. Previous studies have reported that HIF-1α was induced at the mRNA level in gingival and synovial fibroblasts, and that IL-1 increased the binding of heterodimeric HIF-1 to the HIF consensus sequence, suggesting a role in inflammation (26). In addition, IL-1β up-regulated HIF-1α protein through a classical inflammatory signaling pathway involving NF-κB and COX-2, culminating in the up-regulation of vascular endothelial growth factor in A549 cells (22). The expression of HIF-1α was detected in an antigen-induced arthritis model (41) and in macrophages in the rheumatoid synovium (42). Furthermore, HIF-1α-positive immunoreactivity was significantly higher in periodontal pockets than in control gingival samples (25). Taken together, these data implicate HIF-1α as an important mediator of inflammation in periodontal disease.

To examine the role of HIF-1α signaling in the response to nicotine and LPS, we first analyzed the protein levels of HIF-1α. Our results indicate that nicotine and LPS are able to induce HIF-1α expression in a time- and a dose-dependent manner, whereas the HIF-1β protein level is not affected by nicotine or LPS in PDLCs. Following exposure to nicotine and LPS, in parallel with up-regulation of HIF-1α protein, we detected increased protein levels of the HIF-1α target genes *COX2*, *MMP2* and *MMP9*, as well as elevated production of HIF-1α.

Based on our studies showing that the induction of HIF-1α by nicotine and LPS leads to the production of PGE_2 and MMPs, we hypothesized that the induction of HIF-1α by nicotine and LPS would lead to the up-regulated expression of COX-2 and MMPs. To clarify this, we transfected siRNA for the HIF1α gene into PDLCs or treated the cells with chetomin, an HIF-1α inhibitor, to suppress HIF-1α expression or activity. Both treatments significantly suppressed the nicotine- and LPS-induced expression of COX-2, MMP-2 and MMP-9 protein, as well as the production of PGE_2 and MMPs, suggesting that increases in PGE_2 and MMPs by treatment with nicotine and LPS may be mediated by HIF-1α expression.

The production of inflammatory molecules, such as PGE_2 and MMPs, are considered a major target for periodontal therapy because they are inducible and present in cells involved in periodontal disease (1,2,7,8). Thus, we focused on signal transduction pathways related to the expression of HIF-1α, COX-2 and MMPs. The role of COX-2-mediated expression and MMP production was studied using NS-398, a specific inhibitor of COX-2 with the capacity to inhibit PGE_2 biosynthesis induced by inflammatory mediators in PDLCs (43). The finding that NS-398 reduced nicotine- and LPS-induced MMP-2 and MMP-9 production as well as HIF-1α expression suggests that nicotine and LPS up-regulate the functional HIF-1α protein through the COX-2 pathway, a classical inflammatory signaling pathway, culminating in the up-regulation of MMPs in PDLCs. These results are in agreement with a previous report in which a selective COX-2 inhibitor, prophylactic omega-3 fatty acid, and celecoxib inhibited LPS-induced MMP-8 expression in the gingival tissue of experimental periodontitis (44). In addition, the COX-2 inhibitors NS-398, celecoxib and rofecoxib caused decreases in the level of IL-1β-stimulated IL-6 and PGE_2 in HGFs (45).

Given that HIF-1α can be induced via activated PI3K (24), we explored the role of PI3K on nicotine- and LPS-mediated expression of HIF-1α and periodontal tissue destruction in PDLCs. Akt, a serine-threonine kinase and a downstream target of PI3K, is regulated by PTK-1 (46). We found that nicotine and LPS greatly increased the expression of PI3K, as well as Akt

and PDK-1 phosphorylation, in PDLCs. Treatment of cells with the PI3K-specific inhibitor, LY294002, blocked nicotine- and LPS-induced up-regulation of HIF-1α, COX-2, MMP-2 and MMP-9, and subsequently inhibited the production of PGE_2 and MMPs. These results suggest that the PI3K/Akt/PDK-1 pathway is involved in the regulation of HIF-1α, PGE_2 and MMPs in PDLCs.

The MAPK and NF-κB signal transduction pathways also regulate HIF-1α expression and activity (47). The duration and magnitude of MAPK activity is linked to the action of specific phosphatases. The MKPs family, a dual-specificity (threonine/tyrosine phosphatases) group of enzymes, plays an important role in regulating MAPK activity (48). One member of the MKPs family is MKP-1, a nuclear enzyme rapidly induced by growth factors, hormones and stress signals, which displays activity towards ERKs, JNKs and p38 (48). Furthermore, PKC involves Akt activation, a key component of HIF-1α signaling pathways (49). In human PDLCs, nicotine and LPS activated p38, ERK and JNK MAPKs, resulting in the subsequent activation of NF-κB (19). To further understand the pathways that are affected by HIF-1α, we analyzed the pathways downstream of PI3K, namely PKC, MAPK and NF-κB. Specifically, we demonstrated that PKC and MKP-1 were increased in PDLCs treated with nicotine and LPS. Furthermore, pretreatment with p38, ERK, JNK, PKC and NF-κB inhibitors suppressed the induction of HIF-1α, PGE_2, MMP-2 and MMP-9 by nicotine and LPS. Similar findings have been reported in human PDLCs, in which IL-1β-induced MMP-1 and PGE_2 production was significantly blocked by treatment with three MAPKs and an NF-κB inhibitor (50).

To the best of our knowledge, this study is the first to demonstrate that nicotine and LPS lead to the up-regulation of HIF-1α, which subsequently up-regulates PGE_2, MMP-2 and MMP-9 production via the PI3K, p38, ERK, JNK, PKC and NF-κB pathways. Therefore, our results suggest that HIF-1α is an apparent mediator of periodontal tissue destruction and can be considered a novel molecular target for the prevention and treatment of periodontal disease.

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