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Hydrogen sulfide inhibits cell proliferation and induces cell cycle arrest via an elevated $p21^{Cip1}$ level in Ca9-22 cells

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Background and Objective: Volatile sulfur compounds such as hydrogen sulfide (H_2S) and methyl mercaptan (CH_3SH) are the main causes of oral malodor. However, the physiological functions of H_2S have not been investigated in oral tissues. The aim of this study was to evaluate the effect of H_2S on cell proliferation and the cell cycle in oral epithelial-like cells.

Material and Methods: Ca9-22 cells were used in this study. Cells were cultured in 5% CO_2/95% air with (5 or 10 ng/mL) or without H_2S. DNA synthesis was measured using a 5-bromo-2-deoxyuridine enzyme-linked immunosorbent assay. The cell cycle was analyzed using a flow cytometer. The expressions of phosphorylated retinoblastoma protein (Rb), $p21^{Cip1}$ and $p27^{Kip1}$ were evaluated by western blotting.

Results: Exposure to 5 and 10 ng/mL of H_2S significantly decreased DNA synthesis ($p < 0.05$). Cell cycle analysis also showed that exposure to both concentrations of H_2S significantly increased the proportion of cells in G_1 phase ($p < 0.001$) and significantly decreased the proportion of cells in S phase ($p < 0.01$). Western blotting showed that Rb phosphorylation was reduced and $p21^{Cip1}$ was enhanced by exposure to H_2S.

Conclusion: The results indicated that H_2S inhibits cell proliferation and induces cell cycle arrest via the expression of $p21^{Cip1}$ in Ca9-22 cells.

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Oral malodor is principally caused by volatile sulfur compounds such as hydrogen sulfide (H_2S) and methyl mercaptan (CH_3SH) (1–3). These compounds are produced through the putrefaction of proteins by microorganisms in the oral cavity (4,5), mainly in the gingival sulcus, the tongue dorsal surface and other mucous surfaces (6,7). Previous studies have shown that the levels of volatile sulfur compounds in mouth air increase with the severity of periodontal disease (1,6,8), and the levels of volatile sulfur compounds were also shown to be elevated in periodontal pockets (4,9–13). Furthermore, the CH_3SH/H_2S ratio is increased in patients with periodontal disease (6), suggesting physiological functions of CH_3SH in the oral cavity. The synthesis of total protein, DNA and collagen decrease in human gingival fibroblasts exposed to CH_3SH (14–16). Interleukin-1, prostaglandin E_2 and collagenase activities during the immunological response were also shown to increase in human gingival fibroblasts exposed to CH_3SH (17). CH_3SH also activates the production of interleukin-6 in human pulp fibroblasts (18). These studies suggest that CH_3SH might contribute to the initiation and progression of inflammation and periodontal disease. We also reported that CH_3SH inhibits the growth and proliferation of oral epithelial cells (19). However, little is known about the physiological effect of H_2S in the oral cavity.

In recent years, endogenous H_2S was identified as the third gas transmitted, following nitric oxide and carbon monoxide. As a result, many studies on the physiological functions of H_2S have been conducted with regard to systemic diseases. Among these studies, it has been reported that H_2S inhibits cell proliferation in HEK-293 cells (20) and in rat vascular smooth muscle cells (21). However, it remains unclear whether H_2S inhibits cell proliferation in oral epithelial cells, and whether its mechanism is different from that of CH_3SH.

The purpose of the present study therefore was to investigate the effects of H_2S on cell proliferation and the cell cycle in a human gingival epithelial cell line (Ca9-22) with the aim of elucidating the physiological functions of H_2S in the oral cavity.

Material and methods

Cell culture with H_2S

The human gingival epithelial cell line, Ca9-22, was purchased from the Japanese Collection of Research Bioresources (Osaka, Japan). Cells were maintained in Eagle's minimum essential medium supplemented with 10% fetal bovine serum. After 24 h, the culture medium was changed and the cells were exposed to H_2S (5 or 10 ng/mL), as described by Johnson *et al.* (15). Briefly, test (with H_2S) and control (without H_2S) cultures were incubated simultaneously in separate humidified sealed chambers perfused with a 95% air/5% CO_2 gas mixture. The inlet line leading to the test chambers was connected to a Permeater (PD-1B; Gastec Co., Kanagawa, Japan) containing a calibrated permeation tube (Gastec Co.) used to emit a certain concentration of H_2S into the air mixture. As stimulation with H_2S in the present study was independent, the control group was shown, respectively.

Cell viability assay

Cellular viability was determined by Trypan Blue dye exclusion. The cells were seeded in a six-well plate and exposed to H_2S (5 or 10 ng/mL) for 24 h, then trypsinized and resuspended in phosphate-buffered saline. After staining with Trypan Blue, viable cells in five fields of view were counted using a hemocytometer.

Lactate dehydrogenase assay

The cytotoxicity of H_2S was measured using a cytotoxicity detection kit (Roche Diagnostics, Penzberg, Germany). Lactate dehydrogenase activity released from cells was determined according to the manufacturer's instructions. Briefly, cells were seeded in a 96-well plate and exposed to H_2S (5 or 10 ng/mL) for 24 h. Maximum release of lactate dehydrogenase was obtained by adding 100 μL of 2% Triton X-100 to untreated cells. After centrifugation, 100 μL of each sample was incubated with 100 μL of lactate dehydrogenase assay reagent for 30 min at room temperature. The absorbance of samples was then measured at 490 nm. The percentage of lactate dehydrogenase release was determined by dividing the lactate dehydrogenase released by the cells by the maximum lactate dehydrogenase release multiplied by 100.

DNA synthesis

Cells (5×10^4/mL) were seeded in a 96-well plate and exposed to H_2S (5 or 10 ng/mL) for 24 h. DNA synthesis was measured using a 5-bromo-2′-deoxyuridine enzyme-linked immunosorbent assay (ELISA) kit (Cell Proliferation ELISA, 5-bromo-2-deoxyuridine; Roche Diagnostics). 5-Bromo-2-deoxyuridine was added to the medium during the last 2 h of incubation. After fixation, the 5-bromo-2-deoxyuridine incorporated into the cells was assessed using the assay kit, in accordance with the manufacturer's instructions.

Cell cycle analysis

Cells (5×10^4/mL) were seeded in 100-mm dishes, exposed to H_2S (5 or 10 ng/mL) for 24 h, then fixed with ice-cold 70% ethanol. Cells were washed twice in phosphate-buffered saline then treated with 150 μg/mL of RNase (Sigma, St Louis, MO, USA) at room temperature for 30 min and resuspended in phosphate-buffered saline containing 50 μg/mL of propidium iodide (Sigma). DNA fluorescence was measured using a flow cytometer (EPICS XL; Beckman Coulter, Fullerton, CA, USA).

Western blotting

Cells were exposed to H_2S (10 ng/mL) for varying times and then treated with Isogen (Nippon Gene, Tokyo, Japan). Protein concentrations were determined using the bicinchoninic acid Protein Assay Reagent (Pierce, Rockford, IL, USA). Extracts containing the same amounts of protein (20 μg) were separated by 7.5 or 12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred to poly(vinylidene difluoride) membranes (Immobilon; Millipore, Billerica, MA, USA). Membranes were blocked in blocking buffer (Block Ace Powder; Dainippon, Osaka, Japan) then probed with anti-Rb (1 : 500; Pharmingen, San Diego, CA, USA), anti-$p21^{Cip1}$ (1 : 2000; Cell Signaling, Beverly, MA, USA), anti-$p27^{Kip1}$ (1 : 2500; Pharmingen) and anti-β-actin (1 : 4000; Sigma) primary antibodies followed by incubation with alkaline phosphatase-conjugated antimouse secondary antibody (Promega, Madison, WI, USA). The proteins were detected using 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium (Color Development Substrate; Promega) as a substrate of alkaline phosphatase.

Statistical analysis

Data are expressed as means ± standard deviation. The Student's *t*-test for unpaired samples was used to compare mean values between the control and test groups. A *p*-value of less than 0.05 was considered statistically significant.

Results

Necrosis assay

To determine whether necrosis of Ca9-22 was induced by exposure to H_2S, we

Table 1. Necrosis of Ca9-22 cells treated with H_2S

	Cell viability (%)	Cytotoxicity (%)
Control	97.6 ± 0.59	0.53 ± 1.92
H_2S 5 ng/mL	97.4 ± 0.73	1.43 ± 1.49
Control	96.2 ± 0.49	2.68 ± 6.16
H_2S 10 ng/mL	95.5 ± 0.66	2.48 ± 4.43

Necrosis was evaluated 24 h after exposure to 5 or 10 ng/mL of H_2S using the Trypan Blue dye exclusion test and by monitoring the enzymatic activity of lactate dehydrogenase released into the culture medium. Values represent the means ± standard deviation of four replicates. There were no significant differences compared with the control.

examined the results of the Trypan Blue dye-exclusion test and lactate dehydrogenase activity. As shown in Table 1, there were no statistical differences between the groups in cellular viability and cytotoxicity. The most head of stimulation time that we used was 24 hours, therefore necrosis was not induced by H_2S in this study.

DNA synthesis

The Ca9-22 cells were exposed to 5 or 10 ng/mL of H_2S for 24 h. The effect of H_2S on DNA synthesis was determined using the 5-bromo-2-deoxyuridine ELISA. Figure 1A,B shows that both concentrations of H_2S significantly decreased the incorporation of 5-bromo-2-deoxyuridine into DNA.

Cell cycle analysis

To determine whether the observed cell growth inhibition was caused by cell cycle arrest, the distribution of cells in different phases of the cell cycle was examined 24 h after exposure to 5 or 10 ng/mL of H_2S. Flow cytometric analyses of the DNA content revealed that both concentrations of H_2S significantly increased G_1-phase populations and significantly decreased S-phase populations (Fig. 2A,B and Table 2).

Rb phosphorylation and $p21^{Cip1}$ and $p27^{Kip1}$ expression, as determined by western blotting

Western blot analysis was performed to investigate Rb phosphorylation and the expression of $p21^{Cip1}$ and $p27^{Kip1}$ after exposure to 10 ng/mL of H_2S for 0, 6, 12, 18 and 24 h. Rb phosphorylation was inhibited by exposure to H_2S (Fig. 3). The expression of $p21^{Cip1}$ was enhanced after exposure to H_2S for 6 h and elevated in a time-dependent manner. There was no difference in the expression of $p27^{Kip1}$ compared with the control (Fig. 4).

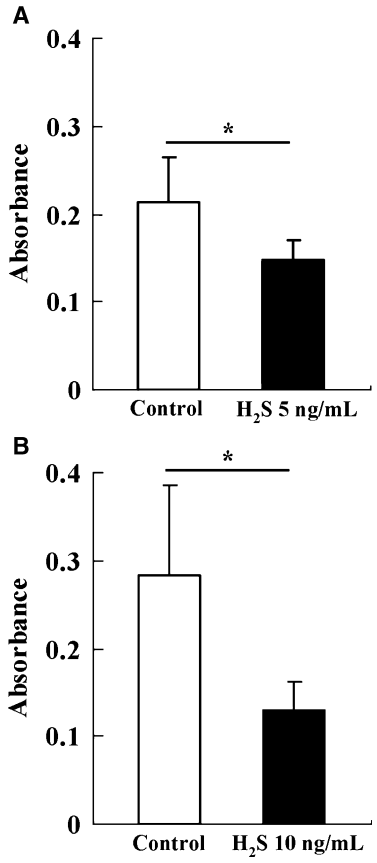

Fig. 1. Effect of H_2S on DNA synthesis in Ca9-22 cells. Cells were cultured with 5 ng/mL (A) or 10 ng/mL (B) of H_2S for 24 h, then 5-bromo-2-deoxyuridine incorporation into the DNA was measured using a 5-bromo-2-deoxyuridine enzyme-linked immunosorbent assay kit. Values represent the means ± standard deviation of five replicates. *, $p < 0.05$ compared with the control.

Discussion

In present study, we aimed to investigate the effects of H_2S on cell proliferation and the cell cycle in oral epithelial cells. Ca9-22 cells have been used in previous studies as a culture model of oral epithelial cells (22–25) because Ca9-22 cells are well-characterized epithelial cells that are epithelial-like in morphology and produce epidermal growth factor receptor extensively (26). We also thought that Ca9-22 cells would be an optimal model of oral epithelial cells in this study.

Recent studies have indicated that volatile sulfur compounds may contribute to the etiology of periodontal disease (27). It has been reported that subgingival bacterial species (*Prevotella intermedia*, *Tannerella forsythus*, *Treponema denticola*) associated with periodontal diseases were detected in sulfide-positive periodontal pockets (28). These studies have described the relationship between CH_3SH and periodontal conditions, because CH_3SH is present at a relatively high concentration in patients with periodontal disease (6). Most reports on H_2S have focused on the toxicological effects rather than on physiological functions, and, furthermore, the physiological functions of H_2S in oral tissues remain almost unknown despite the fact that H_2S is the principal cause of physiological halitosis.

In recent years, a number of studies have demonstrated that endogenous H_2S is a gas transmitter, along with nitric oxide and carbon monoxide (29). The physiological effects of H_2S on the cardiovascular system and neuromodulates have been also reported (e.g. H_2S inhibits cell proliferation by the modulation of extracellular signal-regulated kinase 1/2 phosphorylation and the expression of $p21^{Cip1}$ in HEK-293 cells) (20). Moreover, Du *et al.* (21) reported that H_2S suppresses the proliferation of rat vascular smooth muscle cells through the mitogen-activated protein kinase pathway and suggested that endogenous H_2S is a vital modulator in cardiovascular physiology and pathophysiology. Similarly to the results of these reports,

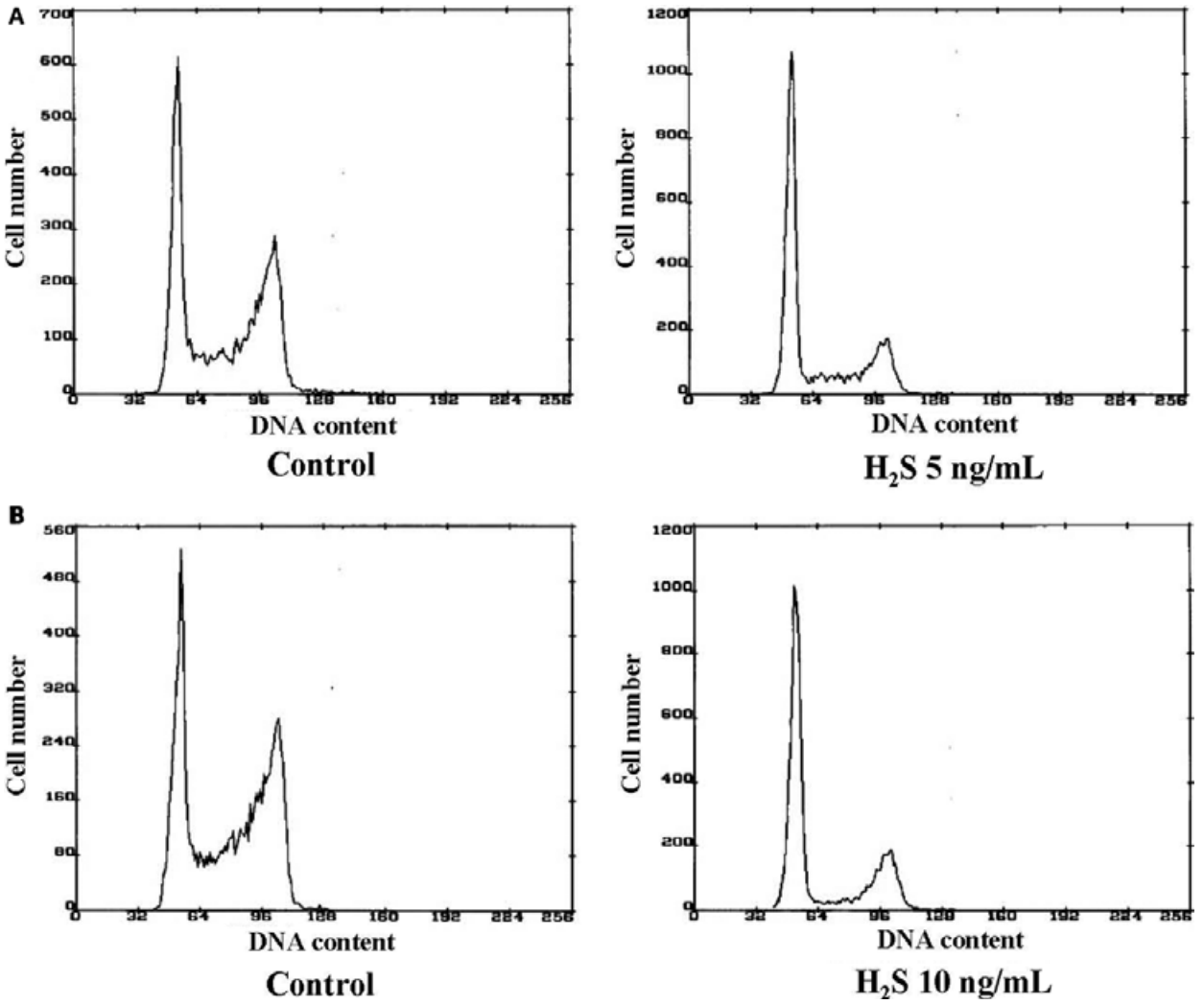

Fig. 2. Effect of H_2S on cell cycle progression in Ca9-22 cells. Cells were cultured with 5 ng/mL (A) or 10 ng/mL (B) of H_2S for 24 h. The cell cycle distribution in the G_1, S and G_2 phases was measured using a flow cytometer. The results shown are representative of those from the four independent experiments.

Table 2. The population of Ca9-22 cells, in the presence or absence of H_2S, in G_1, S and G_2 phases

	$\%G_1$	%S	$\%G_2$
Control	36.7 ± 2.0	34.6 ± 4.0	28.7 ± 3.2
H_2S 5 ng/mL	60.2 ± 2.4***	23.6 ± 3.0**	16.3 ± 3.7**
Control	32.4 ± 3.7	43.6 ± 2.0	24.1 ± 2.1
H_2S 10 ng/mL	55.8 ± 6.2***	18.4 ± 5.8***	25.8 ± 5.8

Cells were exposed to H_2S at the indicated concentrations for 24 h. The cell cycle distribution was measured with a flow cytometer. Values represent the means ± standard deviation of four independent experiments. **, $p < 0.01$; ***, $p < 0.001$ compared with the control.

we indicated that H_2S induced G_1 phase arrest in Ca9-22 cells. Along with nitric oxide and carbon monoxide, it has been implied that H_2S is able to infiltrate the three-dimensional structure of receptors, enzymes and channels, and presumably alter their conformation to affect function (30). In Ca9-22 cells, it was suggested that H_2S penetrated the plasma membrane and mediated the cell cycle.

Rb protein is known to control cell cycle progression during G_1 phase transition, in response to extracellular signals for growth inhibition, and Rb phosphorylation has an important role in the expression of genes with the products affecting the cell cycle (31). The cyclin-dependent kinase complex phosphorylates Rb protein and is negatively regulated by $p21^{Cip1}$ and $p27^{Kip1}$, as both are cyclin-dependent kinase inhibitors (31–36). In the present study, we demonstrated that H_2S inhibited Rb phosphorylation and increased $p21^{Cip1}$ expression, and indicated that H_2S induced cell cycle arrest in Ca9-22 cells. p53 is a known transcription factor of $p21^{Cip1}$ (37) and plays a role in DNA damage and apoptosis (38). In this study, the expression of $p21^{Cip1}$ was elevated, suggesting that H_2S may activate the p53 pathway. H_2S did not affect

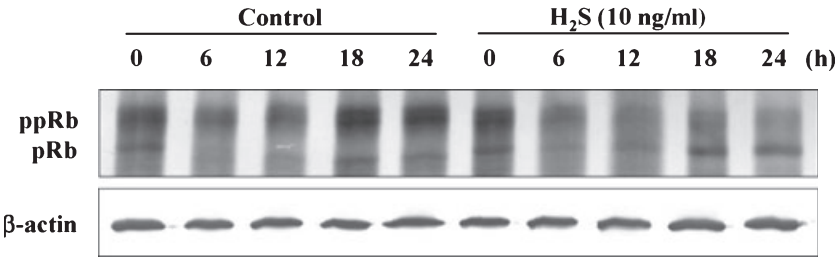

Fig. 3. Effect of H_2S on retinoblastoma protein phosphorylation in Ca9-22 cells. Cells were cultured with 10 ng/mL of H_2S for 0, 6, 12, 18 or 24 h. The expressions of retinoblastoma protein and β-actin were analyzed by western blotting. Extracts containing the same amounts of protein (20 μg) were separated by 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. pRb, hypophosphorylated retinoblastoma protein; ppRb, hyperphosphorylated retinoblastoma protein.

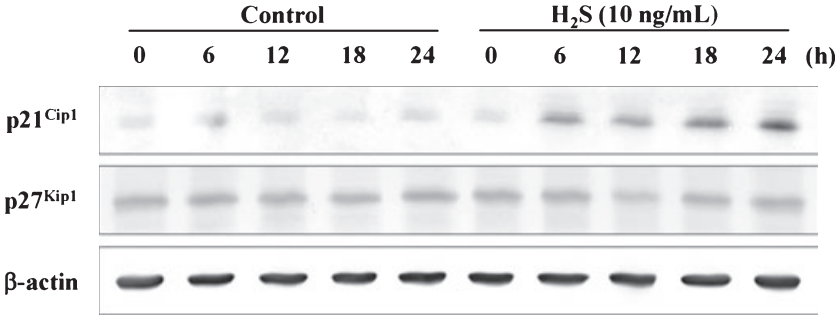

Fig. 4. Effect of H_2S on $p21^{Cip1}$ and $p27^{Kip1}$ levels in Ca9-22 cells. Cells were cultured with 10 ng/mL of H_2S for 0, 6, 12, 18 or 24 h, then the expressions of $p21^{Cip1}$, $p27^{Kip1}$ and β-actin were analyzed by western blotting. Extracts containing the same amounts of protein (20 μg) were separated by 12.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

the expression of $p27^{Kip1}$, possibly indicating a peculiar effect of H_2S that was dependent on the cell line used.

In a previous study we reported that CH_3SH (50 ng/mL) inhibited the cell growth and cell proliferation of oral epithelial cells by inducing necrosis, and suggested that CH_3SH might have a important role in the periodontal pathogenic mechanism by causing the barrier function of the gingival epithelium to be lost (19). However, exposure to CH_3SH (50 ng/mL) for 24 h did not induce cell cycle arrest (data not shown). The present study suggested that H_2S inhibits gingival epithelial cell proliferation by a different mechanism from that of CH_3SH.

The concentrations of H_2S (3.5 and 7 p.p.m.) used in this study were higher than that in normal mouth air, but, although rare, a concentration of 3.5 p.p.m. of H_2S is detected in patients with severe oral malodor. Perrson *et al*. reported that the highest concentration of H_2S in gingival crevicular fluid was 1.9 mM (39), which is equivalent to 64.8 p.p.m. It is therefore thought that H_2S is more concentrated in local areas like deep periodontal pockets and therefore higher than the concentrations used in this study. It is suggested that H_2S might inhibit epithelial cell proliferation in deep periodontal pockets and contribute to delayed epithelial repair. Further study is therefore required to clarify the molecular mechanisms underlying the pathological effects of H_2S.

In conclusion, our study demonstrated that H_2S decreased DNA synthesis and induced G_1 phase arrest via an elevated $p21^{Cip1}$ level in Ca9-22 cells. This study is an initial experiment in investigating the relationship between periodontitis and H_2S and a first step in the investigation of the physiological functions of H_2S in the oral cavity.

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