Anticancer effect of exogenous hydrogen sulfide in cisplatin-resistant A549/DDP cells

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Abstract. Despite huge advances in lung cancer treatment, resistance to cisplatin-based chemotherapy remains one of the major obstacles, and the elucidation of cisplatin resistance remains challenging. As an important biological and pharmacological mediator, hydrogen sulfide (H2S) performs a variety of homeostatic functions related to cancer formation and development. However, the effects of H2S on cisplatin-resistance lung cancer remain largely unknown. In the present study, we investigated the anticancer effects and relevant mechanisms of NaHS (an exogenous donor of H2S) on A549/DDP cells (cisplatin-resistant). The intracellular H2S was first evaluated using a fluorescence probe in A549 (cisplatin-sensitive) and A549/DDP cells. We found that H2S production was markedly decreased in A549/DDP cells compared with that in A549 cells, accomplished by the downregulation of cystathionine β-synthase (CBS), an endogenous H2S-producing enzyme. In view of these findings, we then observed the effects of NaHS treatment on A549/DDP cells. The results showed that NaHS exposure exhibited an inhibitory effect on cell viability and the IC50 of cisplatin in A549/DDP cells decreased markedly during NaHS treatment (800 µmol/l). In addition, our data revealed that NaHS treatment of A549/DDP cells resulted in the induction of apoptosis, cell cycle arrest and inhibition of cell migration and invasion. Finally, we demonstrated that the marked changes in the A549/DDP cell response to NaHS may be triggered by the activation of p53, and overexpression of p21, caspase-3, Bax and MMP-2, as well as the downregulation of Bcl-xL. The findings of the present study provide novel evidence that NaHS administration may represent a new strategy for the treatment of cisplatin-resistant lung cancer.

Introduction

As one of the most prevalent malignancies worldwide, lung cancer is the leading cause of cancer-associated mortality with a 5-year survival rate of only 15% (1,2). Among all lung cancer cases, non-small cell lung cancer (NSCLC) accounts for the most common diagnosed type. Although great progress has been made in NSCLC treatment, cisplatin (DDP), a first-line chemotherapy drug, remains the mainstay of clinical therapy against NSCLC. Unfortunately, during sequential treatment with DDP, its efficacy is often limited due to the development of resistance, which is a major contributor to the relapse and prognosis of NSCLC (3-5). Despite the great efforts in the elucidation of the causes for tumor resistance to DDP, at present, the underlying mechanisms are not clearly understood.

Over the last few decades, due to its importance in a variety of biological functions ranging from physiological and pathophysiological processes, the gasotransmitter H2S has attracted much interest (6-8). Compared with its key role of acting as a vasodilator, neuromodulator and inflammatory signaling mediator, the field of H2S and cancer is a new and expanding research area. Although emerging findings have demonstrated that H2S dysregulation has a significant effect on cell proliferation/cell death, cellular bioenergetic production and cellular redox homeostasis, the role of H2S in cancer development and progression is controversial and paradoxical (9). While many reports show that inhibition of H2S biosynthesis exerts anticancer effects in vitro and in vivo, other studies show that H2S donors of various types exert anticancer actions (10). These inconsistent findings in the field of H2S research indicate that, in different types of human cancers, the underlying mechanisms regulating the delicate balance between the pro-cancer and anticancer effects induced by H2S require further investigation.

In mammalian cells, endogenous H2S is derived primarily from the metabolism of L-cysteine and homocysteine by the catalysis of cystathionine γ-lyase (CSE) and cystathionine β-synthase (CBS). CSE is found predominantly in the cardiovascular system and muscle tissues. In comparison, CBS activity is higher than CSE in the brain, nervous system...
and liver (11,12). In close relation to H₂S generation, it is not surprising that the expression/activity changes in endogenous H₂S-producing enzymes have been found in many cancers, including colon, liver, ovarian, breast, gastric and prostate cancers (13,14). Moreover, recent data have demonstrated that manipulation of H₂S-producing enzymes or administration of H₂S donors can sensitize some types of cancer cells to concomitant chemotherapy (15). These findings imply that there might be a conceivable connection between H₂S and tumor chemoresistance. Given that some laboratory and clinical studies have shown that both CBS and CSE have been simultaneously identified in human lungs (16,17), it is important to assess the effect of H₂S on DDR resistance in NSCLC.

The present study aimed to investigate the role of H₂S in the chemosensitivity of NSCLC cells to cisplatin in vitro. In cisplatin-resistant A549/DDP cells, decreased H₂S production and downregulation of CBS were observed compared with these parameters in A549 cells. The treatment A549/DDP cells with H₂S donor (NaHS) did not only reduce cisplatin resistance through regulation of cell proliferation and apoptosis, but also significantly inhibited the cell migration and invasion capacities. Furthermore, various signaling proteins associated with these key molecular events, including total p53 (t-p53), phosphorylated p53 (p-p53), p21, Bax, Bcl-xl, matrix metalloproteinase-2 (MMP-2) and MMP-9, were detected to explore the potential mechanisms.

Materials and methods

Chemicals and reagents. Sodium hydrogen sulfide (NaHS), a donor of H₂S, was obtained from Sigma Chemical Co. (Merck KGaA, Darmstadt, Germany), stored at 4°C and protected from sunlight. Cisplatin was purchased from Qilu Pharmacy Ltd. Co. (Jinan, Shandong, China). Cy-NO₂ fluorescence probe for H₂S evaluation using a Cy-NO₂ fluorescence probe was performed as previously described (18). Briefly, to compare the intracellular H₂S concentrations under different NaHS treatments in A549/DDP cells, the cells were treated with the indicated doses of NaHS (0, 200, 400, 600, 800 and 1,000 µmol/l) were cultured in RPMI-1640 medium containing 10% FBS at 37°C for 12 h. The medium was then replaced with RPMI-1640 medium containing 10% FBS and the Cy-NO₂ fluorescence probe (10 µmol/l), in which the cells were incubated for 1 h at 37°C. The cells were then harvested and washed twice with ice-cold phosphate-buffered saline (PBS). The fluorescence signal intensity of intracellular H₂S was determined using the FACS FC500 flow cytometer with excitation and emission wavelengths of 755 and 789 nm, respectively. To compare the intracellular H₂S concentrations under different NaHS treatments in A549/DDP cells, the cells treated with the indicated doses of NaHS (0, 200, 400, 600, 800 and 1,000 µmol/l) were cultured in RPMI-1640 medium containing 10% FBS for 24 h at 37°C followed by the aforementioned detection procedures.

Detection of cell viability by MTT assay. To measure the effect of NaHS on the cell viability of A549/DDP cells, the cells were treated with different concentrations of NaHS according to previous publications (19,20). Briefly, the A549/DDP cells were seeded into 96-well flat plates (1.5x10⁴ cells/well) and then treated with or without various concentrations of NaHS (0, 200, 400, 600 and 1,000 µmol/l) for 24 h at 37°C. The group without NaHS treatment was used as a control. Following incubation, 5 mg/ml MTT (10 µl/ml) was added to the media and the cells were further incubated in an atmosphere of 5% CO₂ at 37°C for 4 h. After removal of the supernatant, 100 µl dimethyl sulfoxide was added to determine the OD value at 570 nm using a microtiter plate reader (ELX800; BioTek Instruments, Inc., Winooski, VT, USA). To calculate the percentage of cell viability, the means of the optical density (OD) in the indicated groups in triplicate were used. Cell viability (%) = (OD treatment group/OD control group) x 100%.

Measurement of the chemosensitivity to cisplatin of the A549/DDP cells following NaHS treatment. The half maximal inhibitory concentration (IC₅₀) of the A549/DDP cells was determined by the MTT assay. Briefly, A549/DDP cells with or without NaHS treatment were incubated with the indicated doses of cisplatin (0, 2, 4, 6, 8 and 10 µg/ml) for 24 h followed by the aforementioned detection procedures of the MTT assay. The IC₅₀ value was calculated by nonlinear regression analysis with GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA), using the dose-response with variable slope function. Every step was conducted at least three times.

Analysis of the cell cycle distribution and apoptosis using flow cytometry. For cell cycle analysis, A549/DDP cells were harvested and washed with ice-cold PBS, and then fixed with 70% ethanol (v/v) overnight at -20°C. Fixed cells were washed with ice-cold PBS twice and then resuspended in PBS containing propidium iodide (PI) (50 µg/ml)/RNase A (50 µg/ml) for 10 min. For cell apoptosis analysis, A549/DDP...
cells were double-stained with Annexin V-FITC (5 µg/ml) and PI (5 µg/ml). Finally, both cell cycle and apoptosis were analyzed using a flow cytometer (FACS FC500; Beckman Coulter, Inc., Brea, CA, USA).

Transwell migration and invasion assays. Cell motility was assessed using Transwell chambers (8.0-µm pore size; 6.5-mm diameter insert; Corning Inc., Corning, NY, USA) uncoated (migration assay) or coated (invasion assay) with Matrigel following the manufacturer's instructions. Approximately 2x10^4 A549/DDP cells were seeded into the upper Transwell chambers in 500 µl serum-free medium with or without NaHS (800 µmol/l). The bottom chamber was filled with 10% FBS RPMI-1640 medium which was used as a chemoattractant. After being cultured for 24 or 48 h, cells on the upper side of the inserts were removed and then fixed in 4% paraformaldehyde and finally stained with 0.1% crystal violet solution. For quantification, the migratory and invasive cells were counted in 10 randomly selected fields under a light microscope with x200 magnification. Triplicate experiments were performed with each group, and the means and standard deviations were calculated.

Western blot analyses. A549/DDP cells from a 25 cm^2 flask following the various treatments were washed twice in ice-cold PBS and lysed in 100 µl RIPA lysis buffer on ice. Cell lysates were then centrifuged at 14,000 x g for 20 min at 4°C. The supernatant was recovered and the protein concentration was detected by Coomassie Blue Fast staining solution (Beyotime, Haimen, China). Proteins (20-40 µg) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to polyvinylidene difluoride (PVDF) membranes. After incubation in a blocking buffer (containing 10% non-fat milk and 0.1% Tween-20) for 2 h, the membranes were immunoblotted with specific antibodies overnight with gentle agitation at 4°C. The dilutions for the primary antibodies used are as follows: CBS (1:500), CSE (1:500), t-p53 (1:1,000), p-p53 (1:1,000), p21 (1:1,000), caspase-3 (1:500), Bax (1:500), Bcl-xL (1:500), MMP-2 (1:1,000) and MMP-9 (1:1,000). β-actin (1:500) was used as a control. After being washed for 5 min, the membranes were incubated with secondary antibodies conjugated to horseradish peroxidase (1:3,000) for detection. Finally, images were captured using a FluorChem FC2 gel imaging system (Alpha Innotech, San Leandro, CA, USA).

Statistical analysis. SPSS 19.0 software (IBM SPSS, Inc., Armonk, NY, USA) was used for statistical analysis. Data are expressed as the mean ± SD. The statistical significance of the results between each group was evaluated using one-way ANOVA or t-test. Differences were considered significant at P<0.05. All experiments were repeated at least three times.

Results

Endogenous H_2S production and expression levels of CBS and CSE in A549 and A549/DDP cells. To determine the role of H_2S in NSCLC, using a Cy-NO2 fluorescence probe, we first test whether there are differences in H_2S production between A549 and cisplatin-resistant A549/DDP cells. As shown in Fig. 1A, we found that the production of hydrogen sulfide in the A549/DDP cells was distinctly decreased,
compared with the A549 cells. Then, as the main endogenous H$_2$S-producing enzymes, the expression levels of CSE and CBS were detected in both cell lines. The results showed that the expression of CBS (Fig. 1B) was significantly downregulated in the A549/DDP cells. In contrast, there was no difference in CSE expression between the A549 and A549/DDP cells.

**NaHS inhibits cell viability of the A549/DDP cells.** To test the effect of exogenous H$_2$S on the cell viability of A549/DDP cells, we performed MTT assays using various doses (0, 200, 400, 800 and 1,000 µmol/l) of NaHS (a donor of H$_2$S) for 24 h. The MTT assay showed that NaHS significantly inhibited the viability of the A549/DDP cells in a dose-dependent manner (Fig. 2A). Since the inhibitory effect of H$_2$S on the viability of the A549/DDP cells was most significant following treatment with 800 µmol/l NaHS, this dose of NaHS was used in all subsequent experiments with different treatment. Moreover, the decrease in cell viability was accomplished by increased intracellular H$_2$S after NaHS treatment of A549/DDP cells, which was indicated by enhanced fluorescence signals at 789 nm emission (Fig. 2B).

**Effect of NaHS treatment on cisplatin sensitization of A549/DDP cells.** In order to determine whether NaHS treatment can influence the cisplatin sensitization of A549/DDP cells, the cells were incubated with various concentrations of cisplatin with or without NaHS (800 µmol/l) for 24 h. It was demonstrated that, without NaHS treatment, the IC$_{50}$ of A549/DDP cells for cisplatin was 7.82±0.58 µg/ml. In contrast, when A549/DDP cells treated with NaHS (800 µmol/l) and cisplatin in combination, the IC$_{50}$ decreased to 3.63±0.27 µg/ml (Fig. 3A). These observations indicate that NaHS administration sensitized the A549/DDP cells to cisplatin.

**Pro-apoptotic and antiproliferative effect of NaHS on A549/DDP cells.** To further explore the reasons for the effect of NaHS on the viability of A549/DDP cells, flow cytometric analyses were performed to investigate the differences in cell apoptosis and proliferation with or without NaHS treatment (800 µmol/l) at 12, 24 and 48 h, respectively. As shown in Fig. 3B, compared with the control group, the levels of cell apoptosis in the NaHS-treated groups were gradually increased. After 24 and 48 h of incubation, the apoptosis rates in the NaHS-treated cells were >25.2 and 32.31% with significant changes, respectively. Similarly, in comparison with the control group, the percentage of NaHS-treated cells in the G1 phase was increased (69.98 and 75.97%, respectively) and the percentage in the S stage was decreased (23.15 and 13.94%, respectively) following 24 and 48 h of intervention, and the differences were significant (Fig. 3C).

**Expression levels of apoptosis- and proliferation-associated proteins.** To analyze the molecular changes during the NaHS-induced decrease in cell viability, protein levels of t-p53, p-p53, p21, caspase-3, Bax and Bcl-xL were examined at different times. As shown in Fig. 4A and B, compared with the untreated control group, the levels of t-p53, p-p53, p21, caspase-3 and Bax were considerably increased after NaHS treatment in a time-dependent manner. However, Bcl-xL was markedly downregulated after NaHS incubation (Fig. 4B).

**NaHS inhibits the migration and invasion of A549/DDP cells.** To evaluate the contribution of NaHS to cell migration and invasion, we added NaHS to the upper inserts of Transwell chambers. The results showed that, at various time points (24 and 48 h), the numbers of cells that infiltrated the membrane was significantly reduced after NaHS treatment, indicating the inhibitory effects of NaHS on the migration and invasion of A549/DDP cells (Fig. 5). Moreover, MMP-2 and MMP-9 expression was assessed to further determine the mechanisms involved in the reduced cell migration and invasion following NaHS treatment. As shown in Fig. 6, MMP-2 expression was significantly attenuated following 800 µmol/l NaHS treatment, but there was no significant effect of NaHS observed at the MMP-9 level.
Figure 3. NaHS treatment influences cisplatin sensitization, apoptosis and proliferation of A549/DDP cells. (A) A549/DDP cells were co‑treated with NaHS (800 µmol/l) and serially diluted cisplatin (0, 2, 4, 6, 8 and 10 µg/ml) for 24 h, resulting in a significant decrease in the half maximal inhibitory concentration (IC₅₀) of cisplatin. Data are presented as the mean ± SD; *P<0.05, **P<0.01, NaHS‑treated group vs. NaHS‑untreated group. (B) The apoptosis of A549/DDP cells was significantly increased following treatment of NaHS (800 µmol/l). (C) Treatment of NaHS (800 µmol/l) induced G1/S cell cycle phase arrest of the A549/DDP cells.

Figure 4. Expression levels of apoptosis‑ and proliferation‑associated proteins were detected following NaHS treatment (800 µmol/l) at indicated times (12, 24 and 48 h). (A) NaHS treatment enhanced the expression level of p‑p53, t‑p53 and p21. (B) NaHS treatment upregulated the expression level of caspase‑3 and Bax, and decreased the expression level of Bcl‑xL in the A549/DDP cells. The histograms (right panels) represent the quantitative data of the detected proteins normalized to β‑actin. The group without NaHS treatment was used as a control. Data are expressed as the mean ± SD.
Discussion

As a great challenge in the treatment of lung cancer, drug resistance has become increasingly severe. Thus, it is urgent to elucidate the underlying mechanisms so as to improve the efficacy of chemotherapy drugs, such as cisplatin (DDP) (21). Research over the past few decades has identified that the cause of DDP resistance involves multiple factors, such as drug transport and metabolism, DNA repair, cell survival and apoptosis (22). Although numerous studies have focused on investigations to elucidate the roles of H$_2$S in different types of human cancers in vitro and in vivo, literature concerning the effect of H$_2$S on cisplatin resistance in cancer is limited. Based on the fact that H$_2$S exerts an important role in most biological processes, it can be anticipated that H$_2$S may also contribute to the induction of DDP resistance.

To test our hypothesis, we first detected the concentration of endogenous H$_2$S with a fluorescent probe in A549 and A549/DDP cell lines. It was found that the production of H$_2$S was definitely elevated in A549 cells when compared with the production in A549/DDP cells. Based on these findings, we speculated that the difference in the H$_2$S pathway between A549 and A549/DDP cells may contribute to, at least in part, their phenotypic variance, such as resistance to DDP. To this end, we first observed...
the effect of NaHS, an exogenous H$_2$S donor, at different concentrations on A549/DDP cell viability. MTT assay revealed that the doses of NaHS from 200 to 1,000 µmol/l markedly inhibited cell proliferation, leading to a decrease in cell viability which reached a peak at 800 µmol/l. These observations were confirmed by the observation of apoptosis induction and cell cycle arrest. Taking into account that normal cellular homeostasis is maintained through a balance between the processes of cell proliferation and cell death (e.g. apoptosis), it is reasonable to believe that changes in cell proliferation and/or apoptosis after the treatment of H$_2$S may influence the proportion of surviving cells in the A549/DDP cell population, namely, cell viability. Moreover, our results demonstrated that the significant inhibitory effect was likely related to the increased cisplatin efficacy of A549/DDP cells due to the finding that 800 µmol/l NaHS treatment shifted the IC$_{50}$ of cisplatin from 7.82 to 3.63 µg/ml. In addition, the strong influence of NaHS on the inhibition of migration and invasion abilities of A549/DDP cells was also observed in the present study.

Consistent with our data, previous studies have shown the anticancer activity of H$_2$S in a range of cancers through the role of this gas in triggering cell death and/or inhibiting cell proliferation (23-25). In addition, several investigations have provided evidence that H$_2$S is involved in the efficacy of cancer radiotherapy and chemotherapy. In cultured MDA-MB-231 cells, De Preter et al. found that tumor cells after NaHS administration were more sensitive to irradiation compared with those that received irradiation alone, implying that NaHS may act as a potential radiosensitizer in some solid cancers (26). In contrast, Bhattacharyya and colleagues found high expression of CBS in ovarian tumor samples and inhibition of CBS sensitized several ovarian cancer cell lines to cisplatin (15). Similarly, in lung adenocarcinoma, a recent study reported that a decrease in H$_2$S biosynthesis through inhibition of H$_2$S-producing enzymes can sensitize certain lung cancer cell lines to chemotherapeutic agents in vitro and in vivo (16). These apparent discrepancies might be attributed to several factors which should be a concern in further investigations. Above all, cellular H$_2$S can be metabolized through two pathways, known as enzymatic (endogenous) and nonenzymatic (exogenous) processes. Since an increasing amount of evidence suggests that exogenously administered and/or endogenously produced H$_2$S could exhibit two obviously opposite functions on the growth of cancer cells, there may be a delicate balance between the pro-cancer and anticancer effects induced by H$_2$S (20,27). Therefore, it can be anticipated that the net effects of H$_2$S on cell characteristics are the result of the balance of both pathways. The bell-shaped pharmacology of H$_2$S, whereby lower (endogenous) H$_2$S production tends to promote, while higher (generated from exogenously added H$_2$S donors) tends to inhibit cancer cell proliferation, should also be kept in mind (28). These observations imply that the paradoxical actions of H$_2$S in cancer can also result from the different production of this gasotransmitter. Accordingly, the source and dosage of H$_2$S should be determined for future research to make the data more consistent and explicit.

It has been well recognized that the anticancer activity of cisplatin is largely depended on its ability to increase DNA damage and cell apoptosis, both of which can be controlled by p53 (29-31). Therefore, to provide further insight into the potential mechanisms involved in the effect of H$_2$S on the biological behaviors of A549/DDP cells, we then focused our investigation on p53 pathway members associated with these key molecular events, such as cell cycle, apoptosis and migration. Research demonstrated that p53 is not only a key player in carcinogenesis, but is also associated with resistance to established cytotoxic anticancer drugs, including cisplatin (32,33). In the present study, the enhancement of total p53 and p-p53 in A549/DDP cells after NaHS treatment was consistent with previous reports that restoring p53 apoptotic function in advanced cancer through modulation its expression and activation is often essential for sensitivity toward chemotherapeutic drugs (34,35). Analyses of several downstream effectors of p53 offer more detailed clues to the antitumor potential of NaHS. On the one hand, upregulation of p21 after NaHS exposure indicated that fewer A549/DDP cells can proceed through the G1 checkpoint to S phase because of cell cycle arrest controlled by p21. On the other hand, cell death was promoted by NaHS treatment through changes in apoptosis-related proteins, such as anincreasein-caspase-3 and an increase in the Bax/Bcl-xL ratio. Metastasis is another fatal characteristic of malignant tumors, especially for advanced or late stage cancer. The association of MMPs with cancer metastasis has raised considerable interest due to their ability to cleave the extracellular matrix (ECM) allowing cancer cells to invade adjacent tissues or spread to distant organs. Among the known MMPs, MMP-2 and MMP-9 have been thought to be key enzymes due to their capacity to degrade gelatin as well as type IV collagen, the central component of the basement membrane. Recent in vitro studies using diverse cancer cell lines showed that the inhibitory effects of NaHS or H$_2$S on cell invasion maybe through the downregulation of MMP-2 (36). In line with these previous findings, A549/DDP cells treated with 800 µmol/l NaHS exhibited diminished MMP-2 expression, implying the involvement of MMPs in the participation of invasion and migration of A549/DDP cells.

It should be noted that the present study has some limitations which should be considered for future assessments. Firstly, it is well-known that the development of drug resistance in cancer is complicated and heterogeneous. The present study only indicated that there may be a relation between H$_2$S production and the cisplatin-resistant phenotype in A549/DDP cells. To determine whether there is a causal association between H$_2$S and the development of cisplatin resistance in A549/DDP cells, further intensive studies should be performed (37). Secondly, although the study found that p53 and its downstream signaling members are involved in the effect of NaHS on A549/DDP cells, the detailed mechanism(s) of p53 regulation were not demonstrated. The crosstalk network that exists between p38 MAPK and p53 in NaHS-induced apoptosis of glioma cells may provide a valuable clue to elucidate the reasons for p53 activation under NaHS challenge (19). Finally, further investigations are warranted in order to extend these findings to animal in vivo studies and clinical sample detections.

In summary, this study demonstrated that NaHS exposure can increase the efficacy of cisplatin in A549/DDP cells. The
anticancer effect of NaHS treatment was evidenced by inhibition of proliferation, induction of apoptosis and suppression of invasion. These phenotypic changes in cell functions may be mediated by the activation of p53, which in turn may alter the expression of downstream targets, such as p21, caspase-3, Bax, Bcl-xL and MMP-2. These findings imply that NaHS administration may be a potential therapeutic strategy for the treatment of NSCLC with cisplatin resistance.

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Availability of data and materials

Not applicable.

Authors’ contributions

YM and FJ conceived and designed the study. YM, ZHY, XMD, JQG, JXH and YY performed the experiments. ZHY and FJ wrote the paper. YM, ZHY, YY and FJ reviewed and edited the manuscript. All authors read and approved the manuscript.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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