A Novel Fluorescent Probe for Detecting Hydrogen Sulfide in Osteoblasts During Lipopolysaccharide-Mediated Inflammation Under Periodontitis

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A novel fluorescent probe for detecting hydrogen sulfide in osteoblasts during lipopolysaccharide-mediated inflammation under periodontitis

Title page

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Author contributions

Xiaoya Lu, Yi Chen and Yue Wu performed the bioimaging experiments together.

Hanchuang Zhu synthesized the H2S fluorescence probe.

Shengyun Huang, Baocun Zhu and Dongsheng Zhang conceived the idea and directed the work.

All authors contributed to data analysis, manuscript writing and participated in research discussions.

Abstract

Periodontitis, one of the most common chronic inflammatory diseases, affects the quality of life. Osteogenesis makes an important role of the disease. There is a connection between hydrogen sulfide (H_2S) and periodontitis, but according to the study has been published, the precise role of H_2S in inflammation remains in doubt. The main reason of the lack of research is that H_2S is an endogenous gasotransmitter, difficult to discern through testing. So, we synthesis a novel fluorescence probe which can detective H_2S in vitro. By using the novel H_2S fluorescence probe, we found that H_2S changes in osteoblasts mainly by cystathionine-γ-lyase, and H_2S increases under LPS stimulation. H_2S could be a potential marker for diagnosis of inflammatory diseases of bone, and might help deeper studies of the changes of H_2S level and promote the progression on the researches about pathogenesis of periodontitis.

Keywords

Periodontitis, hydrogen sulfide, fluorescence probe, osteogenesis, LPS

Introduction

Periodontitis as one of the most common chronic inflammatory diseases, afflicting man. It can leading cause of bone resorption, even worse tooth loss. Under normal physiologic conditions, the balance of osteoclasts and osteoblasts is tightly related to avoid the loss of bone. The breakdown of the balance will cause diseases. Avoiding alveolar bone destruction is an important problem to control the periodontitis. However, the detailed mechanism of periodontitis is still largely unknown.

Lipopolysaccharide (LPS), a major toxic factor of gram-negative bacteria, plays a main role in periodontitis. It can cause periodontitis by modulating the activity of the host defenses [1], inducing a hypoxic phase [2] etc., and it eventually stimulates bone resorption [3]. LPS may leads to inflammatory response in osteoblasts and osteoblast, which may results in a disorder in the balance of osteoclasts and osteoblasts even cell death, leading to accelerating bone loss [4]. For experimental researches, LPS stimulated the rat gingival sulcus every day in order to obtain an experimental periodontitis model by immunizing it with the antigen [5]. LPS treated cells are in a similar situation as well. Halitosis is one of the clinical features of periodontitis, and Hydrogen sulfide (H_2S) is the main unbearable stinky smell of periodontitis and may play a significant role in its development.

Biothiols are indispensable in human physiology, which are in a vital branch of reactive sulfur species (RSS) family. H_2S is an endogenous gasotransmitter, which is well-known for its stinky smell like rotten eggs. H_2S is produced by the sulfur-containing materials cysteine, homocysteine or

3-mercaptopyruvate. H_2S is transformed by cystathionine-β-synthase (CBS), cystathionine-γ-lyase (CSE) and 3-mercaptopyruvate sulfurtransferase (3-MST)[6]. Most researchers previously believe that H_2S can promote the pathogenesis of periodontitis, and hugely harm to their periodontal tissue [7]. But recently, there is evidence shows that H_2S might be useful in cell protection. For exogenous H_2S, it can promote LPS-induced apoptosis of osteoblast cells, which might represent a new direction in the treatment of osteomyelitis [8]. When oxidative damage occurs, H_2S can increase cell viability and reduce cell apoptosis. H_2S might have a advantageous effect, because according to the research, NaHS treatment can produce anti-inflammatory effects via NO and TNF-α [9]. Besides, H_2S can protect cell injury by regulating oxidative stress, mitochondrial function, and inflammation. It also have the ability to potentially prevent bone loss in periodontitis [10]. So, there is a connection between H_2S and periodontitis, but until now, the precise role of H_2S in inflammation remains unknown.

Most of the studies focus on the effect of the H_2S, not many about H_2S changes under stimulation. Researchers often use Western blot, immumohistochemical staining, and some other methods to detect the H_2S changes indirectly. Recently, there are some direct techniques to detect H_2S, such as chromatography, electrochemistry and colorimetry [11]. But a technique that can detect H_2S directly in living cells is still needed. H_2S- fluorescence probes, which is high- speed developing, is considered as one of the most helpful instrument areas in the field of H_2S biology [12]. We previously designed a H_2S probe, which consists of a 4-chloro-1,8-naphthalimide as fluorophore

89 *and introduces hydrophobic chains (dodecylamine) and hydrogen sulfide recognition*
90 *groups (azide groups) (Figure1 a). The introduction of the electron-withdrawing azide*
91 *group changes the push-pull system and quenches the fluorescence. It is noteworthy*
92 *that the reaction is easy to carry out and the yield is high. When the probe reacts with*
93 *hydrogen sulfide, the azide group is reduced to an amino group. Because the amino*
94 *group acts as an electron-donating group, the effect of intramolecular charge transfer*
95 *is enhanced, and the fluorescence is recovered. The probe is able to directly measure*
96 *the real time H_2S level in living cells. Overall, because of high resolution and sensitivity*
97 *of the H_2S probe make it a helpful tool. There are some studies showing that H_2S*
98 *fluorescence probe can detect endogenous H_2S in real-time and in situ. However, most*
99 *of the them use tumor cells instead of somatic cells. if the probe could be used in*
100 *somatic cells, it can broaden the diagnose and treatment applications of H_2S. By using*
101 *a novel H_2S fluorescence probe, we found that H_2S changes in osteoblast mainly by*
102 *CSE, and H_2S increases under LPS stimulation.*
103
104 ***Materials and methods***
105 *1. Regents*
106 *The hydrogen sulfide fluorescent probe was provided by Professor Baocun Zhu (School*
107 *of Resources and Environment, University of Jinan, Jinan, China). The mother*
108 *solution of the probe was prepared with DMSO (Sigma-aldrich, USA) and*
109 *dichloromethane. The probe concentration was 1 mM. The test concentration was*
110 *10 μ M and the experiment was carried out at room temperature (25℃).*

DL-propargylglycine (PAG) (cystathionine γ -lyase inhibitor, Sigma-Aldrich), Cysteine (Cys), NaHS, lipopolysaccharide (LPS) (Sigma-aldrich, USA), cell counting kit-8 (CCK-8; Dojindo Molecular Technologies, Tokyo, Japan).

2. MC3T3-E1 cell culture

The murine calvaria-derived MC3T3-E1 osteoblast-like cell line (Procell CL-0378, subclone 14) was provided by Procell Life Science and Technology CO.,Ltd. Cells were seeded at 5×104 cells/ml into 25 cm₂ flasks and maintained in α -MEM, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Cells were maintained in an incubator containing a 5% carbon dioxide/air environment at 37℃.

3. Toxicity Analysis:

The influence of the H_2S probe on MC3T3-E1 cell was examined by CCK-8. Briefly, MC3T3-E1 cells, seeded at a density of 5×104cells·/ml on a 96-well plate, were maintained at 37℃ in a 5% CO_2, 95% air incubator for 24 h. Then the cells were incubated with different concentrations (0, 5, 10, 20 ,25, 37.5, 50, 75 and 100 μM) of probe suspended in culture medium for 24 h. Same as the probe group, the other plate of cells incubated with same concentrations (0, 5, 10, 20 ,25, 37.5, 50, 75 and 100 μM) of solvent. Subsequently, CCK-8 solution was added into each well for 2 h, and measured the absorbance at 450 nm was measured.

4. Application of H_2S probe to access exogenous H_2S levels: The cells were pre-treated with NaHS (50, 100, 150, 500 μM) for 30 minutes, then, treated with the H_2S probe(10 μM) for 30minutes. Fluorescence and bright field images were collected after PBS washing for three times. Green fluorescence was observed under the confocal

microscope at excitation wavelengths of 405 nm. In order to control exposure, Smart Gain was kept at the same voltage in every photographs.

5. Application of H_2S probe to access endogenous H_2S levels:: In the periodontium of mammalian host, H_2S is produced using Cys mainly by CSE and CBS. The cells were pre-treated with Cys (100 μM, 200 μM) for 30 minutes, then, treated with the H_2S probe (10 μM) for 30min. Fluorescence and bright field images were collected after PBS washing for three times. Green fluorescence was observed under the confocal microscope at excitation wavelengths of 405 nm. In order to control exposure, Smart Gain was kept at the same voltage in every photographs.

PAG is an irreversible inhibitor of CSE. It can blocking-up the produce of endogenous H_2S in MC3T3-E1. Therefore, we pre-treated cells with 50 μM PAG, 30min, then cells were treated with or without Cys for 30 min. Last, fluorescence was examined as before, Smart Gain was kept at the same voltage in every photographs.

6. Addition of lipopolysaccharide (LPS) for inducing inflammation and assessment with H_2S probe: The cells were incubated with 1, 2 μg/ml LPS for one day. Subsequently, the culture dish was washed with PBS for three times and incubated with 10 μM probe for 30 min. Then, the cells were washed with PBS, then the fluorescence imaging was examined by confocal microscope, Smart Gain was kept at the same voltage in every photographs.

Results

Toxicity analysis: The cell's viable and healthy during the detection is a key concern. Figure 1 b showed that cell viability was almost not affected by the probe at 10 μM.

Toxicity is mainly introduced by solvent, DMSO and dichloromethane. The result verify that the H_2S probe is harmless to the cell. Thus, the H_2S probe can be used in living cells for fluorescence imaging analysis.

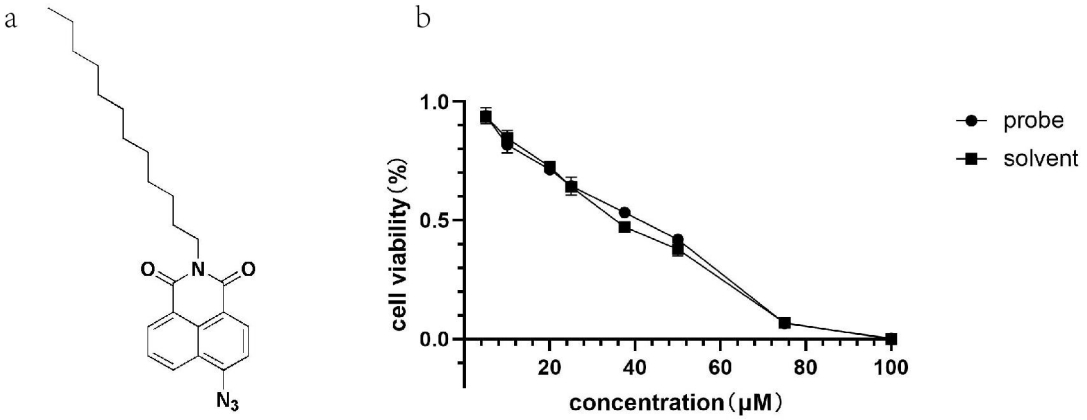
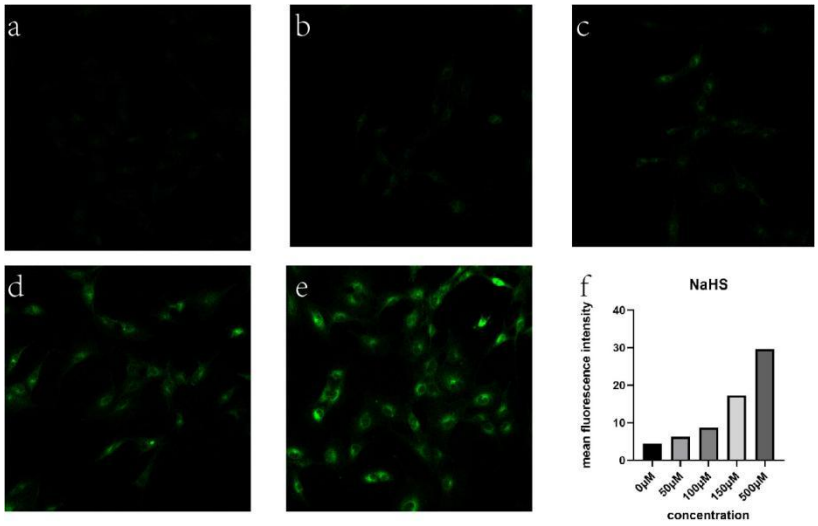


Figure1 **a**.*Structure of H_2S probe.* **b**.*toxicity Analysis.*

Cell fluorescence imaging of different concentrations exogenous H_2S: As shown by figure 2, with the different concentrations (0, 50, 100, 150, 500 μ M) of NaHS, a gradual increase of intensive green fluorescence was observed using 405nm as an excitation wavelength. Consistent with previous studies, the amount of H_2S is one third of exogenous of NaHS. Thus, the probe was estimated detection of accuracy to 10 μ M. Fluorescent intensity is stable during the progress of takeing pictures under the confocal laser scanning microscopy. That indicated that our probe is sensitive to H_2S, and it also prove that H_2S probe was cell membrane permeable and can used in the normal cells for detecting intracellular H_2S.



170

171 *Figure2 Cell fluorescence imaging of different concentrations exogenous H_2S.* **a-e.**

172 *Fluorescence imaging of cells incubated with different concentration of NaHS (0, 50,*

173 *100, 150, 500 μM)* ***f****. Fluorescence intensity analysis.*

174

175 *Cell fluorescence imaging of endogenous H_2S: According to the previous research, for*

176 *osteoblasts, CSE-H_2S might be the major path for the H_2S produced [13]. As shown by*

177 *Figure 3, the incubation of cells with 100 μM Cys produced intensive green*

178 *fluorescence, but the fluorescence decreased when cells were incubated with 200 μM*

179 *Cys. That means that low dose of Cys could increase H_2S production, but high dose of*

180 *Cys inhibited H_2S production. In order to verify whether the CSE-H_2S pathway is the*

181 *main pathway to produce the H_2S, we used PAG as the irreversible inhibitor to CSE.*

182 *Figure 3 showed that the intensity of fluorescence was decreased, which means the H_2S*

183 *was decreased, because of the pretreatment of the inhibitor, and the intensity of PAG*

184 *group was as weak as the control group, indicating that the production of endogenous*

185 *H_2S was significantly inhibited with CSE inhibitor.*

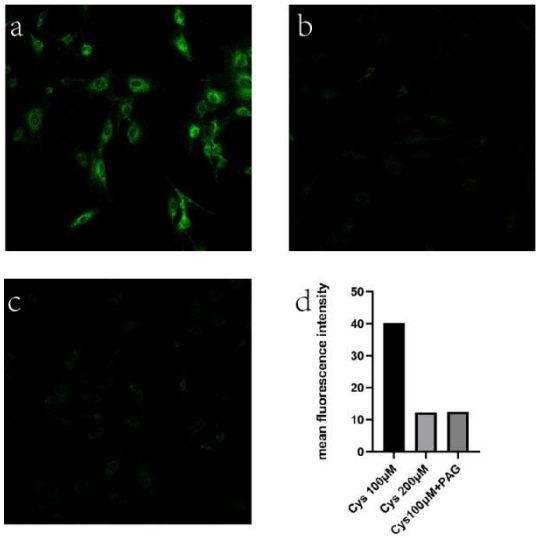


Figure3 Cell fluorescence imaging of endogenous H_2S. **a**. *Cys 100 μ M,* **b**. *Cys 200 μ M,* **c**. *Cys 100 μ M + PAG 50 μ M,* **d**. *Fluorescence intensity analysis.*

Cell fluorescence imaging of LPS induced endogenous H_2S: when cells were treated with LPS (2 μ g/ml) to produce inflammation, as shown by Figure 4, intensive green fluorescence were produced compared to the control group. This indicates that when inflammation occurs, a lot of H_2S was produced. In other words, the increase of H_2S level can serve as an indicator for cells that are under the inflammation state. The production of endogenous H_2S induced by lipopolysaccharide-mediated inflammation was successfully monitored with this H_2S probe.

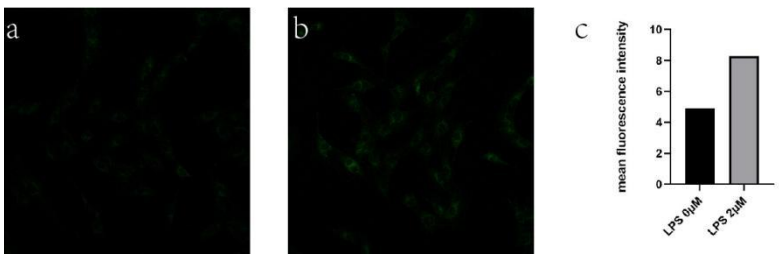


Figure4 Cell fluorescence imaging of LPS induced endogenous H_2S. **a**. *LPS 0 μ M,* **b**. *LPS 2 μ M,* **c**. *Fluorescence intensity analysis.*

Discussion

The main aim of the experiment is to solve the problem of detection of the inflammation of osteoblast, furthermore, we found that H_2S produced by osteoblast is mainly via CSE-H_2S pathway. In our study, we proved that our probe can be used in the normal cell to detect the H_2S changes, which is rarely studied. There are already a lot of fluorescent probes that have been devised to detect intracellular H_2S levels, however, to our knowledge, most of these probes were successfully applied to show alteration of H_2S levels of tumor cells or living animals [14; 15; 16]. But in our study, we used a novel fluorescent probe to detect alteration of H_2S levels in living osteoblast cells with exogenous or endogenous H_2S for the first time. The H_2S probe possesses high sensitivity, selectivity, and an ultrafast response to H_2S, rendering it suitable for detection of H_2S concentration in living cells. In order to determine whether the cell could translate Cys to H_2S, and whether the probe could visualize endogenous H_2S, we treated the osteoblast cells with irreversible inhibitor, PAG. The result proved that H_2S is produced mainly by CSE-H_2S pathway, which had not been proved in a visual way before. Other researchers have proved that (CSE) majorly contributed to endogenous H_2S production in the primary osteoblast by overexpression and knockdown CSE [13]. This is consistent with our results.

For the inflammation of bone, there are two proved sources of H_2S: bacteria and macrophage. When inflammation occurs, some bacteria produced and released H_2S, including various common gram-negative pathogens in osteomyelitis such as

Escherichia coli, Enterococcus faecalis, Enterobacter cloacae, and Klebsiella Pneumoniar. For macrophage, research shows that the level of H_2S was improved and the expression of CSE mRNA increased because of the stimulate of LPS[17]. Our study shows that osteoblasts is the third source of H_2S. Different sources of H_2S might have interaction effect, for example, H_2S production by osteoblast might modulate macrophage polarization and contribute to bone reparation. Keeping physiological level of endogenous H_2S in PDLSCs/periodontal tissue is beneficial to maintain the homeostasis of periodontal tissue [18]. A appropriated level of H_2S may play an vital role in maintaining the homeostasis of the bone marrow system. A previous study have clarify that BMSCs can produce H_2S, regulate osteogenic differentiation and cell self-renewal, and that the lack of H_2S could lead to defects in their differentiation [19]. Exogenous H_2S could protect cell injury by regulating oxidative stress, mitochondrial function, and inflammation. While when inflammation occurs, H_2S from bacteria disturbs the endogenous H_2S of osteoblast cells, leads to a negative effect. In periodontitis studies, drugs that can release H_2S have been used for the treatment, such as ATB-352, a kind of ketoprofen that can releasing H_2S. The main aim is to minimize the presence of side-effect at the gastrointestinal tract. Meanwhile they found that the reduction of the inflammation even had a beneficial effect on bone resorption or tissue damage. ATB-346, releasing H_2S like ATB-352, is beneficial for improving bone quality too [20]. Since H_2S also can promote the development of periodontitis, there are still many questions about the biological mechanisms of H_2S. It is well-know that there are many kinds of cell playing important roles in periodontitis,

244 *such as periodontal ligament stem cells, osteoclasts, and immune cells. Independent*
245 *detection of H_2S changes in living cell might facilitate the study of the role of H_2S in*
246 *diseases.*
247 *It was found previously that CBS and CSE were both increased in human gingival*
248 *tissue during periodontitis through the technology of PCR and Western blot. However,*
249 *H_2S level or H_2S synthesis in gingivitis and periodontitis was detected not increase*
250 *after tissue homogenate [21]. This can be problematic for many reasons, such as the*
251 *synthesis capacity decreased or consume increased of H_2S in inflammation. But as a*
252 *gasotransmitter, half of H_2S can escape from medium in five minutes in tissue culture*
253 *wells, which makes it hard to detect [22]. Under physiological conditions, H_2S presents*
254 *in three chemical ionization forms, about 18.5% H_2S, 81.5% HS^- and minute quantities*
255 *of S^{2-} [23]. Different detection methods might lead to different results. H_2S is more*
256 *permeable in plasma membranes, the solubility of H_2S in lipophilic solvents is*
257 *quintuple greater than in water [24], thus, fluorescence probe in theory could detect*
258 *H_2S more precisely. Our H_2S probe might help deeper studies of the changes of H_2S*
259 *level and promote the progression on the researches about pathogenesis of*
260 *periodontitis.*
261 *Fluorescence techniques is gaining widespread attention as sensors offering excellent*
262 *sensitivity, good selectivity, and rapid response to changes. First of all, our probe has*
263 *been shown to be sensitive for endogenous H_2S detection and real-time monitoring of*
264 *the changes in H_2S in living cells, and it reacts quickly under physiological conditions.*
265 *There are some things that can be improved, for example, a more precise target of*

probes to certain subcellular organelles, certain cells, tissues, or organs, which may be achieved by using near-infrared emit to get a greater tissue penetration and minimize the interference from background auto-fluorescence [25]. For clinical use, H_2S has a potential to be used as an appropriate biomarker for the related investigations of inflammation response. However, it still requires further development.

Conclusion

In conclusion, it is the first experiment using H_2S probe to detect H_2S changes under stimulation in osteoblast in real time. We used a new hypotoxic H_2S probe for exogenous and endogenous H_2S detection in living osteoblast cells. Moreover, the results indicate that in osteoblast cells, H_2S is produced mainly by CSE-H_2S pathway directly, it also shows that under inflammation stimulation, endogenous H_2S production will increase. The results suggest that H_2S could be a potential marker for diagnosis of inflammatory diseases of bone, and it might help further studies for understanding the synthesis and change of H_2S level in pathogenesis of periodontal disease.

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Competing interests

The authors declare that they have no competing interests.

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