Sodium Hydrogen Sulfide Inhibits Nicotine and Lipopolysaccharide–Induced Osteoclastic Differentiation and Reversed Osteoblastic Differentiation in Human Periodontal Ligament Cells

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ABSTRACT

Although previous studies have demonstrated that hydrogen sulfide (H_2S) stimulated or inhibited osteoclastic differentiation, little is known about the effects of H_2S on the differentiation of osteoblasts and osteoclasts. To determine the possible bioactivities of H_2S on bone metabolism, we investigated the in vitro effects of H_2S on cytotoxicity, osteoblastic, and osteoclastic differentiation as well as the underlying mechanism in lipopolysaccharide (LPS) and nicotine-stimulated human periodontal ligament cells (hPDLCs). The H_2S donor, NaHS, protected hPDLCs from nicotine and LPS-induced cytotoxicity and recovered nicotine- and LPS-downregulated osteoblastic differentiation, such as alkaline phosphatase (ALP) activity, mRNA expression of osteoblasts, including ALP, osteopontin (OPN), and osteocalcin (OCN), and mineralized nodule formation. Concomitantly, NaHS inhibited the differentiation of tartrate-resistant acid phosphatase (TRAP)-positive osteoclasts in mouse bone marrow cells and blocked nicotine- and LPS-induced osteoclastogenesis regulatory molecules, such as RANKL, OPG, M-CSF, MMP-9, TRAP, and cathepsin K mRNA. NaHS blocked nicotine and LPS-induced activation of p38, ERK, MKP-1, PI3K, PKC, and PKC isoenzymes, and NF-κB. The effects of H_2S on nicotine- and LPS-induced osteoblastic and osteoclastic differentiation were remarkably reversed by MKP-1 enzyme inhibitor (vanadate) and expression inhibitor (triptolide). Taken together, we report for the first time that H_2S inhibited cytotoxicity and osteoclastic differentiation and recovered osteoblastic differentiation in a nicotine- and periodontopathogen-stimulated hPDLCs model, which has potential therapeutic value for treatment of periodontal and inflammatory bone diseases. J. Cell. Biochem. 114: 1183–1193, 2013.   © 2012 Wiley Periodicals, Inc.

Inflammatory bone diseases frequently exhibit imbalances in the regulation of bone resorption and formation that lead to excessive bone resorption and tissue destruction, as seen in rheumatoid arthritis, periodontitis, implant osteolysis, and osteo-myelitis [Goldring, 2003; Sabokbar et al., 2003]. Periodontal disease is dental plaque-induced inflammation of the periodontal tissues that results in bone loss in the affected teeth. The primary etiologic organisms of this process disease are gram-negative, anaerobic

bacteria that exist within a tooth-associated biofilm under the gum line [Darveau et al., 1997]. *Porphyromonas gingivalis* was suggested to usually be a late colonizer in the oral cavity and to play a central role in subgingival pockets [Lamont and Jenkinson, 1998]. Thus, subgingival plaque biofilms containing *P. gingivalis* appear to be important for the progression of periodontal disease [Lamont and Jenkinson, 1998]. Lipopolysaccharides (LPS) are cell wall components of virtually all subgingival gram-negative organisms. LPSs induce polymorphonuclear leukocyte infiltration, edema, and vascular dilatation in inflamed periodontal tissues [Page, 1991]. Furthermore, LPSs play an important role in the destruction of periodontal tissue, including the gingiva, periodontal ligament (PDL), and alveolar bone through the production of bone-absorbing cytokines, such as interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) [Agarwal et al., 1995].

Tobacco smoking is the main risk factor associated with chronic destructive periodontal disease [Bergstrom and Preber, 1994]. Many studies have shown the effects of continued smoking on persistent, gingival bleeding, vertical bone loss, and poor treatment outcomes [Johnson and Guthmiller, 2007]. Similarly, in vitro studies have demonstrated that nicotine, a major component of cigarette smoke, inhibits the attachment and growth of human gingival fibroblasts (HGFs) and human periodontal ligament cells (hPDLCs) [Tipton and Dabbous, 1995; James et al., 1999]. Recently, we reported that nicotine treatment concomitantly downregulates osteoblastic differentiation markers, such as alkaline phosphatase (ALP), osteocalcin (OCN), and osteopontin (OPN), and upregulates the expression of receptor activator of nuclear factor-κB ligand (RANKL) in hPDLCs [Lee et al., 2009].

The combination of nicotine and LPS stimulates the formation of osteoclast-like cells by increasing the production of macrophage colony-stimulating factor (M-CSF) and PGE_2 by osteoblasts [Tanaka et al., 2006] and increases PGE_2 and cyclooxygenase-2 (COX-2) expression in osteoblasts [Shoji et al., 2007]. Recently, we demonstrated that nicotine and LPS synergistically induce the production of nitric oxide (NO) and PGE_2 and increase inducible nitric oxide synthase (iNOS) and COX-2 expression in hPDLCs [Pi et al., 2010].

Hydrogen sulfide (H_2S) is a novel gaseous mediator, which is synthesized by mammalian tissues via two cytosolic pyridoxal-5′-phosphate-dependent enzymes responsible for metabolism of L-cysteine—cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE)—as well as by a mitochondrial third pathway involves the production from L-cysteine of H_2S via the combined action of 3-mercaptopyruvate sulfurtransferase (3-MST) and cysteine aminotransferase (CAT) [Kamoun, 2004; Kimura, 2011]. Recent studies have implicated that H_2S plays an important role in many physiological and pathological processes, such as vasodilatation, cardio protection, and neuromodulation [Zhao et al., 2001; Cheng et al., 2004]. Increased biosynthesis of H_2S has been demonstrated in animal models of septic/endotoxic and hemorrhagic shock, pancreatitis and carrageenan-evoked hindpaw edema in the rat [Li et al., 2005; Zhang et al., 2007]. Clinical studies demonstrated the elevated H_2S levels in periodontally involved pockets. For instance, positive correlations are noted between the amounts of H_2S in the gingival crevice and the depth of periodontal pockets [Rizzo, 1967]

and between gingival index, gingival crevicular fluid volume, and H_2S production [Solis-Gaffar et al., 1980]. It is also known that sulfide rise significantly with an increase in radiographic bone loss and are correlated with other clinical parameters, such as probing depth, clinical attachment level, and bleeding on probing [Morita and Wang, 2001a,b].

The anti-inflammatory effects of H_2S were previously reported in several animal models, including knee joint synovitis in rats [Ekundi-Valentim et al., 2010] and caerulein-induced acute pancreatitis [Sidhapuriwala et al., 2009] and colitis in mice [Fiorucci et al., 2007]. The observation that administration of an inhibitor of endogenous H_2S synthesis resulted in significant adhesion of leukocytes (primarily neutrophils) to the vascular endothelium in mesenteric venules of rats suggested that H_2S tonically down-regulates leukocyte adhesion and emigration [Zanardo et al., 2006]. Moreover, H_2S inhibits NO production and nuclear factor κB (NF-κB) activation in LPS-stimulated macrophages [Oh et al., 2006] and attenuates LPS-induced inflammation in primary cultured and immortalized rodent microglia cells [Hu et al., 2007]. In contrast, H_2S induces the synthesis of proinflammatory cytokines, such as TNF-α, IL-1β, and IL-6, in human monocyte cell line U937 [Zhi et al., 2007] and promotes IL-8 production in epithelial cells [Chen et al., 2010].

H_2S single application causes a transient increase of osteoclast differentiation with upregulation of RANKL expression in the osteoblasts of rat periodontal tissue [Irie et al., 2009]. Furthermore, H_2S (NaHS, a H_2S donor) and LPS had an additive effect on tartrate-resistant acid phosphate (TRAP)-positive osteoclast differentiation in rats [Irie et al., 2012]. In addition, H_2S attenuates hydrogen peroxide (H_2O_2)-suppressed osteoblastic differentiation in MC3T3-E1 cells and may have a potentially therapeutic value for osteoporosis [Xu et al., 2011]. These data support the hypothesis of inhibitory effects of H_2S on nicotine and LPS stimulated osteoblastic or osteoclastic differentiation in hPDLCs. However, whether H_2S exerts any cytoprotective or cytotoxic and osteoclast- or osteoblast-inducing or inhibitory effects is not fully understood. Therefore, the purpose of this study was to examine the effects and underlying signal transduction pathways of H_2S on nicotine- and LPS-stimulated hPDLCs focusing on osteoblastic and osteoclastic differentiation, since smoking and dental plaque-induced periodontitis stimulate bone resorption and inhibit bone formation.

MATERIALS AND METHODS

REAGENTS

Dulbecco's modified Eagle's medium (DMEM), α-minimum essential medium (α-MEM), fetal bovine serum (FBS), and other tissue culture reagents were obtained from Gibco-BRL (Grand Island, NY). Ultrapure LPS from *P. gingivalis* was purchased from InvivoGen (San Diego, CA). Recombinant RANKL and M-CSF were purchased from Peprotech (London, UK). NaHS, nicotine, and all other chemicals were obtained from Sigma (St. Louis, MO) unless otherwise indicated. Anti-IκBα, phospho-IκBα, and NF-κB p65 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Other antibodies were obtained from Cell Signaling Technology (Beverly, MA).

PRIMARY CULTURE OF hPDLCs

hPDLCs were isolated with an explant culture technique from patients undergoing orthodontic treatment by previously described methods [Somerman et al., 1988]. Briefly, tissues were cut into 1-mm^2 explants and placed on 100-mm culture dishes (Nunc, Naperville, IL) containing 100 U/ml of penicillin G, 100 μg/ml of streptomycin, 25 μg/ml of amphotericin B, and 10% heat-inactivated FBS (Gibco-BRL) at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. After 2 or 3 days, cells were detached with 0.025% trypsin and 0.05% EDTA diluted with culture medium, then subcultured at a ratio of 1:4. Cells between the 4th and 7th passages were used in this study. For osteogenic differentiation, cells were cultured with differentiation medium (10% FBS/DMEM including 50 μg/ml ascorbic acid and 10 mM β-glycerophosphate) as described previously [Fujii et al., 2002]. All experiments were performed in accordance with the guidelines approved by the local ethics committee.

MEASUREMENT OF H$_2$S LEVEL

Intracellular levels of H_2S were measured using a previously described method [Li et al., 2007]. After incubation with NaHS for the indicated times, 75 μl of culture media from each group were collected. They were diluted in deionized water (final volume, 500 μl) and added to an eppendorf already containing zinc acetate (1%, w/v, 250 μl) to trap H_2S. Subsequently, N,N-dimethyl-p-phenylenediamine sulfate (20 μM; 133 μl) in 7.2 mol/L HCl was added, followed by $FeCl_3$ (30 μM; 133 μl) in 1.2 mol/L HCl. Thereafter, trichloroacetic acid (10%, w/v, 250 μl) was used to precipitate any protein that might be present in the culture media and upon centrifugation (10,000g) absorbance (670 nm) of aliquots from the resulting supernatant (300 μl) was determined using a 96-well microplate reader (Bio-Rad, CA).

MTT ASSAY

Cell viability was assessed by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. Cells were seeded and cultured as described above. MTT (0.5 mg/ml) was added for the last 3 h. After removal of the medium and the addition of dimethyl sulfoxide to the flask, the absorbance at 570 nm was measured using a microplate reader (Bio-Rad, Mississauga, ON, Canada).

DETERMINATION OF ALP ACTIVITY

Cells were plated on 96-well microplates at a density of 1×10^4 cells/cm^2 and cultured in 5 mM nicotine and 1 μg/ml LPS with the indicated concentration NaHS for up to 3, 7, or 14 days. Two hundred microliters of enzyme assay solution (8 mM p-nitrophenyl phosphate, 12 mM $MgCl_2$, and 0.1 mM $ZnCl_2$ in 0.1 M glycine–NaOH buffer, pH 10.5) were added to the cells in each well, and the plate was incubated for several minutes at 37°C. The enzyme reaction was terminated by the addition of 50 μl of 0.1 M NaOH. The amount of p-nitrophenol released by the enzyme reaction was determined by measuring the absorbance at 405 nm using a microtiter plate reader.

OSTEOCLAST FORMATION

Mouse bone marrow cells were isolated by flushing the bone marrow spaces of the femora and tibiae of 5- to 6-week-old ICR mice as described previously [Huang et al., 2006]. Cells were incubated with α-MEM containing 10% FBS, penicillin (100 U/ml), and streptomycin (100 mg/ml) for 24 h in 5% CO_2 at 37°C. Nonadherent cells were completely removed by aspiration and remaining cells further cultured for 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 (Oc) precursor cells. To achieve Oc 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 6 days with 30 ng/ml M-CSF and 100 ng/ml RANKL. Following the time course of Oc 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 instruction. TRAP-positive multinucleated cells containing three or more nuclei were counted as Ocs under a light microscope.

ISOLATION OF RNA AND RT–PCR ANALYSIS

Cells were grown in 60-mm culture dishes and incubated for indicated times in fresh medium containing stimuli as indicated. Total RNA was isolated from cells using TRIzol® reagent (Invitrogen Life Technology, Carlsbad, CA) according to the manufacturer's protocol. RNA quality was assessed using 1% agarose gel electrophoresis and spectrophotometric 260/280 nm absorbtion. Reverse transcription of the RNA was performed using AccuPower RT PreMix (Bioneer, Daejon, Korea). Then, 1 μg RNA was reverse-transcribed for first strand cDNA synthesis (Gibco-BRL). 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 reaction conditions for PCR were 30–35 cycles of denaturation at 95°C for 30 s, annealing at 55–60°C for 30 s, and extension at 72°C for 1 min. Primer sequences for differentiation markers are detailed in Table I. The PCR products were resolved on a 1.5% agarose gel and stained with ethidium bromide.

TABLE I. The Primers Used for cDNA Amplification and PCR Conditions

Gene (bp)	Primers	Annealing
ALP (475)	F: 5'-ACGTGGCTAAGAATGTCATC-3' R: 5'-CTGGTAGGCGATGTCCTTA-3'	55°C, 30 s
OPN (347)	F: 5'-CCAAGTAAGTCCAACGAAAG-3' R: 5'-GGTGATGTCCTCGTCTGTA-3'	55°C, 30 s
OCN (310)	F: 5'-CATGAGAGCCCTCACA-3' R: 5'-AGAGCGACACCCTAGAC-3'	55°C, 30 s
OPG (420)	F: 5'-TGACAAATGTCCTCCTGGTA-3' R: 5'-TGTGTTGCATTTCCTTTCTG-3'	60°C, 45 s
RANKL (557)	F: 5'-CTATTTCAGAGCGCAGATGGAT-3' R:5'-TATGAGAACTTGGGATTTTGATGC-3'	60°C, 45 s
M-CSF (437)	F:v5'-ATGACAGACAGGTGGAACTGCCAGTGTAGAGG-3' R:v5'-TCACACAACTTCAGTAGGTTCAGGTGA TGGGC-3'	60°C, 1 min
MMP-9 (312)	F: 5'-GCAGAGATGCGTGGAGAGTC-3' R: 5'-GGCTGTACACGCGAGTGAAG-3'	55°C, 1 min

WESTERN BLOT ANALYSIS

Protein samples (20 µg) were mixed with an equal volume of 2× SDS sample buffer, boiled for 5 min, and then separated through 8–15% SDS–polyacrylamide electrophoresis gels. After electrophoresis, proteins were transferred to polyvinylidene fluoride membranes by electrophoretic transfer. The membranes were blocked in 5% dry milk (1 h), rinsed, and incubated with antibodies (1:1,000 dilution) in Tris-buffered saline (TBS) overnight at 4°C. Primary antibody was then removed by washing the membranes three times in TBS. Primary antibodies were labeled by incubation with 0.1 mg/ml peroxidase-labeled secondary antibodies 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 NaHS ON NICOTINE- AND LPS-INDUCED CYTOTOXICITY

The cytotoxic potential of nicotine and LPS on the hPDLCs was determined by the MTT assay. Nicotine demonstrated a cytotoxic effect on human hPDLCs in a concentration-dependent manner at 2–5 mM concentrations (Fig. 1A). However, 2 µg/ml LPS did not

significantly affect the cell viability (Fig. 1B). As shown in Figure 1C, incubation with 1 µg/ml LPS and various concentrations of nicotine decreased cell viability in concentration- and time-dependent manners. To determine whether H_2S has a cytoprotective effect, NaHS, a H_2S donor, was added simultaneously. The addition of NaHS was able to protect cells from cytotoxicity induced by LPS and nicotine (Fig. 1D). The maximum cytoprotective effect was achieved at a 100 µM concentration of NaHS.

EFFECTS OF NaHS ON MEASUREMENT OF SULFIDE CONCENTRATION IN CULTURE MEDIUM

Since sulfide is volatile in culture media and moreover is efficiently metabolized by cells, sulfide concentrations were monitored after increasing times in aliquots of the culture media bathing the hPDLCs (Fig. 2). In hPDLCs treated with NaHS, we observed a marked time-dependent decrease in H_2S formation. Using 100 µM NaHS present at the onset of incubation, we demonstrated that 50% of sulfide were still present after 24 h incubation but were under 30% after 48 h, whereas 10 µM NaHS was lost after 48 h evidencing a dose-dependent decrease of sulfide in the culture media.

EFFECTS OF NaHS ON NICOTINE AND LPS-SUPPRESSED OSTEOBLASTIC DIFFERENTIATION OF hPDLCs

Since bone loss occurs as the result of an imbalance between bone resorption and bone formation, the changes in mRNA expression for osteoblastic and osteoclastic marker genes in nicotine- and LPS-
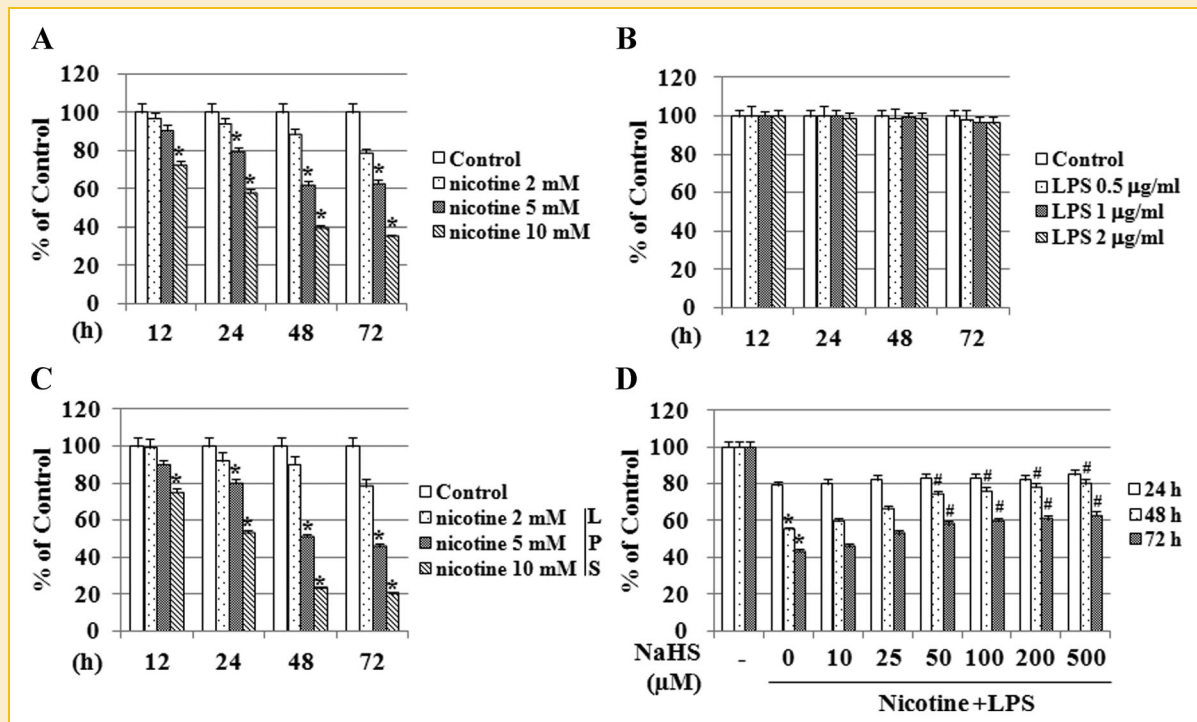

Fig. 1. Effects of NaHS on nicotine- and LPS-induced cytotoxicity in human periodontal ligament cells (hPDLCs). A,C: Cells were incubated for 12–72 h with the indicated concentrations of nicotine (2, 5, and 10 mM), LPS (0.5, 1, and 2 µg/ml) (B). C,D: Cells were incubated with nicotine (5 mM) and LPS (1 µg) or the indicated concentration NaHS for 3 days. Cell viability was determined using the MTT assay. Data were obtained from three independent experiments. Values are means ± SD of four experiments. *Statistically significant difference compared to the control, $P < 0.05$. #Statistically significant difference compared to the nicotine and LPS group, $P < 0.05$.
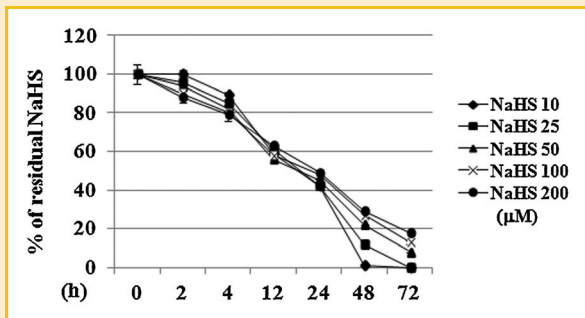
Fig. 2. Effects of NaHS on sulfide concentration in human periodontal ligament cells (hPDLCs). Indicated concentration of NaHS was freshly dissolved in the culture medium bathing hDPLCs and aliquots were tested after different periods of times. Sulfide concentration was calculated using a standard calibration curve. This experiment was repeated three times.

treated hPDLCs were examined. As shown in Figure 3A,B, nicotine and LPS markedly decreased the mRNA expression of osteoblastic differentiation markers, such as ALP, OPN, and OCN, in hPDLCs. The results of RT-PCR presented in Figure 3C,D demonstrate that nicotine and LPS time- and dose-dependently upregulated the mRNA expression of osteoclast markers, including RANKL, M-CSF, and MMP-9, but downregulated OPG.

To investigate the effects of NaHS on the LPS and nicotine-suppressed osteoblastic differentiation in hPDLCs, ALP activity, Alizarin red staining, and osteoblastic marker mRNA expression

were assessed. As shown in Figure 4, NaHS recovered the nicotine- and LPS-suppressed ALP activity, osteoblast marker gene expression, such as ALP, OPN, and OCN, and mineralized nodule formation in a dose-dependent manner (Fig. 4A–C).

To determine the effect of NaHS on osteoclastogenesis in vitro, mouse bone marrow cells were cultured with M-CSF and RANKL. With increasing doses of NaHS, a dose-dependent reduction in the number of osteoclasts occurred (Fig. 5A,B). To analyze the functional changes of osteoclastogenesis induced by NaHS treatment, total RNA from hPDLCs culture was collected and RT-PCR for osteoclast-specific gene expression was performed. NaHS reduced nicotine- and LPS-stimulated upregulation of RANKL, M-CSF, and MMP-9, with a reciprocal increase in OPG mRNA in a dose-dependent manner (Fig. 5C).

EFFECTS OF NaHS ON THE NICOTINE- AND LPS-STIMULATED SIGNALING CASCADE IN hPDLCs

To elucidate the molecular basis of the responses to NaHS, we examined the effects of NaHS on the MAP kinase (MAPK), PI3K, PKC, MKP-1, and NF-κB signaling pathways in hPDLCs. As shown in Figure 6A, treatment of hPDLCs with 50 μM NaHS blocked the time-dependent nicotine- and LPS-induced phosphorylation of p38, ERK, and Akt, but not JNK. The protein levels of p38, JNK, ERK, and Akt were not affected by NaHS treatment. Compared to the nicotine and LPS group, the treatment with NaHS blocked the phosphorylation of p38, ERK, and Akt in a dose-dependent manner (Fig. 6B).
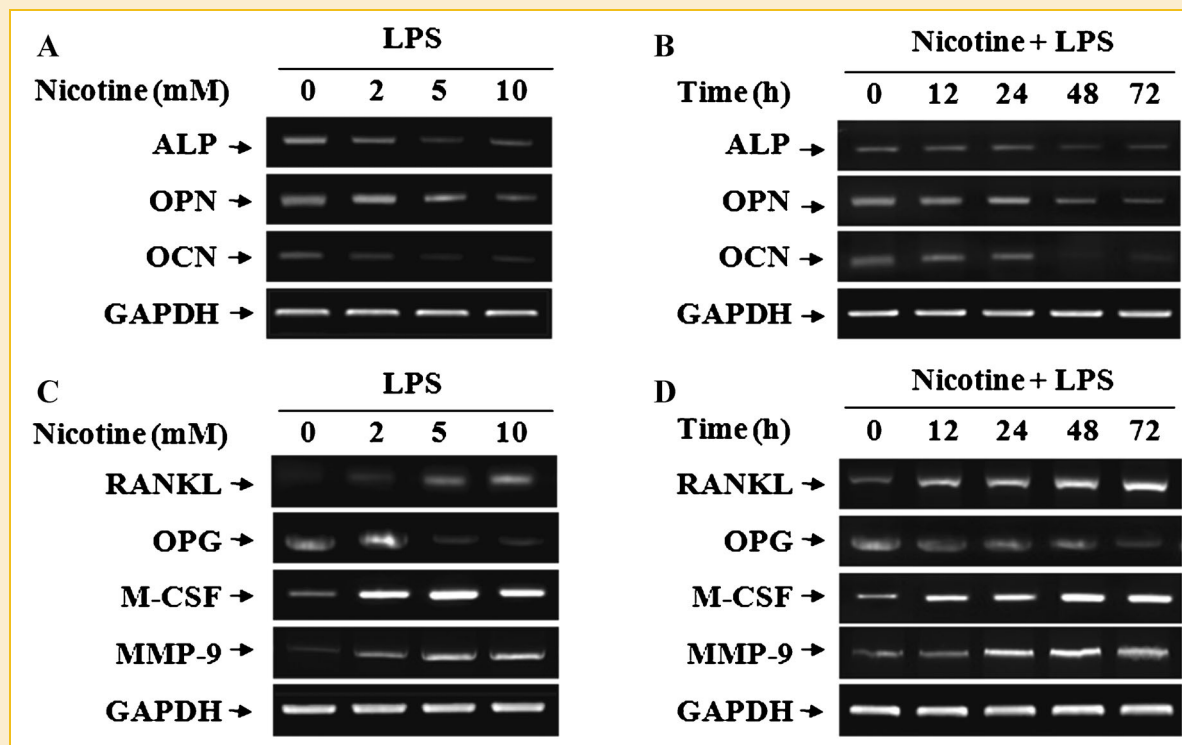

Fig. 3. Effects of nicotine and LPS on mRNA expression of osteoblastic (A,B) and osteoclastic (C,D) differentiation markers in hPDLCs. Cells were incubated with the indicated concentrations of LPS and nicotine (A,C) for 72 h and 5 mM nicotine and 1 μg/ml LPS for the indicated times (B,D). mRNA expression was evaluated by RT–PCR. Results are from three independent representative experiments.
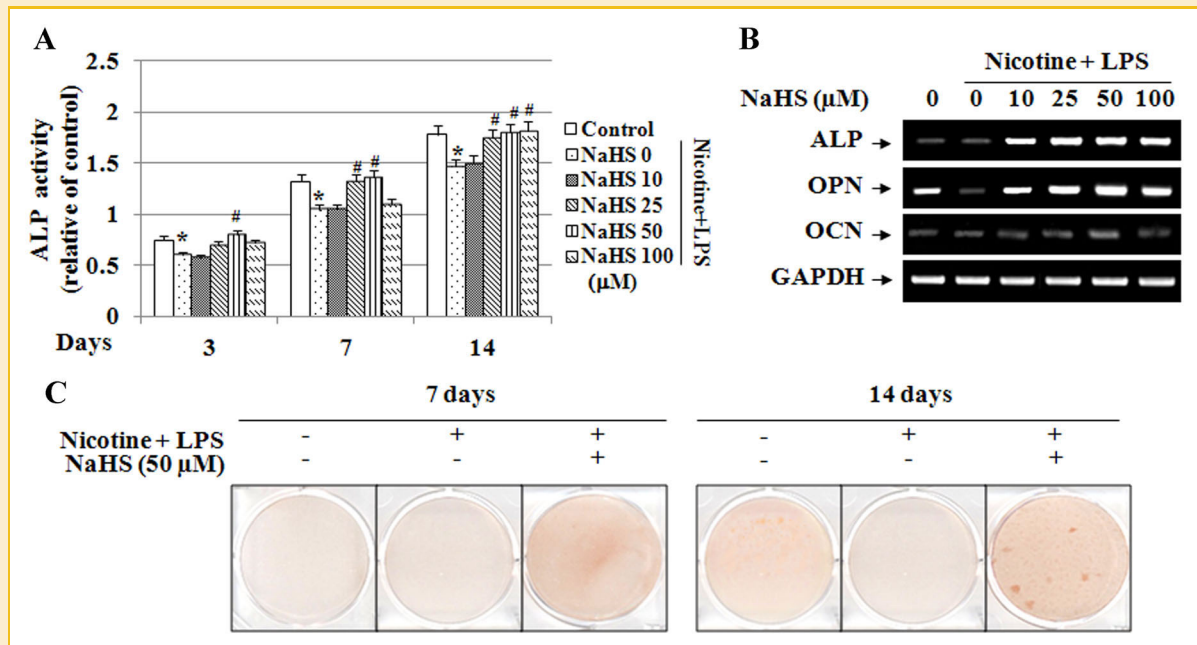
Fig. 4. Effects of NaHS on nicotine and LPS-suppressed osteoblastic differentiation in hPDLCS. Differentiation was evaluated by ALP activity (A), mRNA expression of markers by RT-PCR (B), and mineralized nodule formation by Alizarin red staining (C). Cells were incubated with nicotine (5 mM) and LPS (1 μg) or the indicated concentration NaHS (H_2S) for 3 (A,B), 7, or 14 days (C). ALP activity represents the mean values of four experiments. *Statistically significant difference compared to the control, $P < 0.05$. #Statistically significant difference compared to the nicotine and LPS group, $P < 0.05$. Similar data in RT-PCR (B) and Alizarin red staining (C) were obtained from three independent experiments.
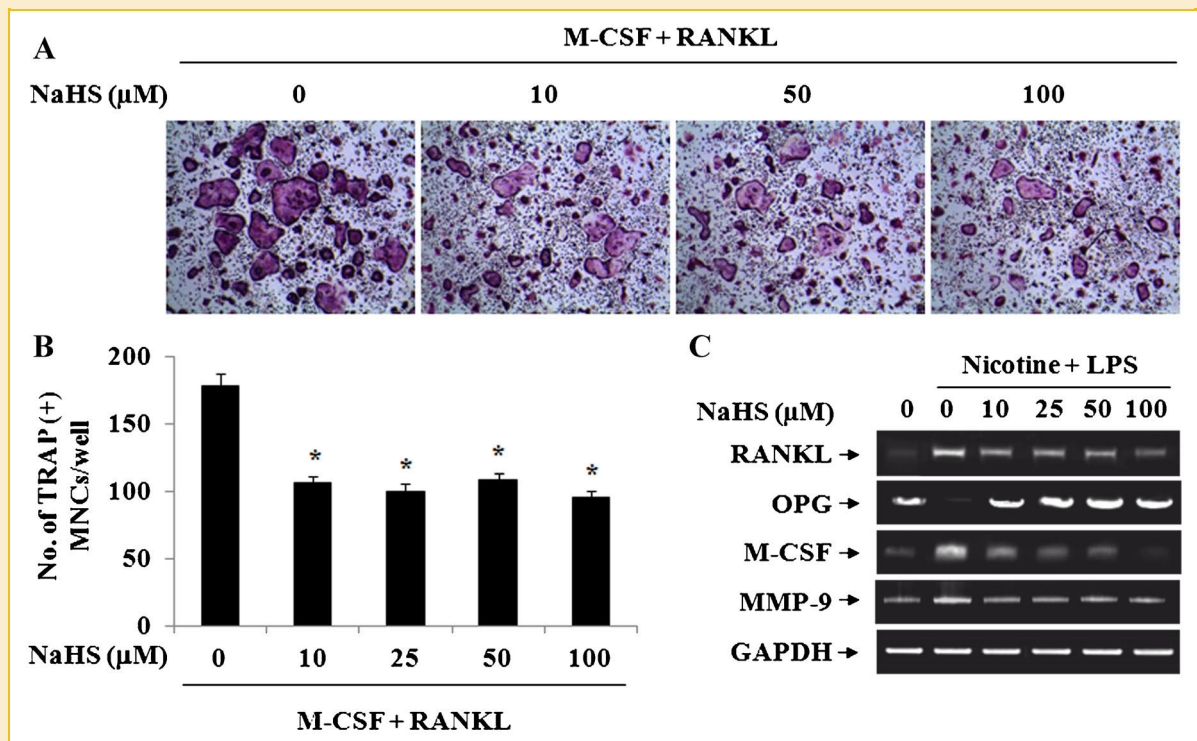

Fig. 5. Effects of NaHS on osteoclastic differentiation in mouse bone marrow cells (A) and hPDLCS (B). A: Mouse bone marrow cells were stimulated with RANKL (100 ng), M-CSF (50 ng), and the indicated concentration of H_2S for 7 days. TRAP (+) cells were determined as described in the Materials and Methods Section. *Statistically significant difference compared to the RANKL and M-CSF group, $P < 0.05$. B: hPDLCs were incubated with LPS, nicotine, and H_2S for 3 days. Similar data from RT-PCR were obtained from three independent experiments.
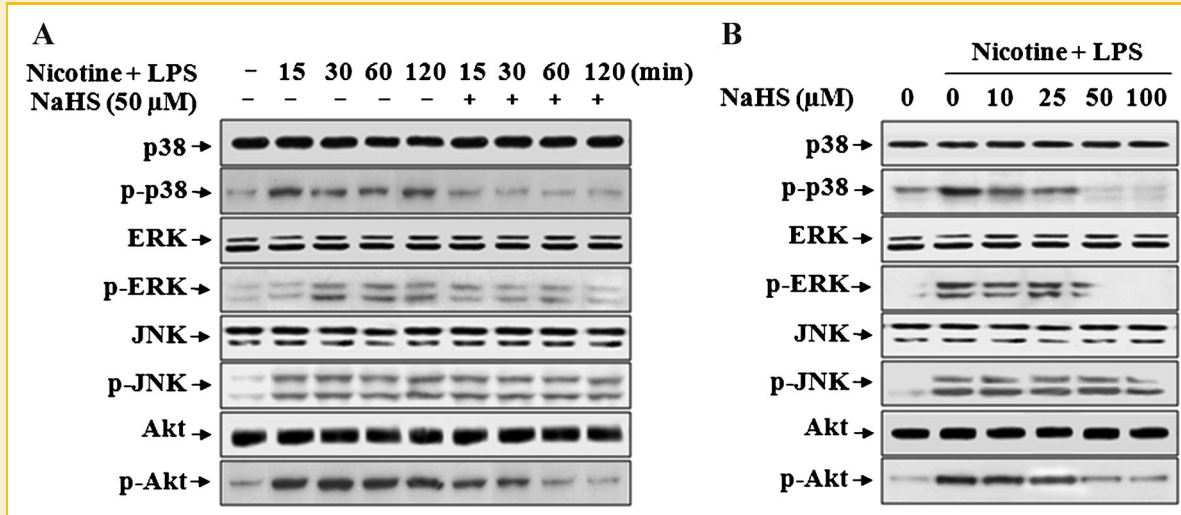
Fig. 6. Effects of H$_2$S on nicotine- and LPS-induced phosphorylation of mitogen-activated protein kinases (MAPKs) and Akt in hPDLCs. Cells were incubated with nicotine and LPS with the indicated concentration of H$_2$S for 1 h (A) and the indicated times for 50 μM H$_2$S (B). Similar data were obtained from three independent experiments.

Nicotine and LPS stimulation of the cells resulted in a decrease in MKP1 protein expression within 1 h, which was reversed by H$_2$S in a dose-dependent manner (Fig. 7A). Because H$_2$S affected nicotine- and LPS-induced MKP-1 expression, we next examined whether the MKP-1 inhibitors triptolide (TP) and vanadate could also affect osteoblastic and osteoclastic differentiation in hPDLCs treated with LPS and nicotine. Pretreatment with 0.5 μM TP and 10 μM vanadate partly reversed the inhibitory effects of nicotine- and LPS-
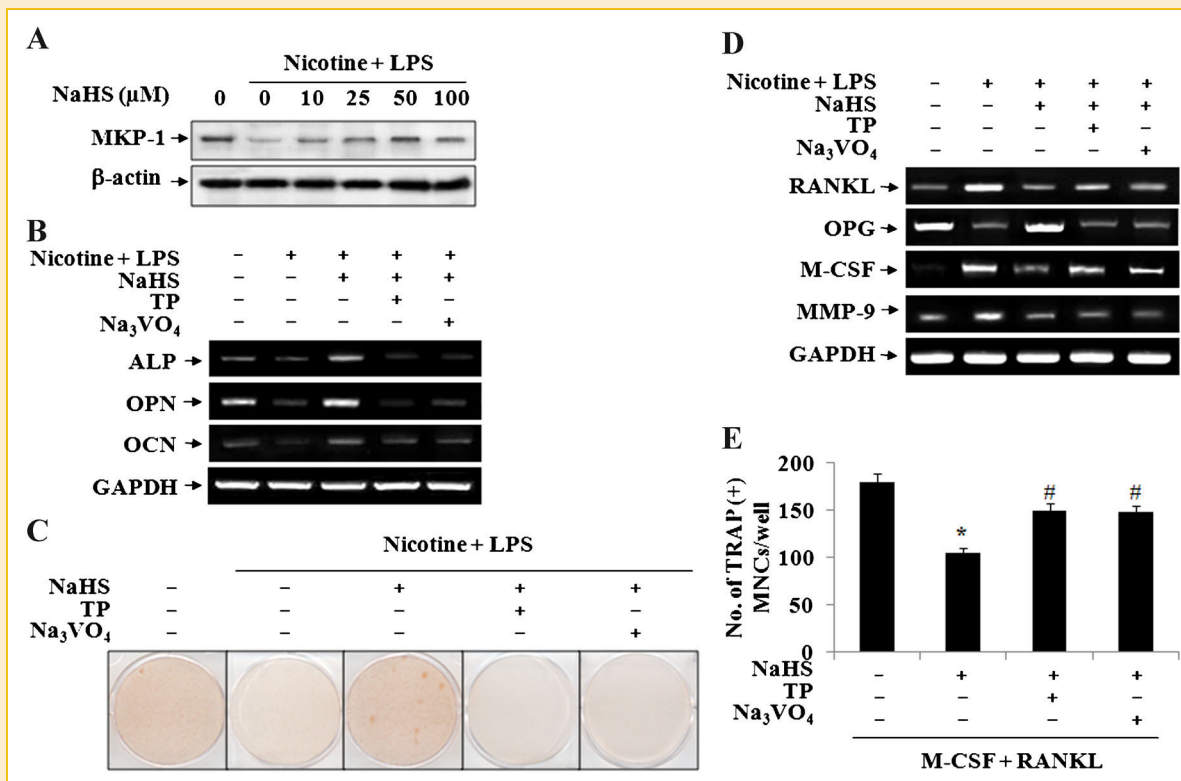

Fig. 7. Involvement of MKP-1 and the effect of H$_2$S on nicotine- and LPS-induced osteoblastic (B,C) and osteoclastic differentiation (D,E) in hPDLCs. A: Cells were incubated with nicotine and LPS for 1 h. B,C: Cells were pretreated with the MKP-1 inhibitors triptolide (TP) or Na$_3$VO$_4$ (vanadate) for 4 h and then posttreated with nicotine (5 mM), LPS (1 μg), and H$_2$S (50 μM) for 3 days (B,D) and 14 days (C). E: Mouse bone marrow cells were stimulated with RANKL (100 ng), M-CSF (50 ng), TP or Na$_3$VO$_4$, and H$_2$S for 7 days. TRAP (+) cells were determined as described in the Materials and Methods Section. Similar data from RT-PCR and Western blots were obtained from three independent experiments. [#]Statistically significant difference compared to the NaHS group.
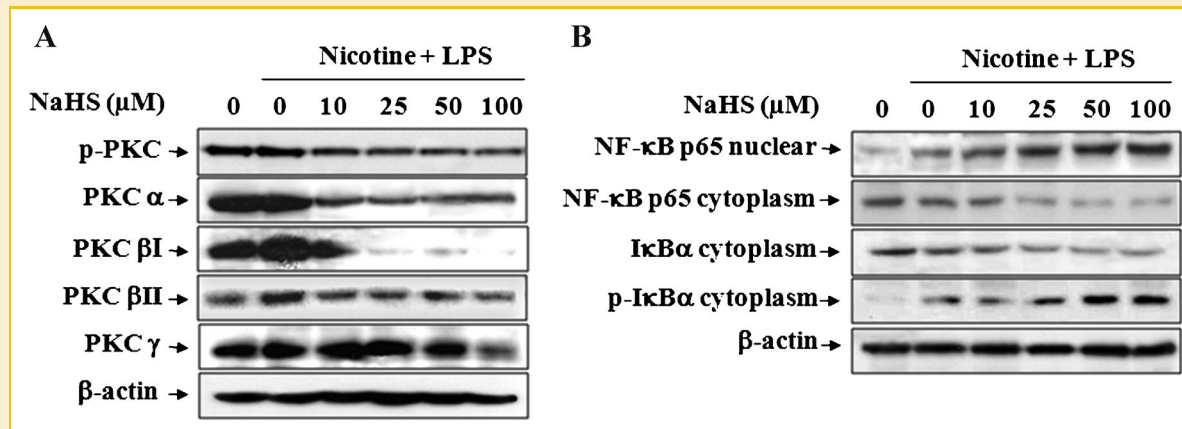
Fig. 8. Effects of H₂S on nicotine- and LPS-stimulated PKC isoform and NF-kB activation in hPDLCs. Protein expression was determined by Western blot analysis. Cells were incubated for 1 h with nicotine (5 mM), LPS (1 μg/ml), and the indicated concentration of NaHS (A,B). Blots shown are representative of three independent experiments.

stimulated expression of osteoblastic differentiation markers, as well as the stimulatory effects of nicotine and LPS on osteoclastic differentiation markers (Fig. 7B–E).

Since the PKC family is essential in signaling steps for cell differentiation, phosphorylation of PKC (p-PKC) and PKC iso-enzymes were assessed by Western blotting. When cells were treated with nicotine and LPS, p-PKC and all four PKC isoenzymes including α, βI, βII, and γ were activated. In addition, incubation with NaHS blocked nicotine- and LPS-induced upregulation of p-PKC and PKC isoenzymes α, βI, βII, and γ in a dose-dependent manner (Fig. 8A).

We next examined whether NaHS affected nicotine- and LPS-induced NF-κB activation. Following cell activation with nicotine and LPS, the degradation of IκBα protein, phosphorylation of IκBα, and the nuclear translocation of p65 (NF-κB) were elevated. However, pretreatment of cells with NaHS enhanced nicotine- and LPS-induced NF-κB activation in a dose-dependent manner (Fig. 8B).

DISCUSSION

Periodontal or alveolar bone loss reflects enhanced osteoclastic activity and suppressed osteoblastic activity during the host response to periopathogenic bacteria. Bisphosphonates [Tenenbaum et al., 2002] and COX2 inhibitors [Moore et al., 1998] block the osteoclastogenesis of hPDLCs through MMP-inhibitory properties. Recently, voltage-gated potassium channel blockers [Alverde et al., 2004] were shown to have therapeutic effects for the management of inflammatory bone resorption in experimental periodontal disease. In addition, simvastatin [Yazawa et al., 2005], enamel matrix derivative [Takayanagi et al., 2006], and chitosan [Pang et al., 2005] were found to promote osteoblastic differentiation of hPDLCs, suggesting their potential as drugs for periodontal regenerative therapy. However, to fully address bone metabolism imbalance, periodontal regenerative therapy should reduce bone resorption and promote bone formation. hPDLCs are thought to play some role in alveolar bone remodeling as well as osteoblasts and osteoclasts.

Therefore, we used hPDLCs because these cells are involved in regulating alveolar bone metabolism. hPDLCs express osteoblast-like properties, including the expression of ALP, OCN, and OPN [Lee et al., 2009]. In the present study, the mRNA expression levels of markers for osteoblastic differentiation, such as ALP, OCN, OPN were determined using a semi-quantitative RT-PCR method.

Although osteoblasts are thought to be the major functional regulators of osteoclast activity, many other cell types also influence osteoclast activity, including hPDLCs, which express RANKL [Hasegawa et al., 2002]. Both OPG and RANKL expression have been identified in hPDLCs, with increased RANKL expression in hPDLCs associated with inflammatory mediators LPS, PGE₂, and IL-1, as well as increased bone resorptive activity [Hasegawa et al., 2002; Wada et al., 2004]. It is therefore also important to observe the expression of RANKL and other osteoclastogenic cytokines in hPDLCs that could be related to the biological function of osteoclasts. M-CSF is constitutively expressed in osteoblasts, while RANKL is controlled by various factors involved in bone resorption. The expression of TRAP is a characteristic of the macrophage/osteoclast lineage and is often used as a lineage marker [Minkin, 1982]. Among MMPs, MMP-9 is known as one of the major proteases produced by osteoclasts [Okada et al., 1995]. Among cysteine proteinases, cathepsin K plays an essential role in osteoclast-mediated degradation of bone organic matrix [Tezuka et al., 1994]. Since bone remodeling is integral processes involving multiple feedback loops between osteoblast and osteoclast [Boyle et al., 2003], mRNA expression of osteoclast phenotypic markers in cultured hPDLCs were detected by semi-quantitative RT-PCR. RT-PCR demonstrated the constitutive expression of osteoclasto-genic cytokines, RANKL, OPG, M-CSF, and MMP-9 mRNA in hPDLCs without any stimulation, which results suggested that these factors might be involved in extracellular tissue matrix destruction of periodontal tissues. Our results was further supported by reports that hPDLCs are able to induce osteoclastogenesis in vitro [Hasegawa et al., 2002; Kanzaki et al., 2002]. In the present study, nicotine and LPS from *P. gingivalis* treatment were chosen because periodontitis is a multifactorial disease in which both host factors

and environmental factors play important roles [Zee, 2009]. In addition, we investigated osteoclast differentiation from mouse BMMs, since our hPDLCs did not showed TRAP$^+$ multinucleated Oc formation by nicotine and LPS.

Because H$_2$S is volatile and metabolized by cells [Levitt et al., 1999], we monitored the kinetics of H$_2$S decay in the culture media in our experimental conditions. In the present study, the decay of H$_2$S was observed in time- and dose-dependant manner, which result is consistent with a previous study in human colonic epithelial cells [Leschelle et al., 2005], neuron [García-Bereguiaín et al., 2008], and cell-free DMEM [Oh et al., 2006]. Our results confirmed that NaHS releases in aqueous medium hydrosulfide anions and then H$_2$S is able to exert marked effects [Reiffenstein et al., 1992]. Further experiments will be necessary to establish the precise role of esterase enzymes in this release of H$_2$S by chemical degradation.

Although H$_2$S exhibits anti-inflammatory [Fiorucci et al., 2007; Sidhapuriwala et al., 2009; Ekundi-Valentim et al., 2010] and proinflammatory effects [Zhi et al., 2007; Chen et al., 2010], little biological information exists about the mechanism by which exogenous H$_2$S acts as a mediator for bone metabolism. Our data demonstrate that nicotine and LPS induce cytotoxicity and concomitantly downregulate osteoblastic differentiation as evidenced by ALP activity, Alizarin red staining, and mRNA expression of osteoblast markers, and upregulates the expression of osteoclast differentiation markers (Figs. 3 and 4). These results are consistent with our previous study of the cytotoxic and osteoclastic-inducing and osteoblastic-inhibiting effects of nicotine [Lee et al., 2009].

In the present study, the addition of the H$_2$S donor, NaHS (50 μM), to hPDLCs blocked nicotine (5 mM)- and LPS (1 μM)-induced cytotoxicity and upregulated osteoclast marker expression in hPDLCs and TRAP activity in mouse bone marrow cells. Moreover, H$_2$S prevented osteoblastic bone loss induced by nicotine and LPS, which was well correlated with ALP activity, mineralized nodule formation, and expression of osteoblastic markers (Fig. 4). These results are consistent with a previous study reporting that pretreatment with NaHS (100 μM, 30 min) inhibited H$_2$O$_2$ (400 μM, 4 h)-induced cytotoxicity and reversed H$_2$O$_2$-suppressed osteoblastic differentiation in MC3T3-E1 osteoblasts [Xu et al., 2011]. However, our data from hPDLCs are opposite to the previous study that 1 M NaHS single application (1-day) caused a transient increase of osteoclast differentiation with up-regulation of RANKL expression in a rats periodontitis model [Irie et al., 2009]. This level was higher than the concentration of H$_2$S in human feces (0.3–3.4 mM) [Florin et al., 1991], that of sulfide in human periodontal pockets (0.025–0.1 mM) [Morita and Wang, 2001a,b], or that of H$_2$S in gingival fluid (1.9 mM) of human [Persson, 1992]. In addition, combined treatment of NaHS (1 mM) and LPS (25 mg/ml) for 1-day had additive effects on osteoclast differentiation in the rat periodontal tissue [Irie et al., 2012]. This discrepancy may be due to different experimental conditions, in vitro or in vivo effects, and dosage. Further studies will be necessary to clarify the long-term studies and molecular mechanisms for investigating detailed effects of H$_2$S on the periodontal tissue.

Since in our previous study on the hPDLCs in which we demonstrated that nicotine and LPS are able to cause MAPK, PI3K, PKC, MKP-1, and NF-κB activation [Pi et al., 2010; Kim et al., 2012], we attempted to further confirm the crucial roles of signal cascades by NaHS. Recently, we reported that nicotine and LPS stimulates the PI3K and MAPK activity necessary for proinflammatory cytokine expression [Pi et al., 2010] in hPDLCs. The requirement of these kinases was shown in osteoblastic or osteoclastic differentiation in hPDLCs [Kook et al., 2009; Lee et al., 2009]. In addition, the protective effects of H$_2$S were mediated by p38 and ERK in H$_2$O$_2$-stimulated MC3T3-E1 osteoblastic cells [Xu et al., 2011]. In this study, we demonstrated that treatment with NaHS blocked nicotine- and LPS-induced phosphorylation of p38, ERK, and Akt but did not affect the activation of JNK in dose- and time-dependent manners. These results suggest that NaHS antagonizes p38, ERK, and Akt activation in nicotine- and LPS-stimulated hPDLCs.

The MKP-1 gene is an immediate early response gene whose expression is induced rapidly by a variety of extracellular stimuli [Wang et al., 2006]. We hypothesized that MKP-1 may be involved in the NaHS regulation of nicotine- and LPS-induced osteoblastic and osteoclastic differentiation in hPDLCs. This study supports our hypothesis by demonstrating that H$_2$S blocked nicotine- and LPS-induced MKP-1 downregulation. We also found that the downregulation of MKP-1 enzyme activity and expression by vanadate and TP reversed the decrease in osteoblast marker expression as well as increased osteoclast marker expression in nicotine- and LPS-stimulated hPDLCs. Consistent with our data, a previous study showed that knockdown of MKP-1 by MKP-1 siRNA increased Runx2 expression and mineralized matrix formation in MC3T3-E1 cells [Wu et al., 2011]. These studies indicate that MKP-1 is a key therapeutic target in controlling inflammation-induced bone loss.

To further understand the pathways that are affected by NaHS, we analyzed the PI3K downstream pathways PKC and NF-κB. PKCα, PKCβ, and PKCγ are the three main isoforms involved in the differentiation of osteoblasts and osteoclasts [Moonga and Dempster, 1998]. In the present study, we confirmed that NaHS treatment reduced the ability of nicotine and LPS to induce pan-PKC and PKC isoenzyme α, βI, βII, and γ expression, which implies that the protective effect of H$_2$S mainly originates from PKCα, β, and γ. These results are similar to a report stating that NaHS treatment significantly reversed the effects of 6-hydroxydopamine on the activities of the three PKC isoforms, including δ, α, and ε, in a human neuroblastoma cell line [Tiong et al., 2010]. Because previous studies have demonstrated that H$_2$S inhibits LPS-induced NF-κB activation through inhibition of phosphorylation and degradation of IκBα, a key step in NF-κB activation in macrophages [Oh et al., 2006], we examined whether H$_2$S pretreatment could inhibit nicotine- and LPS-induced NF-κB. Our findings indicate that the regulatory role of H$_2$S in nicotine- and LPS-treated hPDLCs is mediated by reduction in the activation of NF-κB.

In summary, the present data show for the first time that H$_2$S protects against cytotoxicity, inhibits osteoclastic differentiation, and promotes osteoblastic differentiation in a periodontal pathogen and nicotine-stimulated hPDLC model via the p38, ERK, Akt, MKP-1, PKC, and NF-κB pathways. Our findings suggest that H$_2$S may have a potentially therapeutic value for periodontitis resulting from dental plaque and smoking.

REFERENCES

Agarwal S, Baran C, Piesco NP, Quintero JC, Langkamp HH, Johns LP. 1995. Synthesis of proinflammatory cytokines by human gingival fibroblasts in response to lipopolysaccharides and interleukin-1β. J Periodontal Res 30: 382–389.

Alverde P, Kawai T, Taubman MA. 2004. Selective blockade of voltage-gated potassium channels reduces inflammatory bone resorption in experimental periodontal disease. J Bone Miner Res 19:155–164.

Bergstrom J, Preber H. 1994. Tobacco use as a risk factor. J Periodontol 65:540–550.

Boyle WJ, Simonet WS, Lacey DL. 2003. Osteoclast differentiation and activation. Nature 423:337–342.

Chen W, Kajiya M, Giro G, Ouhara K, Mackler HE, Mawardi H, Boisvert H, Duncan MJ, Sato K, Kawai T. 2010. Bacteria-derived hydrogen sulfide promotes IL-8 production from epithelial cells. Biochem Biophys Res Commun 391:645–650.

Cheng Y, Ndisang JF, Tang G, Cao K, Wang R. 2004. Hydrogen sulfide-induced relaxation of resistance mesenteric artery beds of rats. Am J Physiol Heart Circ Physiol 287:2316–2323.

Darveau RP, Tanner A, Page RC. 1997. The microbial challenge in periodontitis. Periodontol 2000 14:12–32.

Ekundi-Valentim E, Santos KT, Camargo EA, et al. 2010. Differing effects of exogenous and endogenous hydrogen sulphide in carrageenan-induced knee joint synovitis in the rat. Br J Pharmacol 159:1463–1474.

Fiorucci S, Orlandi S, Mencarelli A, Caliendo G, Santagada V, Distrutti E, Santucci L, Cirino G, Wallace JL. 2007. Enhanced activity of a hydrogen sulphide-releasing derivative of mesalamine (ATB-429) in a mouse model of colitis. Br J Pharmacol 150:996–1002.

Florin T, Neale G, Gibson GR, Christl SU, Cummings JH. 1991. Metabolism of dietary sulphate: Absorption and excretion in humans. Gut 32:766–773.

Fujii S, Maeda H, Wada N, Kano Y, Akamine A. 2002. Establishing and characterizing human periodontal ligament fibroblasts immortalized by SV40T-antigen and hTERT gene transfer. Cell Tissue Res 324:117–125.

García-Bereguiaín MA, Samhan-Arias AK, Martín-Romero FJ, Gutiérrez-Merino C. 2008. Hydrogen sulfide raises cytosolic calcium in neurons through activation of L-type Ca2+ channels. Antioxid Redox Signal 10:31–42.

Goldring S. 2003. Pathogenesis of bone and cartilage destruction in rheumatoid arthritis. Rheumatology 42:31–36.

Hasegawa T, Yoshimura Y, Kikuiri T, Yawaka Y, Takeyama S, Matsumoto A, Oguchi H, Shirakawa T. 2002. Expression of receptor activator of NF-kappa B ligand and osteoprotegerin in culture of human periodontal ligament cells. J Periodontal Res 37:405–411.

Hu LF, Wong PT, Moore PK, Bian JS. 2007. Hydrogen sulfide attenuates lipopolysaccharide-induced inflammation by inhibition of p38 mitogen activated protein kinase in microglia. J Neurochem 100:1121–1128.

Huang H, Ryu J, Ha J, Chang EJ, Kim HJ, Kim HM, Kitamura T, Lee ZH, Kim HH. 2006. Osteoclast differentiation requires TAK1 and MKK6 for NFATc1 induction and NF-κB transactivation by RANKL. Cell Death Differ 13:1879–1891.

Irie K, Ekuni D, Yamamoto T, Morita M, Yaegaki K, Ii H, Imai T. 2009. A single application of hydrogen sulphide induces a transient osteoclast differentiation with RANKL expression in the rat model. Arch Oral Biol 54:723–729.

Irie K, Ekuni D, Tomofuji T, Endo Y, Kasuyama K, Yaegaki K, Morita M. 2012. Combined effects of hydrogen sulphide and lipopolysaccharide on osteoclast differentiation in rats. J Periodontol 83:522–527.

James JA, Sayers NM, Drucker DB, Hull PS. 1999. Effects of tobacco products on the attachment and growth of periodontal ligament fibroblasts. J Periodontol 70:518–525.

Johnson GK, Guthmiller JM. 2007. The impact of cigarette smoking on periodontal disease and treatment. Periodontol 2000 44:178–194.

Kamoun P. 2004. Endogenous production of hydrogen sulfide in mammals. Amino Acids 26:243–254.

Kanzaki H, Chiba M, Shimizu Y, Mitani H. 2002. Periodontal ligament cells under mechanical stress induce osteoclastogenesis by receptor activator of nuclear factor κB ligand up-regulation via prostaglandin E2 systhesis. J Bone Miner Res 17:210–220.

Kim YS, Shin SI, Herr Y, Bae WJ, Kim EC. 2012. Nicotine and LPS stimulates the production of MMPs and prostaglandin E2 by hypoxia-inducible factor-1α upregulation in human periodontal ligament cells. J Peridontal Res 47:719–728.

Kimura H. 2011. Hydrogen sulfide: Its production, release and functions. Amino Acids 41:113–121.

Kook YA, Lee SK, Son DH, Kim Y, Kang KH, Cho JH, Kim SC, Kim YS, Lee HJ, Lee SK, Kim EC. 2009. Effects of substance P on osteoblastic differentiation and heme oxygenase-1 in human periodontal ligament cells. Cell Biol Int 33:424–428.

Lamont RJ, Jenkinson HF. 1998. Life below the gum line: Pathogenic mechanisms of Porphyromonas gingivalis. Microbiol Mol Biol Rev 62:1244–1263.

Lee HJ, Pi SH, Kim Y, Kim HS, Kim SJ, Kim YS, Lee SK, Kim EC. 2009. Effects of nicotine on antioxidant defense enzymes and RANKL expression in human periodontal ligament cells. J Periodontol 80:1281–1288.

Leschelle X, Goubern M, Andriamihaja M, et al. 2005. Adaptative metabolic response of human colonic epithelial cells to the adverse effects of the luminal compound sulfide. Biochim Biophys Acta 15:201–212.

Levitt MD, Furne J, Springfield J, Suarez F, De Master E. 1999. Detoxification of hydrogen sulfide and methanethiol in the cecal mucosa. J Clin Invest 104:1107–1114.

Li L, Bhatia M, Zhu YZ, Zhu YC, Ramnath RD, Wang ZJ, Anuar FB, Whiteman M, Salto-Tellez M, Moore PK. 2005. Hydrogen sulfide is a novel mediator of lipopolysaccharide-induced inflammation in the mouse. FASEB J 19:1196–1198.

Li L, Rossoni G, Sparatore A, Lee LC, Del Soldato P, Moore PK. 2007. Anti-inflammatory and gastrointestinal effects of a novel diclofenac derivative. Free Radic Biol Med 42:706–719.

Minkin C. 1982. Bone acid phosphatase: Tartrate-resistant acid phosphatase as a marker of osteoclast function. Calcif Tissue Int 34:285–290.

Moonga BS, Dempster DW. 1998. Effects of peptide fragments of protein kinase C on isolated rat osteoclasts. Exp Physiol 83:717–725.

Moore KD, Goss K, Anglen JO. 1998. Indomethacin versus radiation therapy for prophylaxis against heterotopic ossification in acetabular fractures: A randomised, prospective study. J Bone Joint Surg Br 80:259–263.

Morita M, Wang HL. 2001a. Relationship of sulcular sulfide level to severity of periodontal disease and BANA test. J Periodontol 72:74–78.

Morita M, Wang HL. 2001b. Relationship between sulcular sulfide level and oral malodor in subjects with periodontal disease. J Periodontol 72:79–84.

Oh GS, Pae HO, Lee BS, Kim BN, Kim JM, Kim HR, Jeon SB, Jeon WK, Chae HJ, Chung HT. 2006. Hydrogen sulfide inhibits nitric oxide production and nuclear factor-κB via heme oxygenase-1 expression in RAW264.7 macrophages stimulated with lipopolysaccharide. Free Radic Biol Med 41:106–119.

Okada Y, Naka K, Kawamura K, Matsumoto T, Nakanishi I, Fujimoto N, Sato H, Seiki M. 1995. Localization of matrix metalloproteinase 9 (92-kilodalton

gelatinase/type IV collagenase = gelatinase B) in osteoclasts: Implications for bone resorption. Lab Invest 72:311–322.

Page RC. 1991. The role of inflammatory mediators in the pathogenesis of periodontal disease. J Periodontal Res 26:230–242.

Pang EK, Paik JW, Kim SK, Jung UW, Kim CS, Cho KS, Kim CK, Choi SH. 2005. Effects of chitosan on human periodontal ligament fibroblasts *in vitro* and on bone formation in rat calvarial defects. J Periodontol 76:1526–1533.

Persson S. 1992. Hydrogen sulfide and methyl mercaptan in periodontal pockets. Oral Microbiol Immunol 7:378–379.

Pi SH, Jeong GS, Oh HW, Kim YS, Pae HO, Chung HT, Kim EC. 2010. Heme oxygenase-1 mediates nicotine and lipopolysaccharide-induced COX-2 and iNOS expression in human periodontal ligament cells. J Periodontal Res 45:177–183.

Reiffenstein RJ, Hulbert WC, Roth SH. 1992. Toxicology of hydrogen sulfide. Annu Rev Pharmacol Toxicol 32:109–134.

Rizzo AA. 1967. The possible role of hydrogen sulfide in human periodontal disease. I. Hydrogen sulfide production in periodontal pockets. Periodontics 5:233–236.

Sabokbar A, Kudo O, Athanasou N. 2003. Two distinct cellular mechanisms of osteoclast formation and bone resorption in periprosthetic osteolysis. J Orthop Res 21:73–80.

Shoji M, Tanabe N, Mitsui N, Suzuki N, Takeichi O, Katono T. 2007. Lipopolysaccharide enhances the production of nicotine-induced prostaglandin E_2 by an increase in cyclooxygenase-2 expression in osteoblasts. Acta Biochim Biophys Sin (Shanghai) 39:163–172.

Sidhapuriwala JN, Ng SW, Bhatia M. 2009. Effects of hydrogen sulfide on inflammation in caerulein-induced acute pancreatitis. J Inflamm 30:6–35.

Solis-Gaffar MC, Rustogi KN, Gaffar A. 1980. Hydrogen sulfide production from gingival crevicular fluid. J Periodontol 51:603–636.

Somerman MJ, Archer SY, Imm JR, Foster AR. 1988. A comparative study of human periodontal ligament cells and gingival fibroblast *in vitro*. J Dent Res 67:66–70.

Takayanagi K, Osawa G, Nakaya H, Cochran DL, Kamoi K, Oates TW. 2006. Effects of enamel matrix derivative on bone-related mRNA expression in human periodontal ligament cells *in vitro*. J Periodontol 77:891–898.

Tanaka H, Tanabe N, Shoji M, Suzuki N, Katono T, Sato S. 2006. Nicotine and lipopolysaccharide stimulate the formation of osteoclast-like cells by increasing macrophage colony-stimulating factor and prostaglandin E_2 production by osteoblasts. Life Sci 78:1733–1740.

Tenenbaum HC, Shelemay A, Girard B, Zohar R, Fritz PC. 2002. Bisphosphonates and periodontics: Potential applications for regulation of bone mass in the periodontium and other therapeutic/diagnostic uses. J Periodontol 73:813–822.

Tezuka K, Tezuka Y, Maejima A, et al. 1994. Molecular cloning of a possible cysteine proteinase predominantly expressed in osteoclasts. J Biol Chem 269:1106–1109.

Tiong CX, Lu M, Bian JS. 2010. Protective effect of hydrogen sulphide against 6-OHDA-induced cell injury in SH-SY5Y cells involves PKC/PI3K/Akt pathway. Br J Pharmacol 161:467–480.

Tipton DA, Dabbous MK. 1995. Effects of nicotine on proliferation and extracellular matrix production of human gingival fibroblasts *in vitro*. J Periodontol 66:1056–1064.

Wada N, Maeda H, Yoshimine Y, Akamine A. 2004. Lipopolysaccharide stimulates expression of osteoprotegerin and receptor activator of NF-kappa B ligand in periodontal ligament fibroblasts through the induction of interleukin-1 beta and tumor necrosis factor-alpha. Bone 35:629–635.

Wang Z, Yang H, Tachado SD, Capó-Aponte JE, Bildin VN, Koziel H, Reinach PS. 2006. Phosphatase-mediated crosstalk control of ERK and p38 MAPK signaling in corneal epithelial cells. Invest Ophthalmol Vis Sci 47:5267–5275.

Wu RW, Lin TP, Ko JY, Yeh DW, Chen MW, Ke HC, Wu SL, Wang FS. 2011. Cannabinoid receptor 1 regulates ERK and GSK-3β-dependent glucocorticoid inhibition of osteoblast differentiation in murine MC3T3-E1 cells. Bone 49:1255–1263.

Xu ZS, Wang XY, Xiao DM, Hu LF, Lu M, Wu ZY, Bian JS. 2011. Hydrogen sulfide protects MC3T3-E1 osteoblastic cells against H_2O_2-induced oxidative damage – Implications for the treatment of osteoporosis. Free Radic Biol Med 50:1314–1323.

Yazawa H, Zimmermann B, Asami Y, Bernimoulin JP. 2005. Simvastatin promotes cell metabolism, proliferation, and osteoblastic differentiation in human periodontal ligament cells. J Periodontol 76:295–302.

Zanardo RC, Brancaleone V, Distrutti E, Fiorucci S, Cirino G, Wallace JL. 2006. Hydrogen sulfide is an endogenous modulator of leukocyte-mediated inflammation. FASEB J 20:2118–2120.

Zee KY. 2009. Smoking and periodontal disease. Aust Dent J 54:S44–S50.

Zhang H, Hegde A, Ng SW, Adhikari S, Moochhala SM, Bhatia M. 2007. Hydrogen sulfide up-regulates substance p in polymicrobial sepsis-associated lung injury. J Immunol 179:4153–4160.

Zhao W, Zhang J, Lu Y, Wang R. 2001. The vasorelaxant effect of H_2S as a novel endogenous gaseous K (ATP) channel opener. EMBO J 20:6008–6016.

Zhi L, Ang AD, Zhang H, Moore PK, Bhatia M. 2007. Hydrogen sulfide induces the synthesis of proinflammatory cytokines in human monocyte cell line U937 *via* the ERK, NF-κB pathway. J Leukoc Biol 81:1322–1332.