Published in final edited form as:
Biochem Biophys Res Commun. 2010 January 1; 391(1): 645–650. doi:10.1016/j.bbrc.2009.11.113.

Bacteria-Derived Hydrogen Sulfide Promotes IL-8 Production from Epithelial Cells

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Abstract

Hydrogen sulfide (H_2S), a volatile sulfur compound, is implicated as a cause of inflammation, especially when it is produced by bacteria colonizing gastrointestinal organs. However, it is unclear if H_2S produced by periodontal pathogens affects the inflammatory responses mediated by oral/gingival epithelial cells. Therefore, the aims of this study were 1) to compare the *in vitro* production of H_2S among 14 strains of oral bacteria and 2) to evaluate the effects of H_2S on inflammatory response induced in host oral/gingival epithelial cells. *P. gingivalis* (*Pg*) produced the most H_2S in culture, which, in turn, resulted in the promotion of proinflammatory cytokine IL-8 from both gingival and oral epithelial cells. The up-regulation of IL-8 expression was reproduced by the exogenously applied H_2S. Furthermore, the mutant strains of *Pg* that do not produce major soluble virulent factors, i.e. gingipains, still showed the production of H_2S, as well as the promotion of epithelial IL-8 production, which was abrogated by H_2S scavenging reagents. These results demonstrated that *Pg* produces a concentration of H2S capable of up-regulating IL-8 expression induced in gingival and oral epithelial cells, revealing a possible mechanism that may promote the inflammation in periodontal disease.

Keywords

Hydrogen sulfide; Epithelial cells; IL-8; Bacteria; Porphyromonas gingivalis; Gingipain; Pathogenesis; Hydrogen; Innate immune response; Periodontal disease

Introduction

Halitosis is a characteristic symptom of periodontal disease, and is caused by the production of volatile sulphur compounds (VSCs), such as hydrogen sulphide (H2S) and methyl mercaptan, by sulfate-reducing bacteria [1]. Importantly, the major cultivatable periodontal opportunistic pathogens, *Porphyromonas gingivalis* (*Pg*), *Fusobacterium nucleatum* (*Fn*) and *Tannerella forsythia* (*Tf*), are reported to have produced H_2S in an *in vitro* system as measured by gas chromatography [2]. Also, H_2S produced by gut bacteria is recognized as a pathogenic

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factor in inflammatory bowel diseases (IBD) which present features of mucosal inflammatory lesion similar to periodontal disease [3]. In the host biological system, H_2S is produced authentically in a manner that causes desulphydration of L-cysteine, cystathionine β-synthetase (CBS) and cystathionine γ-lyase (CSE) [4], indicating its association with the regulation of the cardiovascular and nervous systems, as well as inflammation [5]. Although H_2S can be released from either bacteria or the host metabolic system, it is not known whether the H_2S released into solution surrounding periodontal bacteria can reach a concentration higher than the one derived from the host metabolic system.

Gingival epithelial cells represent the first line of defense in the gingival crevice. As such, they play a key role in host innate immune response by protecting the host from bacterial challenge with the production of proinflammatory cytokines, such as IL-8, which is a chemotaxis factor for neutrophils [6,7], and antimicrobial peptides [8], as well as adaptive immune responses [9]. On the other hand, over-expression of these proinflammatory cytokines causes collateral tissue damages. For instance, reactive oxygen species (ROS) produced from the activated neutrophils in response to periodontopathogenic bacteria appears to contribute to tissue destruction in the context of periodontal disease [10]. Therefore, it is thought that over-expression of IL-8 from gingival epithelial cells can promote the local accumulation of neutrophils which results in periodontal tissue damage [11].

Pg is one of the most putatively pathogenic periodontal bacteria, and, in addition to H_2S, it produces several other virulence factors. These include extracellular proteases, or gingipains, [12–15], lipopolysaccharide (LPS) [16,17], and hemagglutinins [18]. However, a recent study discovered that *Pg* and other putative periodontal pathogens not only colonize the diseased periodontal pocket but also the healthy gingival crevice [19]. Nonetheless, the reason why healthy gingival epithelium remains unaffected in the presence of periodontopathogens such as *Pg* has not yet been fully investigated.

Based on the lines of evidence described above, we hypothesized that H_2S produced from periodontal bacteria contributes to the promotion of inflammatory response by the up-regulation of IL-8 production from gingival epithelial cells. Therefore, the aims of the present study were 1) to monitor the concentration of H_2S released from a total of fourteen different oral bacterial strains and 2) to examine the effects of H_2S released from *Porphyromonas gingivalis* (*Pg*) on the production of proinflammatory cytokine IL-8 from gingival epithelial cells.

Materials and Methods

Bacterial strains and culture

The following oral bacterial strains were used in this study: *Porphyromonas gingivalis* (*Pg*) 33277, *Pg* W83, *Tannerella forsythia* (*Tf*) 338, *Prevotella intermedia* (*P. intermedia*) 25611, *Fusobacterium nucleatum* (*Fn*) 10953, *Fusobacterium periodonticum* (*F. periodonticum*) 33693, *Peptostreptococcus micros* (*P. micros*) 33270, *Streptococcus gordonii* (*S. gornodii*) 10558, *Streptococcus sanguis* (*S. sangius*) 10556, *Capnocytophaga ochracea* (*C. ochracea*) 25, *Capnocytophaga gingivalis* (*C. gingivalis*) 27, *Actinobacillus actinomycetemcomitans* (*Aa*) 3826, *Aa* JP2, *Aa* Y4, and *Pg* 33277 mutant strains KDP112 (*rgpA*$^-$/*rgpB*$^-$) [20] and KDP137 (*rgpA*$^-$/*kgp*$^-$/*hagA*$^-$) [21]. Especially, *Pg* 33277 and *Pg* W83 were grown in brain heart infusion (BHI; BBL) broth medium supplemented with hemine and menadoine (Sigma). *T. forsythia* was cultured in BHI broth medium containing hemine, menadoine, heat-inactivated fetal bovine serum (FBS), N-acetyl muramic acid, and yeast extract. The remaining bacterial strains were cultured in BHI medium alone or supplemented with yeast extract. All bacteria were cultured in a 37°C chamber under anaerobic conditions (5% H_2, 10% CO_2 and 85% N_2).

Measurement of hydrogen sulfide and hydrogen released from bacteria

Bacteria were cultured in broth medium until they reached late log growth phase, and the concentration of all strains was then adjusted to an optical density (OD 590 nm) of 0.9. Subsequently, the bacterial suspension was centrifuged at 7,000 rpm for 15 min. The supernatants were immediately subjected to measurement for hydrogen sulfide (H_2S) or hydrogen (H_2) using a needle-type H_2S or H_2 sensor (Unisense A/S, Denmark) [22,23]. For each test, the sensor was allowed to stabilize, and three testing points were taken at 5 second intervals. Each bacterial strain grown in broth was examined for the production of H_2S and H_2 on at least three separate occasions.

Epithelial cell culture

Human gingival epithelial cell line OBA9 [24] and oral (cheek) epithelial cell line OKF6 [25] were used in this study. The cells were cultured in Keratinocyte-Serum Free Medium (K-SFM, Gibco) supplemented with 50 μg/ml bovine pituitary extract, 5 ng/ml epidermal growth factor, 50 μg/ml gentamicin and 50 ng/ml amphotericin B (medium A) in humidified atmosphere of 5% CO_2 at 37°C.

Treatment of epithelial cells with sodium sulfide (Na_2S)

OBA9 or OKF6 cells were seeded at a density of 1×10^4 cells/well in 96-well culture plates coated with type 1 collagen and maintained in medium A until they reached confluence. The cells were treated with or without mitogen phorbol myristate acetate (PMA; 1 μmol/L, Sigma) and immediately exposed to various concentrations of Na_2S (VWR), as a H_2S donor, in K-SFM alone (medium B) for 24 hr. The culture supernatants were then subjected to enzyme-linked immunosorbent assay (ELISA) for detection of TNF-α, IL-6, and IL-8, as described below.

Transwell assay

Transwell culture plates (24-well format, see Fig 3A) with 0.4-μm pores (Corning) were used to test the effects of soluble small factors released from bacteria on epithelial cells. Epithelial cells in medium A were cultured in the bottom compartment of plates which were pre-treated with type 1 collagen. The cells were treated with PMA (1 μmol/L) in medium B, and bacteria, specifically, *Pg* W83, *Pg* KDP112, or *Pg* KDP137, were cultured in the upper chamber of the Transwell system at various concentrations with or without Morpholine coupled to Rast resin (25mg/ml, Sigma) or 4,6-Dichloro-1,3,5-triazine–resin (25mg/ml, Sigma) in medium B for 24 or 48 hr. The culture supernatants of the bottom well were collected, and the IL-8 concentration was determined by ELISA as described below.

Measurement of proinflammatory cytokines

Quantification of the proinflammatory cytokines TNF-α, IL-6 and IL-8 was performed by ELISA kits (PeproTech) following the instructions provided by the manufacturer.

Statistical analysis

Differences between the two groups were analyzed by Student's *t* test.

Results

The concentrations of H_2S produced from fourteen different oral bacterial strains were monitored using an instrument that accurately measures the concentration of small gas molecules dissolved in solution, including hydrogen (H_2) and H_2S [22,23]. Among them, 12 oral bacterial strains are clustered into color-coded complexes as follows: red complex (3 strains), orange complex (4 strains), yellow complex (2 strains) and green complex (3 strains)

in descending order according to their degree of virulence in association with clinically diagnosed adult periodontal disease. Two strains of *Aa* were not classified in any of the color-coded clusters because *Aa* is more associated with aggressive (juvenile) periodontitis than adult periodontal disease. Since it was recently discovered that hydrogen (H_2) produced by intestinal bacteria can provide beneficial effects to the host [22,23], we also monitored the concentration of H_2. The concentrations of H_2S detected from two strains of *Porphyromonas gingivalis*, *Pg* W83 and *Pg* 33277, were prominently high (about 600 – 800 μmol/L), whereas 4 strains of orange complex bacteria, *F. periodonticum33693*, *F. nucpoly338*, *P. intermedia25611*, and *P. micros33270*, also produced a modest concentration of H_2S (50 – 300 μmol/L) in descending order (Fig. 1). In contrast, little or no H_2S production was detected from the yellow complex, green complex and putative pathogens associated with aggressive (juvenile) periodontitis, including *Aa JP2* and *Aa Y4* (Fig. 1). H_2 production was detected in the 4 strains of bacteria belonging to the orange complex, while the concentrations of such H_2 detected from these bacterial strains (30–100 μmol/L) were lower than those of H_2S in respective strain bacteria in the orange complex. Exceptionally, *T. forsythia338* in the red complex showed a higher production of H_2 than H_2S (Fig. 1). These results indicated that the red complex bacterium *Pg* produces a remarkably higher concentration of H_2S compared to the other bacteria and that the production of possibly host-beneficial H_2 from the bacteria tested was low.

To explore whether H_2S can affect the host inflammatory response in the context of periodontal disease, the effect of exogenously applied H_2S on the production of inflammatory cytokine by the human gingival epithelial cell line OBA9 and oral epithelial cell line OKF6 was examined. The addition of H_2S donor, Na_2S, to the culture medium did not affect IL-8 production from OBA9 in the absence of PMA (Fig. 2A). However, the addition of Na_2S did enhance IL-8 production from OBA9 stimulated with PMA in a dose-dependent manner (Fig. 2A). Similar results were obtained when oral epithelial cell line OKF6 was exposed to Na_2S in the presence or absence of PMA (Fig. 2B). Although it was examined whether H_2S affects the production of other proinflammatory cytokines, including IL-6 and TNF-α, from OBA9 in the presence or absence of PMA stimulation, no noticeable effects mediated by H_2S on these cytokines were observed (data not shown). These results demonstrated that H_2S can promote IL-8 production from oral and gingival epithelial cells reacted with PMA, indicating that a predisposed inflammatory condition is required for H_2S-mediated promotion of IL-8 production.

In order to examine the effects of H_2S derived from oral bacteria on the IL-8 production from epithelial cells, a transwell assay (Fig. 3A) was employed. As a test bacterium, *Pg* W83 was used because it showed the highest production of H_2S (Fig. 1). *Pg* W83 applied to the transwell (pore size: Ø 0.4 μm; smaller than the size of bacteria) was placed on the OBA9 cells cultured in the bottom compartment of a 24-well tissue culture plate. After incubation for 24 hr, live *Pg* W83 (10^6, 10^7 and 10^8 bacteria/ml) increased IL-8 production (Fig. 3B). In addition, OKF6 cells co-cultured with *Pg* W83 in the same transwell assay system showed an increase of IL-8 production at a concentration of 10^7 bacteria/ml (Fig. 3C). In contrast, co-culture of both OBA9 and OKF6 with killed *Pg* W83 did not affect their production of IL-8 (Fig. 3B and C). These results suggest that soluble factors released from live bacteria up-regulate the IL-8 production from epithelial cells. The concentration of exogenously applied H_2S equaled the concentration of H_2S produced by *Pg* W83, which resulted in the up-regulation of IL-8 (Fig 2). Next, to test if H_2S released from *Pg* W83 is responsible for the promotion of IL-8, OBA9 and OKF6 cells co-cultured with *Pg* W83 were treated with resin particles coupled to morpholine, which is a H_2S scavenger chemical. As expected, morpholine-resin particles abolished the up-regulation of IL-8 production induced by co-culture with *Pg* W83 in both OBA9 (Fig. 3D) and OKF6 (Fig. 3E). Furthermore, similar results were obtained with 4,6-dichloro-1,3,5-triazine, another H_2S scavenger (data not shown).

It is well known that *Pg* W83 produces arginine-specific cysteine proteinase (Arg-gingipain, RGP) and lysine-specific cysteine proteinase (Lys-gingipain, KGP) extracellularly [26,27] and that these proteases are recognized as major virulent factors [12–15]. Therefore, it is plausible that either RGP or KGP could also affect IL-8 production in the transwell assay system as performed above. To address this possibility, it was tested if *Pg* KDP137 (*rgpA*$^{-}$/*kgp*$^{-}$/*hagA*$^{-}$ mutant strain of ATCC 33277) affects the IL-8 production from epithelial cells. First, *Pg* KDP137 produced H_2S at a level similar to that of wild-type *Pg* W83 (Fig. 4A). Co-culture of OBA9 cells with *Pg* KDP137 in the transwell system for 48 hr increased IL-8 production (Fig. 4C). Furthermore, co-culture of OBA9 cells with *Pg* KDP112 (*rgpA*$^{-}$/*rgpB*$^{-}$ strain) also enhanced IL-8 production (not shown). Most importantly, the addition of morpholine-resin particles to the co-culture between OBA9 and *Pg* KDP137 abrogated the increased IL-8 production induced by each of the mutant *Pg* strains (Fig. 4B), suggesting that H_2S, but not gingipains, released from *Pg* KDP137 is responsible for the increased expression of IL-8 from epithelial cells.

Discussion

The present study demonstrated that *Pg*, a component of the red complex of perio pathogens, releases significantly more hydrogen sulfide than bacteria in the yellow or green complexes, while the levels released by orange complex bacteria fall somewhere in between, suggesting that the pathogenesis mediated by Pg is related to its production of H_2S. Most strikingly, adding Na_2S, as a source of H_2S, up-regulated IL-8 production by OBA9 gingival epithelial cells and by OKF6 oral epithelial cells, both of which were stimulated with PMA. H_2S released from live *Pg*, but not killed *Pg*, appeared to be responsible for the up-regulation of IL-8 production from PMA-stimulated OBA9 and OKF6 cells because addition of hydrogen sulfide scavenging chemicals suppressed IL-8 production from PMA-stimulated epithelial cells. Up-regulation of IL-8 from PMA-stimulated gingival epithelial cells was still found with mutant strains of *Pg* that lacked all three classes of gingipains, indicating that the production of these soluble and potent virulence factors did not contribute to IL-8 up-regulation. Consequently, for the first time, this study has demonstrated that the red complex bacterium *Pg* produces the highest concentration of H_2S among the 14 tested strains of periodontal bacteria and that the H_2S released from *Pg* can promote the inflammatory responses induced in gingival and oral epithelial cells, suggesting a novel pathogenic mechanism underlying the development of periodontal disease mediated by periodontal bacteria.

The classical gas chromatograph may not be useful in measuring the concentration of H_2S released from bacteria to the surrounding culture medium or host biological fluids, such as saliva, because it is designed to measure atmospheric H_2S, not H_2S in solution. However, a cutting-edge instrument, which has recently become available, can accurately measure the concentration of small gas molecules dissolved in solution, including hydrogen (H_2) and H_2S [22,23]. Therefore, in the present study, H_2S produced from a variety of oral bacteria, including both periodontal pathogens and commensal benign bacteria, was first monitored using this instrument. Persson et al. previously reported that red complex bacteria, *Pg* and *Tf*, and orange complex bacteria, *Fn* and *Prevotella intermedia* (*Pi*), produce sulfide in the laboratory culture system, as measured using gas chromatography [2]. Since Persson et al. measured the sulfide released to the atmosphere using gas chromatography [2], our results, which measured H_2S in solution using a special H_2S sensor, as referenced above, (Unisense, Denmark) cannot be compared. Nevertheless, it should be emphasized that both our studies demonstrated that bacteria classified in the red and orange complexes produce a significantly higher concentration of H_2S, when compared to the benign, or moderately pathogenic, groups of bacteria in the green and yellow complexes.

The high concentration of H_2S produced from *Pg* (650–1,150 μmol/L) appeared to affect IL-8 production from PMA-stimulated epithelial cells because a concentration of H_2S less than 400 μmol/L did not show any significant effect on IL-8 production in PMA-stimulated epithelial cells (Fig. 2). Since physiologically generated H_2S is also present at concentrations in the range of 30–100 μM in blood [28], epithelial cells may well tolerate H_2S at concentrations lower than 400 μmol/L. Interestingly, even a high concentration of H_2S (800 or 1,600 μmol/L) did not induce IL-8 production in epithelial cells in the absence of PMA (Fig. 2), indicating that a predisposed inflammatory condition is required for H_2S-mediated promotion of IL-8 production.

The primary biological function mediated by IL-8 is the chemo-attraction of neutrophils, a type of scavenger cell [29]. Neutrophils migrate to the site where IL-8 is released from epithelial cells, and facilitating phagocytosis of bacteria. In general, the production of reactive oxygen species (ROS) released by neutrophils not only kills bacteria but also augments inflammation [30]. Therefore, H_2S-mediated up-regulation of IL-8 from epithelial cells should result in enhancement of inflammation at the site, by recruiting excess numbers of neutrophils. The *in vivo* studies demonstrated that both endogenous H_2S and exogenously supplied H_2S contribute to increased neutrophil migration to the inflammation induced in the pancreas, lung and liver [31,32], supporting our premise that H_2S released from periodontal bacteria could also promote the chemotaxis of neutrophils to the periodontal pocket.

In conclusion, this study has, for the first time, demonstrated that the red complex periodontal bacterium *Pg* produces a concentration of H_2S capable of up-regulating IL-8 expression induced in gingival and oral epithelial cells, revealing a possible mechanism that may promote the inflammation in periodontal disease.

Acknowledgments

This study was supported by a research grant from Skyview Enterprises and NIH grants DE-018310 and DE-015931. We thank Dr. Koji Nakayama at Kyushu University, Japan, for the strains of *Pg* KDP112 and *Pg* KDP137 as well as Lin Martin and Tina Yaskell in the Department of Periodontology at The Forsyth Institute for providing the strains of other periodontal bacteria used in this study. We also acknowledge Olive Tang's assistance in conducting the laboratory assays. Weilin Chen was supported by The Forsyth Education Outreach Program, and Harrison Mackler was supported by the Medical Fellows Program from the Howard Hughes Medical Institute.

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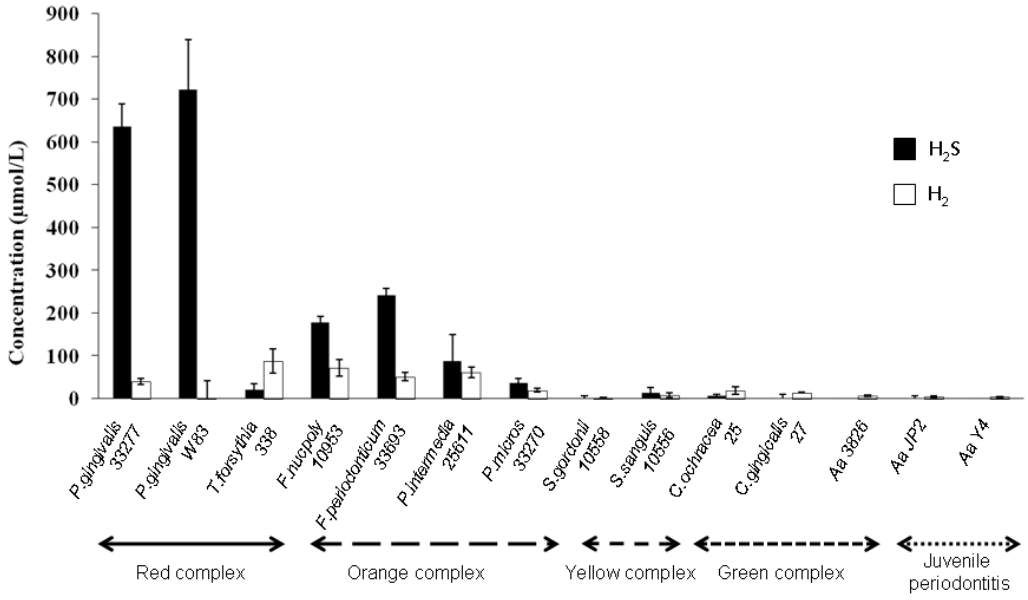

Figure 1. Measurement of H_2Sreleased from oral bacteria

Fourteen bacterial strains that belong to different pathogenesis clustering complexes were cultured in anaerobic conditions until they reached the late log growth phase. The concentration of all strains was adjusted to 0.9 as measured by OD 590 nm. The concentrations of H_2S and H_2 were measured in the bacterial culture broth using a needle-type H_2S or H_2 sensor, as described in the Materials & Methods section. Data are shown as the mean ± SD of three different cultures.

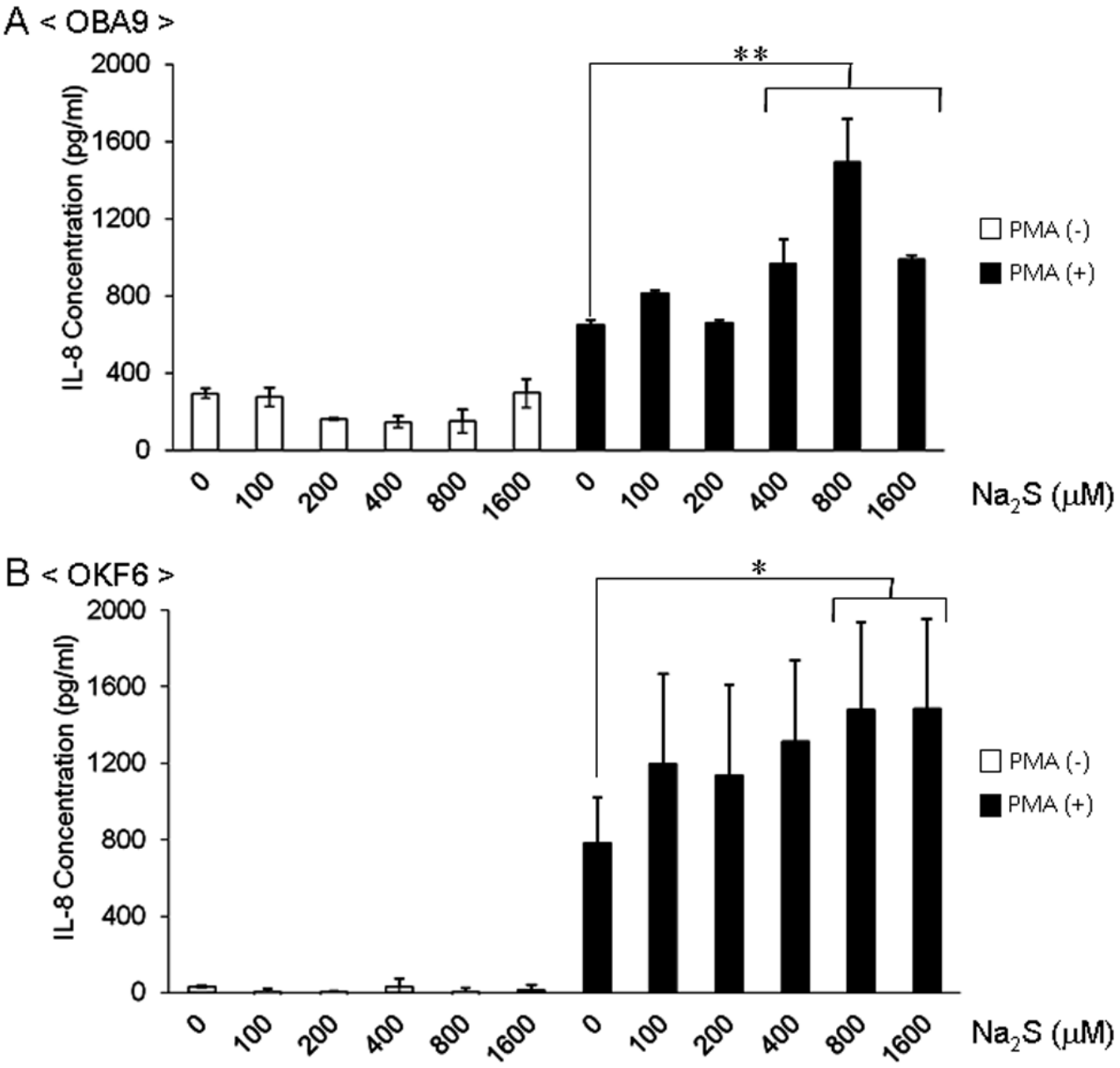

Figure 2. Effect of exogenously applied H_2S on IL-8 production in OBA9 and OKF6 cells

Confluent cultures of gingival epithelial cell line (OBA9) or oral epithelial cell line (OKF6) were exposed to serial dilutions of the H_2S donor, Na_2S, for 24 hr with or without PMA (1 μmol/L) stimulation. The concentrations of IL-8 in the culture supernatant produced from OBA9 (A) or OKF6 (B) were measured by ELISA. Data indicate the mean ± SD of three cultures. *$p < 0.05$, **$p < 0.01$: Values differ significantly (*t*-test).

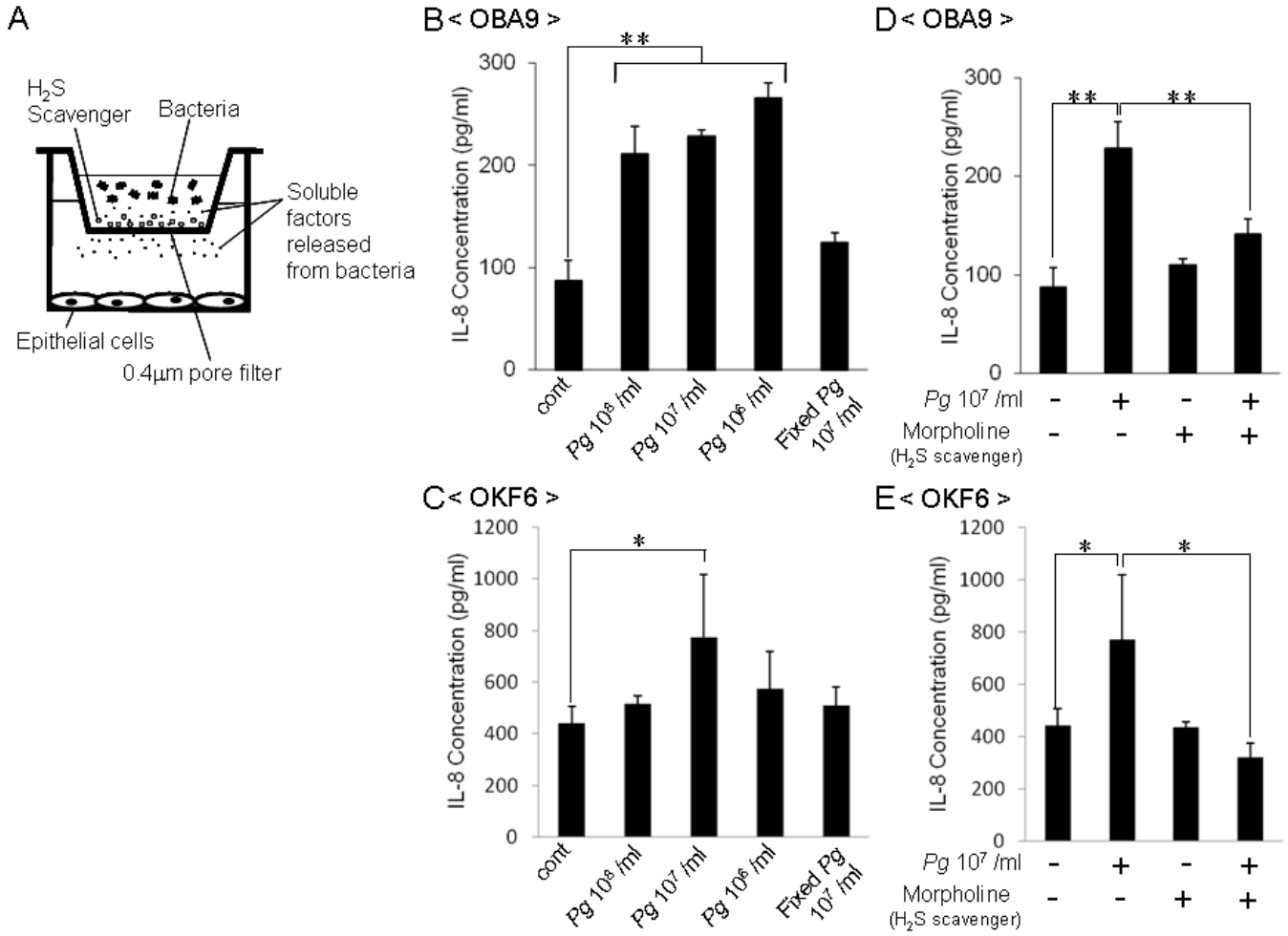

Figure 3. Effect of soluble factors, including H_2S, released from *Pg*W83 on IL-8 production in OBA9 and OKF6

The transwell assay system is illustrated (A). The epithelial cells, OBA9 or OKF6, were cultured in the lower chamber until they reached confluent coverage of surface of tissue culture well. Subsequently, live or fixed *Pg*W83 were applied in the upper chamber with or without H_2S scavenger. They were co-cultured for 24 hr in KSF medium containing inflammatory stimulant PMA (1 μmol/L). The concentrations of IL-8 in the culture supernatant of OBA9 (B and D) or OKF6 (C and E) were measured by ELISA. Data indicate the mean ± SD of three cultures. *$p < 0.05$, **$p < 0.01$: Values differ significantly (*t*-test).

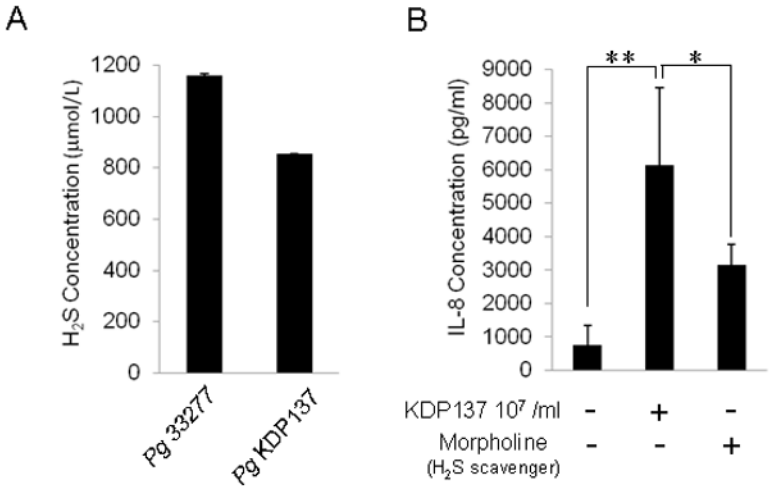

Figure 4. Effects of RGP and KGP deletion mutant strains of *Pg* on IL-8 production in the culture of gingival epithelial cells

(A) The culture broth of wild-type *Pg* 33277 or *Pg* KDP137 (RGP-KGP-HagA triple deficient mutant strain) growing in log growth phase was tested for H_2S concentration using a needle-type H_2S sensor. Data represent the average ± SD of three cultures. (B) OBA9 cells were cultured in the lower chamber, and *Pg* KDP137 was applied to the upper chamber of the transwell co-culture system in the presence of PMA (1*m*mol/L) with or without Morpholine-resin. After co-culture for 48 hr, the concentrations of IL-8 in the culture supernatant were measured by ELISA. Data are the mean ± SD of three cultures. *$p < 0.05$, **$p < 0.01$: Values differ significantly (*t*-test).