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Hydrogen sulfide synergistically upregulates *Porphyromonas gingivalis* lipopolysaccharide-induced expression of IL-6 and IL-8 *via* NF-κB signalling in periodontal fibroblasts

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ABSTRACT

Objectives: The periodontal pathogen *Porphyromonas gingivalis* produces hydrogen sulfide (H_2S). H_2S in the oral cavity is positively correlated with periodontitis but the mechanism by which H_2S contributes to periodontal diseases is obscure. We investigated the effect of H_2S in combination with *P. gingivalis* lipopolysaccharide (LPS) on expression of the pro-inflammatory cytokines interleukin (IL)-6 and IL-8 in periodontal fibroblasts and the underlying mechanism of action.

Material and methods: Gingival fibroblasts (GFs) and periodontal ligament cells (PDLCs) were treated with different concentrations of the H_2S donor NaHS in the presence/absence of *P. gingivalis* LPS for different time periods. Expression of IL-6 and IL-8 was detected by real-time PCR and ELISA. The activity of nuclear factor-kappa B (NF-κB) signalling was investigated using western blotting, EMSA and pathway blockade assays.

Results: Real-time PCR and ELISA results showed that H_2S not only upregulated expression of IL-6 and IL-8 at mRNA and protein levels in a dose- and time-dependent manner, but also aggravated *P. gingivalis* LPS-induced expression of IL-6 and IL-8 in GFs and PDLCs. Western blotting and EMSA showed that NF-κB signalling was activated by NaHS, *P. gingivalis* LPS, and both, which was in accordance with the expression levels of IL-6 and IL-8 in GFs and PDLCs. These results were confirmed using a NF-κB pathway blockade assay.

Conclusions: H_2S synergistically upregulated *P. gingivalis* LPS-induced expression of IL-6 and IL-8 in GFs and PDLCs *via* activation of NF-κB signalling, which could promote the development of periodontitis.

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1. Introduction

Periodontitis is a type of bacteria-initiated inflammatory disease with characteristics of periodontal degradation. Host immune-inflammatory response to foreign pathogens plays a major role in periodontal destruction.[1] Gingival fibroblasts (GFs) and periodontal ligament cells (PDLCs), the predominant resident cells in periodontal tissue, are reported to constitutively express pattern recognition receptor, and consequently recognize periodontal pathogen and their toxic factors in periodontium. Microorganisms stimulated-GFs and PDLCs can produce several pro-inflammatory cytokines, such as interleukin (IL)-6 and IL-8,[2–4] which are involved in the process of periodontal inflammatory response.

Porphyromonas gingivalis, a major pathogenic bacterium of periodontitis, produces various virulence factors, such as lipopolysaccharide (LPS) and gingipains. Through these virulence factors, *P. gingivalis* can induce an inflammatory immune response in the host, and contribute to the pathogenesis of periodontitis. Interestingly, apart from LPS, *P. gingivalis* also produces a gas molecule: hydrogen sulfide (H_2S).[5] H_2S is considered to be (after nitric oxide (NO) and carbon monoxide (CO)[6]) the third gaseotransmitter, and plays a vital role in physiological and pathological process including vasodilatation, neuromodulation, and inflammation.[7–9]

In the oral cavity, a relationship between H_2S and periodontitis has been reported. H_2S is the main contributor to halitosis. High concentrations (2 mM) of H_2S have been found in the periodontal pockets of individuals with periodontitis,[10] and was positively correlated with the gingival index, bleeding upon probing, periodontal pocket depth, and radiographic bone loss.[11–14] Several researchers have shown that H_2S can increase gingival epithelium permeability[15] and induce apoptosis of cells in the periodontium, including gingival epithelial cells,[16] GFs,[17] PDLCs[18] and osteoblastic cells.[19]

Even though many deteriorative effects of H_2S on periodontium have been reported, the effect of H_2S on GFs and PDLCs with regard to inflammation is incompletely understood. Moreover, H_2S was reported to increase epithelial permeability and facilitate the passage of LPS across epithelial barrier.[15,20,21] Given the role of H_2S as a mediator of inflammation[9], we hypothesized that H_2S may promote periodontitis by modulation of pro-inflammatory cytokines like IL-6 and IL-8, and a delicate relationship between *P. gingivalis*-produced H_2S and *P. gingivalis*-derived LPS in triggering expression of IL-6 and IL-8 may exist in GFs and PDLCs.

The aim of the present study was to investigate the effect of H_2S with/without *P. gingivalis* LPS on the expression of the pro-inflammatory mediators IL-6 and IL-8 in GFs and PDLCs as well as the underlying mechanism of action. This was achieved using the real-time polymerase chain reaction (PCR), enzyme-linked immunosorbent assay (ELISA), electrophoretic mobility shift assay (EMSA) and western blotting. Our study is devoted to enrich the pathologic mechanism regarding *P. gingivalis*-induced periodontitis, and provide clues for treatment of periodontitis.

2. Material and methods

The protocol was approved by the Medical Ethics Committee of Peking University School and Hospital of Stomatology (approval number, PKUSSIRB-2012017; Beijing, China). Written informed consent to use teeth was obtained from all subjects.

2.1. Cell culture and treatment

Briefly, GFs and PDLCs were obtained from premolar teeth extracted for orthodontic reasons in three young healthy volunteers. Gingival tissues and middle-third-of-root periodontal ligament tissues, respectively, were harvested and cultured in Dulbecco's modified minimum essential medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with foetal bovine serum (10% v/v; Hyclone, Logan, UT, USA) and antibiotics (100 U/mL penicillin G; 100 μg/mL streptomycin; Invitrogen). Cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2.

GFs and PDLCs (both 4.0×10^5) derived from three donors were use to do the following experiments. Cells were seeded in T25 flasks for 24 h, then treated with the H_2S donor sodium hydrogen sulfide (NaHS, Sigma Aldrich, St. Louis, MO, USA; 0, 250, 500 and 1000 μmol/L) in the absence/presence of 1 μg/mL LPS derived from *P. gingivalis* (Invivogen, Cayla, France). At the indicated time points, cells and culture supernatants were harvested and prepared for real-time PCR, western blotting, EMSA and ELISA.

2.2. RNA extraction and cDNA synthesis

Total RNA from GFs and PDLCs was extracted using Trizol reagent (Invitrogen). Total RNA (2 μg) was used as the template to synthesize cDNA using a Reverse Transcriptase Kit (Promega, Madison, WI, USA).

2.3. Quantitative real-time PCR

Real-time PCR was undertaken in a 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) using SYBR Green Reagent (Roche, Basel Switzerland). The respective upstream and downstream primers for each gene were: IL-6 (5′-GTGAGGAACAAGCCAGAGC-3′ and 5′-TACATTTGCCGAAGAGCC-3′); IL-8 (5′-TTTTGCCAAGGAGTGCTAAAGA-3′ and 5′-AACCCTCTGCACCCAGTTTTC-3′); β-actin (5′-CATGTACGTTGCTATCCAGGC-3′ and 5′-CTCCTTAATGTCACGCACGAT-3′). PCR conditions were 10 min at 95 °C, followed by 40 cycles of 95 °C for 15 s and 60 °C for 60 s. All reactions were carried out in triplicate in two separate experiments. The relative expression of the targets in each sample was calculated by the comparative $2^{-\Delta\Delta Ct}$ method after normalization to the expression of β-actin.

2.4. ELISA

The quantity of IL-6 and IL-8 in cell-culture supernatants was measured using an ELISA kit (R&D Systems, Minneapolis, MN, USA) according to manufacturer's instructions. Culture supernatants were diluted 1:5 and the absorbance was set at

450 nm. All samples were measured in duplicate and repeated independently at least twice.

2.5. Western blotting

Cells were harvested and lysed in cold RIPA buffer with protease inhibitors. After measurement of protein concentration by the bicinchoninic acid method, equal amounts of protein samples were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane by wet blotting. After blocking in 5% non-fat milk for 1 h, membranes were incubated with antibodies against nuclear factor-kappa B (NF-κB)-phosphorylated p65 (p-p65; Cell Signalling, Beverly, MA, USA) or β-actin (ZSGB-BIO, Beijing, China) at 4 °C overnight. Horseradish peroxidase-conjugated immunoglobulin (Ig)G and ECL reagent (Thermo Scientific, Waltham, MA, USA) were used to detect the immunoreactive proteins. The assay was conducted in three separate experiments.

2.6. Blockade of the NF-κB pathway

GFs and PDLCs were pretreated with 5 μM BAY 11-7082 (Sigma Aldrich, St. Louis, MO, USA) for 1 h to inhibit NF-κB signalling, and then exposed to 500 μmol/L NaHS for 5 h. Gene expression of IL-6 and IL-8 was detected by real-time PCR.

2.7. EMSA

Nuclear extracts from GFs and PDLCs were prepared using a Nuclear-Cytosol Extraction kit (Thermo Fisher, Boston, MA, USA). Nuclear extracts (5 μg) of each sample were subjected to EMSA. A chemiluminescent-labelled oligonucleotide probe (5′-AGTTGAGGGGACTTTCCCAGGC-3′) was synthesized for detection of the binding of NF-κB protein to the probe. After incubation of the nuclear extracts and the probe, the binding activity of NF-κB protein to DNA was analyzed using a LightShift Chemiluminescent EMSA kit (Pierce, Rockford, IL, USA). The assay was conducted in three separate experiments.

2.8. Statistical analyses

Data are expressed as means ± SD. Statistical significance was determined by one-way ANOVA by SPSS. $P < 0.05$ was considered significant.

3. Results

3.1. NaHS upregulated expression of IL-6 and IL-8 in a dose- and time-dependent manner in GFs and PDLCs

To evaluate the effect of H_2S on the expression of IL-6 and IL-8 in GFs and PDLCs, these two types of fibroblasts were exposed to NaHS (0, 250, 500 and 1000 μmol/L) for 5 h, or NaHS (500 μmol/L) for 0, 1, 2 and 5 h. Expression of IL-6 and IL-8 was detected by real-time PCR at mRNA level, and by ELISA at protein level, respectively.

Fig. 1 shows that NaHS upregulated the production of mRNA and protein of IL-6 and IL-8 in GFs and PDLCs in a dose-dependent (panel I) and time-dependent manner (panel II). When 500 μmol/L NaHS was used to treat GFs and PDLCs, IL-6 and IL-8 were increased significantly ($P < 0.05$) at mRNA and protein levels after 5-h stimulation. Moreover, 500 μmol/L NaHS did not affect cell morphology, whereas 1000 μmol/L NaHS caused cell shrinkage (data not shown). However, even in this condition, NaHS induced the highest levels of production of IL-6 and IL-8 in the two types of periodontal fibroblasts.

3.2. BAY 11-7082 inhibited NaHS-induced expression of IL-6 and IL-8 in GFs and PDLCs

We undertook a NF-κB pathway blockade assay to ascertain if H_2S upregulated expression of IL-6 and IL-8 in GFs and PDLCs *via* the NF-κB signalling pathway. BAY 11-7082 significantly ($P < 0.05$) blocked H_2S-induced expression of IL-6 and IL-8 genes in the two types of fibroblasts (100% in PDLCs and 67% in GFs), suggesting the key role of NF-κB pathway in H_2S-induced expression of IL-6 and IL-8 (Fig. 2).

3.3. NaHS and P. gingivalis LPS increased expression of IL-6 and IL-8 synergistically in GFs and PDLCs through the NF-κB pathway

To ascertain if H_2S and LPS had a synergistic effect on the expression of IL-6 and IL-8 in GFs and PDLCs, the two types of periodontal fibroblasts were treated with NaHS (500 μmol/L) combined or not with *P. gingivalis* LPS (1 μg/mL) for 5 h. NaHS or *P. gingivalis* LPS alone increased expression of IL-6 and IL-8 in GFs and PDLCs. Combined use of NaHS and *P. gingivalis* LPS induced much higher expression of cytokines than single use of NaHS or *P. gingivalis* LPS, revealing the synergistic role of NaHS and *P. gingivalis* LPS in promoting expression of IL-6 and IL-8 (Fig. 3A–H).

To further explore the underlying signalling mechanism by which NaHS and *P. gingivalis* LPS exerted a synergistic effect upon expression of IL-6 and IL-8, the activity of NF-κB signalling was assessed. Fibroblasts were treated as described above and expression of NF-κB p-p65 (by western blotting) and DNA binding activity of NF-κB (by EMSA assay) were assessed. Expression of p-p65 was upregulated by NaHS and/or *P. gingivalis* LPS treatment, and the highest expression was found in combination-treated cells (Fig. 3I and J). These results were confirmed further by EMSA, which was designed to detect the binding activity of NF-κB to DNA (Fig. 3K and L).

4. Discussion

The inflammatory response of host to bacterial challenge is essential to not only periodontal defense but also periodontium destruction if the inflammatory response is excessive. IL-6 and IL-8 are known to play important roles in the inflammatory reaction and degradation of periodontal tissues. IL-6 can induce osteoclastogenesis.[22–24] IL-8 is a potent chemotactic factor for neutrophils,[25] and can produce proteases such as cathepsin, elastase, or matrix metalloproteinase-8, leading to tissue degradation. In the present study, H_2S generated from NaHS promoted the expression of IL-6 and IL-8

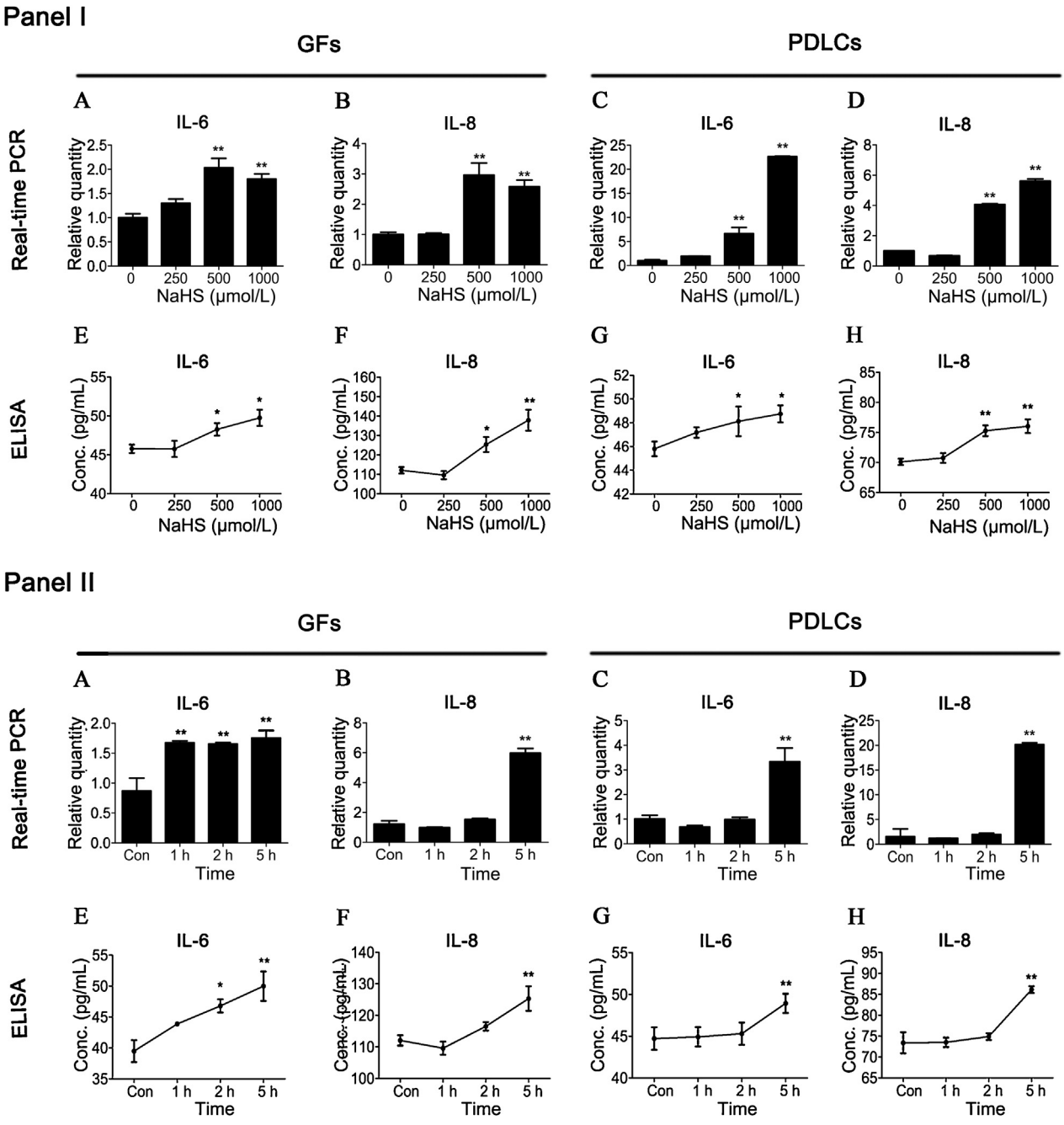

Fig. 1 – Dose- and time-dependent effects of NaHS on expression of IL-6 and IL-8 in GFs and PDLCs. Panel I (dose-dependent assay): fibroblasts were treated with 0, 250, 500 and 1000 μmol/L NaHS for 5 h. Expression of IL-6 and IL-8 was examined by real-time PCR (A–D) and by ELISA (E–H), respectively. The results showed that NaHS upregulated expression of IL-6 and IL-8 in a dose-dependent manner. Panel II (time-dependent assay): fibroblasts were treated with 500 μmol/L NaHS for 1, 2 and 5 h, and expression of IL-6 and IL-8 was examined by real-time PCR (A–D) and by ELISA (E–H). The results showed that NaHS upregulated expression of IL-6 and IL-8 in a time-dependent manner, $^{*}P < 0.05$, $^{**}P < 0.01$.

in GFs and PDLCs in a dose- and time-dependent manner. In the presence of *P. gingivalis* LPS, the promoting effect of H_2S on expression of IL-6 and IL-8 in GFs and PDLCs was more apparent. Our results showed that H_2S-induced expression of IL-6 and IL-8 may involve in the destruction of periodontal tissue.

In addition to IL-6 and IL-8, we also detected gene expression of other classical inflammatory mediators including IL-1β, TNF-α, and IL-10 (Supplementary Figs. S1 and S2). The expression pattern of pro-inflammatory cytokines (IL-1β and TNF-α) under stimulation of H_2S in the presence/absence of *P. gingivalis* LPS was similar as that of IL-6 and IL-8. In

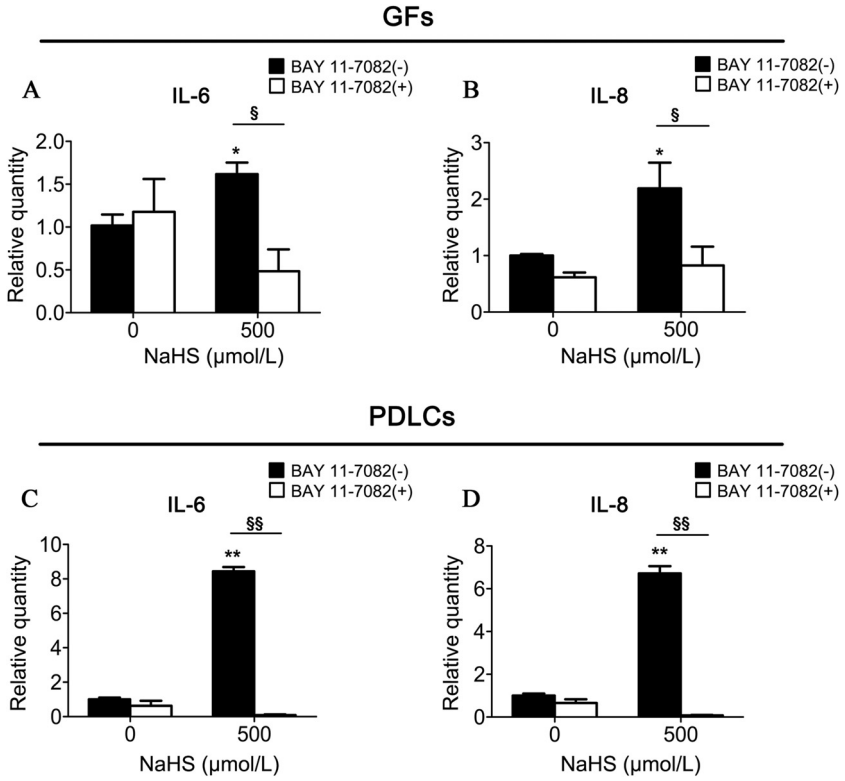

Fig. 2 – BAY 11-7082 inhibited NaHS-induced expression of IL-6 and IL-8 mRNA in GFs and PDLCs. GFs and PDLCs were pre-treated with 5 μmol/L BAY 11-7082 for inhibition of NF-κB signalling and then exposed with 500 μmol/L NaHS for 5 h. Gene expression of IL-6 and IL-8 examined by real-time PCR in GFs (A, B) and PDLCs (C, D). $^{*}P < 0.05$, $^{**}P < 0.01$ compared with corresponding non-treated cells, $^{§}P < 0.05$, $^{§§}P < 0.01$ compared with corresponding NaHS-treated cells.

contrast, the anti-inflammatory cytokine IL-10 expression showed a time- and dose-dependent decrease when GFs and PDLCs were treated with NaHS. This finding suggests that H_2S serves as a pro-inflammatory factor, and perhaps plays a role in the pathogenesis of periodontitis.

NF-κB signalling is one of the most important pathways mediating generation of IL-6 and IL-8 in GFs and PDLCs.[26,27] However, whether NF-κB signalling involved in H_2S-induced expression of IL-6 and IL-8 in GFs and PDLCs was unknown. We found for the first time that inhibition of NF-κB signalling by BAY 11-7082 significantly inhibited expression of IL-6 and IL-8 induced by H_2S in GFs and PDLCs. In fibroblasts treated with H_2S and/or *P. gingivalis* LPS, NF-κB signalling activity was also in accordance with expression of IL-6 and IL-8 (Fig. 3). Taken together, our findings showed that activation of NF-κB signalling was one of the mechanisms contributing to the synergistic effect of H_2S and *P. gingivalis* LPS on expression of IL-6 and IL-8 in GFs and PDLCs. These results are consistent with studies in other cells which showed that H_2S regulated expression of pro-inflammatory cytokines *via* signalling pathways including the NF-κB pathway.[28,29]

Interestingly, we found that PDLCs appeared to be more sensitive to H_2S exposure than GFs in terms of IL-6 expression. NaHS (500 μmol/L) yielded a twofold increase in IL-6 mRNA expression in GFs and a sevenfold increase in PDLCs. Moreover, inhibition of NF-κB signalling inhibited gene expression of IL-6 and IL-8 almost completely in PDLCs, while partially in GFs. These results suggest different characteristics between GFs and PDLCs even though they are very close in anatomic location. Our results are supported by those of Koka et al., which described the heterogeneity of periodontal fibroblasts when challenged by *P. gingivalis* LPS. They found a more sensitive response of PDLCs to LPS stimulation with regard to IL-6 production compared with GFs.[30] The mechanism by which H_2S exerts effects on physiological and pathological processes is not known, and the specific receptors of H_2S have yet to be discovered. Hence, the reason for this difference in response between these two cell populations is not clear. One could speculate that GFs and PDLCs have different (or have a different density of) H_2S receptors, or that H_2S binds to the receptors or diffuses into specific cells at different speeds in GFs and PDLCs. These mechanisms would lead to the different characteristics of GFs and PDLCs as well as different sensitivities of GFs and PDLCs to H_2S. It has been reported that many cell-surface receptors are expressed differentially in GFs and PDLCs. Han et al. showed that PDLCs expressed SLC26A3 and SLC16A4 (the membrane proteins for sulfate transportation) at 3.9- to 4.3-times greater levels than those in GFs.[31]

H_2S has been proposed to be a novel mediator of inflammation. However, in recent years, the notion that H_2S has a dual role in regulating inflammation has become acceptable. That is, H_2S can be a pro-inflammatory mediator as well as an anti-inflammatory molecule. H_2S has been

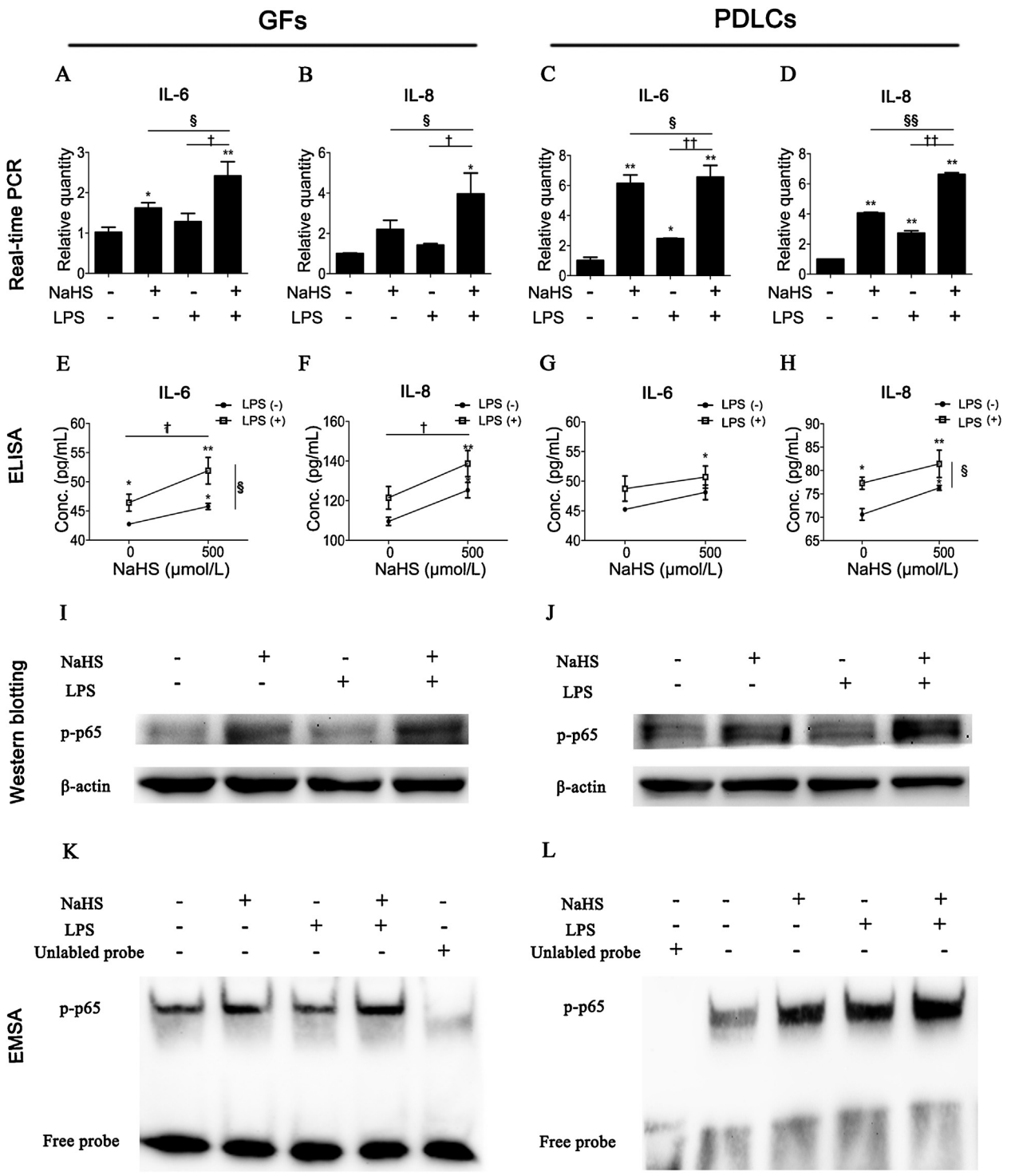

Fig. 3 – Combined effect of NaHS and *P. gingivalis* LPS on expression of IL-6 and IL-8 and NF-κB signalling activity in GFs and PDLCs. GFs and PDLCs were treated with 500 μmol/L NaHS in the presence/absence of 1 μg/mL *P. gingivalis* LPS for 5 h. Expression of IL-6 and IL-8 was examined by real-time PCR (A–D) and by ELISA (E–H), respectively. NaHS and *P. gingivalis* LPS synergistically upregulated expression of IL-6 and IL-8 in GFs and PDLCs. The activity of NF-κB signalling was assessed by western blotting (I and J) and EMSA (K and L). NF-κB signalling was activated by 500 μmol/L NaHS, and the signalling activity was much higher when GFs and PDLCs were treated with NaHS combined with LPS. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with corresponding non-treated cells, $^{\S}P < 0.05$, $^{\S\S}P < 0.01$ compared with corresponding NaHS-treated cells, $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ compared with LPS-treated cells.

reported to inhibit NO production and the NF-κB pathway in Raw 264.7 macrophages treated with LPS,[32] and to protect mice from oleic acid-induced acute lung injury *via* regulation of expression of IL-6, IL-8 and IL-10.[33] Li et al. evaluated the effect of different administrations of exogenous H_2S on complete Freund's adjuvant (CFA)-treated acute joint inflammation in mice. They found that a H_2S donor injected 1 h before CFA administration resulted in marked swelling in the knee joints. Conversely, a H_2S donor injected 6 h and 18 h after CFA administration could induce relatively slight inflammation in knee joints. The anti-inflammatory role of H_2S was postulated to be through reduction of the activity of myeloperoxidase and N-acetyl-b-D-glucosaminidase in synovial fluid, as well as expression of IL-6 and IL-8, in this model.[34] Hua et al. showed that the switch of H_2S from one role to another was dependent upon the cell subsets involved; the crosstalk between H_2S and NO as well as the spatial and temporal formation of H_2S was involved in this process.[35] In the present study, H_2S had a synergistic role together with *P. gingivalis* LPS in triggering expression of IL-6 and IL-8 in GFs and PDLCs. Therefore, we speculate that different cell types and different treatment methods may determine the different (pro-/anti-) inflammatory responses to H_2S. Further study is needed to elucidate the underlying mechanism.

In conclusion, the present study has, for the first time, demonstrated that H_2S can, and has a synergistic effect with, *P. gingivalis* LPS on upregulating expression of IL-6 and IL-8 in GFs and PDLCs *via* activation of NF-κB signalling, which could promote the pathogenesis of periodontitis.

Funding

None

Conflict of interest statement

The authors declare that they have no conflicts of interest.

Ethical approval

The protocol was approved by the Medical Ethical Committee of Peking University School and Hospital of Stomatology (Ethics Approval No. PKUSSIRB-2012017).

Author contributions

Xiaopei Chi contributed to the performance of the study and the manuscript writing. Xiangying Ouyang and Yixiang Wang contributed to the design of the study and manuscript writing. All authors have read and approved the final manuscript.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.archoralbio.2014.05.022.

REFERENCES

1. Cekici A, Kantarci A, Hasturk H, Van Dyke TE. Inflammatory and immune pathways in the pathogenesis of periodontal disease. *Periodontology 2000* 2014;**64**(1):57–80.
2. Liu JR, Wang YX, Ouyang XY. Beyond toll-like receptors: *Porphyromonas gingivalis* induces IL-6, IL-8, and VCAM-1 expression through NOD-mediated NF-κB and ERK signaling pathways in periodontal fibroblasts. *Inflammation* 2014;**37**(2):522–33.
3. Tabeta K, Yamazaki K, Akashi S, Miyake K, Kumada H, Umemoto T, et al. Toll-like receptors confer responsiveness to lipopolysaccharide from *Porphyromonas gingivalis* in human gingival fibroblasts. *Infect Immun* 2000;**68**(6):3731–5.
4. Sun Y, Shu R, Li CL, Zhang MZ. Gram-negative periodontal bacteria induce the activation of Toll-like receptors 2 and 4, and cytokine production in human periodontal ligament cells. *J Periodontol* 2010;**81**(10):1488–96.
5. Persson S, Edlund MB, Claesson R, Carlsson J. The formation of hydrogen sulfide and methyl mercaptan by oral bacteria. *Oral Microbiol Immunol* 1990;**5**(4):195–201.
6. Wagner F, Asfar P, Calzia E, Radermacher P, Szabo C. Bench-to-bedside review: hydrogen sulfide-the third gaseous transmitter: applications for critical care. *Crit Care* 2009;**13**(3):213–21.
7. Zhao W, Zhang J, Lu Y, Wang R. The vasorelaxant effect of H_2S as a novel endogenous gaseous K (ATP) channel opener. *EMBO J* 2001;**20**(21):6008–16.
8. Whiteman M, Armstrong JS, Chu SH, Jia-Ling S, Wong BS, Cheung NS, et al. The novel neuromodulator hydrogen sulfide: an endogenous peroxynitrite 'scavenger'? *J Neurochem* 2004;**90**(3):765–8.
9. Bhatia M. Role of hydrogen sulfide in the pathology of inflammation. *Scientifica* 2012;**2012**:159680.
10. Persson S. Hydrogen sulfide and methyl mercaptan in periodontal pockets. *Oral Microbiol Immunol* 1992;**7**(6):378–9.
11. Rizzo AA. The possible role of hydrogen sulfide in human periodontal disease I. Hydrogen sulfide production in periodontal pockets. *Periodontics* 1967;**5**(5):233–6.
12. Solis-Gaffar MC, Rustogi KN, Gaffar A. Hydrogen sulfide production from gingival crevicular fluid. *J Periodontol* 1980;**51**(10):603–6.
13. Morita M, Wang HL. Relationship of sulcular sulfide level of severity of periodontal disease and BANA test. *J Periodontol* 2001;**72**(1):74–8.
14. Morita M, Wang HL. Relationship of sulcular sulfide level and oral malodor in subjects with periodontal disease. *J Periodontol* 2001;**72**(1):79–84.
15. Ng W, Tonzetich J. Effect of hydrogen sulfide and methyl mercaptan on the permeability of oral mucosa. *J Dent Res* 1984;**63**(7):994–7.
16. Calenic B, Yaegaki K, Murata T, Imai T, Aoyama I, Sato T, et al. Oral malodorous compound triggers mitochondrial-dependent apoptosis and causes genomic DNA damage in

human gingival epithelial cells. *J Periodontal Res* 2010;**45**(1):31–7.
17. Yaegaki K, Qian W, Murata T, Imai T, Sato T, Tanaka T, et al. Oral malodorous compound causes apoptosis and genomic DNA damage in human gingival fibroblasts. *J Periodontal Res* 2008;**43**(4):391–9.
18. Zhang JH, Dong Z, Chu L. Hydrogen sulfide induces apoptosis in human periodontium cells. *J Periodontal Res* 2010;**45**(1):71–8.
19. Aoyama I, Calenic B, Imai T, Ii H, Yaegaki K. Oral malodorous compound causes caspase-8 and -9 mediated programmed cell death in osteoblasts. *J Periodontal Res* 2012;**47**(3):365–73.
20. Tonzetich J, Preface, Rosenberg M, editors. *Bad breath research perspectives*. Tel Aviv: Ramot Publishing-Tel Aviv University; 1995. xi-viii.
21. Tonzetich J. Effect of volatile sulphur compounds on periodontal tissues and cellular metabolism: an overview. In: van Steenberghe SD, Rosenberg M, editors. *Bad breath, a multidisciplinary approach*. Leuven: Leuven University Press; 1996. p. 79–91.
22. Bartold PM, Haynes DR. Interleukin-6 production by human gingival fibroblasts. *J Periodontal Res* 1991;**26**(4):339–45.
23. Patil C, Rossa Jr C, Kirkwood KL. Actinobacillus actinomycetemcomitans lipopolysaccharide induces interleukin-6 expression through multiple mitogen-activated protein kinase pathways in periodontal ligament fibroblasts. *Oral Microbiol Immunol* 2006;**21**(6):392–8.
24. Kurihara N, Bertolini D, Suda T, Akiyama Y, Roodman GD. IL-6 stimulates osteoclast-like multinucleated cell formation in long term human marrow cultures by inducing IL-1 release. *J Immunol* 1990;**144**(11):4226–30.
25. Okada H, Murakami S. Cytokine expression in periodontal health and disease. *Crit Rev Oral Biol Med* 1998;**9**(3):248–66.
26. Herath TD, Darveau RP, Seneviratne CJ, Wang CY, Wang Y, Jin L. Tetra- and penta-acylated lipid A structures of *Porphyromonas gingivalis* LPS differentially activate TLR4-mediated NF-κB signal transduction cascade and immuno-inflammatory response in human gingival fibroblasts. *PLoS ONE* 2013;**8**(3):e58496.
27. Tang L, Zhou XD, Wang Q, Zhang L, Wang Y, Li XY, et al. Expression of TRAF6 and pro-inflammatory cytokines through activation of TLR2, TLR4, NOD1, and NOD2 in human periodontal ligament fibroblasts. *Arch Oral Biol* 2011;**56**(10):1064–72.
28. Kloesch B, Liszt M, Krehan D, Broell J, Kiener H, Steiner G. High concentrations of hydrogen sulphide elevate the expression of a series of pro-inflammatory genes in fibroblast-like synoviocytes derived from rheumatoid and osteoarthritis patients. *Immunol Lett* 2012;**141**(2):197–203.
29. Zhi L, Ang AD, Zhang H, Moore PK, Bhatia M. Hydrogen sulfide induces the synthesis of proinflammatory cytokines in human monocyte cell line U937 via the ERK-NF-kappaB pathway. *J Leukoc Biol* 2007;**81**(5):1322–32.
30. Koka S, Reinhardt RA. Periodontal pathogen-related stimulation indicates unique phenotype of primary cultured human fibroblasts from gingiva and periodontal ligament: implications for oral health disease. *J Prosthet Dent* 1997;**77**(2):191–6.
31. Han X, Amar S. Identification of genes differentially expressed in cultured human periodontal ligament fibroblasts vs. human gingival fibroblasts by DNA microarray analysis. *J Dent Res* 2002;**81**(6):399–405.
32. Oh GS, Pae HO, Lee BS, Kim BN, Kim JM, Kim HR, et al. Hydrogen sulfide inhibits nitric oxide production and nuclear factor-kappaB via heme oxygenase-1 expression in RAW264.7 macrophages stimulated with lipopolysaccharide. *Free Radic Biol Med* 2006;**41**(1):106–19.
33. Li T, Zhao B, Wang C, Wang H, Liu Z, Li W, et al. Regulatory effects of hydrogen sulfide on IL-6, IL-8 and IL-10 levels in the plasma and pulmonary tissue of rats with acute lung injury. *Exp Biol Med (Maywood)* 2008;**233**(9):1081–7.
34. Li L, Fox B, Keeble J, Salto-Tellez M, Winyard PG, Wood ME, et al. The complex effects of the slow-releasing hydrogen sulfide donor GYY4137 in a model of acute joint inflammation and in human cartilage cells. *J Cell Mol Med* 2013;**17**(3):365–76.
35. Hua W, Zhou SL, Gong FQ. Biphasic regulation of hydrogen sulfide in inflammation. *Chin Med J (Engl)* 2013;**126**(7):1360–3.