Hydrogen sulfide-induced DNA damage and changes in apoptotic gene expression in human lung fibroblast cells

Rajamanickam Baskar, Ling Li, and Philip Keith Moore
Cardiovascular Biology Research Group, Department of Pharmacology, Yong Loo Lin School of Medicine, National University of Singapore, Singapore

ABSTRACT Hydrogen sulfide (H₂S) has been shown previously to exert proapoptotic activity. However, the mechanism(s) by which H₂S affects cell growth and function have not been addressed adequately. In this study, cultured human lung fibroblasts were treated with the H₂S donor NaHS (10–75 μM; 12–48 h). NaHS caused a concentration-dependent increase in micronuclei formation (indicating DNA damage) and cell cycle arrest (G1 phase). NaHS increased expression of ku 70 and ku 80 but did not affect the expression of other DNA repair proteins such as proliferating cell nuclear antigen (PCNA) or replication protein A (RPA). NaHS treatment also resulted in stabilization of p53 coupled with induction of downstream products such as p21, Bax, and cytochrome c, as well as translocation of Bax from the cytosol to the mitochondria and release of cytochrome c from mitochondria. NaHS did not up-regulate cell levels of the antiapoptotic protein, Bcl-2. We propose that the genotoxic action of H₂S propels the cell toward apoptotic death triggered initially by stabilization of p53 and subsequently involving a cascade of downstream products. These results are of significance as they uncover a hitherto unknown and very fundamental role for H₂S in determining cell fate. —Baskar, R., Li, L., Moore, P. K. Hydrogen sulfide-induced DNA damage and changes in apoptotic gene expression in human lung fibroblast cells. FASEB J. 21, 247–255 (2007)

Key Words: cell cycle • p53 • mitochondria • cytochrome c • translocation

In recent years, evidence has accumulated that hydrogen sulfide (H₂S) may play a number of putative biological roles and indeed H₂S has been suggested to function as a novel third gasotransmitter in the body alongside NO and carbon monoxide (CO) (1). H₂S is most commonly encountered occupationally as an environmental contaminant and is also found in varying amounts in unrefined natural gas and petroleum, sulfur deposits, volcanic gases, well water, and sulfur springs (2). However, it is now becoming increasingly clear that H₂S is also synthesized naturally from L-cysteine in many mammalian and nonmammalian tissues in a reaction catalyzed by two main enzymes, cystathionine-γ-lyase (CSE) and cystathionine-β-synthetase (CBS) (3, 4) and that this novel gas may play potentially important roles in numerous aspects of normal physiology and in disease (5, 6). In this respect, recent studies have focused on the role of H₂S in, for example, the cardiovascular system (5–7, 8), inflammation (9, 10), diabetes mellitus (11, 12), and on N-methyl-D-aspartate (NMDA) receptor-mediated responses in neurons (4, 13).

Perhaps of greater significance for the present work, H₂S also induces serum-independent proliferation of nontransformed rat intestinal epithelial cells (14) and enhances cell death of human aorta smooth muscle cells most probably by up-regulating the extracellular, signal-regulated kinase (ERK) (15). Cell growth is controlled by multiple, interrelated signaling pathways with many cell cycle regulatory molecules involved. However, the precise mechanism(s) by which H₂S leads to cell death and also its cellular effect on DNA is not well understood. Thus, in this study we have investigated the genotoxic activity of H₂S and its molecular role in the growth of normal human lung fibroblast cells. Specifically, we assess here the effect of H₂S (released from the donor agent, sodium hydrosulfide NaHS) on normal human lung fibroblast (MRC-5) cell cycle distribution and DNA damage by flow cytometry, micronuclei formation, and determination of apoptosis. The effect of H₂S on gene clusters involved in the control of cell cycle check points, DNA repair and cell death/apoptosis proteins in MRC-5 fibroblast cells have also been evaluated.

MATERIALS AND METHODS

Cell culture and treatment

Normal human lung primary fibroblast (MRC-5, IMR-90, and WI-38) cells derived from a healthy individual were obtained...
from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were grown in Eagle minimal essential medium (EMEM) supplemented with 10% v/v FBS (Life Technologies, Inc., Rockville, MD, USA), vitamins, essential amino acids, nonessential amino acids, and antibiotics. The cultures were maintained at 37°C in a humidified 5% CO₂ atmosphere as described previously (16). A stock solution of NaHS was freshly prepared by dissolving sodium hydrosulfide (NaHS, Sigma-Aldrich, St. Louis, MO, USA) using the complete medium immediately before use. Solutions of NaHS prepared in this way provide ~30% free H₂S. In all experiments, cells with 75–80% confluence were treated with NaHS (0–100 μM).

**Cell death by trypan blue**

Cells were plated in T-25 flasks for 24 h after which, various concentrations of NaHS (0–100 μM) were added to the culture medium and incubations performed at 37°C for different time periods. At 12, 24, and 48 h after such treatment, cells were harvested and incubated with 0.4% w/v trypan blue solution (Sigma-Aldrich) for 5 min. Cell viability was determined by trypan blue exclusion assay using a hemocytometer. The percentage cell survival for each treatment group was determined by counting the dead cells (blue) and the live cells (clear). Data are expressed as percentage of control living cells.

**Measurement of cell cycle distribution**

For cell cycle analysis, exponentially growing MRC-5 fibroblast cells cultured in T-25 flasks with the complete medium were treated with NaHS (0–75 μM). At 12, 24, and 48 h after such treatment, cells were harvested, washed once in PBS, and fixed in ice-cold 70% v/v ethanol overnight at 4°C. Cells were then mixed with 0.5 ml of propidium iodide (PI) solution (10 mM Tris-HCl, 0.7 mg of RNase per ml, 10% Nonidet P-40, 1 M NaCl, 0.05 mg/ml of PI) and incubated for 30 min at 4°C. Cell cycle distribution was analyzed using a FACS Caliber System (Becton Dickinson, Germany) flow cytometer (10,000 events recorded for each sample). Apoptotic cells were determined by quantitating the sub-G₁ DNA peak. The cell profile was assessed using cell Quest software.

**Analyses of micronuclei (MN) and apoptosis**

Two commonly used biological endpoints, MN and apoptosis, were assessed to evaluate the effect of H₂S on DNA damage. The cytokinesis-block technique was used to assay for MN in situ. MRC-5 cells in the exponential growth phase were treated with different concentrations of NaHS (0–75 μM). After 24 h, cytochalasin B (5 μg/ml in PBS, Sigma-Aldrich), a cytokinesis inhibitor that generates binucleated cells, was added to the cells. After a further 24 h, cells were harvested and fixed in methanol-acetic acid (3:1 v/v) and the cell suspension was dropped on to clean microscope slides. Slides were then stained with acridine orange (AO, 0.03 mg/ml in PBS; exhibits a differential staining of cytoplasm-red and nucleus-green) for the analysis of MN formation. Micronuclei formation was scored in at least 750 randomly selected mononucleated and binucleated cells for each concentration of NaHS treatment. To detect apoptosis, cells were fixed in ice-cold 70% v/v ethanol overnight at 4°C and stained with the DNA intercalating dye PI (as described for cell cycle analysis experiments). Cells with sub-G₁ DNA content were analyzed flow cytometrically. To characterize nuclear morphology, cells were also stained with AO (on the same slide used for scoring MN) and cells with condensed and/or fragmented DNA were viewed and scored. Cells with MN and apoptotic images were also captured using a laser fluorescence microscope (Zeiss, Germany).

**Protein isolation, SDS-PAGE, and Western blot analysis**

Total cellular proteins were isolated using radio-immuno-precipitation assay (RIPA) buffer (1% w/v Nonidet P-40, 1% w/v sodium deoxycholate, 0.1% w/v SDS, 0.15 M NaCl, 0.01 M sodium phosphate, 2 mM EDTA, 50 mM sodium fluoride, 0.2 mM sodium vanadate, and 100 U/ml aprotinin, pH 7.2) from control and NaHS-treated cells. The proteins were recovered by centrifugation at 14,000 rpm for 10 min. Protein concentration was determined by the bicinchoninic acid (BCA) method using a commercially available kit (Pierce Biotechnology, Inc., Rockford, IL, USA) with BSA as a standard. Western blot analyses of p53, replication protein A (rNase protection assay), ku 70 and ku 80 (NeoMarkers, Fremont, CA, USA), p21, proliferating cell nuclear antigen (PCNA), Bcl-2, Bax, cytochrome c, and actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA), cystathionine-γ-lyase (CSE), and cystathionine-β-synthetase (CBS) (Abnova, Taipei, Taiwan) were performed using specific antibodies. Equal amounts of cell lysates were fractionated using 4–20% polyacrylamide gradient gel electrophoresis and blotted onto PVDF membranes using the manufacturer’s standard protocol (Invitrogen Corporation, Carlsbad, CA, USA). The membranes were immersed in TBST (20 mM Tris-HCl, pH 7.4; 137 mM NaCl; 0.2% Tween) buffer containing 5% v/v nonfat dried milk (NFDM) for 60 min at room temperature. Membranes were then incubated with the primary antibody (Ab) at 1:1000 v/v dilution in TBST-5% NFDM overnight and thereafter washed three times (5 min each) in TBST buffer. Membranes were then incubated with horseradish peroxidase conjugated secondary antibodies (1:2000 dilution v/v in TBST-5% NFDM, Vector Laboratories, Inc., Burlingame, CA, USA) for 1 h followed by repeated washing in TBST buffer. The protein signal was detected by chemiluminescence following the manufacturer’s protocol (Pierce Biotechnology). The densities of the bands were quantified using Kodak 1D image analysis software.

**Detection of cytochrome c and Bax translocations**

Mitochondria were isolated from NaHS-treated cultured cells using the mitochondria isolation procedure of the Pierce protocol (Pierce Biotechnology). Briefly, 2×10⁷ exponentially growing cells were cultured and treated with NaHS (10 and 50 μM) and then harvested at 0.5, 6 and 24 h. The pellet with mitochondria was lysed as described earlier for the whole cell lyses. The cytosolic and mitochondrial fractions were analyzed by Western blot using cytochrome c and Bax antibodies as described above.

**Statistical analysis**

All values are expressed as mean ± sem. Each value is the mean of at least three separate experiments. Significant differences between test and control data were analyzed by one-way ANOVA followed by Tukey’s multiple comparison test. A value of $P<0.05$ was considered statistically significant.

**RESULTS**

In a preliminary study, we first investigated the effect of NaHS (0–75 μM) on cell survival using the trypan blue
exclusion assay in three different, normal human lung primary fibroblast (IMR-90, WI-38, and MRC-5) cells. NaHS caused a very similar concentration-dependent decrease in cell survival in all three primary fibroblasts (Fig. 1A–C). Therefore, we further investigated the effect of NaHS (50 μM) on p53 protein (a well-documented indicator of cell death and damage) and noted a time-dependent up-regulation of p53 in all three fibroblast cells (Fig. 1D). Since all three fibroblast cells appeared to respond similarly to NaHS, we chose MRC-5 cells for further, detailed investigation of the genotoxic effects of H$_2$S. Like IMR-90 and WI-38 cells, exposure of MRC-5 cells to NaHS resulted in a concentration-dependent increase in cell death, i.e., reduced cell survival (Fig. 1C). This effect was more profound when studied at 12 h post-treatment and was statistically significant at all concentrations of NaHS studied. Interestingly, a time-dependent partial recovery in cell survival was noted after 24 and 48 h, which may indicate the replicating nature of the surviving cells. Figure 2 illustrates the density of MRC-5 cells as determined by phase contrast microscopy both before and after treatment with NaHS for 12, 24, and 48 h. Again, exposure to NaHS caused a concentration-dependent decrease in cell survival in these experiments. Since this study aimed to investigate the effects of exogenous H$_2$S on MRC-5 cells, we also considered it of interest to determine whether MRC-5 cells expressed CSE and CBS and thus perhaps could synthesize their own endogenous H$_2$S. In separate preliminary experiments, both CSE and CBS were identified by Western blotting in MRC-5 cells (Fig. 1E).

**NaHS induces micronuclei (MN) formation, an indicator of DNA damage**

In an attempt to probe further the cause(s) of NaHS-induced cell death observed in MRC-5 cell we measured the formation of MN as a marker of DNA damage. Cells

![Image](image_url)

**Figure 1.** Viability of three fibroblast cells (MRC-5, IMR-90, and WI-38) was determined by trypan blue dye exclusion assay following NaHS exposure. A, B) IMR-90 and WI-38 cell survival after 12 and 24 h of culture with NaHS (0–75 μM). C) Viability of MRC-5 cells after 12, 24, and 48 h following NaHS (0–75 μM) exposure. Data represent the mean ± sem of three independent experiments. *$P < 0.05$ compared with control. D) Time course of expression of p53 protein after treatment of MRC-5, IMR-90 and WI-38 cells with NaHS. Cells grown to 75–80% of confluence in T-75 flasks were exposed to NaHS (50 μM). Cells were harvested at 0, 3, 9, and 24 h post-treatment. Whole cells were lysed; equal amounts of proteins were separated using 4–20% SDS-PAGE, transferred to PVDF membrane and immunoreacted with Ab against p53. E) Untreated MRC-5 cells express cystathionine-γ-lyase (CSE) and cystathionine-β-synthetase (CBS). Actin was used as loading control.
were treated with different concentrations of NaHS (0–75 μM) and MN formation was analyzed (Table 1). A concentration-dependent increase in MN was observed in both mononucleated and binucleated cells. This effect was statistically significant at concentrations of NaHS in excess of 50 μM (Table 1). However, when both mononucleated and binucleated cells were scored together, a significant increase in MN formation was detected after treatment with an even lower concentration of NaHS (10 μM, Table 1). These observations suggest that NaHS causes DNA damage as evidenced by MN formation in replicating human fibroblast cells.

Role of NaHS in cell cycle distribution and apoptosis

Subconfluent, cycling MRC-5 cells were treated with NaHS (0–50 μM) and the cells were collected at 12, 24, and 48 h of treatment. The percentage of cells in each cell cycle phase (G1, S, and G2/M) of DNA content was determined by flow cytometry. Treatment with NaHS decreased the percentage of cells in the G1 phase (Fig. 3A). This effect was statistically significant ($P<0.05$) at both 12 h (NaHS concentration of 50 μM) and 24 h (NaHS concentrations of 30 and 50 μM). NaHS caused a reduction in number of cells in the S phase and this was significant only at 12 h after exposure of 30 μM (6.9±0.19%, $P<0.05$) and 50 μM (5.8±0.48%, $P<0.05$) of NaHS (data not shown). At 24 and 48 h, NaHS treatment caused a decrease in the number of cells in the S phase (data not shown). However, when compared to control at 12, 24, and 48 h after NaHS treatment showed a slightly higher incidence of cells in the G2/M phase (data not shown).

H2S-induced apoptotic cell death in the present experiments was assessed in two ways, *i.e.*, by flow cytometry and microscopically. The PI stained cells of the sub-G1 phase (Fig. 3B) analyzed by flow cytometry represent an apoptotic cell population. Flow cytometric analysis revealed a concentration-dependent increase in apoptotic cells at all time points after treatment with NaHS (Fig. 3B). Apoptosis was also assessed microscopically by estimating morphological features of the cells with fragmented nuclei and condensed chromatin in the AO stained cells. NaHS (50 and 75 μM) treatment

### Table 1. Analysis of micronuclei (MN) formation after NaHS treatment to MRC-5 cells

<table>
<thead>
<tr>
<th>Treatment (μM)</th>
<th>0</th>
<th>10</th>
<th>50</th>
<th>75</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total no. of cells scored</td>
<td>2816</td>
<td>2756</td>
<td>2869</td>
<td>2283</td>
</tr>
<tr>
<td>Total No. of micronuclei (%)</td>
<td>142 (5.05)</td>
<td>223 (8.09)*</td>
<td>309 (10.77)*</td>
<td>273 (11.96)*</td>
</tr>
<tr>
<td>Mononucleated cells</td>
<td>2601</td>
<td>2469</td>
<td>2480</td>
<td>1957</td>
</tr>
<tr>
<td>Micronuclei (%)</td>
<td>134 (5.15)</td>
<td>213 (8.63)</td>
<td>289 (11.65)*</td>
<td>256 (13.08)*</td>
</tr>
<tr>
<td>Binucleated cells</td>
<td>73</td>
<td>64</td>
<td>80</td>
<td>53</td>
</tr>
<tr>
<td>Micronuclei (%)</td>
<td>8 (10.95)</td>
<td>10 (15.63)</td>
<td>20 (25.0)*</td>
<td>17 (32.07)*</td>
</tr>
</tbody>
</table>

MRC-5 cells in exponential growth phase were exposed to NaHS. Cells were treated with cytochalasin B 24 h thereafter to generate binucleated cells. Cells were harvested, fixed, and analyzed by differential staining with acridine orange of cytoplasm (red) and nuclei (green). Data represent the mean of three independent experiments. *$P < 0.05$ compared with control.
significantly ($P < 0.05$) increased the number of apoptotic cells (control, 1.85 ± 0.34%; 10 μM, 2.65 ± 0.44%; 50 μM, 4.29 ± 0.52%; and 75 μM, 6.92 ± 0.38%).

**Induction of cell cycle checkpoint and stress-response proteins after NaHS treatment**

In an attempt to probe the molecular mechanism(s) involved in the DNA damaging effect of $H_2S$, we investigated key cell cycle regulatory and stress response proteins present in normal human lung fibroblasts. Cells treated with NaHS (10 and 50 μM) were harvested at 0.5, 2, 6, and 24 h post-treatment and the time course of expression of p53, p21, ku 70, and ku 80 proteins was studied by Western blot analysis (Fig. 4A). Among the cell cycle checkpoint proteins investigated, p53 was rapidly induced after NaHS exposure, followed by p21 (Fig. 4A). Both proteins showed a concentration-dependent increase in a time-dependent manner following NaHS exposure (Fig. 4B). In the present study, ku 70 and ku 80 showed higher expression after NaHS (10 and 50 μM) treatment at 0.5, 2, 6, and 24. However, ku 80 showed a greater sensitivity to the stimulating effect of NaHS than did ku 70 (Fig. 4A, C).

To determine whether $H_2S$-induced genotoxicity affects DNA repair kinetics, we also studied rNase protection assay (RPA) and PCNA proteins. In the present study, Western blot analysis did not reveal any significant increase in either RPA (1.06-fold increase) or PCNA (1.08-fold increase) protein levels following exposure of cultured fibroblasts to NaHS (50 μM) (data not shown).

![Figure 3. Cell cycle distributions of MRC-5 cells exposed to NaHS (0–50 μM) for 12, 24, or 48 h. DNA of the cells was stained by propidium iodide and analyzed by flow cytometry. DNA content was divided by cell cycle phases. A) Percentage of cells in G1 phase. B) DNA content in sub-G1. Data represent the mean ± SEM of three independent experiments. *$P < 0.05$ compared with control.](image)

**Figure 3.** Cell cycle distributions of MRC-5 cells exposed to NaHS (0–50 μM) for 12, 24, or 48 h. DNA of the cells was stained by propidium iodide and analyzed by flow cytometry. DNA content was divided by cell cycle phases. A) Percentage of cells in G1 phase. B) DNA content in sub-G1. Data represent the mean ± SEM of three independent experiments. *$P < 0.05$ compared with control.

![Figure 4. Time course of expression of p53, p21, ku 70, and ku 80 proteins after treatment of cells with NaHS. MRC-5 cells grown to 75–80% of confluence in T-75 flasks were exposed to NaHS (10 or 50 μM). Cells were harvested at 0, 0.5, 2, 6, and 24 h post-treatment. A) Whole cells were lysed, and equal amounts of proteins were separated using 4–20% SDS-PAGE, transferred to PVDF membrane, and immunoreacted with antibodies against p53, p21, ku 70, and ku 80. Actin was used as loading control. The band intensity was quantified using Kodak 1D image analysis software. B, C) Histogram showing the time-dependent induction of p53, p21, ku 70, and ku 80 proteins. Data show fold induction and are expressed as mean ± SEM (n=3). *$P < 0.05$ compared with control.](image)

**Figure 4.** Time course of expression of p53, p21, ku 70, and ku 80 proteins after treatment of cells with NaHS. MRC-5 cells grown to 75–80% of confluence in T-75 flasks were exposed to NaHS (10 or 50 μM). Cells were harvested at 0, 0.5, 2, 6, and 24 h post-treatment. A) Whole cells were lysed, and equal amounts of proteins were separated using 4–20% SDS-PAGE, transferred to PVDF membrane, and immunoreacted with antibodies against p53, p21, ku 70, and ku 80. Actin was used as loading control. The band intensity was quantified using Kodak 1D image analysis software. B, C) Histogram showing the time-dependent induction of p53, p21, ku 70, and ku 80 proteins. Data show fold induction and are expressed as mean ± SEM (n=3). *$P < 0.05$ compared with control.
after NaHS treatment (Fig. 5).

However, proapoptotic proteins such as Bax and cytochrome c also isolated mitochondria and measured the translocation of cytochrome c. Cells were harvested at 0, 0.5, 2, 6, and 24 h thereafter. As shown in Fig. 6A–C, cytochrome c was released into the cytoplasm in a time-dependent manner after exposure to NaHS. In addition, NaHS treatment of fibroblasts led to an increased translocation of Bax from the cytosol to the mitochondria (Fig. 6A, B, D), indicating that H$_2$S may act via the Bax and cytochrome c pathway to induce apoptotic cell death.

**DISCUSSION**

In this study, we have examined the effects of H$_2$S on DNA lesions and a wide range of proteins associated with cell growth and apoptosis in human lung primary fibroblast (MRC-5) cells. We show here for the first time that the H$_2$S donor NaHS induces p53 and causes cell cycle alteration and formation of MN, and also triggers important markers of apoptosis, including the release of cytochrome c from the mitochondrial membrane into the cytoplasm and also the translocation of Bax from cytosol to the mitochondria.

Specifically, we demonstrate here that NaHS increased fibroblast (MRC-5, IMR-90, and WI-38) cell death in a concentration-dependent manner. The effect was more profound after 12 h of treatment. Exposure of cells to NaHS (10–50 µM) resulted in a time-dependent recovery when studied at 24 and 48 h, indicating that the surviving cells are still able to replicate. However, at the highest concentration of NaHS used (i.e., 75 µM), no recovery of cell survival was apparent at 48 h (Fig. 2A), indicating that at this concentration the effect of NaHS is greater. It should perhaps be noted at this stage that the concentration of H$_2$S generated from NaHS (75 µM) is likely to be ~25 µM and, as such, the effects of H$_2$S, which are observed here, may occur naturally in the body, since the plasma H$_2$S concentration, for example, is reportedly ~30–50 µM (1).

To gain a better understanding of the nature of H$_2$S-mediated cell death, formation of micronuclei (MN) after NaHS treatment of MRC-5 cells was assessed. We observed a concentration-dependent increase in MN in both mononucleated and binucleated cells exposed to NaHS, indicating that NaHS treatment increases the amount of DNA lesions that are left unrepaired before replication of the cells. Thus, the present study shows, for the first time, that H$_2$S is a potent clastogenic agent, which produces a range of DNA lesions when incubated with normal human lung fibroblast (MRC-5) cells. Broadly similar conclusions were recently noted by Attene-Ramos et al. (17), who used single-cell gel electrophoresis (SCGE) to show that sodium sulfide (Na$_2$S: 750 µM/L) caused DNA damage in the DNA repair impaired Chinese hamster ovary (CHO) and human colon cancer cells (HT29). In this study, the effect of NaHS was observed only after DNA repair was inhibited by a combination of hydroxyurea and 1-B-arabino-furanosylcytosine (AraC) as opposed to the present work in which H$_2$S exhibits genotoxicity in otherwise normal cells. Furthermore, in the present study, formation of MN was used to identify the genotoxic effect of H$_2$S, whereas SCGE was employed as evidence of DNA damage by Attene-Ramos and colleagues. The formation of MN represents fragments of the chromosome or whole chromosomes resulting from clastogenic or aneugenic events (18, 19, 20) and as such is considered as a reliable marker for DNA damage.
The cell cycle profile after NaHS treatment studied at 12, 24, and 48 h showed a decrease in the percentage of cells in the G1 phase. The G1 phase of the cell cycle was found to be more affected than the S and G2/M phases. On the one hand, the only other relevant report available in the literature (14) suggests that exposure of rat intestinal crypt IEC cells to NaHS (0.2–5 mM) for 4 h decreased the G0/G1 cells and increased the fraction of cells in S and G2/M cell cycle phases. On the other hand, in the present study, normal human lung primary fibroblasts showed significantly fewer cells in the S phase at 12 h after NaHS treatment, but thereafter a partial recovery of cell counts was found in the S phase at 24 and 48 h. To the best of our knowledge, the present study is the first to analyze the effect of H2S at different time points of the cell cycle rather than at a single time point (14). Cell cycle checkpoints are important control mechanisms that maintain the proper execution of cell cycle events. In general, after DNA damage, cells arrest at the transition from G1 to S phases or from G2 to M phase (21). Cell cycle arrest at these check points prevents DNA replication and mitosis in the presence of unrepaired chromosomal alterations (22). The proportion of cells that arrest at G1 to S or G2 to M phases depends on the cell type, the growth conditions, and the checkpoint controls operating in the cell (23, 24); the arrested cells may lead to cell death. In the present study, NaHS arrests the cell cycle of fibroblasts in the G1 phase and such cells might be eliminated by the process of apoptosis, as indicated by a higher incidence of DNA content in the sub-G1 phase.

The mechanism(s) underlying the decision of cells to undergo cell cycle arrest or apoptosis in response to H2S are not well understood. Given that p53 plays a pivotal role in DNA damage (25), we studied the induction of p53 and also genes involved in DNA repair, growth arrest, and apoptosis after NaHS treatment of fibroblasts. We demonstrate here that NaHS inhibits human lung fibroblast cell proliferation and also activates various cell cycle regulators, such as p53, p21, ku 70, and ku 80 in a time-dependent manner. p53, along with its target p21, and ku proteins (ku is a heterodimer composed of 70 and 86 kDa subunits, known as ku 70 and ku 80, respectively) play a role in cell signaling, proliferation, DNA repair, replication, transcriptional activation, and apoptosis (26, 27). Treatment of fibroblasts with NaHS did not increase either PCNA or RPA proteins, which suggests that at least these two DNA repair proteins were not activated under the present experimental conditions. However, clearly this does not rule out the possibility that other DNA repair proteins (28) may be involved in the cellular response to H2S. In this study, greater induction of p53 was observed when compared to the other proteins investigated, indicating that NaHS may...
induce p53, a guardian of the genome (29), and thereby prevent further degradation of genomic DNA and remove damaged cells by the process of apoptosis.

Mounting evidence indicates that, on DNA damage, p53 promotes apoptosis by activating Bax (30), which enhances apoptosis by stimulating the release of cytochrome c and the formation of apoptosomes (31). However, the precise molecular mechanism(s) involved in H2S-induced apoptosis are, as yet, unknown. The antiapoptotic Bcl-2 protects the cells against diverse cytotoxic insults, such as γ and UV-irradiation, cytokine withdrawal, dexamethasone, staurosporine, and various cytotoxic drugs (32). In NaHS-treated cells, we did not observe any increase in the quantity of Bcl-2 protein in the whole cell. Bcl-2 inhibits the translocation activity of Bax and the release of cytochrome c from the mitochondria. Interestingly, we found a concentration- and time-dependent increase in Bax and cytochrome c that led us to study those apoptosis signaling proteins in mitochondria, which play important roles in cell death (33). After NaHS treatment, cytosolic and mitochondrial fractions were analyzed by immunoblotting with antibodies to proapoptotic cytochrome c and Bax. The release of cytochrome c from mitochondria and also the translocation of Bax from the cytoplasm to mitochondria are central events in apoptotic signaling (34, 35). In this study, cytochrome c was released to the cytoplasm in a time-dependent manner. Also, we observed that NaHS treatment increased the translocation activity of Bax, a proapoptotic Bcl-2 family protein (36, 37), from the cytosol to the mitochondria, which suggests that H2S may act through the cytochrome c and Bax pathway to induce cell death via the apoptosis signaling pathway. Both mitochondrial accumulation of Bax from cytosol and cytochrome c release from mitochondria to cytosol were not prevented by the antiapoptotic protein of Bcl-2. In the present study, it is likely that DNA damage caused by H2S induces p53, which then activates Bax (30). Induction of Bax might then promote apoptosis by targeting the mitochondria and releasing cytochrome c to cytosol.

Previous studies have suggested that H2S exhibits proapoptotic activity. For example, Yang et al. (15, 38) showed that H2S causes human aorta smooth muscle cells apoptosis via the MAP kinase pathway. Recently, Cao et al. (39) reported that NaHS could induce apoptosis in mouse pancreatic acinar cells by both death receptor and mitochondrial pathways. However, to the best of our knowledge, no previous concerted attempts have been made to investigate the effect of H2S on those cellular events, which lead to or predispose to the apoptotic event. To the best of our knowledge, this evidence is the first to suggest that H2S causes DNA lesions and up-regulates the genome guardian p53, which leads, in turn, to translocation of proapoptotic Bax and the release of cytochrome c and ultimately results in apoptotic cell death.

In conclusion, the ability of NaHS to promote formation of MN, cell death by apoptosis, and reduction in the number of cells in the G1 phase (detected by cell cycle analysis) suggests that H2S inhibits cell growth perhaps—by damaging cellular DNA—although this was not directly addressed in the present work. A time- and concentration-dependent induction of p53, p21, Ku 70, and Ku 80 and release of cytochrome c into the cytosol from the mitochondrial membrane, as well as Bax from cytosol to mitochondria, were also observed. Our findings suggest a molecular basis for cell cycle-dependent alterations by H2S and indicate that p53 may play a critical role in apoptosis induced by this gaseous molecule.

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**H2S: MECHANISM OF GENOTOXICITY**