

# Hydrogen Sulfide Induces Direct Radical-Associated DNA Damage

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## Abstract

Hydrogen sulfide (H<sub>2</sub>S) is produced by indigenous sulfate-reducing bacteria in the large intestine and represents an environmental insult to the colonic epithelium. Clinical studies have linked the presence of either sulfate-reducing bacteria or H<sub>2</sub>S in the colon with chronic disorders such as ulcerative colitis and colorectal cancer, although at this point, the evidence is circumstantial and underlying mechanisms remain undefined. We showed previously that sulfide at concentrations similar to those found in the human colon induced genomic DNA damage in mammalian cells. The present study addressed the nature of the DNA damage by determining if sulfide is directly genotoxic or if genotoxicity requires cellular metabolism. We also questioned if sulfide genotoxicity is mediated by free radicals and if DNA base oxidation is involved. Naked nuclei from untreated Chinese hamster ovary cells were treated with sulfide; DNA damage was induced by concentrations as low as 1 μmol/L. This damage was effectively quenched by cotreatment with butylhydroxyanisole. Furthermore, sulfide treatment increased the number of oxidized bases recognized by formamidopyrimidine [fapy]-DNA glycosylase. These results confirm the genotoxicity of sulfide and strongly implicate that this genotoxicity is mediated by free radicals. These observations highlight the possible role of sulfide as an environmental insult that, given a predisposing genetic background, may lead to genomic instability or the cumulative mutations characteristic of colorectal cancer. (Mol Cancer Res 2007;5(5):455–9)

## Introduction

Hydrogen sulfide (H<sub>2</sub>S) is produced in the intestine by indigenous sulfate-reducing bacteria (SRB) as an end product

of their anaerobic respiration (1). Sulfide concentrations range from 0.2 to 1 mmol/L H<sub>2</sub>S in mouse intestine (2) to 0.3 to 3.4 mmol/L H<sub>2</sub>S in human feces (3–5). In clinical studies, the presence of either H<sub>2</sub>S or SRB in the intestine has been associated with certain chronic diseases including inflammatory bowel disease and colorectal cancer (6–11), although a causal relationship has not been established for either disorder. Pitcher et al. (12) did not detect differences between counts and carriage rates of SRB in feces of ulcerative colitis patients versus control subjects. Moreover, Fite et al. (13) present evidence that SRB may be ubiquitously present in the human colon. However, an understanding of potential interindividual differences in the density, diversity, and metabolic activity of SRB populations in the human colon is far from complete. Regardless, understanding the effects of H<sub>2</sub>S on the colonic epithelium and the extent of interindividual differences in the responsive pathways is crucial to determine how this intestinal insult may contribute to chronic disorders. Published data indicate that H<sub>2</sub>S can damage the intestinal epithelium, leading to the chronic inflammation (10, 11, 14–16), as well as perturb the delicate balance between cellular proliferation and apoptosis (17–19).

The toxic properties of H<sub>2</sub>S, which were first mentioned 300 years ago (20), have been extensively studied (21, 22). However, surprisingly little is known about H<sub>2</sub>S-induced DNA damage and its possible role as a mutagen or carcinogen, or its involvement in chromosomal instability. We previously showed that sulfide at concentrations similar to those found in the human and mouse intestine caused genomic DNA damage in Chinese hamster ovary (CHO) and human HT-29 colonic epithelial cells (23). Intriguingly, genotoxicity was only observed when DNA repair was inhibited with hydroxyurea and 1-β-D-arabinofuranosylcytosine.

Earlier studies on the genotoxicity of H<sub>2</sub>S reported mixed results. Negative results were reported for the *his* reversion assay in *Salmonella typhimurium* strains TA97, TA98, or TA100 with and without aroclor-induced hamster or rat hepatic S9 fractions with H<sub>2</sub>S concentrations of 17 to 1,750 μg per plate (24). Conversely, Na<sub>2</sub>S at concentrations of 0.1 to 5 μmol per plate were reported to be weakly mutagenic in *S. typhimurium* strain TA1535 (25).

Mutation studies with 0.05% Na<sub>2</sub>S were negative in *Micrococcus aureus* (26). In eukaryotes, sulfide was not genotoxic in *Drosophila melanogaster* eggs (27), nor did 24 to 96.1 mg/kg Na<sub>2</sub>S induce micronuclei in bone marrow cells of mice (25). At very high concentrations (50 mmol/L), X-linked recessive lethal mutations were observed in early

Received 1/2/07; revised 3/13/07; accepted 3/20/07.

**Grant support:** Environmental Protection Agency grant CR83069501 (M.J. Plewa and E.D. Wagner) and David H. and Lorraine A. Baker Graduate Fellowship (M.S. Attene-Ramos).

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doi:10.1158/1541-7786.MCR-06-0439

broods of *Drosophila*. These data indicated that spermatozoa were more sensitive to sulfide than spermatids or spermatoocytes (25).

In the present study, we employed the alkaline single-cell gel electrophoresis (SCGE) assay or comet assay that quantitatively measures genomic DNA damage as single- and double-strand breaks or lesions that can be converted to strand breaks (28, 29). SCGE is a sensitive genotoxicity assay and it has a high correlation in predicting carcinogens (30). The purpose of this research was to determine if  $H_2S$  was a direct-acting genotoxin and, if so, its mechanism of action and the general type of DNA lesions induced. This objective was motivated by the working hypothesis that at least some forms of sporadic colorectal cancer may represent multifactorial interactions among polymorphic alleles of sulfide-responsive genes and other genes or environmental factors (e.g., diet) that may influence the density, diversity or metabolic activity of colonic SRB.

## Results

### *Sulfide Directly Causes DNA Strand Breaks in Naked Nuclei at Very Low Concentrations*

The direct effects of sulfide on genomic DNA were examined using a modified SCGE assay, where naked CHO cell nuclei embedded in a layer of low melting point agarose were treated with different concentrations of  $Na_2S$ . The measure of direct DNA damage was the SCGE tail moment, the integrated value of migrated DNA density multiplied by the migration distance. A concentration-response curve was observed for sulfide concentrations between 1 and 25  $\mu\text{mol/L}$  (Fig. 1). All concentrations were significantly different from the negative control ( $P < 0.001$ ). For sulfide concentrations greater than 50  $\mu\text{mol/L}$ , the treated nuclei were completely degraded.

### *Butylhydroxyanisole Protects Genomic DNA from Sulfide*

To further define the mechanism of sulfide toxicity, slides were pretreated for 30 min with butylhydroxyanisole, a hydroxyl radical scavenger (31), and a known quencher of peroxy and alkoxy radicals (32). The treated slides were exposed to butylhydroxyanisole and sulfide together for 2 h. Butylhydroxyanisole (5  $\mu\text{mol/L}$ ) prevented DNA damage induced by 25  $\mu\text{mol/L}$  sulfide (Fig. 2). Concentrations as low as 250 nmol/L butylhydroxyanisole significantly decreased DNA damage induced by 25  $\mu\text{mol/L}$  sulfide, a 1:100 ratio (Fig. 2). The averaged median tail moment values for sulfide- and butylhydroxyanisole-treated slides were significantly different from sulfide-treated (positive controls) slides.

### *Sulfide Produces DNA Lesions that Are Recognized by Formamidopyrimidine [fapy]-DNA Glycosylase*

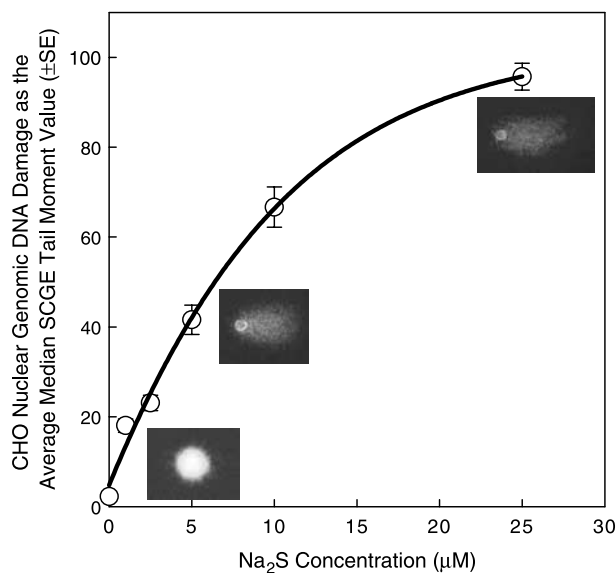
To define the nature of the DNA lesions generated by sulfide, we followed the sulfide treatment with formamidopyrimidine [fapy]-DNA glycosylase (Fpg), a DNA repair enzyme involved in base excision repair (33). Fpg releases both purine bases with damaged imidazole rings and a deoxyribose derivative, leaving a gap bordered by 5'- and 3'-phosphoryl groups (33, 34). If sulfide generates DNA lesions recognized by Fpg, then the median SCGE tail moment in slides treated

with sulfide and Fpg should be larger than the summation of each effect separately, after subtracting the negative control in all cases [ $F-A > (B-A) + (E-A)$  or  $D-A > (B-A) + (C-A)$ ; Fig. 3]. When slides were treated with 2.5  $\mu\text{mol/L}$  sulfide and Fpg, a significant difference between the median tail moment was observed compared with the separate effects of sulfide and Fpg ( $P < 0.001$ ). This indicates that in addition to inducing DNA strand breaks, sulfide generates significant oxidative damage of DNA.

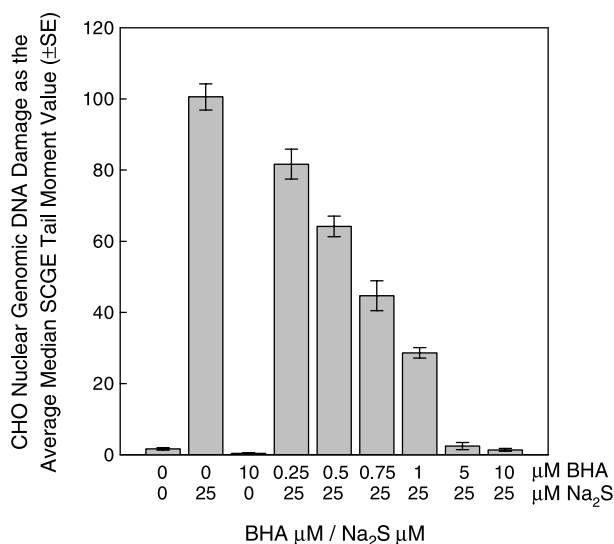
## Discussion

We previously showed for the first time that sulfide was genotoxic in mammalian cells using a modified SCGE assay where DNA repair was inhibited with hydroxyurea and 1- $\beta$ -D-arabinofuranosylcytosine (23). The present data not only confirm these findings but further show that sulfide is directly genotoxic, independent of cellular metabolism, and strongly suggests that this genotoxicity is mediated by free radicals.

We showed that  $Na_2S$  was genotoxic in CHO and human intestinal HT-29 cells, but little is known about the nature of this toxicity (23). In a recent study, Baskar et al. (35) supported our previous findings that  $H_2S$  was genotoxic. The present study shows that sulfide induces DNA damage in the absence of cellular metabolism, which indicates that sulfide, or some oxidation byproduct, reacts directly with DNA. In aqueous solution, sulfide spontaneously oxidizes generating oxygen and sulfur center radicals (36). This oxidation is catalyzed by free (37) or protein-bound divalent metals (36). Recently, Truong et al. (37) showed in a cell-free system, that NaHS depletes



**FIGURE 1.** Modified SCGE analysis of  $Na_2S$  in naked nuclei from CHO cells illustrating a plot of the genotoxic concentration-response curve. Naked nuclei from CHO cells were embedded in agarose and treated with  $Na_2S$  in PBS for 2 h. Compared with the negative control, a significant difference of the average median SCGE tail moment values at all sulfide concentrations was observed ( $F_{5,56} = 283.4$ ,  $P < 0.001$ ). For the data presented, at least eight microgels were analyzed per concentration with 25 nuclei measured per microgel. Inset, examples of SCGE images of CHO nuclei treated with 0 (bottom), 5  $\mu\text{mol/L}$  (middle), and 25  $\mu\text{mol/L}$  (top)  $Na_2S$ .



**FIGURE 2.** Concentration effect of butylhydroxyanisole (BHA) on Na<sub>2</sub>S-treated naked nuclei. Agarose-embedded naked nuclei from CHO cells were incubated with butylhydroxyanisole for 30 min and then treated with Na<sub>2</sub>S and butylhydroxyanisole for 2 h. Butylhydroxyanisole concentrations  $\geq 0.25$   $\mu\text{mol/L}$  significantly reduced the damage generated by 25  $\mu\text{mol/L}$  Na<sub>2</sub>S ( $F_{8,54} = 149.3$ ;  $P < 0.001$ ). Concentrations of 5 or 10  $\mu\text{mol/L}$  butylhydroxyanisole abrogated the sulfide-induced damage and were not significantly different from the negative control or from the 10  $\mu\text{mol/L}$  butylhydroxyanisole control. For the data presented, at least eight microgels were analyzed per concentration with 25 nuclei measured per microgel.

reduced glutathione concentrations in a pH-dependent manner, and that reduced glutathione depletion can be avoided when iron chelators or antioxidant enzymes are added to the reaction mixture, suggesting the generation of reactive sulfur and reactive oxygen species in sulfide toxicity.

We also observed that butylhydroxyanisole at a very low concentration (250 nmol/L) significantly reduced the damage produced by 25  $\mu\text{mol/L}$  Na<sub>2</sub>S. Butylhydroxyanisole, which acts as a peroxy and alkoxy as well as a hydroxyl radical scavenger (31, 32), protects the DNA from the radicals formed from sulfide in solution. Reactive oxygen species formation was observed by others after sulfide treatment in cell cultures and tissue explants (38-40). To further test the hypothesis that oxidative species are involved in sulfide genotoxicity and to enhance the specificity of resulting DNA damage, we conducted experiments using [fapy]-DNA glycosylase (41).

Consistent with involvement of pro-oxidants, we observed an increase in base lesions recognized by Fpg protein. [Fapy]-DNA glycosylase removes ring-opened imidazole derivatives of purines such as 7-methyl-fapy-guanine, FAPY-guanine, FAPY-adenine, and 7,8-dihydro-8 oxoguanine (42). In an alkaline buffer, strand breaks will be generated due to  $\beta$ -elimination of abasic DNA lesions, making it detectable by the SCGE assay. The 7,8-dihydro-8 oxoguanine is formed when 2'-deoxyguanosine reacts with oxidants or ionizing radiation, making it one of the most common oxidative base lesions (43). A previous study reported that sulfur radicals are responsible for 7,8-dihydro-8 oxoguanine formation (44). The bacterial [fapy]-DNA glycosylase has a eukaryotic functional homologue, the oxoguanine glycosylase (OGG1; refs. 45-47). This

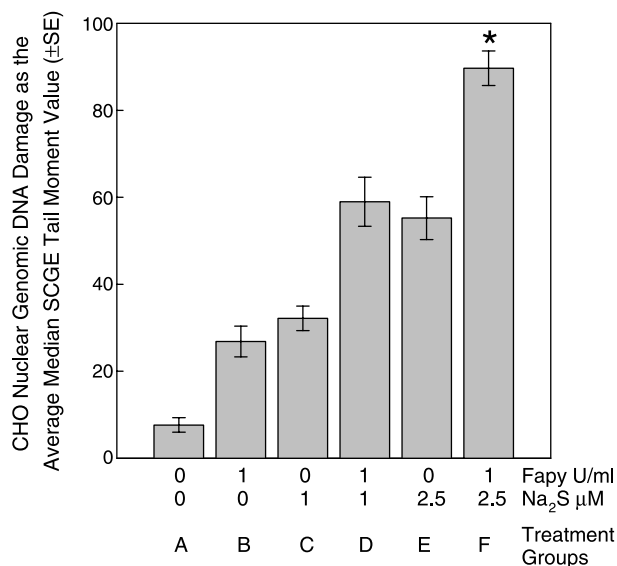
enzyme was up-regulated in *Donax variabilis* after exposure to H<sub>2</sub>S (40).

Intriguingly, sulfide can damage DNA at a very low concentration (1  $\mu\text{mol/L}$ ) relative to concentrations in the molar range that colonic epithelial cells routinely encounter. Similarly, genotoxic concentrations of sulfide are also lower than those reported to be endogenously generated as a signaling molecule by a variety of cell types (48). Our previous study showed sulfide genotoxicity in whole CHO cells only in the presence of DNA repair inhibitors. These data support the notion that detoxifying and DNA repair enzymes play a vital role in the protection of cells routinely exposed to high concentrations of sulfide. Any compromise in either sulfide detoxification or DNA repair (e.g., generated by polymorphic alleles of genes contributing to these mechanisms) could increase the susceptibility of an individual harboring such alleles to sulfide-generated DNA damage. Accordingly, it is relatively easy to envision how multifactorial interactions among polymorphic alleles of sulfide-responsive genes and other genes or environmental factors (e.g., diet) that promote colonization by SRB may contribute to chronic diseases such as colorectal cancer.

## Materials and Methods

### Reagents, Cell Culture Medium, and Biologicals

General laboratory reagents were purchased from Fisher, Sigma, or Life Technologies. Na<sub>2</sub>S·9H<sub>2</sub>O and butylhydroxyanisole were obtained from Sigma, and Fpg was purchased from New England Biolabs. Media supplies and fetal bovine serum were purchased from Hyclone Laboratories. Clone 11-4-8 of CHO cell line AS52 was maintained in Ham's F12 medium



**FIGURE 3.** Sulfide increases the number of lesions recognized by Fpg. Naked nuclei were treated with Na<sub>2</sub>S and washed, and then Fpg was added for 45 min at 37°C. The damage generated by 2.5  $\mu\text{mol/L}$  Na<sub>2</sub>S + Fpg (F-A) was greater than the addition of the damage generated by 2.5  $\mu\text{mol/L}$  Na<sub>2</sub>S (E-A) and Fpg (B-A) independently. \*,  $P < 0.001$ . For 1  $\mu\text{mol/L}$  Na<sub>2</sub>S, the effect was not statistically significant ( $P = 0.085$ ). For the data presented, at least eight microgels were analyzed per concentration with 25 nuclei measured per microgel.

containing 5% fetal bovine serum, 1% antibiotics (100 units/mL sodium penicillin G, 100 µg/mL streptomycin sulfate, 0.25 µg/mL amphotericin B, 0.85% saline), and 1% glutamine at 37°C in a humidified atmosphere of 5% CO<sub>2</sub> (49).

#### *Naked Nuclei SCGE Assay*

CHO cells were grown in glass Petri dishes. After reaching 60% to 90% confluency, cells were harvested with 1 mL trypsin 0.05% in EDTA (Life Technologies) for 1 min and lifted in Ham's F12 medium containing 5% fetal bovine serum (4 mL). Cells were counted using a Coulter Counter (Beckman Z<sub>1</sub> Coulter Particle Counter) and diluted to  $2 \times 10^5$  per mL. Equal volumes of the cell suspension and 1% low melting point agarose prepared with PBS were mixed, and 90 µL were placed on clear microscope slides that were previously coated with a layer of 1% normal melting point agarose prepared with deionized water and dried overnight. After the gel hardened, a second layer of 0.5% low melting point agarose was applied. Detailed methods for preparing and electrophoresing the SCGE microgels were published previously (50-52). Cellular membranes were removed by an overnight immersion in lysing solution at 4°C. The microgels were washed once with water and thrice with a neutralization buffer. The nuclei were treated in Coplin jars for 2 h with different concentrations of Na<sub>2</sub>S in PBS at pH 7. After the treatment, the microgels were placed in an alkaline buffer (pH 13.5) in an electrophoresis tank, and the DNA was denatured for 20 min at 4°C. The microgels were electrophoresed at 25 V, 300 mA (0.72 V/cm) for 40 min at 4°C. The microgels were removed, neutralized with Tris buffer (pH 7.5), rinsed in cold water, dehydrated in cold methanol, dried at 50°C, and stored at room temperature in a covered slide box. For analysis, the microgels were hydrated in cold water for 20 min and stained with 65 µL of 20 µg/mL ethidium bromide for 5 min. The microgels were rinsed in cold water and analyzed with a Zeiss fluorescence microscope with an excitation filter of BP 546/10 nm and a barrier filter of 590 nm. The slides were coded, and 25 randomly chosen nuclei were analyzed in each slide using a charge coupled device camera. A computerized image analysis system (Komet version 3.1, Kinetic Imaging Ltd.) was used to determine the SCGE tail moment (integrated value of migrated DNA density multiplied by the migration distance) of the nuclei as an index of DNA damage. The digitalized data were automatically transferred to a computer-based spreadsheet for subsequent statistical analysis. The microgel, and not the nucleus, is the unit of measure. We follow this procedure to reduce the possibility of type 1 statistical errors. We analyze 25 nuclei per microgel, and with these data, we determined the median SCGE tail moment value. These median values are averaged and used for our statistical analysis. For each treatment group, we analyzed at least eight microgels. Details of the statistical analysis for SCGE data were published in refs. (50, 52, 53).

#### *Naked Nuclei SCGE Assay; Butylhydroxyanisole Treatment*

Microgels were prepared and cell membranes lysed and washed as described above. Before treatment, microgels were pretreated for 30 min in Coplin jars with PBS or different solutions of butylhydroxyanisole in PBS at pH 7. Then the

microgels were treated in Coplin jars with PBS or different solutions of Na<sub>2</sub>S and/or butylhydroxyanisole in PBS for 2 h. After the treatment, the microgels were dipped twice in water and placed in an alkaline buffer, and the protocol was continued as described above.

#### *Modified Naked Nuclei SCGE Assay with Fpg Treatment*

CHO cells were grown, harvested, and counted as described previously. The cell suspension was mixed with an equal volume of 1% low melting point agarose prepared with PBS, and two 70-µL aliquots were placed on the same SCGE microscope slides described previously, making two microgels per slide. A second layer of low melting point agarose was not applied in this case. Detailed methods for preparing the slides were published previously (54). The microgels were kept in lysing solution at 4°C overnight, washed, and treated. After the treatment, the microgels were rinsed in water, and 70 µL of reaction buffer or 1 unit/mL Fpg in reaction buffer were added. The microgels were incubated for 45 min at 37°C, and then the reaction was stopped by placing the slides on a tray with ice for 10 min. The microgels were processed for electrophoresis and microscopic analysis of the nuclei.

#### *Safety and Data Handling*

Manipulations of toxic and mutagenic chemicals were conducted in certified biological/chemical stage 2 safety hoods. At least three independent experiments were conducted with eight microgels analyzed per treatment group. The median SCGE tail moment value for each microgel was determined, and the data from all of the microgels representing each treatment concentration were averaged. Averaged median values express a normal distribution according to the central limit theorem and were used with a one-way ANOVA test (55). A Holm-Sidak method for multiple comparisons versus the control group analysis was conducted, and the power of the test statistic ( $1 - \beta$ ) was  $\geq 0.80$ .

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