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HIF-2 Inhibition Supresses Inflammatory Responses and Osteoclastic Differentiation in Human Periodontal Ligament Cells

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ABSTRACT

Recent reports suggest that hypoxia inducible factor-2α (HIF-2α) is a key regulator of osteoarthritis cartilage destruction. However, the precise role of HIF-2α in the inflammatory response and osteoclast differentiation remains unclear. The purpose of this study was to investigate the effect of HIF-2α on inflammatory cytokines, extracellular matrix (ECM) destruction enzymes, and osteoclastic differentiation in nicotine and lipopolysaccharide (LPS)-stimulated human periodontal ligament cells (PDLCs). HIF-2α was upregulated in chronically inflamed PDLCs of periodontitis patients, and in nicotine- and LPS-exposed PDLC in dose- and time-dependent manners. HIF-2α inhibitor and HIF-2α siRNA attenuated the nicotine- and LPS- induced production of NO and PGE_2, upregulation of iNOS, COX-2, pro-inflammatory cytokines (IL-1β, TNF-α, IL-1β, IL-6, IL-8, IL-10, IL-11, and IL-17), and matrix metalloproteinases (MMPs; MMP-1, -8, -13, -2 and -9), and reversed the effect on TIMPs (TIMP-1 and -2) in PDLCs. The conditioned medium produced by nicotine and LPS-treated PDLCs increased the number of TRAP-stained osteoclasts, TRAP activity and osteoclast-specific genes, which has been blocked by HIF-2α inhibition and silencing. HIF-2α inhibitor and HIF-2α siRNA inhibited the effects of nicotine and LPS on the activation of Akt, JAK2 and STAT3, ERK and JNK MAPK, nuclear factor-κB, c-Jun, and c-Fos. Taken together, this study is the first to demonstrate that HIF-2α inhibition exhibits anti-inflammatory activity through the inhibition of inflammatory cytokines and impairment of ECM destruction, as well as blocking of osteoclastic differentiation in a nicotine- and periodontopathogen-stimulated PDLCs model. Thus, HIF-2α inhibition may be a novel molecular target for therapeutic approaches in periodontitis. J. Cell. Biochem. 116: 1241–1255, 2015.

KEY WORDS: HIF-2α; INFLAMMATORY CYTOKINE; OSTEOCLAST; PERIODONTAL LIGAMENT CELLS; PERIODONTITIS

Periodontal disease is caused by microorganisms of the biofilm and by environmental factors, and features inflammatory destruction of tooth-supporting tissue including gingiva, periodontal ligament and alveolar bone [Negrato et al., 2013]. Lipopolysaccharides (LPS), derived from Gram-negative bacteria that accumulate on the tooth surfaces, penetrate into periodontal tissues and subsequently recruit and activate immune cells [Page et al., 1997]. LPS play a key role in periodontal disease and alveolar bone destruction through the production of pro-inflammatory mediators such as interleukins-1β (IL-1β), tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) [Agarwal et al., 1995].

Cigarette smoking is a major risk factor for the development and progression of periodontal disease [Johnson et al., 2007]. Nicotine, a major component and the most pharmacologically active agent in

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tobacco, has been detected in the saliva and gingival crevicular fluid of smokers [McGuire et al., 1989]. Moreover, nicotine can increase the collagen-degrading ability of gingival fibroblasts [Zhou et al., 2007], potentiating LPS-stimulated human peripheral blood monocyte secretion of prostaglandin E2 (PGE_2) [Payne et al., 1996] and causing direct adverse effects on various functions of the periodontal ligament cells (PDLCs) [Ginnopoulou et al., 1999]. We previously reported that nicotine upregulates cellular antioxidant phase II enzymes and receptor activator of nuclear factor-kappa B ligand (RANKL) expression in PDLCs, which contributes to cellular defense [Lee et al., 2009].

The combination of nicotine and LPS induces a synergistic effect on the production of nitric oxide (NO) and PGE_2, and increase inducible nitric oxide synthetase (iNOS) and COX-2 expression via heme oxygenase-1 (HO-1) in PDLCs [Pi et al., 2010]. In addition, we reported that nicotine and LPS cause degradation of PDLCs or gingival fibroblasts via multiple mechanisms including Nrf-2 [Lee et al., 2009], endoplasmic reticulum stress [Lee et al., 2012], and SIRT-1[Park et al., 2013]. Furthermore, we showed that hydrogen sulfide inhibited cytotoxicity and osteoclastic differentiation and recovered osteoblastic differentiation in a nicotine- and LPS-stimulated human PDLCs model [Lee et al., 2013].

Hypoxia and reoxygenation are known to stimulate the production of VEGF, IL-6, IL-1β, and PGE_2 in PDLCs [Motohira et al., 2007], which could result in the resorption of alveolar bone in periodontitis. Mammalian cells adapt to changes in O_2 availability primarily through hypoxia-inducible factors (HIFs) whose activity is regulated by their subunits (HIF-1α and HIF-2α) [Majmundar et al., 2010]. HIF-1α is upregulated in periodontal pockets of periodontitis [Ng et al., 2011], and by IL-1 in human gingival and synovial fibroblasts [Thornton et al., 2000]. In addition, we recently reported that nicotine and LPS lead to upregulation of HIF-1α, which subsequently upregulates PGE_2, MMP-2, and MMP-9 production via the PI3K, p38, ERK, JNK, PKC, and NF-κB pathways in human PDLCs [Kim et al., 2012].

HIF-2α (endothelial PAS domain protein 1/EPAS-1) has been implicated in regulating basal transcription of Runx2 [Hirata et al., 2012]. Overexpression of a constitutively active form of HIF-1α inhibited in vitro osteoblastic differentiation [Hiraga et al., 2007]. HIF-1α knockout mice demonstrated markedly decreased trabecular bone volume, reduced bone formation rate, and altered cortical bone architecture [Shomento et al., 2010]. In contrast, mice lacking HIF-2α had only a modest decrease in trabecular bone volume [Shomento et al., 2010]. Thus, HIF-1α and HIF-2α exert both distinct and overlapping functions in long bone development [Shomento et al., 2010]. HIF-2α was recently demonstrated to be a catabolic regulator of cartilage destruction which acts by regulating the expression of various catabolic factors, including matrix-degrading enzymes and inflammation mediators [Yang et al., 2010]. In addition, intra-articular injection of an Epas1-expressing adenovirus (Ad-Epas1) triggers articular cartilage destruction in mice and rabbits [Yang et al., 2010]. Moreover, HIF-2α stimulates chondrocyte apoptosis by upregulating Fas expression and amplifying downstream signaling which is associated with osteoarthritis cartilage destruction [Ryu et al., 2012].

We hypothesized that HIF2-α inhibition could contribute to the suppression of the inflammatory response and the pathological development of periodontal disease by decreasing inflammatory cytokine profiles, impairing connective tissue destruction, and blocking osteoclastic differentiation. Therefore, the aim of this study was to investigate whether HIF-2α up-regulation was observed in periodontitis patients and nicotine- and LPS-stimulated HPDLCs, and whether HIF-2α inhibitor and HIF-2α small interfering RNA (siRNA) could suppress nicotine- and LPS-induced inflammatory response in HPDLCs and osteoclastic differentiation in mouse bone marrow-derived macrophages (BMMs). To reveal the molecular mechanism, associated signaling pathways were also examined.

MATERIALS AND METHODS

REAGENTS

LPS (from *P. gingivalis*), nicotine and 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (PGJ_2) were purchased from Sigma-Aldrich Chemical Co. (St Louis, MO). Recombinant RANKL and M-CSF were purchased from Peprotech (London, UK). Affnity purified mouse anti-COX-2 monoclonal antibody (mAb), was purchased from Cayman Chemical (Ann Arbor, MI). Polyclonal antibodies against HIF-2α, phospho-STAT3, phospho-Akt, phospho-ERK5, NF-κB/p65, phospho-IκBα, IκBα, c-jun and c-fos were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against iNOS, phospho-JAK2, phospho-ERK, phospho-p38 and phospho-JNK were purchased from Cell Signaling Technology (Danvers, MA).

CELL CULTURE

Immortalized human dental periodontal ligament cells (HPDLCs), transfected with human telomerase catalytic component (hTERT), were kindly provided by Professor Takashi Takata (Hiroshima University, Japan: Kitagawa et al., 2007). Cells were cultured in α-MEM supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin in a humidified atmosphere of 5% CO_2 at 37 °C. All experiments were approved by the local ethics committee.

PRIMARY CULTURE OF HPDLCS

Pieces of PDL were obtained exclusively from the middle of tooth roots extracted for orthodontic reasons, to exclude intermixture of gingivae and dental pulp. Then, ligament samples were cultured in 10% FBS-MEM supplemented with antibiotics in a 35-mm primary culture dish (Falcon Becton Dickinson, Franklin Lakes, NJ). Cells that proliferated from the extracts were passaged. The HPDLCs used in these experiments underwent four to eight passages.

HIF-2α SIRNA TRANSFECTION

siRNA was synthesized in duplex and purified forms (Bioneer, Daejeon, Korea). The sense and anti-sense strands of the human HIF-2α siRNA were 5′-GCA UCA UGU GUG UCA ACU AUU-3′ (sense) and 5′-AAU AGU UGA CAC ACA UGA UGC-3′ (antisense). HPDLCs were transfected using lipofectamine 2000 (Gibcoen Ltd, Paisley, UK) following the manufacturer's instructions.

TABLE I. Reverse Transcriptase-polymerase Chain Reaction (RT-PCR) Primers and Conditions

Gene	Sequence (5'-3')	Size (bp)	Tm (°C)
HIF-2	Forward: TCTTGGGAGCAGCGCCGTTG Reverse: TGCGCTCAGTGGCTTGTCCG	293	65
HIF-1α	Forward: GTCTTACAGCCTCACCAAACAGAGC Reverse: GTTAACTTGATCCAAAGCTCTGAG	486	60
TNF-α	Forward: GGAAGACCCCTCCCAGATAG Reverse: CCCCAGGGACCTCTCTCTAA	413	52
IL-1β	Forward: GGA TAT GGA GCA ACA AGT GG Reverse: ATG TAC CAG TTG GGG AAC TG	288	60
IL-6	Forward: TAG CCG CCC CAC ACA GAC AG Reverse: GGC TGG CAT TTG TGG TTG GG	408	60
IL-8	Forward: ATGACTTCCAAGCTGGCCGTGG Reverse: TGAATTCTCAGCCCTCTTCAAAAAC	297	62
IL-10	Forward: TACGGCGCTGTCATCGATTT Reverse: AAGGTTTCTCAAGGGGCTGG	273	60
IL-11	Forward: CTGCTGCTGCTGAAGACTCGGCTGTGA Reverse: ATGGGGAAGAGCCAGGGCAGAAGTCTGT	321	59
IL-17	Forward: CGATGACTCCTGGGAAGACCTC Reverse: GTGTGGGCTCCCCAGAGCTCTTA	524	62
MMP-1	Forward: CTGCCCTTGCTGTCCTCCTCTG Reverse: CTGCCGGCTTGGCTTGGTTA	197	55
MMP-2	Forward: ATCCGTGGTGAGATCTTCTTCTT Reverse: AGCCAGGATCCATTTTCTTCTT	435	60
MMP-8	Forward: TGAACGTCAGGGTGCTCGCC Reverse: GGCACTCCACAGCGAGGCTTT	366	60
MMP-9	Forward: TGCCAGTTTCCATTCATCTTCCAA Reverse: CTGCGGTGTGGTGGTGGTT	519	60
MMP-13	Forward: CTCCAAGCATGGCATTGTAGTG Reverse: TTCACCCACATCAGGCACTC	265	60
TIMP-1	Forward: GGCATCCTGTTGTTGCTGTGGC Reverse: CGGATGTCAGCGGCATCCCCTA	218	60
TIMP-2	Forward: GGAATCGGTGAGGTCCTGTCCTG Reverse: GGCGTCACATGCAGAAAGCCC	296	60
-actin	Forward: CATGGATGATGATATCGCCGCG Reverse: ACATGATCTGGGTCATCTTCTCG	371	55
Mouse Cathepsin K	Forward: TGGATCTTTTAGCGTCTTGTTCC Reverse: CCACGCTTGAGACAGGCTTA	226	60
Mouse CR (Calcitonin receptor	Forward: TGGTTGAGGTTGTGCCCAATGGAGA Reverse: CTCGTGGGTTTGCCTCATCTTGGTC	503	65
Mouse GAPDH	Forward: GAGAGTGTTTCCTCGTCCCG Reverse: ACTGTGCCGTTGAATTTGCC	201	59

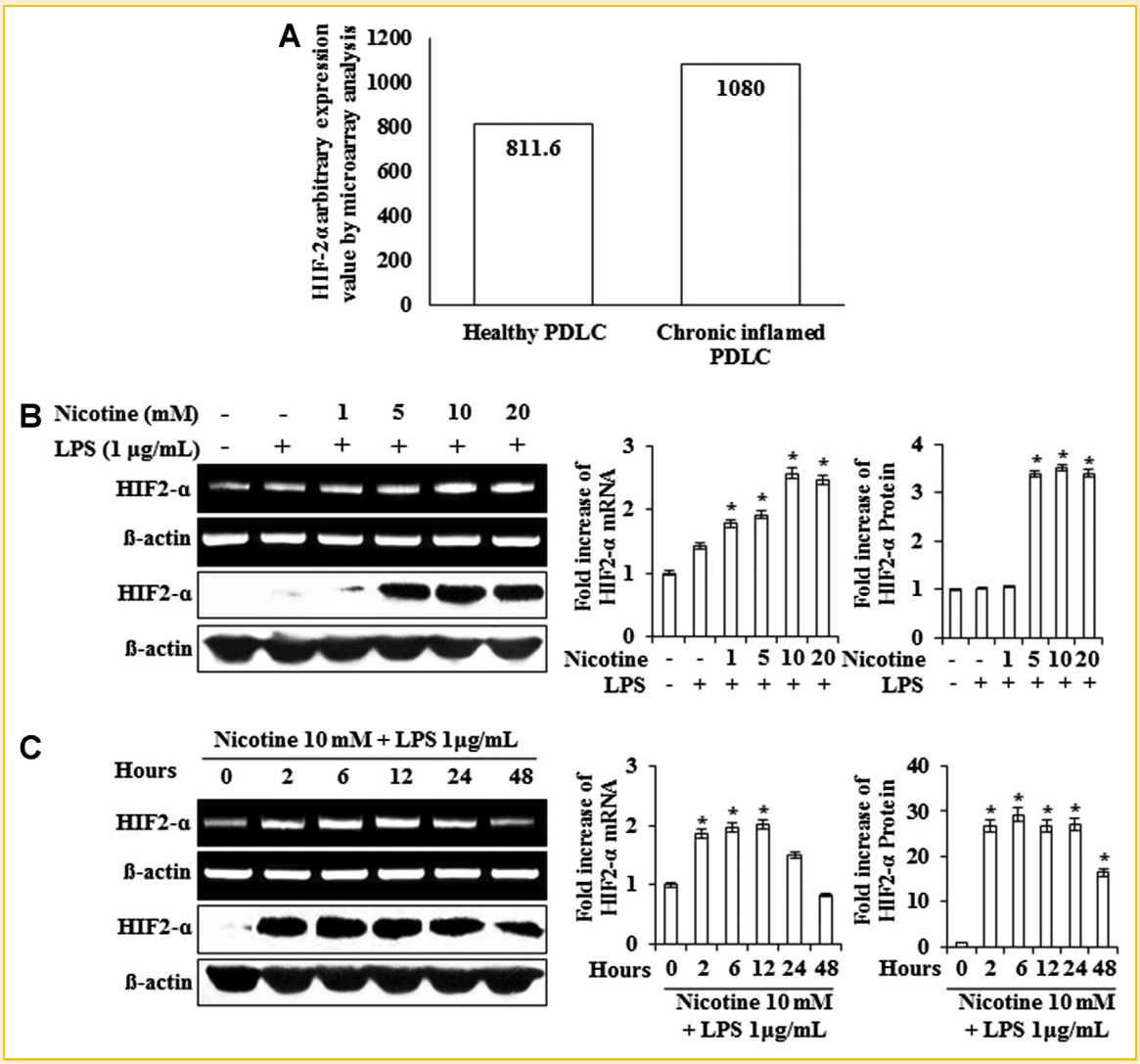

Fig. 1. (A) HIF-2α gene level in PDLCs of patients with chronic periodontitis and healthy subjects, from public expression databases by microarray analysis. (B, C) Effects of LPS and nicotine on mRNA and protein expression of HIF-2α. Cells were incubated for 24 h with the indicated concentrations of nicotine and LPS (B), and for the indicated times with 10 mM nicotine and 1 μg/mL LPS (C). Data are representative of three independent experiments. The histogram (Right) shows the quantification of mRNA and protein expression by densitometry, and is presented as fold increases compared to non-stimulated control cells (B, C). *Statistically significant differences compared with the control, $P < 0.05$ as determined by Duncan's multiple range test.

QUANTIFICATION OF NITRIC OXIDE (NO)

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

DETERMINATION OF PROSTAGLANDIN E_2 (PGE_2) LEVELS

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

CONDITIONED MEDIUM (CM) PREPARATION

After 24 h of HPDLCs culture (10 mM nicotine and 1 μg/mL LPS in serum-free media), the CM were harvested, centrifuged (2300g, 25 min), passed through a 0.2 μm filter (Gelman Sciences, Ann Arbor, MI), and kept at −80 °C.

OSTEOCLAST FORMATION

BMCs were isolated by flushing the bone marrow spaces of the femora and tibiae of 5- to 6-week-old ICR mice as described previously (Azuma et al., 2000). Cells were incubated with α-MEM

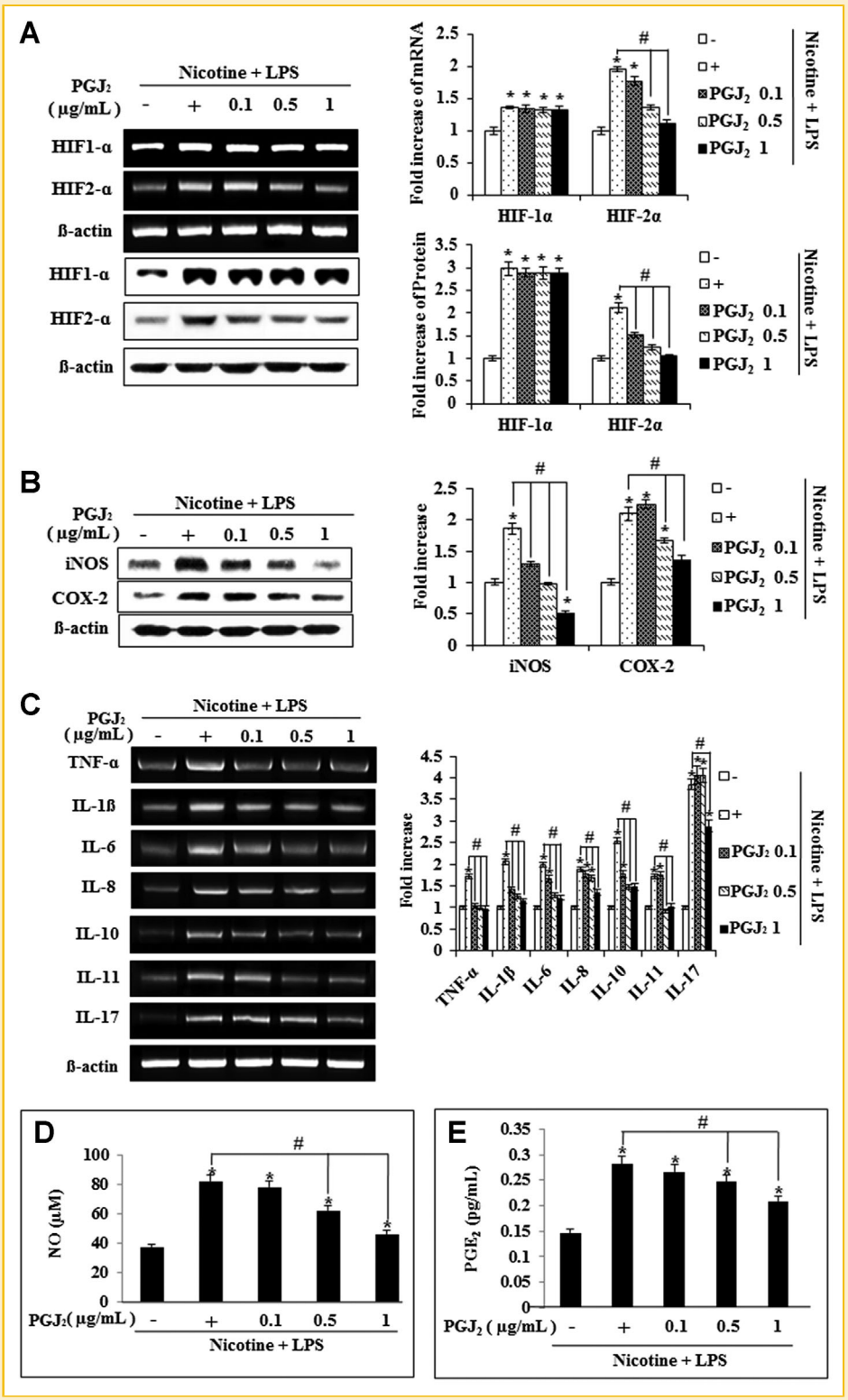

Fig. 2. Effects of HIF2-α inhibitor, 15-Deoxy-Δ12,14-prostaglandin J2 (PEJ_2), on expression of HIF-2α (A), protein expression of iNOS and COX-2 (B), mRNA expression of pro-inflammatory cytokines (C), and production of NO (D) and PGE_2 (E) in PDLCs. Cells were pretreated for 6 h with PEJ_2 and then post-treated with 10 mM nicotine and 1 μg/mL LPS in serum-free media for 24 h (A–E). Protein and mRNA expression were assessed by RT-PCR (A, B, C) and Western blot analysis (A,B), respectively. The production of NO (D) and PGE_2 (D) were measured by Griess reaction and ELISA respectively. Data are replicas of quantification of NO and PGE_2 with the standard deviation of at least three experiments (n = 4). The histogram shows the quantification of protein or mRNA expression by densitometry, and is presented as fold increases compared to control cells (A–C). *Statistically significant differences compared with the control, $P < 0.05$. # Statistically significant difference compared with the nicotine- and LPS- treated group, $P < 0.05$ as determined by Duncan's multiple range test.

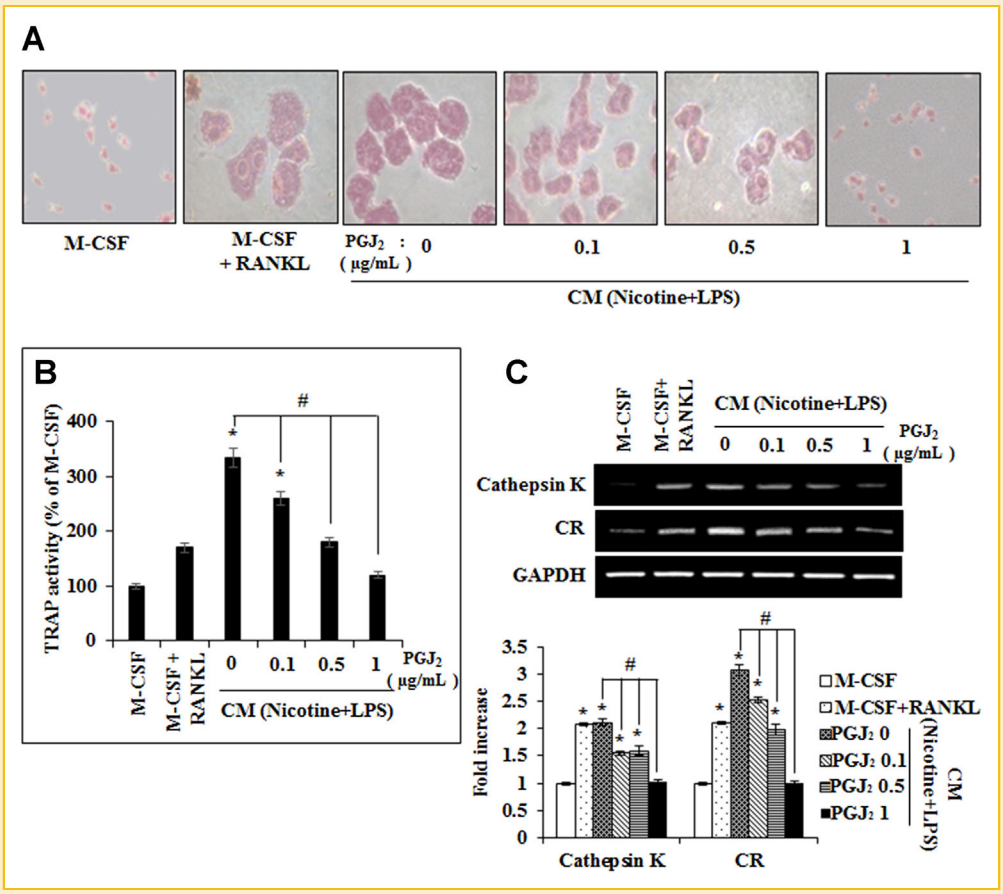

Fig. 3. Effects of HIF2-α inhibitor, 15-Deoxy-Δ12,14-prostaglandin J2 (PEJ_2) on pro-osteoclastogenic activity of PDLCs. The nicotine- and LPS-treated PDLCs were cultured for 24 h in the presence or absence of PEJ_2 and conditioned medium was collected. The bone-marrow derived macrophage (BMM) cells were incubated with M-CSF (10 ng/mL) and RANKL (50 ng/mL) or 20% CM collected from PDLCs. After 48 h of culture, the cells were fixed and osteoclast-like cells were identified by TRAP staining. Data are replicas of quantification of TRAP activity with the standard deviation of at least three experiments ($n = 4$). (A) Representative pictures of TRAP staining. (B) TRAP activity and (C) mRNA expression of osteoclast-specific marker genes were accessed. The histogram shows the quantification of mRNA expression by densitometry, and is presented as fold increases compared to control cells (C). * Statistically significant differences compared with the control, $P < 0.05$. #Statistically significant difference compared with the conditioned medium collected from nicotine- and LPS- treated PDL cells, $P < 0.05$ as determined by Duncan's multiple range test.

containing 10% FBS, penicillin (100 U/mL), and streptomycin (100 mg/mL) for 24 h in 5% CO_2 at 37 °C. Non-adherent cells were completely removed by aspiration and remaining cells were cultured for a further 3 days in the presence of 30 ng/mL M-CSF. Adherent cells were considered to be bone marrow-derived macrophages (BMMs) and were used as osteoclast precursor cells. To achieve osteoclast differentiation, BMMs were seeded in 96-well plates at 1×10^5 cells/well or in six-well plates at 2×10^6 cells/well. Cells were cultured for 5 days with 30 ng/mL M-CSF and 100 ng/mL RANKL. Following the time course of osteoclast differentiation, cells were washed with PBS and fixed with 3.7% formaldehyde. Then, the cells were incubated with 0.1% Triton X-100 for 5 s and stained with the Leukocyte Acid Phosphatase Assay kit (Sigma) following the manufacturer's instructions. TRAP-positive multinucleated cells containing three or more nuclei were counted as osteoclasts under a light microscope. To access the osteoclastogenic activity of CM from HPDLCs, pre-osteoclasts (BMMs treated for 2 days with M-CSF and RANKL) were further cultured up to 4 days in the presence of CM obtained from HPDLCs with PGJ_2 or HIF-2α siRNA as previously described [Lee et al., 2014].

TRAP ACTIVITY

TRAP activity was evaluated in the supernatants collected from wells. TRAP activity was measured by the conversion of α-naphthyl phosphate (4 mM) (Sigma) to α-naphthol in the presence of 2 mM l-tartrate solution (Sigma). Absorbance was measured at 405 nm using a microplate reader (model 550) (Bio-Rad Labs).

WESTERN BLOT ANALYSIS

Cells were lysed in RIPA buffer, phenyl-methylsulphonyl fluoride (PMSF, 0.1 mM), sodium orthovanadate (1 mM) and aprotinin and leupeptin (2 μg/mL). The lysate was centrifuged at 12,000 × g for 20 min at 4 °C, and the supernatant was removed. The protein concentration was measured using a Bio-Rad protein assay kit (Bio-Rad Labs). After boiling for 10 min in the presence of 2-mercaptoethanol, samples containing cell lysate proteins were

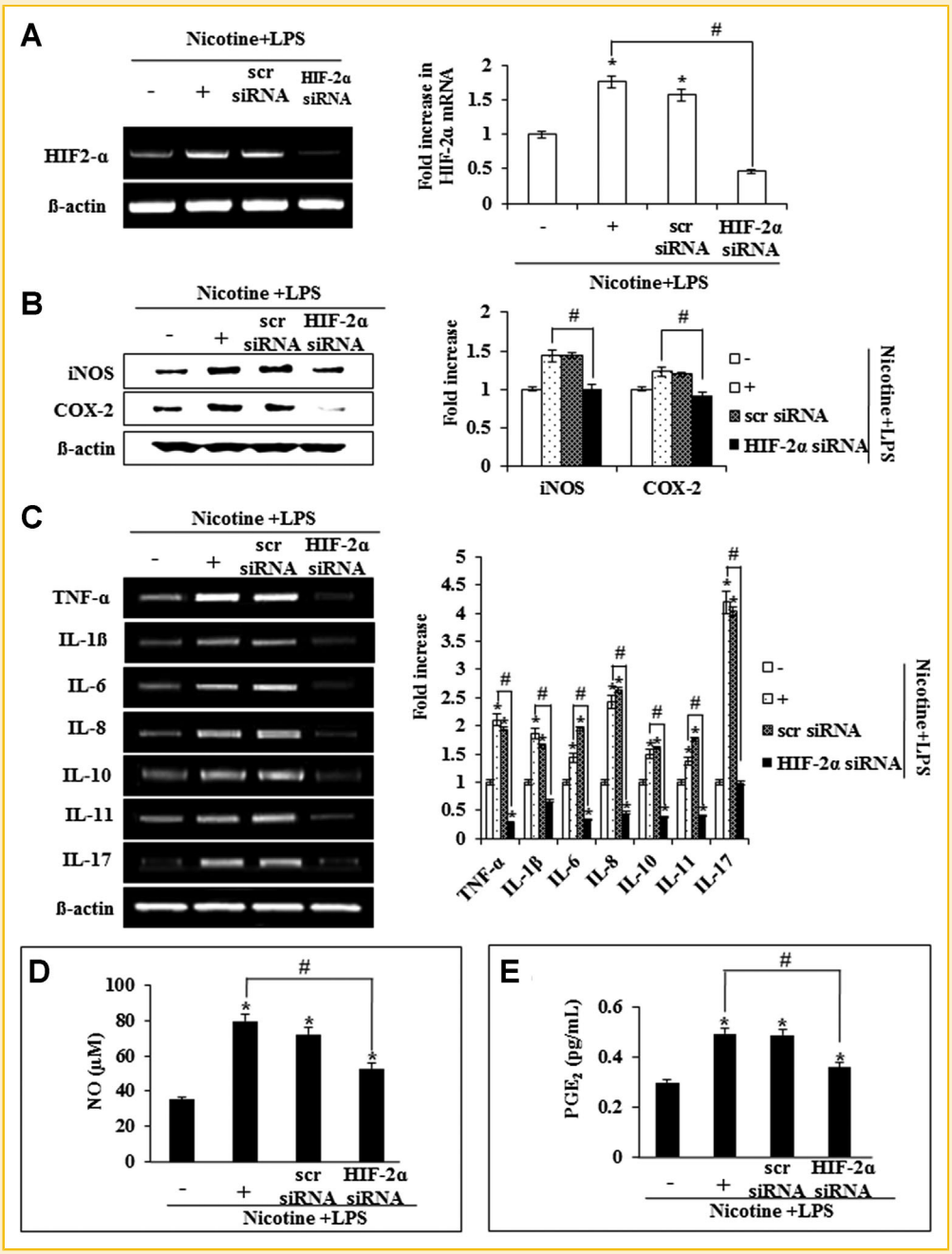

Fig. 4. (A) Effects of HIF2-α silencing by siRNA (PEJ_2) on nicotine- and LPS- induced HIF2-α mRNA and protein expression (A), protein expression of iNOS and COX-2 (B), mRNA expression of pro-inflammatory cytokines (C), and production of NO (D) and PGE_2 (E) in PDLCs. Cells were transfected with 30 nM of HIF2-α siRNA and control siRNA for 12 h and incubated with 10 mM nicotine and 1 μg/mL LPS for 24 h (A). Protein and mRNA expression were assessed by RT-PCR (A, C) and Western blot analysis (B), respectively. The production of NO (D) and PGE_2 (E) were measured by Griess reaction and ELISA, respectively. Data are replicas of quantification of NO and PGE_2 with the standard deviation of at least three experiments (n = 4). Histogram is densitometric quantitation of mRNA or protein expression (A–C). *Statistically significant differences compared with the control, $P < 0.05$. #Statistically significant difference compared with the nicotine- and LPS- treated group, $P < 0.05$ as determined by Duncan's multiple range test.

separated on a 10% or 15% sodium dodecyl sulphate-polyacrylamide (SDS) gel and then transferred onto equilibrated PVDF membranes. After blocking with skim milk, the membranes were incubated with primary antibodies (1:1000 dilution). The bound antibodies were detected with horseradish peroxidase-labeled sheep anti-mouse IgG or horseradish peroxidase labeled anti-rabbit IgG (1:5000 dilution). The immunoblots were then developed with enhanced chemiluminescence reagents (Amersham, Arlington Heights, IL) according to the manufacturer's recommendations. Densitometric analysis of each blot was performed with a computerized image processing system (Quantity One; Bio-Rad, Hercules, CA).

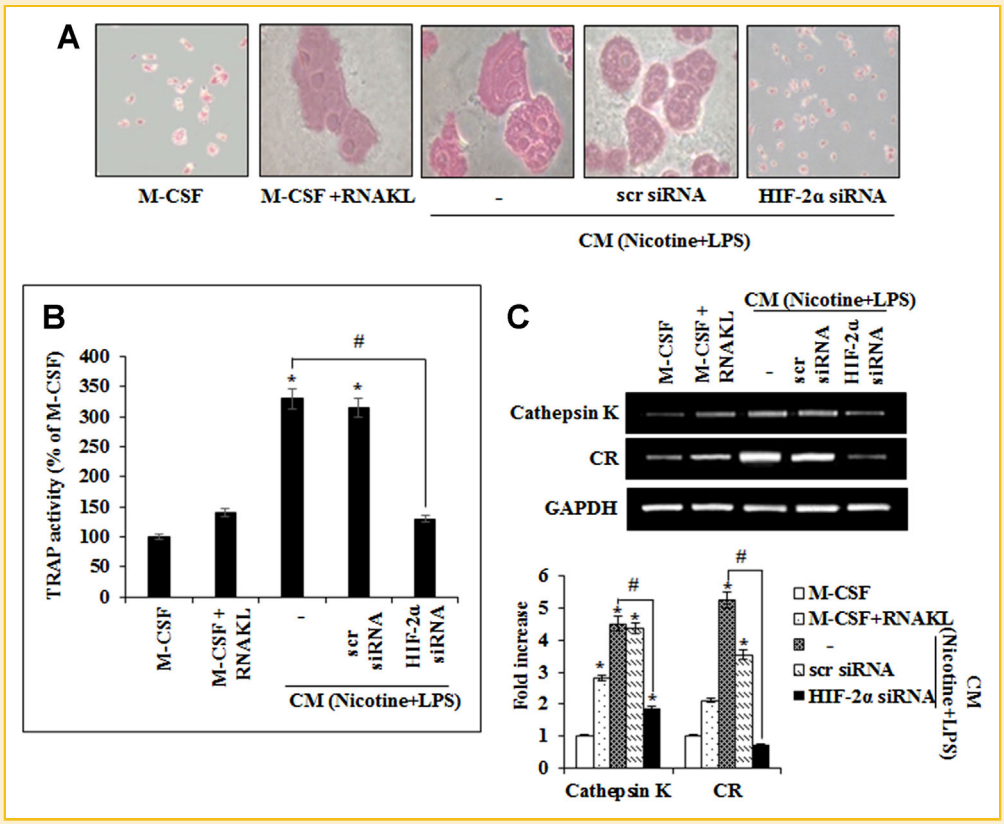

Fig. 5. Effects of HIF2-α siRNA on pro-osteoclastogenic activity of PDLCs. The nicotine- and LPS- treated PDL cells were cultured for 24 h in the presence or absence of 30 nM HIF2-α siRNA and conditioned medium was collected. The bone-marrow derived macrophage (BMM) cells were incubated with M-CSF (10 ng/mL) and RANKL (50 ng/mL) or 20% CM collected from PDLCs. After 48 h of culture, the cells were fixed and osteoclast-like cells were identified by TRAP staining. (A) Representative pictures of TRAP staining. (B) TRAP activity and (C) mRNA expression of osteoclast-specific marker genes were accessed. Data are replicas of quantification of TRAP activity with the standard deviation of at least three experiments (n = 4). Histogram is densitometric quantitation of mRNA expression (C). *Statistically significant differences compared with the control, $P < 0.05$. #Statistically significant differences compared with CM collected from nicotine- and LPS- treated PDL cells, $P < 0.05$ as determined by Duncan's multiple range test.

ISOLATION OF RNA AND REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION (RT-PCR)

Cells were grown in 60-mm culture dishes and were incubated for the indicated times in fresh medium containing stimuli as indicated. Total RNA was extracted from the cells using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Then, 1 μg RNA was reverse transcribed for first-strand cDNA synthesis (Gibco-BRL, Rockville, MD). The cDNA was amplified in a final volume of 20 μL containing 2.5 mM magnesium dichloride, 1.25 units Ex Taq polymerase (Bioneer) and 1 μM specific primers. The sequences of the specific primers used in this study are detailed in Table I. RT-PCR products were electrophoresed on 1.5% agarose gels. Bands were detected by UV illumination of ethidium bromide-stained gels. The intensity of each band after normalization with beta-actin mRNA was quantified on the photographed gels with a densitometer (Quantity One; Bio-Rad, Hercules, CA).

GENE-EXPRESSION PROFILING

In order to compare the HIF-2α mRNA expression in normal and periodontal diseased tissues, we analyzed the transcriptional profiles of previously reported PDL cells [Gersdorff et al., 2008]. The raw data from corresponding experiments were retrieved from National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) data base (accession no. GSE7321, http://www.ncbi.nlm.nih.gov/geo/), which is currently the largest fully public gene expression resource. The GEO repository at the NCBI archives and freely disseminates microarray and other forms of high-throughput data generated by the scientific community [Barrett et al., 2007]. Statistical analysis of microarrays expression data was performed using GEO2R web application (http://www.ncbi.nlm.nih.gov/geo/geo2r/) [Barrett et al., 2013].

GSE7321 is available microarray data set, which was obtained from 32 patients at the Dental Medical School of University of Goettingen (12 men, 20 women, aged between 18 and 72 years) from March 2005 to December 2005. The periodontal tissue was scraped off the roots of the teeth and total RNA from chronically inflamed and healthy PDLCs was isolated. The genome-wide gene expressions of chronically inflamed and healthy PDLCs were compared by microarray analysis.

STATISTICAL ANALYSIS

Data are expressed as means ± standard deviations (SD). Comparisons were made using a one-way analysis of variance (ANOVA). When ANOVA indicated statistical significance, Duncan's multiple

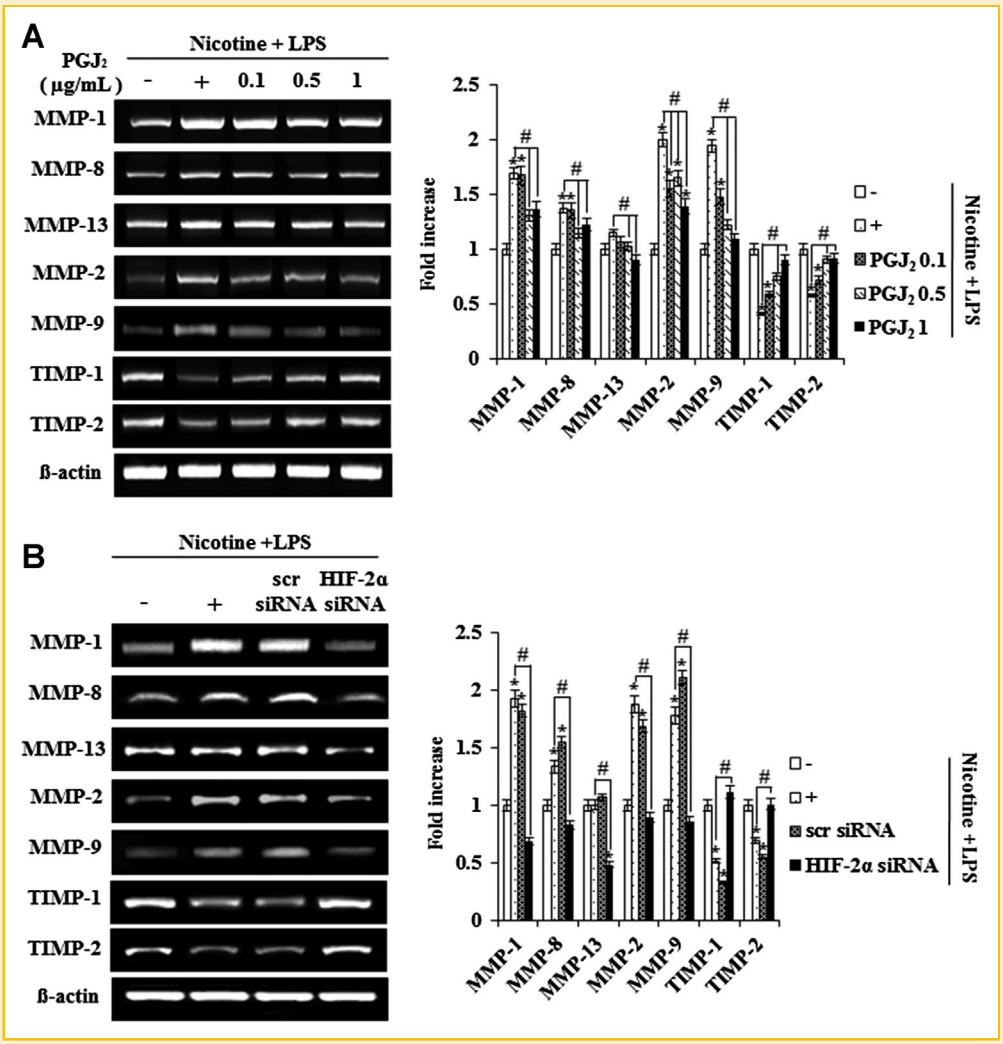

Fig. 6. Effect of HIF2-α inhibitor and HIF2-α siRNA on nicotine and LPS-induced matrix metalloproteinase (MMP) and tissue inhibitor metalloproteinase (TIMP) expression in PDLCs. Cells were pretreated with PEJ_2 for 6 h or HIF2-α siRNA for 12 h and then post-treated with 10 mM nicotine and 1 μg/mL LPS in serum-free media for 24 h. mRNA expression of MMPs and TIMPs were analyzed by RT-PCR (A, B). Data are representative of three independent experiments. Histogram is densitometric quantitation of mRNA expression. (A, B). *Statistically significant differences compared with the control, $P < 0.05$. #Statistically significant difference compared with the conditioned medium collected from nicotine- and LPS- treated PDL cells, $P < 0.05$ as determined by Duncan's multiple range test.

range test was used to determine which means were significantly different. A probability value of $P < 0.05$ was used as the criterion for statistical significance. All experiments were repeated at least three times, and representative experiments are shown.

RESULTS

MRNA EXPRESSION OF HIF-2α IN HEALTHY CONTROLS AND PERIODONTITIS PATIENTS

In order to analyze HIF-2α mRNA expression in normal and periodontal diseased tissues, we searched for expression data in public expression databases (endothelial PAS domain protein 1 (EPAS1) and probe set 200879_s_at on Affymetrix U133 2.0+ platform in Gene Expression Omnibus, GEO, NCBI (http://www.ncbi.nlm.nih.gov/geo/). According to the expression datasets (GEO, series GSE7321), mRNA expression of HIF-2α was significantly higher in chronic inflamed PDLCs from patients with periodontitis than in the healthy PDLCs (Fig. 1A).

EFFECTS OF NICOTINE AND LPS ON MRNA EXPRESSION OF HIF2-α IN HUMAN PDLCS

HPDLCs were treated for 48 h to examine the effects of nicotine and LPS on the expression levels of HIF-2α. PDL cells exposed to different concentrations of nicotine plus 1 μg/mL LPS for 24 h showed a concentration-dependent increase in HIF-2 α mRNA and protein expression, compared to LPS treatment alone (Fig. 1B). As shown by the time-course data in Figure 1C, the combination of LPS and nicotine upregulated HIF-2 expression, with maximal induction at 1 μg/mL LPS and 10 mM nicotine for 12 h.

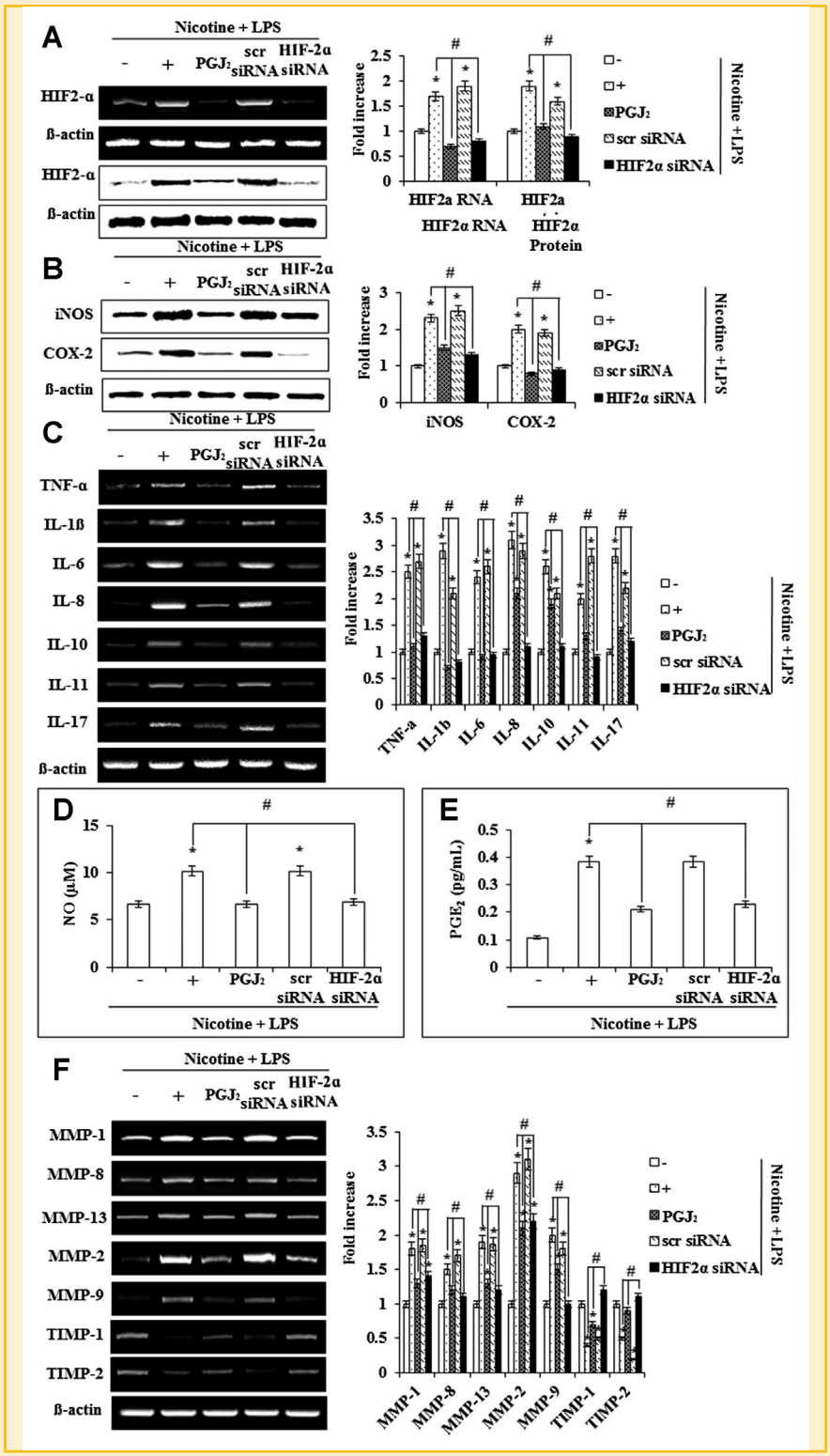

Fig. 7. Effect of HIF2-α inhibitor and HIF2-α siRNA on nicotine and LPS-HIF2-α mRNA and protein expression (A), protein expression of iNOS and COX-2 (B), mRNA expression of pro-inflammatory cytokines (C) and MMPs (F), and production of NO (D) and PGE_2 (E), in primary cultured PDLCs. Cells were transfected with 30 nM of HIF2-α siRNA and control siRNA for 12 h and incubated with 10 mM nicotine and 1 μg/mL LPS for 24 h (A–F). Protein and mRNA expression were assessed by RT-PCR (A, C, F) and Western blot analysis (A, B), respectively. The production of NO (D) and PGE_2 (E) were measured by Griess reaction and ELISA, respectively. Data are replicas of quantification of NO and PGE_2 with the standard deviation of at least three experiments ($n = 4$). Histogram is densitometric quantitation of mRNA or protein expression (A–C, F). *Statistically significant differences compared with the control, $P < 0.05$. #Statistically significant difference compared with the nicotine- and LPS- treated group, $P < 0.05$ as determined by Duncan's multiple range test.

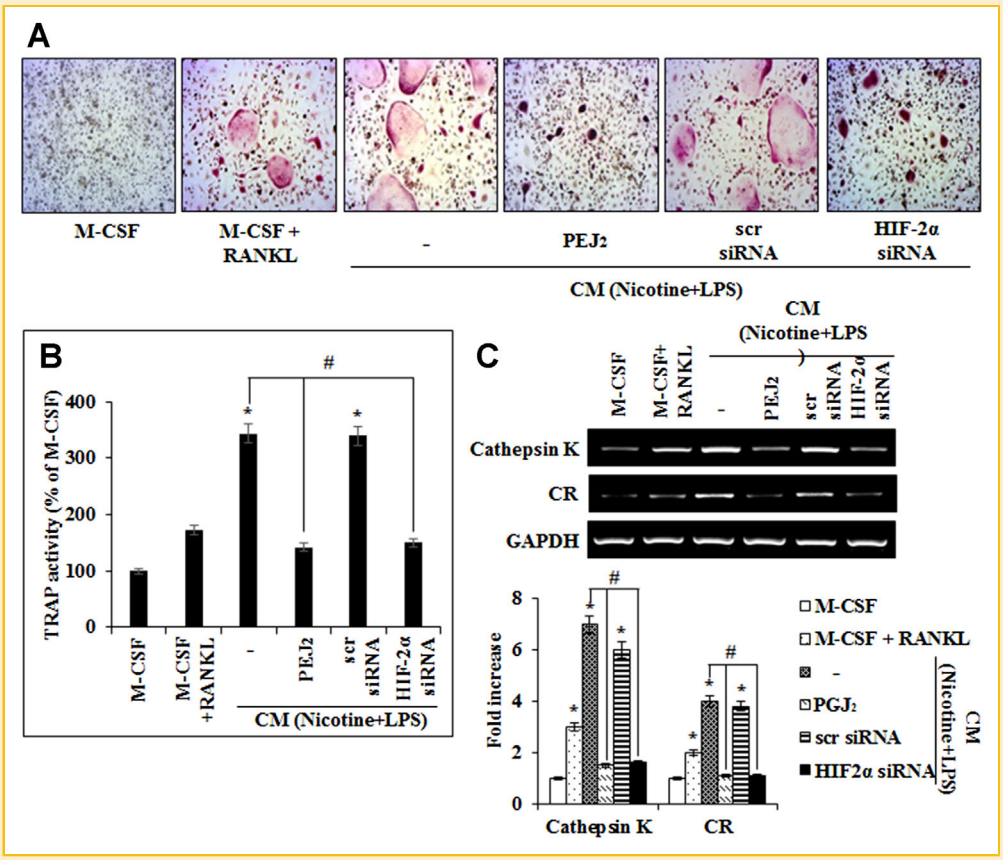

Fig. 8. Effect of HIF2-α inhibitor and HIF2-α siRNA on pro-osteoclastogenic activity of primary cultured PDLCs. The nicotine- and LPS- treated primary PDL cells were cultured for 24 h in the presence or absence of 30 nM HIF2-α siRNA and conditioned medium was collected. The BMM cells were incubated with M-CSF (10 ng/mL) and RANKL (50 ng/mL) or 20% CM collected from primay PDLCs. After 48 h of culture, the cells were fixed and osteoclast-like cells were identified by TRAP staining. (A) Representative pictures of TRAP staining. (B) TRAP activity and (C) mRNA expression of osteoclast-specific marker genes were accessed. Data are replicas of quantification of TRAP activity with the standard deviation of at least three experiments (n = 4). Histogram is densitometric quantitation of mRNA expression (C). *Statistically significant differences compared with the control, $P < 0.05$. #Statistically significant differences compared with CM collected from nicotine- and LPS- treated PDL cells, $P < 0.05$ as determined by Duncan's multiple range test.

EFFECTS OF HIF-2α INHIBITOR, 15-DEOXY-Δ12,14-PROSTAGLANDIN J_2 (PGJ_2) ON NICOTINE- AND LPS-INDUCED INFLAMMATORY RESPONSE IN PDLCS

To study whether nicotine and LPS are involved in inflammatory mediators production regulated by HIF-2α, cells were pre-treated with HIF-2α inhibitor, PGJ_2 for 6 h. PGJ_2 dose-dependently blocked nicotine- and LPS-induced HIF-2α expression, as well as the production of inflammatory mediators such as protein expression of COX-2 and iNOS, production of NO and PGE_2, and mRNA expression of TNF- α, IL-1β, -6, -8, -10, -11, and -17 (Fig. 2 A–E). However, LPS and nicotine-induced HIF1-α mRNA and protein level was not affected by PGJ_2 in PDLCs (Fig. 2A).

EFFECTS OF HIF-2α INHIBITOR ON OSTEOCLASTOGENESIS IN MOUSE BONE-MARROW DERIVED MACROPHAGE (BMM) CELLS

To elucidate the effect of HIF-2α inhibitor PGJ_2 on osteoclastogenesis in vitro, BMM cells were cultured with M-CSF and RANKL for 3 days or with conditioned medium from PDLCs treated with 1 μg/mL LPS and 10 mM nicotine and LPS for 24 h. CM from PDLCs treated with nicotine and LPS increased the amount of TRAP-positive multinucleated cells and TRAP activity, compared to M-CSF and RANKL treated cells. CM-induced TRAP- multinucleated cells and TRAP activity were blocked by PGJ_2 in a dose-dependent manner (Fig. 3A, B). To determine whether the inhibitory effect of PGJ_2 correlated with the expression of the osteoclast-specific genes, total RNA was prepared and analyzed by RT-PCR for osteoclast-specific genes expression in mouse BMM cells. PGJ_2 resulted in dose-dependent attenuation of CM-induced cathepsin-K and calcitonin receptor (CR) mRNA upregulation (Fig. 3C).

EFFECTS OF HIF-2α SIRNA ON NICOTINE- AND LPS-INDUCED INFLAMMATORY MEDIATORS IN PDL CELLS AND OSTEOCLASTIC DIFFERENTIATION IN BMM CELLS

To verify the potential role of HIF-2α in the LPS- and nicotine-induced inflammatory response and osteoclastic differentiation, PDLCs were transiently transfected with either control vector or HIF-2α siRNA, followed by addition of nicotine and LPS for 24 h. As shown in Figure 4, inhibition of HIF-2α expression by HIF-2α siRNA significantly counteracted the effects of nicotine and LPS on COX-2 and iNOS protein expression, TNF-α, IL-1β, -6, -8, -10, -11, and -17

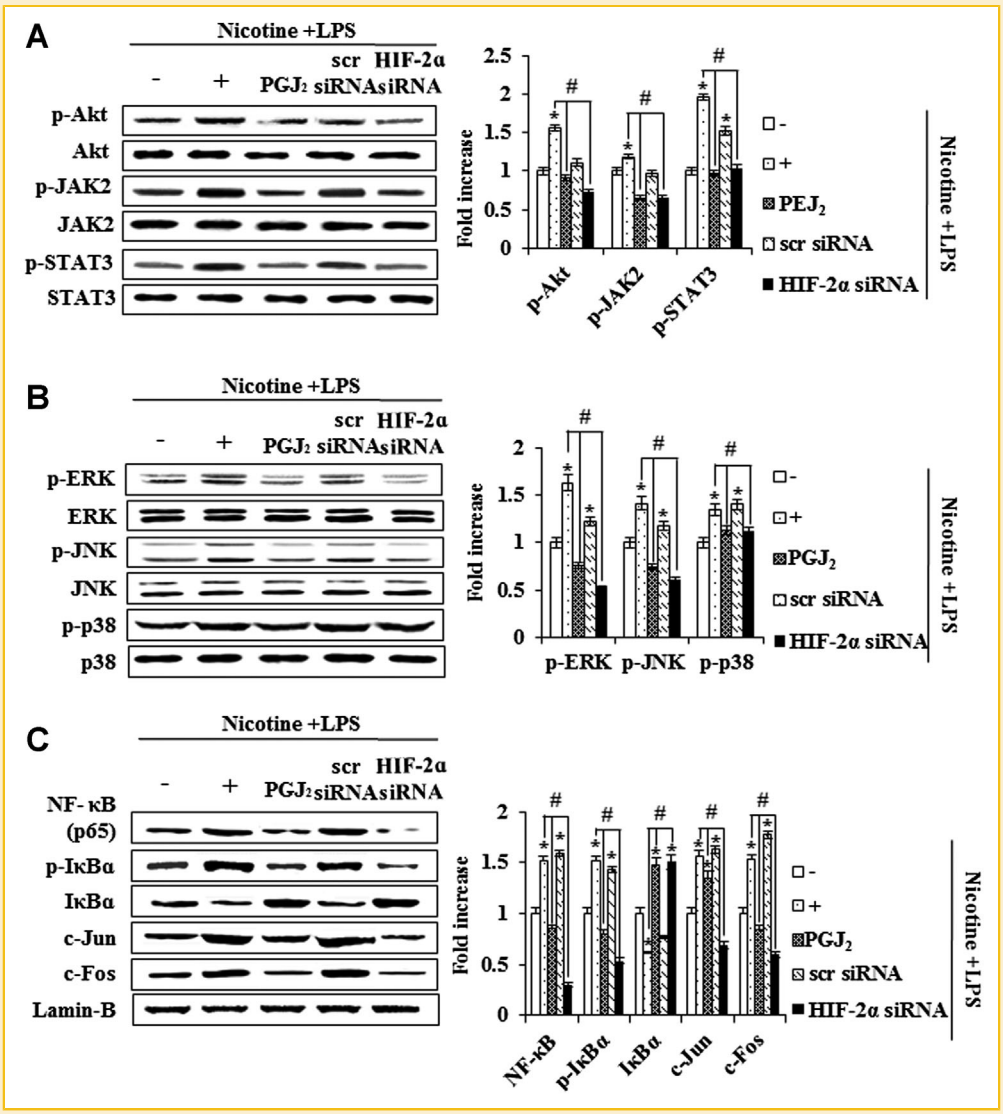

Fig. 9. Effect of HIF2-α inhibitor and HIF2-α siRNA on nicotine and LPS-induced activation of Akt and JAK2/STAT3 (A), mitogen-activated protein kinase (MAPK) (B), and transcription factors (C) in PDLCs. Cells were pretreated with PEJ_2 for 6 h or HIF2-α siRNA for 12 h and then incubated with 10 mM nicotine and 1 μg/mL LPS in serum-free media for 30 min (A–C). Blots are representative of three independent experiments. Histogram is densitometric quantitation of protein expression (A–C). *Statistically significant differences compared with the control, $P < 0.05$. #Statistically significant difference compared with the conditioned medium collected from nicotine- and LPS- treated PDL cells, $P < 0.05$ as determined by Duncan's multiple range test.

mRNA upregulation, and NO and PGE_2 production (Fig. 4 A–E), whereas control siRNA showed no effect in PDLCs.

CM with HIF-2α siRNA had significant inhibitory effects on osteoclast number and TRAP activity as well as osteoclast-specific genes in BMM cells, compared to CM treated with scr siRNA or M-CSF and RANKL (Fig. 5 A–C).

EFFECTS OF HIF2-α SIRNA ON NICOTINE AND LPS- INDUCED MMPS AND TIMPS IN PDLCS

To examine whether HIF2-α expression induced by nicotine and LPS could affected HIF2-α-mediated ECM turn-over molecules, PDLCs were either transfected with HIF2-α siRNA or pretreated for 1 h with the HIF2-α inhibitor PJG_2, following a 24-h exposure to nicotine and LPS. HIF2-α siRNA and PGJ_2 blocked nicotine- and LPS-upregulated collagenases (MMP-1, -8, and -13) and gelatinases (MMP-2 and -9), but downregulation of TIMPs (TIMP-1 and -2) was recovered (Fig. 6A, B).

EFFECTS OF HIF2-α INHIBITION ON NICOTINE- AND LPS-INDUCED INFLAMMATORY MEDIATORS IN PRIMARY CULTURED PDLCS AND OSTEOCLASTIC DIFFERENTIATION

To make comparisons between the PDLCs cell line and primary PDLCs, primary human PDLCs were utilized. Similar to the PDLCs cell line, inhibition of HIF-2α expression by HIF-2α siRNA and PGJ_2 blocked nicotine- and LPS-induced HIF-2α expression (Fig. 7 A), as well as the production of inflammatory mediators such as protein

expression of COX-2 and iNOS (Fig. 7 B), production of NO and PGE_2 (Fig. 7 D, E), and mRNA expression of TNF- α, IL-1β, -6, -8, -10, -11, and -17 in primary cultured cells (Fig. 7C). In addition, HIF2-α siRNA and PGJ_2 blocked nicotine- and LPS-upregulated collagenases (MMP-1, -8, and -13) and gelatinases (MMP-2 and -9), but reversed TIMPs (TIMP-1 and -2) in primay PDLCs (Fig. 7F). CM with HIF-2α siRNA and PGJ_2 from primary PDLCs reduced RANKL-induced osteoclast number and TRAP activity as well as cathepsin K and CR mRNA in BMM cells (Fig. 8 A–C).

EFFECTS OF HIF2-α INHIBITION ON NICOTINE AND LPS-INDUCED ACTIVATION OF SIGNAL PATHWAY IN PDLCS

To investigate the intracellular signaling mechanisms responsible for the effect of HIF2-α in PDLCs, phosphorylation of Akt, STAT3, JAK2, MAP kinase (MAPK), and activation of transcriptional factors were examined. Nicotine and LPS stimulation of the cells resulted in phosphorylation of Akt, Jak-2, and STAT3 within 1 h, which was reversed by HIF2-α siRNA and PGJ_2 , but not by control siRNA (Fig. 9 A). As shown in Fig. 9B, HIF2-α inhibition by siRNA and PGJ_2 blocked the nicotine- and LPS-induced phosphorylation of well-characterized subfamilies of MAPKs including the extracellular signal-regulated kinases (ERK1/2) and c-Jun NH_2-terminal kinases (JNK), but not p38. In addition, pretreatment of PDL cells with HIF2-α inhibition attenuated nicotine- and LPS-induced nuclear translocation of p65 (NF-κB), degradation of IκBα protein, and phosphorylation of IκBα as well as AP-1 components c-Fos and c-Jun (Fig. 9C).

DISCUSSION

HIF has been proposed as a therapeutic molecular target for anti-inflammatory interventions [Cramer et al., 2003; Imtiyaz et al., 2010]. In addition, HIF2-α is a target for therapeutic approaches to prevent cartilage destruction in osteoarthritis, especially in *Epas1*-knockout mice [Yang et al., 2010]. Smokers saliva may have high levels of carbon monoxide (CO), which is predicted to induce oxygen sensors like HIF-1α and HIF-2α to behave as in the presence of high levels of oxygen because of the tight binding of CO with heme proteins. Although hypoxia increases osteoclast formation in human peripheral blood mononuclear cells (hPBMCs) [Utting et al., 2010], it is not known whether HIF-2α regulates pro-inflammatory mediators and osteoclastogenesis in periodontal disease.

Our study is the first to investigate the involvement of HIF2-α in periodontal disease, demonstrating that nicotine and LPS upregulated HIF-2α mRNA and protein expression in time- and dose-dependent manners in PDL cell line and primary cultured PDLCs. Furthermore, HIF2-α mRNA expression was upregulated in chronically inflamed PDL cells, compared with healthy individuals from pubic microarray data [Gersdorff et al., 2008]. The over-expression of HIF-2α was also observed in a nicotine-stimulated fetal-derived immortalized chromaffin cell line [Salman et al., 2012], and in pro- and anti-inflammatory cytokine-treated mouse articular chondrocytes and human osteoarthritis-affected cartilage [Yang et al., 2010].

PGJ2 has dual role in regulating HIF-mediated metabolic responses. It inhibits HIF-2α expression but leads to accumulation of HIF-1α protein in human kidney HK-2 cells [Olmos et al., 2009]. But, PGJ2 is non-functional in HIF-1α expression in human renal cell carcinoma cell line [Zimmer et al., 2010]. Therefore, we believe that the PGJ2 obviously inhibits HIF-2α expression/activity and it is not a cell-specific phenomenon, however the effect of PGJ2 on HIF-1α is still controversial or at least it is a cell context-dependent phenomenon. In PDLCs, PGJ2 treatment did not result in change of HIF-1α compare with significant reduction of HIF-2α expression. Thus, it is highly unlikely that the observed effects of PGJ2 were caused by the indirect regulation of HIF-1α protein because the level of HIF-1α was remaining nearly unaltered upon the use of PGJ_2.

PGJ_2 inhibits translation of the HIF-2α message in a mammalian target of rapamycin-independent manner by promoting the binding of iron regulatory protein-1 (IRP1) to a noncanonical iron responsive element (IRE) embedded within the 5′-untranslated region of the HIF-2α message [Zimmer et al., 2010]. However, our present study demonstrated that PGJ_2 inhibited LPS and TNF-α -induced HIF-2α expression at the transcriptional and translational levels in PDL cell line and primary cultured PDLCs. Therefore, the diminished mRNA expression of HIF-2α upon PGJ2 treatment seems to be one of the indirect effects of PGJ_2. Indeed, PGJ2 was also known to inhibit NK-κB activation directly [Boisvert et al., 2008] or indirectly by reducing HIF-1β expression [van Uden et al., 2011]. In addition, NF-κB activation was prominently decreased in the presence of PGJ_2. From our results, it would be predicted that reduced NF-κB activation was largely caused by the inhibition of HIF-2 α; however, an additional mode of suppression in NF-κB pathway could be exists in part. Accordingly, we believe that the transcriptional down-regulation of HIF-2α was attributed to functional NF-κB inhibitory nature of PGJ_2 in human PDLCs.

In this study, nicotine- and LPS-induced inflammatory mediators such as protein expression of COX-2 and iNOS, production of NO and PGE_2, and mRNA expression of TNF- α, IL-1β, -6, -8, -10, -11, and -17 were abolished in the presence of the HIF-2α inhibitor PGJ_2 or HIF-2α siRNA. These findings suggest that HIF-2α induction by nicotine and LPS leads to up-regulation of these inflammatory mediators. These results are consistent with previous studies which found that pretreatment with PGJ_2 inhibited LPS/IFN-γ-induced TNF- α, IL-1β, and IL-6 in rat astrocytes [Giri et al., 2004], and LPS-induced IL-6 in osteoblast-like cells MC3T3E-1 [Jung et al., 2009].

Hypoxia stimulates osteoclastogenesis and resorption pit formation via stabilization of both HIF-1α and HIF-2α proteins in human peripheral blood mononuclear cells [Utting et al., 2010]. Osteoblasts express both HIF-1α and HIF-2α and their overexpression through the disruption of *Vhl* results in a striking and progressive increase in bone volume and a corresponding increase in skeletal vascularity [Wang et al., 2007]. We found that CM increased the number of TRAP-positive multinucleated cells and TRAP activity in BMM cells, compared to the levels in RANKL and M-CSF only treated cells. These results are consistent with a previous study using CM from nicotine- and LPS-treated osteoblasts [Tanaka et al., 2006]. Since osteoclasts express a number of molecular markers including cathepsin K and calcitonin receptor [Mbalaviele et al., 1999], we next investigated the mRNA expression of important genes for proper osteoclast formation. HIF-2α inhibitor PGJ_2 and HIF-2α siRNA attenuated CM-induced osteoclast differentiation (TRAP activity and multinucleated osteoclast number) as well as the expression of osteoclast markers such as

cathepsin-K and calcitonin receptor in BMM cells. Consistent with our data, a previous study showed that PGJ_2 inhibited TNF-α-mediated osteoclast differentiation in human peripheral monocytes [Hounoki et al., 2008]. These studies indicate HIF-2α as a key therapeutic target in controlling inflammation-induced bone loss.

MMPs and TIMPs are expressed in healthy periodontal tissues, and play a role in tissue destruction in periodontal disease [Reddy et al., 2003; Negrato et al., 2013]. Thus, synthetic MMP-inhibitor such as doxycycline can be used as adjunctive treatment to both support periodontal treatment and systematically reduce inflammation [Reddy et al., 2003]. Furthermore, we showed that nicotine upregulated MMP-1, -2, -8, and -9 by in PDLCs [Lee et al., 2012], and nicotine and LPS upregulated MMP-2 and MMP-9 in PDLCs [Kim et al., 2012]. Our data demonstrated that HIF-2α siRNA and inhibitor abolished nicotine LPS-induced MMP expression, and restored TIMP expression. These results are in agreement with previous studies showing that PGJ_2 inhibited IL-1β-induced MMP-13 in human chondrocytes [Fahmi et al., 2001].

Signal transduction pathways closely involved in inflammation or osteoclast differentiation include the MAPK pathway, phosphatidylinositol-3 protein kinase (PI3) pathway, janus kinase-signal transducer and activator of transcription (JAK-STAT) and NF-κB pathway [Kim et al., 2012; Lee et al., 2013]. Previously, we reported that nicotine and LPS stimulates PI3K, MAPK, and NF-κB activity which is necessary for proinflammatory cytokine expression in PDLCs [Lee et al., 2013]. In this study, we found that nicotine and LPS treatment induced phosphorylation of Akt, JAK2 and STAT3 in PDL cells, and phosphorylation was blocked by HIF-2α inhibitor and siRNA. Consistent with our findings, PGJ_2 has been shown to inhibit IL-10-induced p-STAT3 in macrophages and THP-1 cells [Ji et al., 2005]. Members of the mitogen-activated protein kinase (MAPK) pathway have been implicated in increasing the activity of HIF in response to various stimuli [Motohira et al., 2007; Majmundar et al., 2010]. NF-κB signal is an upstream mechanism that regulates HIF-2α, since IL-1β and TNF-α, putative ligands of the NF-κB signal, increase HIF-2α expression in fibroblasts [Jung et al., 2003] and chondrocytes [Yang et al., 2010]. AP-1 is a dimeric transcription factor composed of the products of Jun and Fos proto-oncogenes (c-Jun, JunB, JunD, c-Fos, FosB, Fra-1 and Fra-2) [Giri et al., 2004]. We demonstrated that treatment with HIF-2α inhibitor and HIF-2α silencing blocked nicotine- and LPS-induced phosphorylation of ERK and JNK, and activation of NF-κB and AP-1 components, suggesting that Akt, JAK2 and STAT3, MAPK, NF-κB, and AP-1 are involved in these responses. Similar signaling mechanisms have been observed where PGJ_2 inhibits LPS-stimulated Akt and NF-κB activation in osteoblasts [Jung et al., 2009], and blocks LPS-induced p38 and NF-κB activation in cardiomyocytes [Hovsepian et al., 2010]. The limitation of the present study is that we did not examined the effects and pharmacological studies of HIF2-α inhibition in animal models of inflammation, periodontitis and bone resorption. Further detailed in vivo experiments are necessary to clarify this anti-inflammatory and bone resorption effect of HIF2-α.

In summary, we demonstrated for the first time that upregulation of HIF2-α is observed in patients with chronic periodontal disease, and in an in vitro model of nicotine plus LPS-stimulated PDL cell line and primary cultured PDLCs. HIF-2α inhibition and silencing of HIF-2α had the ability to ameliorate LPS plus nicotine-induced inflammatory cytokines/ mediators/ proteolytic enzymes, and osteoclast differentiation via Akt, JAK2 and STAT3, MAPK, NF-κB, and AP-1pathways in PDLCs. These findings support HIF-2α inhibition as an attractive biological therapeutic molecular target for the treatment of periodontal disease.

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