Hydrogen sulfide promotes osteogenic differentiation of human periodontal ligament cells via p38-MAPK signaling pathway under proper tension stimulation

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Abstract

Objective: Hydrogen sulfide (H₂S), one of endogenous gaseous signalling molecules, can be induced by mechanical force stimulation on human periodontal ligament cells (hPDLCs). Little is known about the mechanism of H₂S on the osteogenic differentiation although previous studies have demonstrated that H₂S stimulated or inhibited osteoclastic differentiation. The present study was to investigate whether H₂S played a regulatory role in osteogenic differentiation of the periodontal tissue remodeling and the involvement of **mitogen-activated protein kinase (MAPK) signaling** in this process.

Design: hPDLCs were applied with cycle tension force for 6 h, 12 h, 24 h or 48 h to select the optimal time for force application. Then the effects of H₂S on hPDLCs osteogenic differentiation were investigated. Signal-regulated kinases **p38-MAPK and ERK** activities with H₂S treatment were measured. Finally, specific MAPK inhibitors SB203580 and U0126 were employed to investigate the involvement of the two kinases in hPDLCs osteogenic differentiation with H₂S pre-treatment.

Results: Tension stimulation promoted mRNA and protein expression of ALP, OCN and Runx2 in hPDLCs. The expression of ALP, OCN and Runx2 increased in a concentration-dependent manner with H₂S pre-treatment. Importantly, p38-MAPK and ERK were activated in different ways upon induction by H₂S. Furthermore, expression of Runx2, ALP and OCN, the osteogenic regulators, was reversed by SB203580 and U0126.

Conclusions: H₂S could promote osteogenic differentiation of hPDLCs by activating p38-MAPK and ERK signaling pathways.

Keywords: hydrogen sulfide; human periodontal ligament cells; cyclic tension; osteogenic differentiation; p38-MAPK; ERK;

1. Introduction

Orthodontic tooth movement is induced by mechanical stimuli, with changes in the

periodontium consisting of periodontal ligament (PDL), cementum, alveolar bone, and gingiva (Bartold, & Narayanan, 2006; Krishnan, & Davidovitch, 2006). The mechanical stimuli applied to the teeth are transmitted through the PDL to the alveolar bone, leading to bone resorption in the pressure side and bone formation in the tension side. Currently, studies have suggested that orthodontic tooth movement was modulated by PDLCs through the production of various active cytokines, such as osteoprotegerin, the receptor activator of NF- κ B and its ligand (Nishijima, Yamaguchi, Kojima, Aihara, Nakajima, & Kasai, 2006; Yamaguchi, Aihara, Kojima, & Kasai, 2006). In addition, runt-related transcription factor 2 (Runx2), alkaline phosphatase (ALP) and osteocalcin (OCN) play very important roles in modulation of osteogenic differentiation and bone formation (Shi et al., 2011).

Hydrogen sulfide (H₂S) is considered to be the third endogenous gaseous signaling molecule after nitric oxide and carbon monoxide (Wagner, Asfar, Calzia, & Szabo, 2009), which plays multiple physiological and pathological roles including neuromodulation, inflammation, and anti-inflammation (Zhao, Zhang, Lu, & Wang, 2001; Whiteman et al., 2004; Bhatia, 2012; Mirandola, 2011). We have observed that hPDLCs could produce H₂S by cystathionine- γ -lyase (CSE) and the mechanical stimuli could promote the mRNA expression of CSE (Liao, & Hua, 2013), suggesting mechanical stimuli could promote the production of H₂S in hPDLCs. It was reported that H₂S caused a transient increase of osteoclast differentiation in rat periodontal tissue and upregulation of RANKL expression in the osteoblasts (Irie, Ekuni, Yamamoto, Morita, Yaegaki, Ii, & Imai, 2009). Tension force enhanced H₂S up-regulated OPG/RANKL expression ratio in hPDLCs (Liao, & Hua, 2013). In addition, a low concentration of H₂S protected MC3T3-E1 osteoblastic cells against H₂O₂-induced oxidative damage (Xu et al., 2011).

Bone formation, associated with the differentiation of PDL cells, is regulated by multiple signaling pathways. Mitogen-activated protein kinases (MAPKs), which consist of p38-MAPK, the extracellular signal-regulated protein kinase (ERK), are highly conserved signalling kinases. It can respond to a wide range of stresses (Raman, Chen, & Cobb, 2007; Chun, 2004). Recent studies have demonstrated that H₂S promoted osteoblastic differentiation in a periodontal pathogen and nicotine-stimulated hPDLCs model via the p38, ERK, and NF- γ B pathways (Lee et al., 2013). It has been reported that p38-MAPK pathway and ERK1/2 MAPK signaling were related to the osteogenetic differentiation (Wang, Y. et al., 2016; Wang, Q. et al., 2016). However, the involvement of p38-MAPK and ERK pathways in the hPDLCs response to hydrogen sulfide (H₂S) remains to be investigated.

Therefore, there exists a great interest in H₂S in the regulation of osteogenic differentiation in human periodontal ligament cells (hPDLCs) during the process of orthodontic tooth movement, including the mechanism for releasing H₂S under mechanical stimuli, the interaction between H₂S and other signaling molecules, such as nitric oxide, during orthodontic treatment, and signaling mechanism associated with PDL cell responses to tension force-induced H₂S. The aim of the present study was to explore the effect of H₂S on the expression levels of Runx2, ALP and OCN with or without cyclic tension stimulation and investigate the role of p38-MAPK and

ERK pathways during the processes. We hope the results of this study might provide a theoretical basis for us to further understand the mechanisms of how H₂S impacts the orthodontic tooth movement.

2. Materials and methods

2.1 Primary culture of hPDLs

Disease-free first premolars (n = 18) extracted for orthodontic purposes, were collected from 8 patients aged from 12 to 18 years old at the stomatology hospital affiliated to Tongji university. All of the donors were provided written informed consent prior to tooth extraction, according to the guidelines of Tongji University. This study was approved by the Ethics Committee of Tongji University (2013-NSFC002). hPDLs were isolated and cultured according to previously reported protocols (Howard, Kucich, Taliwal, & Korostoff, 1998). Briefly, tissues were cut into 1-mm² explants and maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco, Grand Island, NY, USA) containing 20 % foetal bovine serum (FBS; Hyclone, Logan, UT, USA) and antibiotic solution (100 Uml⁻¹ penicillin and 100 Uml⁻¹ streptomycin) at 37°C in a 5 % CO₂ incubator.

2.2 Application of cyclic strain

hPDLs were plated into flexible-bottomed six-well plates coated with type I collagen (Sigma, Saint Louis, MO, USA) and cultured to confluence, to investigate the optimal application time, cyclic tension force (5 % deformation) was applied to the hPDLs at a frequency of 0.1 Hz (5-s stretch and 5-s relaxation) for 6, 12, 24 or 48 hours using a computer-controlled vacuum stretch apparatus (Flexcell, FX-5000 Tension System, Hillsborough, NC, USA) (Kook, Jang, & Lee, 2011). The effect of H₂S on the expression of Runx2, ALP and OCN in hPDLs was investigated with pre-treatment of H₂S followed by application of cycle tension force for 24 h. The control group cells were cultured in an incubator.

2.3 Real-time quantitative polymerase chain-reaction assay (qPCR)

Total RNA was isolated with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) after performing mechanical loading on the hPDLs. Samples with an A260:A280 ratio $\geq$ 1.9 were reverse transcribed to cDNA using a PrimeScriptTM RT reagent kit according to the manufacturer's protocol. The respective upstream and downstream primers for each gene were as follow: GAPDH (5'-AGAAGGCTGGGGCTCATTTG-3' and 5'-AGGGGCCATCCACAGTCTTC-3'); ALP (5'-CTACACGGTCCTCCTATAC-3' and 5'-CTCGCTCTCGGTAACATC-3'); OCN (5'-GTGCAGAGTCCAGCAAAGGT-3' and 5'-AGACTGCGCCTGGTAGTTGT-3'); Runx2 (5'-GCAGTTCCCAAGCATTTTCAT-3' and 5'-CACTCTGGCTTTGGGAAGAG-3'). Real-time polymerase chain reaction (qPCR) was performed using SYBR Green PCR

Master Mix and an ABI 7500 Real-time PCR Detection System (Applied Biosystems, Grand Island, NY, USA). The reactions were run in triplicate and the results were averaged. The relative expression of the targets in each sample was evaluated by using a modification of the $2^{-\Delta\Delta CT}$ method (Livak, & Schmittgen, 2001).

2.4 Western blotting

Specimens were collected at indicated time points and washed with PBS, then lysed with protein extraction reagent. The BCA Protein Assay Kit (Pierce, Rockford, IL, USA) was used to measure the protein concentration, Lysates were separated by SDS–polyacrylamide electrophoresis gels and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). Blots were probed with antibodies to ALP(Abcam,ab154100,UK), OCN (Abcam,ab13418,UK)), Runx2 (Abcam, ab23981, UK) and anti-p38 antibody(Abcam, ab45381, UK), anti-phosphorylated-p38(p-p38) antibody(Santa Cruz Biotechnology,sc-7973,USA) , ERK(Abcam, ab196883. UK), p-ERK1/2(Cell Signaling, NO.14227,USA) overnight at 4 °C and incubated with HRP-conjugated secondary antibody (Santa Cruz Biotechnology, sc-516087, USA) at 37 °C for 1 h. Blots were visualized using an electrochemiluminescence (ECL) system (Millipore, Billerica, MA, USA); band intensities were measured by the ChemiDoc XRS Gel Documentation System and Quantity One software (Bio-Rad, Hercules, CA, USA) and calculated as absorbance (A). The kinases activity were analysed by band intensity ratio (anti-phospho-ERK/anti-ERK antiserum (Ap-ERK/AERK), Ap-p38 MAPK/Ap38 MAPK).

2.5 Blockade of the MAPK pathway

PDLCs were pretreated with 10 μ M SB203580 or 10 μ M U0126 (Shanghai beyotime Co. Ltd., Shanghai, China) for 1 h to inhibit p38-MAPK or ERK signaling, before being exposed to 50 μ M/L NaHS. Then the cells were subjected to the cyclic tensile force for 24 h. Gene expression of Runx2, ALP and OCN was detected by real-time PCR.

2.6 Statistical analysis

Data are expressed as means $\pm$ standard deviation (SD) from three independent experiments. Variance (ANOVA) was performed for statistical comparisons using SPSS software, version 20.0 (IBM Corporation, Armonk, NY, USA). $P < 0.05$ was considered statistical significant.

3. Results

3.1 Effect of tension force on the expression of Runx2, ALP and OCN

Treatment of hPDLs with application of cyclic tension stimulation for 6 h, 12 h, 24 h and 48 h significantly increased the mRNA and protein expression of ALP, OCN and Runx2 in a time-dependent manner (Fig. 1). The peak of ALP, OCN and Runx2 expression were detected at 24 h.

3.2 NaHS dose-dependently increased mRNA and protein expression of ALP, OCN and Runx2 in hPDLs

To understand the role of H₂S in hPDLs, we investigated the mRNA and protein expression of ALP, OCN and Runx2 induced by H₂S with or without cycle tension force application. H₂S upregulated the mRNA expression of ALP, OCN and Runx2 with or without tension force application and 50 μM H₂S significantly up-regulated the mRNA expression of Runx2, ALP and OCN (Fig. 2a, b and c).

The Western blotting results also showed that 50 μM H₂S dramatically up-regulated the protein expression of ALP, OCN and Runx2 with or without tension force application (Fig. 2d).

3.3 NaHS up-regulated the expression of ALP, OCN and Runx2 in hPDLs through the p38-MAPK and ERK pathway

To further explore the underlying signaling mechanism, the activity of p38-MAPK and ERK signaling (by Western blotting) were assessed. Expression of p-p38 and p-ERK was upregulated by tension force and NaHS treatment, and 50 μM H₂S significantly increased the expression of p38-MAPK and ERK signalling (Fig. 3).

3.4 SB203580, U0126 inhibited NaHS-induced expression of ALP, OCN and Runx2 in hPDLs

We undertook a p38-MAPK and ERK pathway blockade assay respectively to ascertain whether H₂S up-regulated the expression of ALP, OCN and Runx2 in hPDLs via the p38-MAPK and ERK signaling pathway. SB203580 and U0126 significantly ($P < 0.05$) blocked H₂S-induced expression of ALP, OCN and Runx2 genes in hPDLs suggesting the key role of p38-MAPK and ERK pathway in H₂S-induced expression of ALP, OCN and Runx2 (Fig. 4).

4. Discussion

Orthodontic tooth movement is the process of alveolar bone rebuilding under proper mechanical stimulation. hPDLs are thought to play important roles in alveolar bone remodeling as well as osteoblasts and osteoclasts. hPDLs express osteoblastlike properties, including the expression of Runx2, ALP and OCN (Lee, & Kim, 2009). ALP, as an indicator of new bone formation, has been commonly studied as an early marker of the osteogenic differentiation in tension force experiments. Earlier studies have found that mechanical strain stimulation increased the expression

of ALP in PDLCS (Ku, Chang, Chae, Kim, Park, Jung, & Choi, 2009). OCN and Runx2 are also key transcription factors in the modulation of osteogenic differentiation and bone formation (Shi et al., 2011). In our study, both tension force and H₂S administration promoted the expression of Runx2, ALP and OCN in hPDLCS. The results indicate that the enzymatic reaction might mediate mechanical force and H₂S induced osteogenic differentiation and bone remodeling. The findings are consistent with our previous study that mechanical stimulation and H₂S could enhance the osteogenic differentiation of periodontal stem cells (Shen, Qiu, Chang, Yang, Jian, Xiong, Zhou, & Dong, 2014; Lee et al., 2013).

In recent years, as a signaling molecule, the potential role of H₂S in many physiological processes has been widely studied. For example, H₂S protects cells against cytotoxicity caused by β -amyloid, peroxynitrite, H₂O₂ and hypochlorous acid (Laggner et al., 2007; Lu et al., 2008; Tang et al., 2008). However, little biological information is known about how H₂S acts as a mediator for bone metabolism. It was reported that bone marrow mesenchymal stem cells (BMMSCs) can produce H₂S to regulate their osteogenic differentiation and cells' self-renewal, and H₂S deficiency resulted in defects in BMMSC differentiation (Liu et al., 2014). Our data demonstrated that H₂S upregulated the mRNA and protein expression of osteoblast markers, Runx2, OCN and ALP, with or without tension force application and 50 μ M H₂S significantly upregulated the expression of osteoblast markers. These results support our previous observation that H₂S could promote osteogenic differentiation by up-regulates the expression level of OPG/RANKL expression ratio in hPDLCS (Liao, & Hua, 2013).

The MAPK signaling mediates the signal transduction of external stimuli into intracellular signals, which can regulate cell growth and differentiation (Xiao et al., 2000). The role of MAPK signaling components, such as ERK and p38-MAPK has been shown to favor osteoblastic cell proliferation and differentiation (Xiao et al. 2000; Sowa, Kaji, Yamaguchi, Sugimoto, & Chihara, 2002; Ziros et al., 2002). Centrifugal force has been reported to induce ERK1/2 activation in MC3T3-E1 osteoblasts (Hatton, Pooran, Li, Luzzio, & Hughes-Fulford, 2003). Jessop et al. (Jessop, Rawlinson, Pitsillides, & Lanyon, 2002) reported that ERK1/2 activation was induced in osteoblast-like cells by mechanical stimulation. Cyclic tensile force-induced p38-MAPK activation in osteoblasts may play an important role in bone remodeling (Kusumi et al., 2005). It is reported that the MAPK (ERK1/2) signaling is activated in proliferation of neural stem cells (NSCs) induced by H₂S (Liu et al., 2014). Moreover, MAPKs were indicated to activate and phosphorylate Runx2, which subsequently initiate osteogenesis-specific gene transcription in vitro (Xiao et al. 2000). Therefore, we hypothesized that H₂S may stimulate p38-MAPK and ERK signaling in PDLCS and induce osteogenic differentiation. Our investigation showed that expression of p-p38 and p-ERK was upregulated by NaHS treatment, and 50 μ M H₂S significantly increased the expression of p38-MAPK and ERK signaling. Furthermore, we performed p38-MAPK and ERK signaling blockade assay to ascertain if H₂S upregulated expression of above genes in PDLCS via the p38-MAPK and ERK pathways. We found that SB203580 and U0126 significantly ($P < 0.05$)

blocked H₂S-induced expression of Runx2, ALP and OCN genes in hPDLs, which suggest the key role of p38-MAPK and ERK pathway in H₂S-induced expression of Runx2, ALP and OCN. Our results are consistent with the previous study, which reported that the protective effects of H₂S were mediated by p38 and ERK in H₂O₂-stimulated MC3T3-E1 osteoblastic cells (Xu et al., 2011).

In summary, the present studies indicate that H₂S promotes osteogenic differentiation in hPDLs via the p38-MAPK and ERK pathways. Our findings suggest that H₂S might be a novel potential candidate to accelerate orthodontic tooth movement. However, further investigations are needed to explore the molecular mechanisms of the role of H₂S in the alveolar bone reconstruction.

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Conflict of Interest statement

The authors declare that we have no conflict of interest.

Ethical approval

The protocol for this experiment was reviewed and approved by the Ethics committee (2013-NSFC002) of Tongji University.

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