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Hydrogen Sulfide Induces Direct Radical-Associated DNA Damage

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Abstract

Hydrogen sulfide (H_2S) 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_2S 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_2S) 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_2S in mouse intestine (2) to 0.3 to 3.4 mmol/L H_2S in human feces (3-5). In clinical studies, the presence of either H_2S 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_2S 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_2S 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_2S, which were first mentioned 300 years ago (20), have been extensively studied (21, 22). However, surprisingly little is known about H_2S-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_2S 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_2S concentrations of 17 to 1,750 μg per plate (24). Conversely, Na_2S 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_2S 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_2S 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

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broods of *Drosophila*. These data indicated that spermatozoa were more sensitive to sulfide than spermatids or spermatocytes (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 μmol/L (Fig. 1). All concentrations were significantly different from the negative control ($P < 0.001$). For sulfide concentrations greater than 50 μmol/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 μmol/L) prevented DNA damage induced by 25 μmol/L sulfide (Fig. 2). Concentrations as low as 250 nmol/L butylhydroxyanisole significantly decreased DNA damage induced by 25 μmol/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 μmol/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-β-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

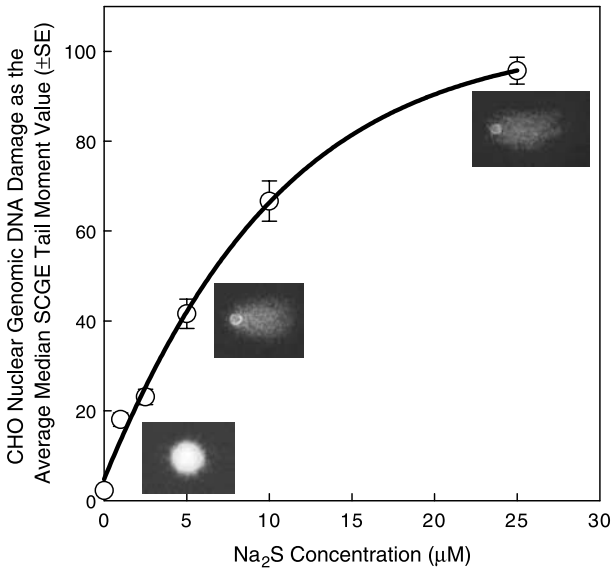

FIGURE 1. Modified SCGE analysis of Na_2S in naked nuclei from CHO cells illustrating a plot of the genotoxic concentration-response curve. Naked nuclei from CHO cells were embedded in agarose and treated with Na_2S in PBS for 2 h. Compared with the negative control, a significant difference of the average 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 μmol/L (*middle*), and 25 μmol/L (*top*) Na_2S.

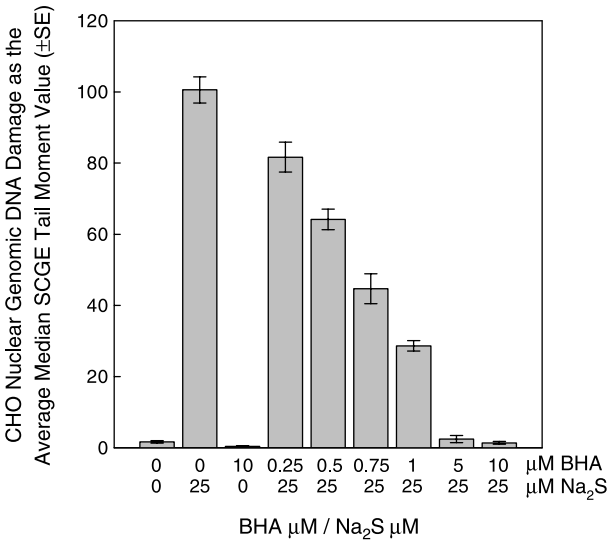

FIGURE 2. Concentration effect of butylhydroxyanisole (*BHA*) on Na_2S-treated naked nuclei. Agarose-embedded naked nuclei from CHO cells were incubated with butylhydroxyanisole for 30 min and then treated with Na_2S and butylhydroxyanisole for 2 h. Butylhydroxyanisole concentrations $\geq$0.25 μmol/L significantly reduced the damage generated by 25 μmol/L Na_2S ($F_{8,54} = 149.3$; $P < 0.001$). Concentrations of 5 or 10 μmol/L butylhydroxyanisole abrogated the sulfide-induced damage and were not significantly different from the negative control or from the 10 μmol/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 μmol/L Na_2S. 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 β-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_2S (40).

Intriguingly, sulfide can damage DNA at a very low concentration (1 μmol/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. $Na_2S \cdot 9H_2O$ 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

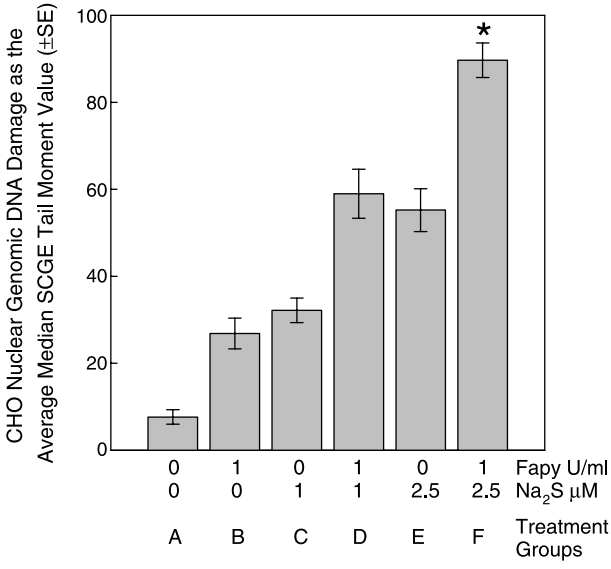

FIGURE 3. Sulfide increases the number of lesions recognized by Fpg. Naked nuclei were treated with Na_2S and washed, and then Fpg was added for 45 min at 37°C. The damage generated by 2.5 μmol/L Na_2S + Fpg (F-A) was greater than the addition of the damage generated by 2.5 μmol/L Na_2S (E-A) and Fpg (B-A) independently. *, $P < 0.001$. For 1 μmol/L Na_2S, 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_2 (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_1 Coulter Particle Counter) and diluted to 2×10^5 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_2S 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_2S 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 ≥ 0.80.

References

1. Beerens H, Romond C. Sulfate-reducing anaerobic bacteria in human feces. Am J Clin Nutr 1977;30:1770–6.

2. Deplancke B, Finster K, Graham WV, Collier CT, Thurmond JE, Gaskins HR. Gastrointestinal and microbial responses to sulfate-supplemented drinking water in mice. Exp Biol Med 2003;228:424–33.

3. Florin T, Neale G, Gibson GR, Christl SU, Cummings JH. Metabolism of dietary sulphate: absorption and excretion in humans. Gut 1991;32:766–73.

4. Magee EA, Richardson CJ, Hughes R, Cummings JH. Contribution of dietary protein to sulfide production in the large intestine: an *in vitro* and a controlled feeding study in humans. Am J Clin Nutr 2000;72:1488–94.

5. Pochart P, Dore J, Lemann F, Goderel I, Rambaud JC. Interrelations between populations of methanogenic archaea and sulfate-reducing bacteria in the human colon. FEMS Microbiol Lett 1992;77:225–8.

6. Gibson GR, Cummings JH, Macfarlane GT. Growth and activities of sulphate-reducing bacteria in gut contents of healthy subjects and patients with ulcerative colitis. FEMS Microbiol Lett 1991;86:103–11.

7. Pitcher MCL, Beatty ER, Gibson GR, Cummings JH. Incidence and activities of sulphate-reducing bacteria in patients with ulcerative colitis. Gut 1995;36:A63.

8. Kanazawa K, Konishi F, Mitsuoka T, et al. Factors influencing the development of sigmoid colon cancer. Bacteriologic and biochemical studies. Cancer 1996;77:1701–6.

9. Levitt MD, Furne J, Springfield J, Suarez F, DeMaster E. Detoxification of hydrogen sulfide and methanethiol in the cecal mucosa. J Clin Invest 1999;104:1107–14.

10. Roediger WE, Moore J, Babidge W. Colonic sulfide in pathogenesis and treatment of ulcerative colitis. Dig Dis Sci 1997;42:1571–9.

11. Huycke MM, Gaskins HR. Commensal bacteria, redox stress, and colorectal cancer: mechanisms and models. Exp Biol Med 2004;229:586–97.

12. Pitcher MC, Beatty ER, Cummings JH. The contribution of sulphate reducing bacteria and 5-aminosalicylic acid to faecal sulphide in patients with ulcerative colitis. Gut 2000;46:64–72.

13. Fite A, Macfarlane GT, Cummings JH, et al. Identification and quantitation of mucosal and faecal desulfovibrios using real time polymerase chain reaction. Gut 2004;53:523–9.

14. Babidge W, Millard S, Roediger W. Sulfides impair short chain fatty acid beta-oxidation at acyl-CoA dehydrogenase level in colonocytes: implications for ulcerative colitis. Mol Cell Biochem 1998;181:117–24.

15. Roediger WE, Duncan A, Kapaniris O, Millard S. Reducing sulfur compounds of the colon impair colonocyte nutrition: implications for ulcerative colitis. Gastroenterology 1993;104:802–9.

16. Moore DM, Kotake Y, Huycke MM. *Enterococcus faecalis* produces thiyl radicals while colonizing the colon. Free Radic Biol Med 2003;35:S175.

17. Leschelle X, Goubern M, Andriamihaja M, et al. Adaptative metabolic response of human colonic epithelial cells to the adverse effects of the luminal compound sulfide. Biochim Biophys Acta 2005;1725:201–12.

18. Deplancke B, Gaskins HR. Hydrogen sulfide induces serum-independent cell cycle entry in nontransformed rat intestinal epithelial cells. FASEB J 2003;17:1310–2.

19. Christl SU, Eisner HD, Dusel G, Kasper H, Scheppach W. Antagonistic effects of sulfide and butyrate on proliferation of colonic mucosa: a potential role for these agents in the pathogenesis of ulcerative colitis. Dig Dis Sci 1996;41:2477–81.

20. Ramazzini B. On the diseases of artificers (De morbis artificum diatriba), 1713. In: James R, editor. Health preserved, in two treatises. 2nd ed. London: Whiston, J; 1750.

21. Reiffenstein RJ, Hulbert WC, Roth SH. Toxicology of hydrogen sulfide. Annu Rev Pharmacol Toxicol 1992;32:109–34.

22. Beauchamp RO, Jr., Bus JS, Popp JA, Boreiko CJ, Andjelkovich DA. A critical review of the literature on hydrogen sulfide toxicity. Crit Rev Toxicol 1984;13:25–97.

23. Attene-Ramos MS, Wagner ED, Plewa MJ, Gaskins HR. Evidence that hydrogen sulfide is a genotoxic agent. Mol Cancer Res 2006;4:9–14.

24. Hughes TJ, Sparacino C, Frazier S. Validation of chemical and biological techniques for evaluation of vapors in ambient air testing of twelve (12) vapor-phase compounds (project summary). Research Triangle Park (NC): Environmental Protection Agency; 1984. Report No. EPA-6001.

25. Gocke E, King MT, Eckhardt K, Wild D. Mutagenicity of cosmetics ingredients licensed by the European Communities. Mutat Res 1981;90:91–109.

26. Clark J. The mutagenic action of chemicals on *Micrococcus aureus*. Proc Okla Acad Sci 1953;34:114.

27. Matter BE, Wurgler FE, Ulrich H. On the radioprotective effect of hydrogen sulphide in *Drosophila melanogaster*. Int J Radiat Biol Relat Stud Phys Chem Med 1969;15:557–62.

28. Tice RR, Agurell E, Anderson D, et al. Single cell gel/comet assay: guidelines for *in vitro* and *in vivo* genetic toxicology testing. Environ Mol Mutagen 2000;35:206–21.

29. Rundell MS, Wagner ED, Plewa MJ. The comet assay: genotoxic damage or nuclear fragmentation? Environ Mol Mutagen 2003;42:61–7.

30. Anderson D, Yu TW, McGregor DB. Comet assay responses as indicators of carcinogen exposure. Mutagenesis 1998;13:539–55.

31. Thompson D, Moldeus P. Cytotoxicity of butylated hydroxyanisole and butylated hydroxytoluene in isolated rat hepatocytes. Biochem Pharmacol 1988;37:2201–7.

32. Robertson DG, Epling GA, Kiely JS, Bailey DL, Song B. Mechanistic studies of the phototoxic potential of PD 117596, a quinolone antibacterial compound. Toxicol Appl Pharmacol 1991;111:221–32.

33. Krokan HE, Standal R, Slupphaug G. DNA glycosylases in the base excision repair of DNA. Biochem J 1997;325:1–16.

34. Graves RJ, Felzenszwalb I, Laval J, O'Connor TR. Excision of 5′-terminal deoxyribose phosphate from damaged DNA is catalyzed by the Fpg protein of *Escherichia coli*. J Biol Chem 1992;267:14429–35.

35. Baskar R, Li L, Moore PK. Hydrogen sulfide-induces DNA damage and changes in apoptotic gene expression in human lung fibroblast cells. FASEB J 2007;21:247–55.

36. Tapley DW, Buettner GR, Shick JM. Free radicals and chemiluminescence as products of the spontaneous oxidation of sulfide in seawater, and their biological implications. Biol Bull-Us 1999;196:52–6.

37. Truong DH, Eghbal MA, Hindmarsh W, Roth SH, O'Brien PJ. Molecular mechanisms of hydrogen sulfide toxicity. Drug Metab Rev 2006;38:733–44.

38. Julian D, April KL, Patel S, Stein JR, Wohlgemuth SE. Mitochondrial depolarization following hydrogen sulfide exposure in erythrocytes from a sulfide-tolerant marine invertebrate. J Exp Biol 2005;208:4109–22.

39. Eghbal MA, Pennefather PS, O'Brien PJ. H2S cytotoxicity mechanism involves reactive oxygen species formation and mitochondrial depolarisation. Toxicology 2004;203:69–76.

40. Joyner-Matos J, Downs CA, Julian D. Increased expression of stress proteins in the surf clam *Donax variabilis* following hydrogen sulfide exposure. Comp Biochem Physiol A Mol Integr Physiol 2006;145:245–57.

41. Friedberg EC, Walker GC, Siede W. DNA repair and mutagenesis. Washington (DC): ASM Press; 1995.

42. David SS, Williams SD. Chemistry of glycosylases and endonucleases involved in base-excision repair. Chem Rev 1998;98:1221–62.

43. Grollman AP, Moriya M. Mutagenesis by 8-oxoguanine: an enemy within. Trends Genet 1993;9:246–9.

44. Shi X, Mao Y. 8-Hydroxy-2′-deoxyguanosine formation and DNA damage induced by sulfur trioxide anion radicals. Biochem Biophys Res Commun 1994;205:141–7.

45. van der Kemp PA, Thomas D, Barbey R, de Oliveira R, Boiteux S. Cloning and expression in Escherichia coli of the OGG1 gene of *Saccharomyces cerevisiae*, which codes for a DNA glycosylase that excises 7,8-dihydro-8-oxoguanine and 2,6-diamino-4-hydroxy-5-*N*-methylformamidopyrimidine. Proc Natl Acad Sci U S A 1996;93:5197–202.

46. Lu R, Nash HM, Verdine GL. A mammalian DNA repair enzyme that excises oxidatively damaged guanines maps to a locus frequently lost in lung cancer. Curr Biol 1997;7:397–407.

47. Nash HM, Bruner SD, Scharer OD, et al. Cloning of a yeast 8-oxoguanine DNA glycosylase reveals the existence of a base-excision DNA-repair protein superfamily. Curr Biol 1996;6:968–80.

48. Fiorucci S, Distrutti E, Cirino G, Wallace JL. The emerging roles of hydrogen sulfide in the gastrointestinal tract and liver. Gastroenterology 2006;131:259–71.

49. Wagner ED, Rayburn AL, Anderson D, Plewa MJ. Analysis of mutagens with single cell gel electrophoresis, flow cytometry, and forward mutation assays in an isolated clone of Chinese hamster ovary cells. Environ Mol Mutagen 1998;32:360–8.

50. Plewa MJ, Wagner ED, Jazwierska P, Richardson SD, Chen PH, McKague AB. Halonitromethane drinking water disinfection byproducts: chemical characterization and mammalian cell cytotoxicity and genotoxicity. Environ Sci Technol 2004;38:62–8.

51. Cemeli E, Wagner ED, Anderson D, Richardson SD, Plewa MJ. Modulation of the cytotoxicity and genotoxicity of the drinking water disinfection byproduct iodoacetic acid by suppressors of oxidative stress. Environ Sci Technol 2006;40:1878–83.

52. Plewa MJ, Kargalioglu Y, Vankerk D, Minear RA, Wagner ED. Mammalian cell cytotoxicity and genotoxicity analysis of drinking water disinfection by-products. Environ Mol Mutagen 2002;40:134–42.

53. Muellner MG, Wagner ED, McCalla K, Richardson SD, Woo YT, Plewa MJ. Haloacetonitriles vs. regulated haloacetic acids: are nitrogen containing DBPs more toxic? Environ Sci Technol 2007;41:645–51.

54. Rundell MS, Muellner MG, Wagner ED, Plewa MJ, Berhow MA, Vaughn SF. Isolation of antigenotoxic components and antioxidants from commercial corn processing by-products. Environ Molecular Mutagen 2003;41:201.

55. Box GEP, Hunter WG, Hunter JS. Statistics for experimenters: an introduction to design, data analysis and model building. New York (NY): Wiley & Sons, Inc.; 1978.