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Heme oxygenase-1 mediates nicotine- and lipopolysaccharide-induced expression of cyclooxygenase-2 and inducible nitric oxide synthase in human periodontal ligament cells

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Background and Objective: Although heme oxygenase-1 (HO-1) 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 HO-1 on the proinflammatory mediators activated by nicotine and lipopolysaccharide (LPS) stimulation in human periodontal ligament (PDL) cells.

Material and Methods: The production of nitric oxide (NO) and prostaglandin E_2 (PGE_2) was evaluated using Griess reagent and an enzyme immunoassay, respectively. The expression of inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2) and HO-1 proteins was evaluated by Western blot analysis.

Results: Lipopolysaccharide and nicotine synergistically induced the production of NO and PGE_2 and increased the protein expression of iNOS, COX-2 and HO-1. Treatment with an HO-1 inhibitor and HO-1 small interfering RNAs blocked the LPS- and nicotine-stimulated NO and PGE_2 release as well as the expression of iNOS and COX-2.

Conclusion: Our data suggest that the nicotine- and LPS-induced inflammatory effects on PDL cells may act through a novel mechanism involving the action of HO-1. Thus, HO-1 may provide a potential therapeutic target for the treatment of periodontal disease associated with smoking and dental plaque.

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Nicotine is a major component of the particulate phase of tobacco smoke and induces vascular changes in gingival tissue (1–3). Although previous reports have detected nicotine on the root surfaces of teeth (4), in the saliva and in the gingival crevicular fluid of smokers (5), little is known about the effect of nicotine on inflammatory

responses in periodontal ligament (PDL) cells.

The PDL cells not only function as support cells for periodontal tissues but also produce inflammatory mediators that recognize various molecules, including lipopolysaccharide (LPS) (6). Bacterial products, particularly endotoxins and LPS, play a key role in the destruction of periodontal tissue, including gingiva, PDL and alveolar bone (7).

The release of large amounts of proinflammatory cytokines, 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) (8). Inducible nitric oxide synthase is one of the three isoforms of nitric oxide synthase, which catalyzes the oxidative deamination of L-arginine to produce NO and is thus responsible for prolonged NO production (9). Cyclooxygenase-2 is induced by several stimuli and is responsible for the production of large amounts of proinflammatory prostaglandins at the site of inflammation (10). Based on these observations, it was hypothesized that the inhibition NO and PGE_2 production by blockadeof iNOS and COX-2 could serve as the basis for the development of anti-inflammatory drugs to treat periodontitis.

Heme oxygenase-1 (HO-1) is a key cytoprotective enzyme and is the rate-limiting enzyme involved in the degradation of heme to bilirubin, carbon monoxide (CO) and iron (11). Recently, HO-1 was suggested to play a regulatory role in the resolution phase of inflammation and is considered to be a potential therapeutic target for the treatment of inflammatory diseases (12,13). However, the role of HO-1 as a mediator of inflammation has not been reported in PDL cells. Nicotine and LPS stimulate the production of PGE_2, NO, COX-2 and inflammatory mediators (14–17), and HO-1 may be involved in the inflammatory response of PDL cells to LPS and nicotine.

The present study investigated the effects of nicotine and LPS, which result from smoking and dental plaque-induced periodontitis, on the production of proinflammatory mediators in human PDL cells. We aimed to explore the therapeutic potential of HO-1 in LPS- and nicotine-induced iNOS and COX-2 expression, as well as NO and PGE_2 production, in the context of relevant signaling pathways in human PDL cells.

Material and methods

Reagents

Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and other tissue culture reagents were obtained from Gibco BRL Co. (Grand Island, NY, USA). Ultrapure LPS from *P. gingivalis* was purchased from InvivoGen (San Diego, CA, USA). The HO-1 activator cobalt (III) protoporphyrin IX chloride (CoPP) and HO-1 inhibitor tin protoporphyrin (SnPP) were purchased from Porphyrin Products (Logan, UT, USA). Anti-I-κBα, anti-phospho-I-κBα, anti-iNOS, anti-COX-2 and anti-HO-1 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Nicotine and all other chemicals were obtained from Sigma Chemical Co. (St Louis, MO, USA) unless otherwise indicated.

Cell culture

Our previously described HPV16-immortalized human periodontal ligament (IPDL) cells were used (18). Cells were cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin (BioWhittaker, Walkersville, MD, USA) in a humidified atmosphere of 5% CO_2 (95% air) at 37°C.

Determination of PGE_2 levels

The culture medium of control and treated cells was collected, centrifuged, and stored at −70°C until tested. The level of PGE_2 released into culture medium was quantified using a specific enzyme immunoassay according to the manufacturer's instructions (Amersham, Arlington Heights, IL, USA).

Quantification of nitric oxide

Thawed 50 mL aliquots of culture supernatant were mixed with 50 mL Griess reagent, comprising: 5% phosphoric acid (Fisher Scientific, Fair Lawn, NJ, USA), 1% sulfanilamide and 0.1% *N*-naphthylethylenediamine (Sigma Aldrich, St Louis MO, USA). Samples were incubated at room temperature for approximately 10 min and then read on an enzyme-linked immunosorbent assay microplate plate reader (Bio-Rad, Hercules, CA, USA) at 570 nm.

Transfection with HO-1 small interfering RNAs (siRNA) into PDL cells

The siRNA target sequence for HO-1 was: forward, 5′- GCA GAG AAU GCU GAG UUC AUG AGG A-3′ and reverse, 3′-UCC UCA UGA ACU CAG CAU UCU CUG C-5′. Cells were transfected with siRNA (30 nM) using Lipofectamine™ RNAiMAX (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Silencer negative control siRNA (Invitrogen) was used as a negative control and was introduced into the cells using the same protocol. After transfection, cells were cultured for an additional 24 h with fresh medium and harvested or further treated with LPS and nicotine, when necessary.

Western blot analysis

Protein samples (50 μg) were mixed with an equal volume of 2× sodium dodecyl sulfate (SDS) sample buffer, boiled for 5 min, and then separated through 8–15% SDS-polyacrylamide gel electrophoresis gels. After electrophoresis, proteins were transferred to nylon membranes by electrophoretic transfer. The membranes were blocked in 5% dry milk (1 h), rinsed, and incubated with anti-HO-1 antibodies (1:1000 dilution) in Tris-buffered saline (TBS) overnight at 4°C. Primary antibody was then removed by washing the membranes four times in TBS. Primary antibodies were labeled by incubation with 0.1 mg/mL peroxidase-labeled

secondary antibodies (against mouse and rabbit) for 1 h. Following three washes in TBS, bands were visualized by chemiluminescence and exposed to X-ray film.

Statistical analysis

Differences among groups were analyzed using one-way analysis of variance combined with the Bonferroni test. All values were expressed as means ± standard deviations, and differences were considered significant at $p < 0.05$.

Results

Effects of LPS and nicotine on iNOS/COX-2 expression and NO/PGE_2 production

To evaluate the effects of nicotine and LPS on iNOS expression and NO production, human PDLs were treated with various concentrations of nicotine and LPS for 24 h (Fig. 1A). Between 0 and 1 μg/mL LPS, the iNOS and NO induction effects were concentration dependent (data not shown). In addition, nicotine induced iNOS expression and NO production in a concentration-dependent manner at 0–5 mM concentrations (data not shown). Maximal iNOS expression and NO production in PDL cells were achieved using 1 μg/mL LPS and 5 mM nicotine (Fig. 1A).

Next, we examined the time course of LPS- and nicotine-induced changes in iNOS/COX-2 levels and NO/PGE_2 production in PDL cells. Co-treatment with LPS (1 μg/mL) and nicotine (5 mM) resulted in a time-dependent increase of iNOS (Fig. 1B) and COX-2 expression, with maximal induction after 18 or 24 h of incubation (Fig. 1C). This combination of LPS and nicotine also increased iNOS-derived NO (Fig. 1D) and COX-2-derived PGE_2 (Fig. 1E), with maximal induction after 18 or 24 h of incubation.

Effects of HO-1 inhibition on LPS- and nicotine-induced NO and PGE_2 production

We examined human PDL cells for HO-1 protein expression induced by LPS and nicotine, because the induction of HO-1 by various stress stimuli has been implicated in inflammation. As shown in Fig. 2A, the HO-1 expression induced by nicotine or LPS was comparable to that in the control cultures; in contrast, the combination of LPS and nicotine had a synergistic effect on HO-1 expression in PDL cells. In addition, to assess the potential role of HO-1 expression in the production of NO and PGE_2, human PDL cells were co-treated for 18 h with a combination of nicotine and LPS in the presence of protoporphyrin (SnPP), a competitive inhibitor of HO-1. As expected, SnPP pretreatment blocked the LPS- and nicotine-induced HO-1 expression (Fig. 2B). Moreover, SnPP pretreatment revealed a significant inhibitory effect on LPS- and nicotine-induced NO and PGE_2 production in PDL cells (Fig. 2C,D). In addition, SnPP decreased the LPS- and nicotine-induced synthesis of iNOS and COX-2 proteins in PDL cells (Fig. 2C,D).

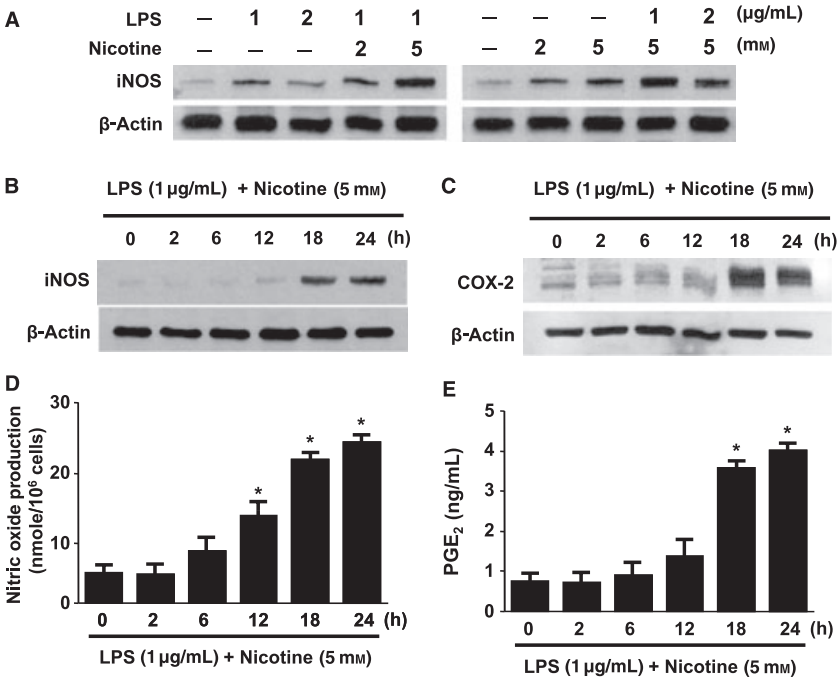

Fig. 1. Effect of LPS and nicotine on the expression of iNOS and COX-2 and the production of NO and PGE_2. Cells were incubated for 24 h with the indicated concentrations of LPS and nicotine. Western blot analysis for iNOS and COX-2 expression was performed as described in the Material and methods section. The concentrations of nitrite and PGE_2 were determined as described in the Material and methods section. Shown are the mean ± SD values of three experiments. *$p < 0.05$ with respect to untreated control group.

Effect of silencing HO-1 with siRNA on LPS- and nicotine-induced NO/PGE_2 production

To examine whether HO-1 expression is involved in nicotine- and LPS-mediated induction of NO and PGE_2 production, PDL cells were transfected with HO-1 siRNA, following an 18 h exposure to LPS and nicotine. As shown in Fig. 3, HO-1 siRNA abolished the induction effects of nicotine and LPS on PGE_2 and NO production (Fig. 3B,C). Moreover, HO-1 siRNA blocked LPS- and nicotine-induced iNOS and COX-2 expression (Fig. 3A).

Effects of LPS and nicotine on nuclear factor-κB (NF-κB) activation

We examined the effects of nicotine and LPS on NF-κB activation by measuring I-κBα degradation and I-κBα phosphorylation as well as RelA/p65 regulation of NF-κB in PDL cells. Maximal phosphorylation and

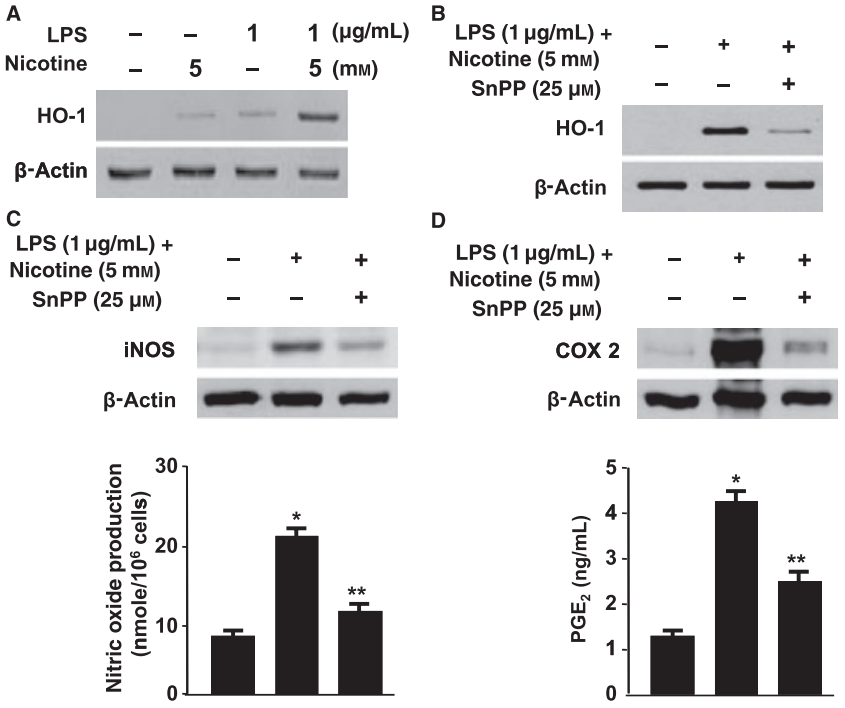

Fig. 2. Effect of LPS and nicotine on the expression of HO-1 and inhibition of HO-1 activity by SnPP. Cells were incubated for 18 h with the indicated concentrations of LPS and nicotine (A). Cells were pretreated for 3 h with SnPP, a competitive inhibitor of HO-1, and then incubated with 1 μg/mL of LPS and 5 mM of nicotine (B–D). $*p < 0.05$ with respect to untreated control group; $**p < 0.05$ with respect to the group treated with 1 μg/mL of LPS and 5 mM of nicotine.

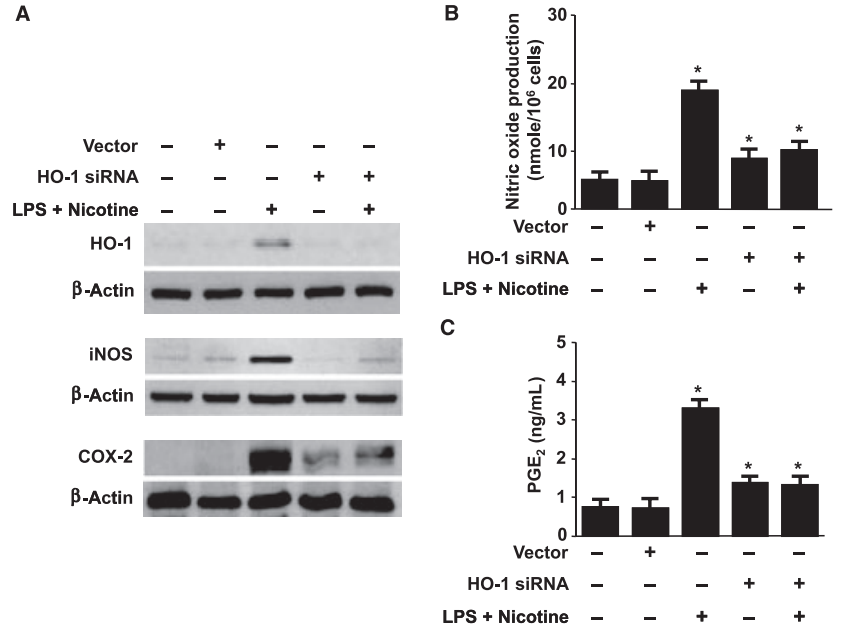

Fig. 3. Roles of HO-1 in inhibition of LPS- and nicotine-induced NO and PGE_2 production. Cells were transiently transfected with control vector and HO-1 siRNA, following treatment with LPS and nicotine for 18 h. Western blot analysis was performed as described in the Material and methods section. The concentrations of nitrite and PGE_2 were determined as described in the Material and methods section. Shown are the mean ± SD values of three experiments. $*p < 0.05$ with respect to the group transfected with control vector.

degradation of I-κBα and maximal expression of the p65 protein were detected after PDL cells were incubated for 60 min in nicotine plus LPS (Fig. 4A). By 2 h, however, p65 protein levels had returned to baseline (Fig. 4B).

Effects of various signal pathway inhibitors on LPS- and nicotine-induced iNOS and COX-2 expression

We used pharmacological inhibitors to confirm that mitogen-activated protein kinases (MAPKs) and phosphoinositide 3-kinase (PI3K) are involved in the LPS- and nicotine-induced production of iNOS and COX-2 in PDL cells. Lipopolysaccharide- and nicotine-mediated iNOS protein expression and NO induction were significantly inhibited by the specific c-JUN NH -terminal protein kinase (JNK) inhibitor SP600125 (25 μM) and by the PI3K inhibitor wortmannin (2 μM). The extracellular signal-regulated kinase (ERK) inhibitor U0126 (10 μM) and the p38 inhibitor SB203580 (20 μM) did not affect production (Fig. 5A). In contrast, LPS- and nicotine-mediated COX-2 protein expression and induction of PGE_2 were significantly inhibited by SP600125, SB203580 and wortmannin. Again, the ERK inhibitor U0126 did not affect production (Fig. 5B).

Discussion

It has been reported that nicotine can induce c-*fos*, COX-2 and HO-1 expression in fibroblasts derived from the periodontium (19–21). Lipopolysaccharide from a periodontopathogenic bacterium stimulated interleukin-1β, tumor necrosis factor-α and PGE_2 production through the induction of COX-2 gene expression in gingival fibroblasts and PDL cells (22–24). Previous studies have reported nicotine- and LPS-mediated induction of proinflammatory cytokines in gingival fibroblasts (15), COX-2 expression in osteoblasts (14) and U937 cells (17), and NO and PGE_2 production in RAW264.7 cells (16). However, the induction of HO-1, COX-2 and iNOS by LPS and nicotine has not been verified in human PDL cells. We

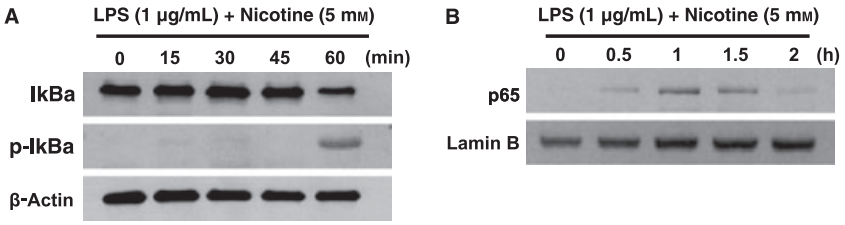

Fig. 4. Effects of LPS and nicotine on LPS- and nicotine-induced NF-κB activation (A,B) in human PDL cells. Results are from a representative experiment.

studied the role of HO-1 in regulating COX-2 and iNOS expression as a therapeutic target in periodontal disease; we also examined NO and PGE_2 synthesis in LPS- and nicotine-stimulated PDL cells. This study is the first to demonstrate that the inhibition of HO-1 in LPS- and nicotine-stimulated human PDL cells results in the suppression of iNOS and COX-2 expression, as well as the reduction of NO and PGE_2.

Lipopolysaccharide has been demonstrated to potently induce NO production through activation of iNOS gene expression (16). However, nicotine is known to suppress inflammation by inhibiting the proinflammatory factors that control NO synthesis (25,26). Moreover, it was previously reported that nicotine inhibits NO synthesis and iNOS expression in H19-7 cells (27). However, the pro- and anti-inflammatory effects of nicotine are not yet fully understood. Notably, nicotine has been shown to enhance production of proinflammatory factors such as COX-2 and PGE_2 in LPS-treated human gingival fibroblasts and peripheral microglial cells (16,28). Our results also showed that nicotine enhanced LPS-induced NO, iNOS, COX-2 and PGE_2 synthesis in PDL cells. In contrast, nicotine inhibits both COX-2 and interleukin-1b expression in LPS-stimulated cells from the U937 human monocytic cell line (24). This differential effect of nicotine might be explained by a biphasic role in inflammation, dependent on concentration (29) and cell type (25).

Heme oxygenase-1 has been shown to exert cytoprotective and anti-inflammatory effects in stress conditions (30). We previously showed that HO-1 is induced by proinflammatory cytokines, nitric oxide, mechanical stress and hydrogen peroxide and that it may have a cytoprotective role in human pulp and PDL cells (31–37). Thus, we hypothesized that the induction of HO-1 by LPS and nicotine in PDL cells may be responsible for the COX-2- and iNOS-inducing effects of treatment with LPS and nicotine. Our first finding was that LPS and nicotine synergistically induced HO-1 expression in PDL cells. In addition, either the down-regulation of HO-1 expression by HO-1 siRNA transfection or the arrest of HO-1 activity by the HO-1 inhibitor SnPP blocked the LPS- and nicotine-induced increases in iNOS/COX-2 expression and NO/PGE_2 production. These results suggest that LPS and nicotine induced iNOS and COX-2 as well as NO and PGE_2 production via the HO-1-dependent pathway in PDL cells. Based on these findings, we propose that HO-1 as an inflammatory mediator represents a novel preventive or therapeutic target in periodontitis.

Regarding the mechanisms of COX-2 and iNOS induction, several studies have suggested the involvement of MAPK, NF-κB and PI3K/amino kinase terminal pathways (38). It is known that inactive NF-κB generally binds to I-κBα in the cytosol and that NF-κB can be activated by various

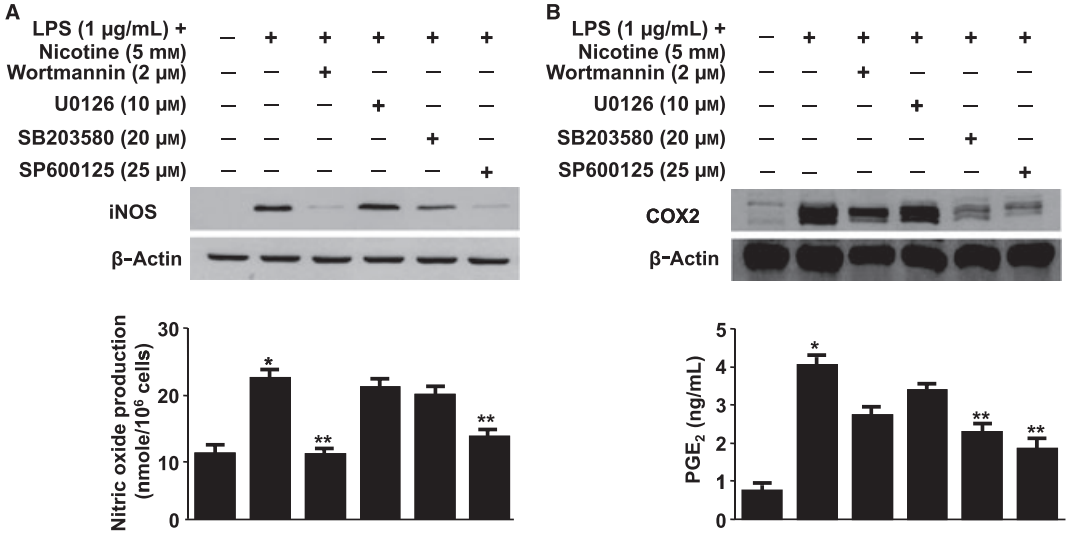

Fig. 5. Effects of various signal pathway inhibitors on LPS- and nicotine-induced iNOS (A) and COX-2 expression (B) in human PDL cells. Cells were pretreated with various key signal pathway inhibitors (PI3K inhibitor wortmannin, ERK1/2 inhibitor U0126, p38 MAPK inhibitor SB203580 and JNK inhibitor SP600125), then incubated with 1 μg/mL of LPS and 5 mM of nicotine. Shown are the mean ± SD values of three experiments. *$p < 0.05$ with respect to untreated control group; **$p < 0.05$ with respect to the group treated with 1 μg/mL of LPS and 5 mM of nicotine.

factors such as infection, cytokines and reactive oxygen species, which induce increased protein phosphorylation and proteolysis of I-κBα (38). Nuclear factor-κB is also a crucial transcription factor expression of iNOS (9) and its response elements are on the promoter regions of the iNOS gene (32,33). In the present study, LPS and nicotine induced NF-κB activation, demonstrated by cytosolic I-κBα phosphorylation, degradation, as well as increased expression of nuclear p65, the major component of NF-κB (Fig. 4A). 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. The MAP kinases are also known to be important for the activation of NF-κB and the induction of HO-1 (27,38). In the present study, we found that a specific JNK inhibitor and PI3K inhibitor effectively reduced LPS- and nicotine-induced COX-2 protein and PGE_2 levels. A specific ERK1/2 inhibitor and p38 MAP kinase inhibitor had no effect on COX-2/PGE_2 levels; a p38 MAP kinase inhibitor also failed to inhibit the LPS- and nicotine-induced iNOS and NO production in PDL cells (Fig. 5). This suggests that, even though LPS- and nicotine-activated MAPKs play differential roles in iNOS and COX-2 induction, the iNOS induction by LPS and nicotine is not affected by inhibition of p38 MAPK through modulation of the MAPK pathway.

In summary, we demonstrate the inhibitory role of HO-1 on iNOS/COX-2 expression and NO/PGE_2 production following LPS and nicotine treatment in human PDL cells. Therefore, targeted HO-1 regulation during inflammation, by modulating NO and PGE_2 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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