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Endogenous hydrogen sulfide is involved in osteogenic differentiation in human periodontal ligament cells

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ABSTRACT

Objective: Endogenous hydrogen sulfide (H_2S) has recently emerged as an important intracellular gaseous signaling molecule within cellular systems. Endogenous H_2S is synthesized from L-cysteine via cystathionine β-synthase and cystathionine γ-lyase and it regulates multiple signaling pathways in mammalian cells. Indeed, aberrant H_2S levels have been linked to defects in bone formation in experimental mice. The aim of this study was to examine the potential production mechanism and function of endogenous H_2S within primary human periodontal ligament cells (PDLCs).
Design: Primary human PDLCs were obtained from donor molars with volunteer permission. Immunofluorescent labeling determined expression of the H_2S synthetase enzymes. These enzymes were inhibited with D,L-propargylglycine or hydroxylamine to examine the effects of H_2S signaling upon the osteogenic differentiation of PDLCs. Gene and protein expression levels of osteogenic markers in conjunction with ALP staining and activity and alizarin red S staining of calcium deposition were used to assay the progression of osteogenesis under different treatment conditions. Cultures were exposed to Wnt3a treatment to assess downstream signaling mechanisms.
Results: In this study, we show that H_2S is produced by human PDLCs via the cystathionine β-synthase/cystathionine γ-lyase pathway to promote their osteogenic differentiation. These levels must be carefully maintained as excessive or deficient H_2S levels temper the observed osteogenic effect by inhibiting Wnt/β-catenin signaling.
Conclusions: These results demonstrate that optimal concentrations of endogenous H_2S must be maintained within PDLCs to promote osteogenic differentiation by activating the Wnt/β-catenin signaling cascade.

1. Introduction

Periodontitis is a common chronic inflammatory disease leading to destruction of the supporting structures of the teeth including the alveolar bone, periodontal ligament and cementum (Trofin, Monsarrat, & Kemoun, 2013; Wolf et al., 2013). This degeneration can lead to early tooth loss in affected individuals (Preshaw et al., 2012; Reich & Hiller, 1993). Periodontal ligament (PDL) is a specialized connective tissue that maintains and supports teeth *in situ* (Nanci & Bosshardt, 2006; Seo et al., 2004). Current periodontal treatments rely in the main on the osteogenic differentiation of PDL, which is responsible for the regeneration of the adjacent periodontal structure (Acil et al., 2015; Sun & Liu, 2014). Periodontal ligament cells (PDLCs) are endogenous multipotential progenitors, which form a major cellular component of the PDL (Liu, Zha, Xuan, Xie, & Zhang, 2010; Wei, Wu, Ling, & Liu, 2008). The osteogenic differentiation of PDLCs is mediated through diverse signaling mechanisms by a vast array of signaling molecules, such as nitric oxide (Klein-Nulend, Semeins, Ajubi, Nijweide, & Burger, 1995), prostaglandin E1 (Bakker, Soejima, Klein-Nulend, & Burger, 2001; Jessop, Rawlinson, Pitsillides, & Lanyon, 2002), prostaglandin E2 (Ryder & Duncan, 2001) and mechano- and voltage-sensitive Ca^{2+} channels (Kolluru, Shen, Bir, & Kevil, 2013).

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Hydrogen sulfide (H_2S), as the most recently discovered gasotransmitter, following nitric oxide and carbon monoxide, has aroused considerable interest as a potential signaling molecule (Li, Rose, & Moore, 2011). In mammalian cells, H_2S is endogenously synthesized from L-cysteine via the action of cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE). It has been suggested that H_2S may regulate a variety of signaling pathways[17] and abnormal metabolism links to a number of diseases, such as hypertension, coronary heart disease, inflammation (Kamoun, 2004). It has also been shown to be involved in the regulation of body temperature, metabolic levels (Lowicka & Beltowski, 2007; Zhang, Dong, & Chu, 2010) and the induction of apoptosis in PDLCs (Irie et al., 2009).

Recently the role of endogenous H_2S in regulating ossification has generated a great deal of controversy. Whereas Koichiro et al. showed that H_2S could transiently promote osteoclast differentiation through the up-regulation of RANKL expression in osteoblasts (Irie et al., 2009), Liu et al. revealed that H_2S-deficient mice displayed an osteoporotic phenotype (Liu et al., 2014).

It is currently unknown whether PDLCs produce endogenous H_2S or whether H_2S can exert osteoblastic or osteoclastic effects within this cell type. In this study, we investigated the potential of primary human PDLCs to endogenously synthesize H_2S and whether this occurs via the typical CBS/CSE pathway in this cell type. Additionally, we have attempted to clarify the signaling mechanism responsible for the osteogenic effects of H_2S within PDLCs.

2. Materials and methods

The experiments were approved by the Medical Ethics Committee of Wenzhou Medical University and the Hospital of Stomatology. Informed written consent was obtained from all donors.

2.1. Primary culture and identification of PDLCs

Human PDLCs (hPDLCs) were obtained from third molars extracted for impaction in six healthy volunteers (three male and three female) aged 18–28. The teeth were infiltrated with sterile phosphate-buffered saline (PBS) upon separation, and rinsed gently with running sterile PBS. Periodontal ligament tissue was gently scraped from middle-third of roots, and minced into 1-mm^2 cubes. The obtained periodontal ligament tissues were digested by treatment with type I collagenase (Sigma Aldrich, St. Louis, MO, USA, 3 mg/ml dissolved in hanks) for 30 min at 37 °C and cultured in complete medium, α-minimum essential medium (α-MEM, Gibco, Grand Island, NY, USA) supplemented with fetal bovine serum (10% v/v, Millipore Corporation, Billerica, MA, USA) and antibiotics (100 U/ml penicillin G, 100 μg/ml streptomycin, Solarbio Life Sciences, Beijing, China), at 37 °C in a humidified atmosphere of 5% CO_2. When confluence reached 80%, cells were detached using Trypsin-EDTA (0.25%, Gibco; diluted with culture medium), and subcultured at a ratio of 1:3. These cells were used for experiments between passages 3 and 5. Staining for vimentin and cytokeratin were used to identify PDLCs.

2.2. Treatment of PDLCs

Donor PDLCs were divided into six groups, all of which were then seeded within a 6-well cell culture plate, and treated with the H_2S donor, sodium hydrogen sulfide (NaHS, Sigma Aldrich; 100 μmol/L) in the absence or presence of the CSE inhibitor D, L-propargylglycine (PAG, Sigma Aldrich; 100 μmol/L) or CBS inhibitor hydroxylamine (HA, Sigma Aldrich, St. Louis, MO; 100 μmol/L). Control cultures were treated with either PAG or HA alone, or cultured with complete medium alone. Thus, the six groups were as follows; control; NaHS; PAG; HA; combinative stimulation of PAG and NaHS; and HA and NaHS. For osteoblast induction, cells were cultured in differentiation medium (complete medium including 50 μg/mL ascorbic acid (Sigma Aldrich), 5 mmol/L β-glycerophosphate (Sigma Aldrich) and 10 nmol/L dexamethasone (Sigma Aldrich)). The reagents involved in this study were all dissolved in PBS, and the concentration of NaHS used was 100 μmol/L, if not otherwise specified. The medium was refreshed every three days. Cells in each group were collected for analysis at 48 h.

2.3. MTT assay

Cell viability under NaHS stimulation was assayed by the addition of 5 mg/mL tetrazolium salt [MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, Sigma Aldrich] to the treated cultures. Briefly, cells were seeded in 96-well cell culture plates at a density of 2×10^3 cells/well and cultured with PAG (100 μmol/L), HA (100 μmol/L) and NaHS (10, 100, and 1000 μmol/L) for 1, 3, 5, and 7 d, respectively, with medium refreshed every three days. Cells without stimulation acted as control groups. 100 μL complete medium and 20 μL MTT (5 mg/mL) replaced the previous liquid in each well of plates, and the cells were incubated for 4 h at 37 °C. After removal of the medium and addition of dimethyl sulfoxide (DMSO, Sigma Aldrich) to each well, the absorbance was measured at 490 nm by a microplate reader (Infinite M200 PRO, Tecan).

2.4. Immunofluorescent staining

The endogenous expression of CBS and CSE in PDLCs was confirmed by immunocytochemical staining. After being washed twice with PBS, the cultures were fixed in 4% paraformaldehyde (Solarbio Life Sciences) for 10 min at 4 °C. The cultures were then incubated with 0.2% TritonX–100 at 37 °C for 15 min, followed by blocking reagent (Super sensitive TM IHC detection system kit, Bioworld St. Louis, MN, USA) at 37 °C for 1 h. Antibody incubations with anti-CBS rabbit antibody (2 μg/mL; Abcam) were carried out at 4 °C overnight and goat anti-rabbit secondary antibody (5 μg/mL; MaiBio, Shanghai, China) at 37 °C for 2 h or anti-cystathionase mouse antibody (5 μg/mL; Abcam) at 4 °C overnight and goat anti-mouse secondary antibody (5 μg/mL; MaiBio) at 37 °C for 2 h. Between incubations, cells were washed twice with PBS, air dried and then observed under an inverted fluorescent phase contrast microscope (Nikon Eclipse Ti).

2.5. Measurement of H_2S level

To assess H_2S production by PDLCs under the different culture treatments examined, 500 μL supernatants were obtained from each cell culture treatment and mixed with 250 μL Zn acetate (1%, Sigma Aldrich) and 450 μL water for 10 min at room temperature. Trichloroacetic acid (TCA, 250 μL, 10%, Sigma Aldrich) was then added, and the mixture was centrifuged for 10 min at 14,000*g*, at 4 °C. The supernatant collected was mixed with 133 μL N,N-dimethyl-*p*-phenylenediamine sulfate (20 μmol/L, Sigma Aldrich) in 1.2 mol/L HCl and 133 μL $FeCl_3$ (30 μmol/L, Sigma Aldrich) in 1.2 mol/L HCl for 20 min at 37 °C. The absorbance was measured at 650 nm by a microplate reader (Infinite M200 PRO, Tecan).

2.6. ALP activity assay

Following PDLC culture in osteoblastic medium for 7 d and 24 h treatment with different H_2S analysis conditions cells from each group were washed twice, and lysed using 1% TritonX-100 and