Effects of hydrogen sulfide on the expression of alkaline phosphatase, osteocalcin and collagen type I in human periodontal ligament cells induced by tension force stimulation

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Abstract. Periodontal ligament cells (PDLCs) are important in homeostasis and remodeling in the mechanically-stimulated periodontium. The aim of the present study was to investigate the effects of hydrogen sulfide (H_2S) on periodontal tissue remodeling by examining the mRNA and protein expression levels of alkaline phosphatase (ALP), osteocalcin (OCN) and collagen type I (COL-1) in human (h)PDLCs induced by tension force application. Cultured hPDLCs were treated with H_2S for 24 h, followed by application of a tension force for 1, 3 and 6 h. Cell proliferation and apoptosis were determined using a Cell Counting Kit 8 assay and flow cytometric analysis, respectively. The mRNA expression levels of ALP, OCN and COL-1 were quantified using reverse transcription-quantitative polymerase chain reaction analysis, and western blot analysis was used to detect the protein levels of ALP, OCN and COL-1. The results demonstrated that the mRNA and protein expression levels of ALP, OCN and COL-1 increased with H_2S treatment in a concentration-dependent manner, which was enhanced by the application of tension force in a relatively short period of time. These findings suggested that H_2S may be important in periodontal tissue remodeling during orthodontic tooth movement via increasing hPDLC differentiation, tissue mineralization, bone formation and collagen synthesis.

Introduction

The periodontium is composed of gingival tissue, periodontal ligament, alveolar bone and cementum. During orthodontic tooth movement, periodontal ligament cells (PDLCs) are directly subject to mechanical stress, and are critical in regulating the processes of periodontal tissue repair and remodeling (1). Previously, several studies have shown that orthodontic tooth movement is regulated by PDLCs via modulation of the activity of alkaline phosphatase (ALP), production of osetocalcin (OCN) and synthesis of collagen type I (COL-1). In addition, the biological characteristics of PDLCs can be altered under the action of mechanical force (2-5). It is generally accepted that ALP is involved in the process of calcification in various mineralizing tissues (6). OCN is considered to be a marker of bone formation (7,8). Collagen fibers are the predominant component of the periodontal ligament extracellular matrix (ECM), and collagen types I and III are important in periodontal tissue remodeling (9). Therefore, the synthesis and degradation of ECM are key processes in the regulation of bone remodeling (10).

Hydrogen sulfide (H_2S) is an endogenous gaseous signaling molecule, which has been traditionally classified as a toxic gas (11). In previous years, it was reported that low concentrations of H_2S have anti-inflammatory, cytoprotective and chemopreventative potential (12), and have shown anticancer effects (13,14). H_2S is synthesized endogenously from L-cysteine in mammals by at least two pyridoxal-5'-phosphate-dependent enzymes, cystathionine-γ-lyase and cystathionine β-synthase, in various organs (15,16). This molecule can permeate the cellular membrane without the assistance of a specific transporter. There are limited reports regarding with the effects of H_2S on the biological activity of human PDLCs (hPDLCs), particularly during mechanical stress. Our previous results showed that H_2S upregulated the expression ratio of OPG/receptor activator of nuclear factor-κB ligand (RANKL) in hPDLCs, with the maximum effect being observed at 0.5 mM, and tension force enhanced the effect of H_2S on the expression of OPG/RANKL (17). The present study, investigated the effect of H_2S on the expression levels of ALP, OCN and COL-1 in hPDLCs with and without tension force in order to further understand the effects of H_2S on periodontal tissue remodeling.

Materials and methods

Cell isolation and culture. The hPDLCs were obtained from 21 patients (10 male and 11 female) aged between 12

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and 16 years who required premolar extraction during the course of orthodontic treatment between August 2014 and October 2014 at Affiliated Stomatology Hospital of Tongji University (Shanghai, China). All patients signed informed consent and the study was approved by the Ethics Committee (2013-NSFC002) of Tongji University (Shanghai, China). The teeth, which were absent of inflammation, were immediately placed into a tube containing 1% Dulbecco's modified Eagle's medium (DMEM; Hyclone; GE Healthcare Life Sciences, Logan, UT, USA), 100 U/ml penicillin and 100 U/ml streptomycin. The cells were scraped from the middle third of the root and maintained in DMEM supplemented with 15% charcoal-stripped serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 U/ml penicillin and 100 mg/ml streptomycin at 37˚C in a 5% CO_2 incubator, with replacement of the medium every 2 days. On reaching confluence, the cells were detached with 0.25% trypsin without EDTA and subcultured at a 1:3 ratio (18). In the subsequent experiments, cells between the third and eighth passages were used.

Cell viability assay. To determine cell viability, the hPDLCs were seeded into two 96-well plates with a cell density of $5x10^3$ cells per well. Each treatment group was evaluated in triplicate. Sodium hydrosulfide (NaHS), as an exogenous donor (19), has been used to examine various biological activities of H_2S. In the present study, NaHS (Aladdin, Shanghai, China) was dissolved in phosphate-buffered saline (PBS) and diluted into four concentrations (0.01, 0.05, 0.1 and 0.5 mM) with DMEM supplemented with 2% charcoal-stripped serum. The cells were then treated with H_2S (0.01-0.5 mM) for 1-5 days and incubated at 37˚C in 5% CO_2. A Cell Counting Kit 8 (CCK 8) was then used to determine the viability of the cells. The optical density (OD) values of the media were measured at λ=450 nm using a microplate reader (Infinite™ 200; Tecan Austria GmbH, Grödig, Austria).

Flow cytometric analysis. The hPDLCs ($1x10^6$ cells/ml) were seeded in six-well plates and treated with H_2S (0.01, 0.05, 0.1 and 0.5 mM) for 1-5 days at 37˚C in 5% CO_2. An Annexin V-fluorescein isothiocyanate (FITC) kit (BioVison, Inc., Mountain View, CA, USA) was used to assess the apoptosis of cells following treatment with H_2S for different durations.

Tension force stimulation. The cells ($1x10^6$ cells/ml) were seeded into four flexible plates and pre-treated with H_2S (0, 0.01, 0.05, 0.1 and 0.5 mM) in DMEM containing 2% charcoal-stripped serum for 24 h at 37˚C in 5% CO_2. The four flexible plates were subjected to a wave of 5% elongation, 0.5Hz (2 sec) (20) for 1, 3 and 6 h every time. The cells were collected for western blot and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analyses.

RT-qPCR analysis. Total cellular mRNA was obtained using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Complementary DNA was synthesized using a PrimeScript 1st Strand cDNA Synthesis kit (Takara Bio, Inc., Tokyo, Japan). The qPCR was performed using a SYBR Premix Ex Taq II (Tli RNase H Plus) kit (Takara Bio, Inc.) in an ABI Prism 7500 Detection System (Applied Biosystems; Thermo Fisher Scientific, Inc.). The primer sequences are listed in Table I. The thermocycling conditions were as follows: 37˚C for 15 min and 85˚C for 5 sec (for the reverse transcription); followed by 40 cycles of 95˚C for 5 sec and 60˚C for 34 sec. Relative fold changes were calculated using the $2^{-\Delta\Delta Cq}$ method (21) and standard curves were produced. The Cq values of the samples were normalized to the appropriate endogenous housekeeping gene, GAPDH. Each measurement was performed in triplicate.

Western blot analysis. The cells were lysed using radioimmunoprecipitation assay buffer (Beyotime Institute of Biotechnology, Haimen, China) and stored at -80˚C. The cell lysates were separated on 10% (ALP and COL-1) and 15% (OCN) SDS-polyacrylamide electrophoresis gels and transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA) using a semi-dry transfer cell (Tannon Science & Technology Co., Ltd., Shanghai, China). The membranes were blocked for 1 h in 5% dry milk, rinsed and incubated with rabbit polyclonal anti-ALP antibody (cat. no. ab95462), rabbit monoclonal anti-OCN antibody (cat. no. ab133612), rabbit monoclonal anti-COL 1 antibody (cat. no. ab138492; all obtained from Abcam, Cambridge, MA, USA) at 1:1,000 dilutions in Tris-buffered saline (TBS) overnight at 4˚C. The primary antibodies were then removed by washing the membranes three times in TBS. The primary antibodies were labeled by incubation with 0.1 mg/ml horseradish peroxidase-labeled goat anti-rabbit secondary antibodies (1:2,000; cat. no. KGAA35; Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) at room temperature for 1 h. Following three washes in TBS, the antibody-bound proteins were detected using an enhanced chemiluminescence system (EMD Millipore).

Statistical analysis. All data in the present study are presented as the mean ± standard deviation from three independent experiments. Data was analyzed using Student's *t*-test with SAS 8.2 (SAS Institute, Cary, NC, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Effect of H_2S on cell proliferation. The cells were treated with different concentrations of H_2S for 1-5 days. H_2S had no significant effect on cell proliferation when the hPDLCs were treated for 3 days. However, H_2S reduced the proliferation of the hPDLCs in a concentration-dependent manner following 4 and 5 days of incubation (Fig. 1).

Effect of H_2S on cell apoptosis. No effects of H_2S on cell apoptosis were detected using flow cytometry when the cells were treated up to 4 days. However, H_2S significantly induced apoptosis at the concentration of 0.5 mM following 5 days of treatment (Fig. 2A-E).

Effect of H_2S on mRNA expression levels of ALP, OCN and COL-1. Data on the mRNA expression levels were obtained using RT-qPCR analysis, and the results showed that H_2S significantly upregulated the mRNA expression levels of ALP, OCN and COL-1 in the hPDLCs at the concentration of 0.5 mM (Fig. 3A-C). Treatment with 0.1 and 0.5 mM H_2S increased the mRNA expression levels of ALP and COL-1

Table I. Primer sequences for ALP, OCN, COL-1 and GAPDH.

Gene	Primer sequence (5'-3')	Length (bp)
ALP	F: CTACACGGTCCTCCTATAC	19
	R: CTCGCTCTCGGTAACATC	18
OCN	F: CAGAGTCCAGCAAAGGTG	18
	R: CCAGCCATTGATACAGGTA	19
COL-1	F: GCTGTCTTATGGCTATGATGAGAA	24
	R: GACCACGAGGACCAGAGG	18
GAPDH	F: AGAAGGCTGGGGCTCATTTG	20
	R: AGGGGCCATCCACAGTCTTC	20

F, forward; R, reverse; ALP, alkaline phosphatase; OCN, osteocalcin; COL-1, collagen type I.

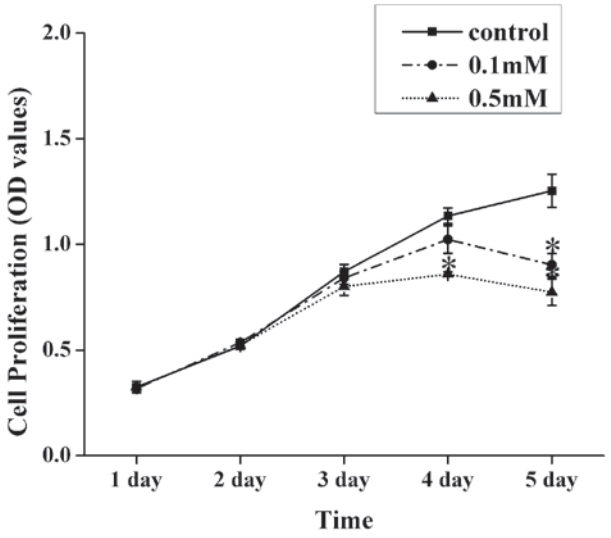

Figure 1. Effects of H_2S on cell proliferation. hPDLCs were treated with H_2S (0.1 and 0.5 mM), OD values were measured using a Cell-Counting Kit 8 assay. The viability of the hPDLCs was significantly reduced following treatment of the cells with 0.1 mM H_2S for 5 days, and 0.5 mM H_2S for 4 and 5 days. Data are presented as the mean ± standard deviation. *P<0.05, vs. control. hPDLCs, human periodontal ligament cells; OD, optical density; H_2S, hydrogen sulfide.

induced by 1 h tension force stimulation (Fig. 3A and C). Treatment with 0.05-0.5 mM H_2S upregulated the mRNA expression levels of ALP and COL-1 following tension force application for 3 and 6 h (Fig. 3A and C). The mRNA expression levels of OCN induced by tension force stimulation for 1, 3 and 6 h significantly increased following pretreatment with 0.05, 0.1 and 0.5 mM H_2S (Fig. 3B).

Effect of H_2S on protein expression levels of ALP, OCN and COL-1. To investigate the role of H_2S in hPDLCs, the protein expression levels of ALP, OCN and COL-1 were determined using western blot analysis following H_2S pretreatment with or without subsequent tension force application (Fig.4 A-D). The results showed that treatment with 0.05-0.5 mM H_2S significantly upregulated the protein expression levels of ALP, OCN and COL-1 (Fig. 4E-G). Treatment with 0.01-0.5 mM H_2S significantly upregulated the protein expression levels of ALP and OCN in the cells subjected to 1 and 3 h of tension force application, and the protein expression levels of ALP and OCN induced by 6h tension force application significantly increased in the cells pre-exposed to H_2S 0.05-0.5 mM; Fig. 4E and F). Treatment with 0.01-0.5 mM H_2S significantly upregulated the protein expression of COL-1 in the cells induced by tension force application for 1 h, and treatment with 0.05-0.5 mM H_2S significantly upregulated the protein expression of COL-1 in the cells subjected to tension force for 3 and 6 h. However, protein expression levels of OCN and COL-1 induced by 3 h of tension force was more marked, compared with those subjected to 1 and 6 h tension force application following pretreatment with 0.5 mM H_2S (Fig. 4F and G).

Discussion

H_2S has been recognized as a gasotransmitter, which has multiple physiological and pathophysiological functions in various mammalian systems (22,23). It has been reported that H_2S has potential anti-inflammatory, anti-apoptotic, anti-cancer and neuroprotective effects (24,25). Previous studies have shown that the exogenous donor of NaHS significantly protects PC12 cells against formaldehyde-induced cytotoxicity and apoptosis through attenuating the accumulation of reactive oxygen species (ROS), upregulating levels of B cell lymphoma-2 (Bcl-2) and downregulating the expression of Bcl-2-associated X protein (26,27). In the present study, cytotoxicity was observed in response to H_2S at a high concentration (Fig. 2). A high concentration of H_2S may increase ROS formation and mitochondrial depolarization (28), decreasing the concentration of oxygen and leading to hypoxia and cell death. By contrast, low concentrations of H_2S were protective and relatively safe to hPDLCs, suggesting <0.5 mM H_2S is useful in hPDLCs.

hPDLCs produce ECM components, including collagen, which build up the periodontal ligament to secure attachment of the root cementum to the surrounding alveolar bone and is important in the restoration of mineralized tissue (29-31). In the present study, hPDLCs were isolated and characterized for their mesenchymal origin, with confirmation of their fibroblast-like morphology, as in our previous report (17). hPDLCs are capable of producing ALP, OCN and COL-1, suggesting osteoblast- and fibroblast-like features, which is consistent with previous studies (32,33). hPDLCs can differentiate into either osteoblasts or cementoblasts in response to mechanical force (34-38). In the present study, in order to investigate the effect of H_2S on hPDLCs during orthodontic tooth movement, an appropriate tension force was applied to the hPDLCs following H_2S treatment. As mentioned previously, our previous study demonstrated that H_2S had a regulatory role within the periodontal remodeling process by promoting osteogenic differentiation via upregulating the expression ratio of OPG/RANKL in the hPDLCs. This promoting effect of H_2S on OPG/RANKL was enhanced by tension force application (17). In the present study, it was observed that H_2S upregulated the expression levels of ALP, OCN and COL-1 in a concentration-dependent manner, and pre-treatment with H_2S enhanced the expression levels of ALP, OCN and COL-1 induced by tension force application.

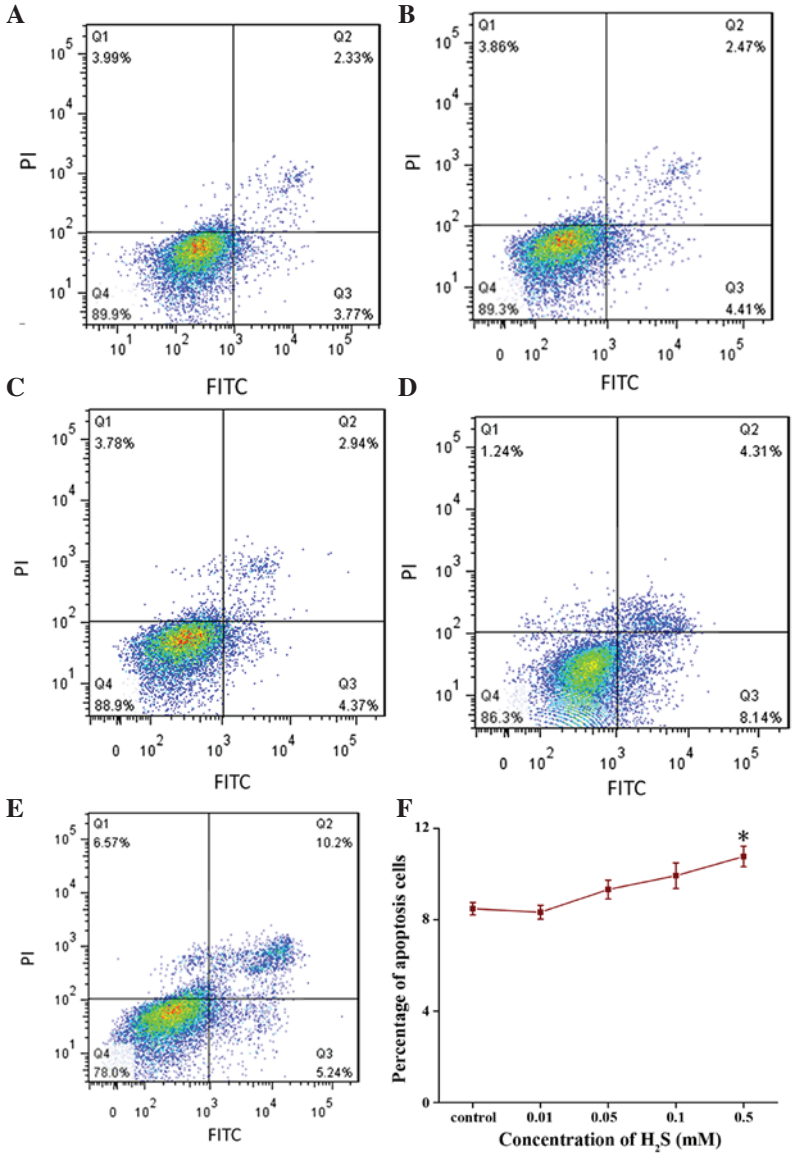

Figure 2. Effects of H_2S on hPDLC apoptosis. Cells were treated with H_2S (0.01-0.5 mM) for 5 days and flow cytometry was used to detect cell apoptosis. Representative flow cytometry results are shown for the (A) control group and the (B) 0.01, (C) 0.05, (D) 0.1 and (E) 0.5 mM H_2S groups. Q2 and Q4 represent early and late apoptosis, respectively. (F) Apoptotic cells are expressed as a percentage of the total cell number. A significant increase in apoptosis was observed in the 0.5 mM H_2S group. *P<0.05, vs. control. hPDLCs, human periodontal ligament cells; H_2S, hydrogen sulfide; FITC, fluorescein isothiocyanate; PI, propidium iodide.

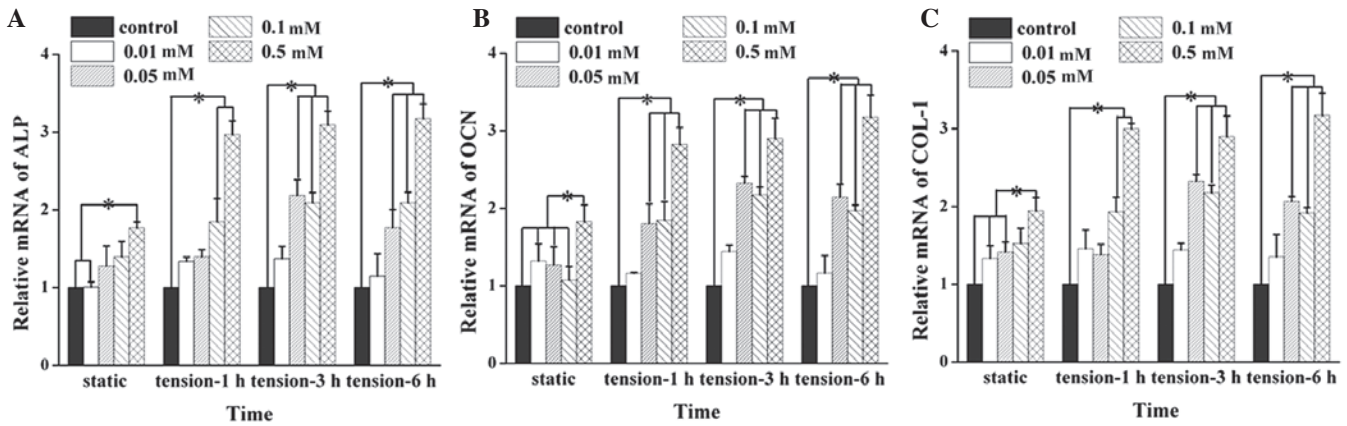

Figure 3. mRNA expression levels of ALP, OCN and COL-1 following H_2S treatment. Treatment with 0.5 mM H_2S significantly upregulated the mRNA expression of (A) ALP, (B) OCN and (C) COL-1 in the cells. Treatment with 0.1 and 0.5 mM H_2S enhanced mRNA expression of ALP and COL-1 following 1 h mechanical tension stimulation. (B) Pretreatment with H_2S (0.05-0.5 mM) significantly upregulated the mRNA expression of OCN induced by tension force (1, 3 and 6 h). Treatment with 0.05-0.5 mM H_2S significantly upregulated the mRNA expression of ALP and COL-1 following 3 and 6 h tension force. Data are expressed as the mean ± standard deviation (*P<0.05). H_2S, hydrogen sulfide; ALP, alkaline phosphatase; OCN, osteocalcin; COL-1, collagen type I.

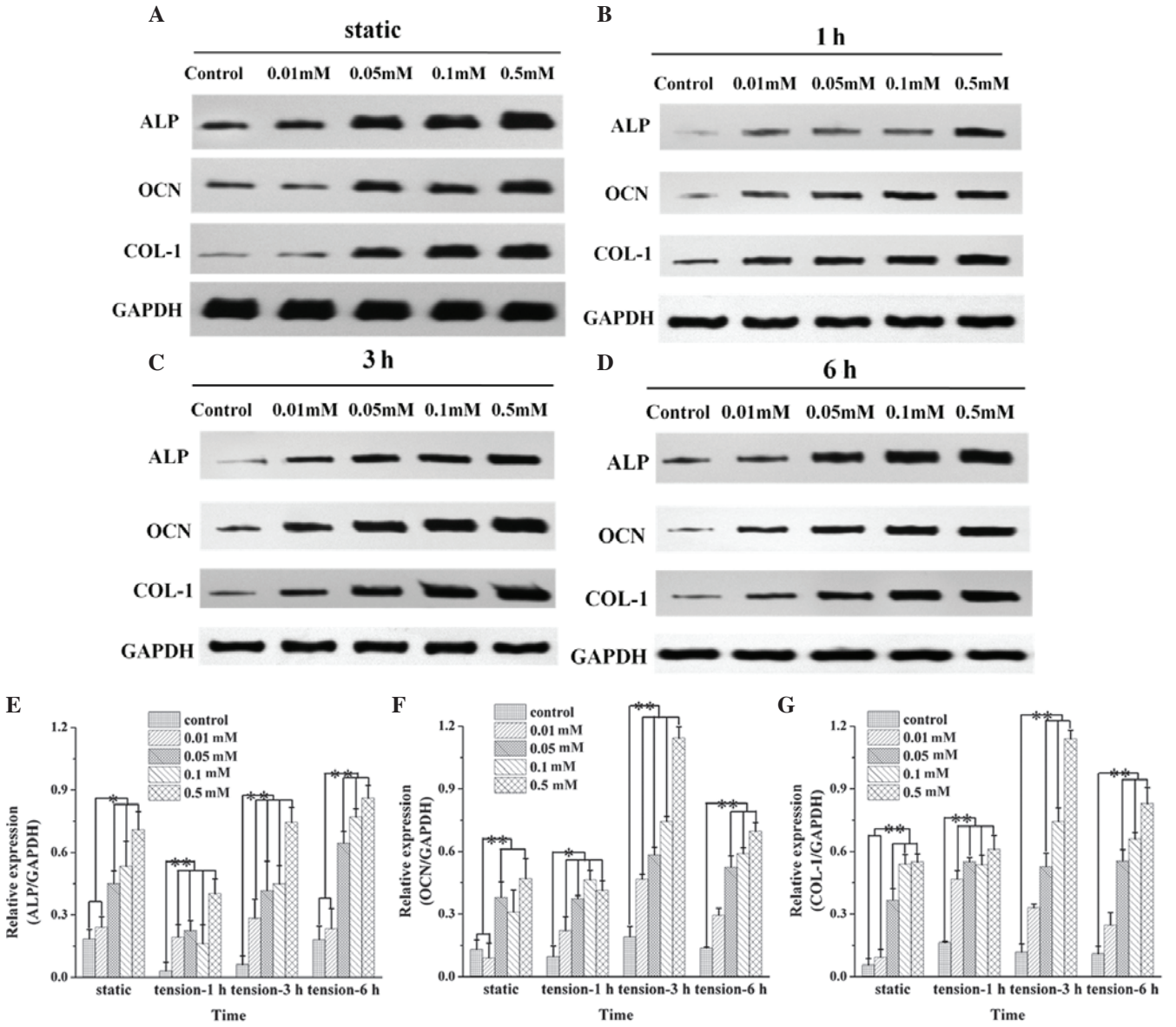

Figure 4. Protein expression of ALP, OCN and COL-1 in hPDLCs following H_2S pretreatment. The hPDLCs were treated with H_2S (0-0.5 mM) and subjected to tension force application for (A) 0, (B) 1, (C) 3 or (D) 6 h. The protein expression levels of (E) ALP, (F) OCN and (G) COL-1 in the cells were examined using western blot analysis. Prior to application of tension force, the protein expression of ALP and COL-1 was significantly increased at concentrations of 0.05-0.5 mM. The protein expression of OCN was significantly increased at 0.05 and 0.5mM. Following tension force application, 0.01-0.5 mM H_2S significantly upregulated the protein expression levels of ALP and OCN in response to 1 and 3 h tension force, and 0.05-0.5 mM H_2S H_2S significantly increased the protein expression levels of ALP and OCN induced by 6 h tension force. Pretreatment with 0.01-0.5 mM H_2S significantly upregulated the protein expression of COL-1 following tension force stimulation for 1 h. Treatment with 0.05-0.5 mM H_2S significantly increased the protein expression of COL-1 in response to 3 and 6 h tension force. Protein expression levels of OCN and COL-1 were higher in response to 3 h tension, compared with 1 and 6 h tension following incubation with 0.5 mM H_2S. Data are expressed as the mean ± standard deviation. (*P<0.01 and **P<0.01). H_2S, hydrogen sulfide; ALP, alkaline phosphatase; OCN, osteocalcin; COL-1, collagen type I.

ALP is a marker for the calcification and osteoblastic differentiation of PDLCs (39,40). OCN is a vitamin K-dependent Ca^{2+}-binding protein of the bone matrix, which is synthesized by osteoblast-like cells and is considered to be a marker for bone formation (7,8). The collagen molecules in the periodontal ligament are known to respond to mechanical stimuli (41,42). COL-1 is one of the major fibrous elements of the periodontal ligament. It forms solid fibers anchored to the cementum and alveolar bone, and protects the periodontal ligament from tensile stress and masticatory loading (34,35). Therefore, the present study hypothesized that H_2S regulates hPDLC differentiation, tissue mineralization and collagen synthesis through modulation of the mRNA and protein levels of ALP, OCN and COL-1.

The data obtained in the present study showed that the H_2S-induced mRNA and protein expression levels of ALP, OCN and COL-1 were upregulated following tension force application (Fig. 4E). The mechanism underlying this H_2S-induced expression of ALP, OCN and COL-1 in response to tension-force was not determined in the present study. It has been demonstrated that light mechanical strain induces biological changes in hPDLCs, including the release of various types of adhesion molecules and osteogenic factors, including ALP, OCN and COL-1 (2-4). Studies have shown that static mechanical deformation of PDLCs activates c-Jun and c-Fos, and increases the binding of activator protein-1 to the promoter of ALP, which is a major effector of osteoblastic

differentiation (43). The gene expression of ALP in hPDLCs as a response to tension stimulation is associated with the intensity of the mechanical force and the culture conditions used (44-47). These findings indicate a potential synergetic effect between H_2S and tension force application.

In conclusion, the present study demonstrated that exposure of hPDLCs to a high concentration and prolonged duration with H_2S reduced the viability of the hPDLCs. It was observed that H_2S significantly upregulated the expression levels of ALP, OCN and COL-1 in the hPDLCs, and pretreatment of the cells with H_2S enhanced the expression levels of the proteins induced by tension force stimulation. These results suggested that H_2S may be important in remodeling and functional regulation of periodontal tissue. Further investigations are required to elucidate the underlying cellular mechanism.

Acknowledgements

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