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Cystathionine gamma lyase-H_2S contributes to osteoclastogenesis during bone remodeling induced by mechanical loading

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ABSTRACT

Hydrogen sulfide (H_2S), a gaseous signaling molecule produced by cystathionine γ-lyase (CSE) and cystathionine β-synthase (CBS), influences bone remodeling in many ways. Osteoclasts play an important role in bone remodeling during tooth movement induced by mechanical loading, which increases the rate of tooth movement. Recently, we found that osteoclasts could produce H_2S. However, whether H_2S modulates bone remodeling by affecting osteoclasts remains unclear. In this study, we found that CSE-H_2S was a dominant H_2S generating system in osteoclasts, while CBS did not generate H_2S. A significant increase in CSE mRNA expression and H_2S production was observed in periodontal ligament (PDL) tissues of wild-type (WT) mice after 3 days of mechanical loading. CSE gene knockout led to a significant reduction in the number of maxillary osteoclasts and in the amount of tooth movement. The number of RANKL-induced TRAP-positive osteoclasts and the mRNA expression of osteoclast markers were downregulated after 6 days of incubation in monocytes extracted from $CSE^{-/-}$ mice. The expression of IL-1, IL-6 and TNF-α, which can stimulate osteoclastogenesis in periodontal tissue and serum samples, was lower in $CSE^{-/-}$ mice after mechanical loading. Application of the H_2S donor GYY4137 increased the number of RANKL-induced osteoclasts, the number of osteoclasts in periodontal tissues and tooth movement distance in $CSE^{-/-}$ mice. The results suggested that endogenous H_2S and CSE play vital roles in the osteoclastogenesis and alveolar bone resorption induced by mechanical loading.

1. Introduction

Orthodontic tooth movement (OTM) induced by orthodontic force application is characterized by remodeling in alveolar bone and periodontal tissues. In the process of bone remodeling induced by mechanical loading, mechanical force is applied to the tooth; the surrounding alveolar bone is absorbed by osteoclasts at the site of compression, and new bone is formed by osteoblasts at the sites of tension, which leads to migration of the tooth [1,2]. Mechanical loading results in the local synthesis and release of various molecules, such as neurotransmitters, cytokines, growth factors, colony-stimulating factors, etc. [3,4]. The released molecules evoke cellular responses in the various cell types in and around the tooth, providing a favorable microenvironment for tissue resorption and deposition.

Hydrogen sulfide (H_2S) is an endogenous gaseous signaling molecule that is synthesized by cystathionine γ-lyase (CSE), the predominant H_2S-producing enzyme in mammalian cells; however, other systems, including those that are supported by cystathionine β-synthase (CBS), exist [5]. H_2S is an important mediator of many physiological processes, such as neurodegeneration, and is involved in the regulation of inflammation, blood pressure and metabolism [6]. In addition, endogenous H_2S influences bone remodeling by affecting bone-forming cells [7,8]. In a rat model of bone fractures, the overexpression of CSE promotes the fracture healing by facilitating osteoblast activity via the RUNX2 pathway [9]. However, whether H_2S modulates bone remodeling by affecting osteoclasts remains unclear. It was reported that the inhibition of CSE expression resulted in reduced osteoclast differentiation and the downregulation of osteoclast markers, such as tartrate-resistant acid phosphatase (TRAP), cathepsin K (CTSK) and MMP-9 [10]. Therefore, we hypothesized that endogenous H_2S modulated bone remodeling by affecting osteoclast activity.

Periodontal inflammation and alveolar bone remodeling induced by mechanical loading are considered regional reactions,

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and they both contribute to tooth movement [11,12]. Inflammatory monocytes are recruited to the site of compression in periodontal tissues and directly participate in tooth movement by undergoing osteoclast differentiation [13]. In the human monocyte cell line U937, treatment with the H_2S donor NaHS resulted in significant increases in the mRNA and protein expression of TNF-α, IL-1β, and IL-6 [14]. Inflammatory cytokines, such as TNF-α, IL-1β and IL-6, are thought to play roles in bone remodeling, bone resorption, and new bone deposition [15]. In this study, we revealed that H_2S endogenously synthesized by CSE was produced during osteoclast formation and OTM and played a proinflammatory role. The results also demonstrated that the bone remodeling and osteoclast activity induced by mechanical loading were regulated by CSE and H_2S.

2. Materials and methods

2.1. Animals

CSE knockout ($CSE^{-/-}$) mice were generated at the Shanghai Biomodel Organisms Center, Inc. The experiments were performed on 6- to 8-week-old, wild-type (WT, $CSE^{+/+}$) mice and $CSE^{-/-}$ mice, which were obtained by mating CSE $^{\pm}$ males and females. The animal experiments were approved by the Animal Use and Care Committee of Tongji University, according to their guidance. To evaluate the effects of H_2S on tooth movement, $CSE^{-/-}$ mice were intraperitoneally administered the H_2S donor GYY4137 (1 mg/mouse, Sigma-Aldrich, USA) once every other day starting with the day prior to the application of mechanical loading.

2.2. Orthodontic tooth movement

To confirm the function of CSE during bone remodeling induced by mechanical loading, we used a mouse OTM model. The experimental protocol for OTM was modified as previously described [16]. Briefly, a nickel-titanium coil spring (Smart Technology, China) was bonded between the maxillary left first molar and incisor with resin (Z350XT, 3 M). The force produced was approximately 0.35 N [16], as measured by a dynamometer (HF-2; ALIYIOI). The right side was used as the control.

2.3. Micro-CT scanning

The maxillae of mice were scanned by Micro-CT (Scanco Medical, Bassersdorf, Switzerland). To determine OTM, the distance between the distal height of the contour of the first molar and the mesial height of the contour of the second molar was measured.

2.4. Osteoclast culture

Cells were harvested from the bone marrow of 6-week-old $CSE^{-/-}$ mice and WT mice and cultured for 3 days in α-minimum essential medium (SH30265.01, HyClone,USA) containing 10% fetal bovine serum (Gibco, Rockville, MD) and 1% penicillin -streptomycin (SV30010, HyClone, USA). Bone marrow macrophages (BMMs), which were not adherent, were seeded in 96-well plates ($4–6 \times 10^4$ cells/well) or 6-well plates (1×10^6 cells/well) with mouse macrophage colony-stimulating factor (M-CSF) (50 ng/ml; PeproTech) for 2 days. The precursors of osteoclasts were then cultured with RANKL (50 ng/mL or 100 ng/mL; PeproTech) and M-CSF (50 ng/mL) for 6 days. To understand the role of H_2S in osteoclastogenesis, GYY4137 was added to cultures of osteoclast precursors at a concentration of 200 μM with RANKL and M-CSF for 6 days. At the end of the experiment, TRAP and PCR assays were performed.

2.5. Histological analysis

The maxillae were collected on the 3rd day of mechanical loading, fixed with 4% paraformaldehyde and decalcified in 10% EDTA (pH 7.4) for 20 days. The samples were embedded in paraffin and cut into horizontal sections at a thickness of 4 μm. The region from the first molar bifurcation to 300 μm toward the root apex was selected [17]. The activated osteoclasts were labeled by TRAP staining (Sigma-Aldrich, USA). The total number of osteoclasts (osteoclast number per bone surface) were counted at the alveolar bone compression site on the distal buccal root of the upper first molar.

2.6. Quantitative real-time polymerase chain reaction (PCR)

The periodontal ligaments and surrounding alveolar bone near the upper first molars were collected. The total RNA of the molars and osteoclasts was extracted using TRIzol® reagent (Invitrogen, USA) and was then used to obtain cDNA with a Transcription First Strand cDNA Synthesis kit (Roche, Basel, Switzerland). Real-time PCR was performed with a Fast Start Essential DNA Green Master kit (Roche, Switzerland) and a LightCycler®96 instrument (Roche, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was analyzed as the housekeeping gene. The sequences of the primers are listed in Appendix Table 1.

2.7. Western Blot analysis

The cultured osteoclasts were washed with PBS and lysed with RIPA buffer. The cell lysates were separated on a 10% SDS-PAGE gel and transferred to a PVDF membrane (Millipore, USA). The membrane was blocked with 5% non-fat dry milk in TBST for 1 h and then incubated with primary antibodies of CSE (Ab151769, Abcam) at 1:500 dilution in PBS overnight at 4 °C. Primary antibodies were labeled by incubation with secondary antibodies (1:8000, Ab181602, Abcam) at room temperature for 1 h. Antibody-bound proteins were detected using an ImageQuant LAS 4000 mini system (Sagecreation, China). GAPDH antibody levels were used for normalization.

2.8. Enzyme-linked immunosorbent assay (ELISA)

After 3 days of mechanical loading, the maxillary bone, from the first molar to the third molar, was removed and trimmed. The tissue was homogenized by TissueLyser II (Qiagen, Germany). The supernatant was collected and measured with an ELISA, according to the manufacturer's instructions. The ELISA was performed with kits for H_2S (AE98327Mu, Lianshuo Biological, shanghai), IL-1 (AE98022, Lianshuo Biological, Shanghai), IL-6 (AE90247, Lianshuo Biological, Shanghai), and TNF-α (AE90301Mu, Lianshuo Biological, Shanghai).

2.9. Statistical analysis

The data are presented as the mean ± SEM. Comparisons among the groups were analyzed by independent 2-tailed Student's t tests or one-way analysis of variance (ANOVA); $p < 0.05$ was considered significant.

3. Results

3.1. Levels of mechanical-loading-induced H_2S and CSE in periodontal tissues and expression of CSE and CBS in bone marrow-derived osteoclasts

We used a mouse OTM model to test the in vivo function of mechanical-loading-induced endogenous H_2S production (Fig. 1A).

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