Inhibition of invasion and epithelial-mesenchymal transition of human breast cancer cells by hydrogen sulfide through decreased phospho-p38 expression

MIN LV1*, YAN LI2*, MING-HUA JI3, MING ZHUANG2 and JIN-HAI TANG4

1Department of Scientific Research, Nanjing Medical University Affiliated Cancer Hospital, Cancer Institute of Jiangsu Province, Nanjing, Jiangsu 210009; 2Department of Oncology, Lianyang First People’s Hospital, Lianyangang, Jiangsu 222002; Departments of 3Radiotherapy and 4General Surgery, Nanjing Medical University Affiliated Cancer Hospital, Cancer Institute of Jiangsu Province, Nanjing, Jiangsu 210009, P.R. China

Received July 29, 2013; Accepted January 21, 2014

doi: 10.3892/mmr.2014.2161

Abstract. Sodium hydrosulfide (NaHS) is an exogenous hydrogen sulfide (H2S)-releasing molecule and has antitumor potential against a wide variety of human cancer types. The effect of exogenous H2S on the invasion of breast cancer and the possible underlying mechanisms remain unknown. The present study aimed to investigate the in vitro effects of H2S on transforming growth factor-β1 (TGF-β1)-induced human breast cancer cells and the associated mechanisms. MCF-7 cells were incubated with TGF-β1 to induce epithelial-mesenchymal transition (EMT) and an MTT assay was performed to detect cell viability. Flow cytometry, using propidium iodide (PI) staining, was used to determine the stages of the cell cycle. Apoptosis was detected with Annexin V-fluorescein isothiocyanate and PI double staining. Western blotting was performed to detect the protein expression of cystathionine γ-lyase (CSE, an endogenous H2S producer), phospho-p38 (a signaling protein associated with apoptosis), and SNAI1 (Snail, associated with the induction of EMT). A Boyden chamber invasion assay was performed to detect tumor invasion. The results demonstrated that when NaHS was administered to TGF-β1-treated MCF-7 cells, the cells exhibited decreased proliferation, G0/G1 phase cell cycle arrest and increased apoptosis. NaHS treatment following TGF-β1 administration also resulted in decreased cell invasion and decreased EMT, which was indicated by decreased Snail protein expression. In addition, incubation with NaHS increased endogenous CSE protein expression and decreased p38 mitogen-activated protein kinase phosphorylation in MCF-7 cells stimulated by TGF-β1. Furthermore, the inhibition of endogenous CSE by DL-propargylglycine increased EMT in the MCF-7 cells treated with NaHS and TGF-β1. In conclusion, the present study provides insights into a novel anticancer effect of H2S on breast cancer cells through activation of the CSE/H2S pathway and decreased expression of phospho-p38.

Introduction

Hydrogen sulfide (H2S) gas exhibits numerous physiological and pathological effects. H2S is endogenously produced from L-cysteine by cystathionine γ-lyase (CSE) and cystathionine β-synthase in mammalian tissues, and endogenous hydrogen sulfide exists in the sodium hydrosulfide (NaHS, 2H2S) and H2S (1/3) forms (1). H2S has been demonstrated to exert a therapeutic effect in a wide range of diseases, including neuronal injury (2), hypertension (3), myocardial infarction (4) and hypoxic pulmonary hypertension (5). In addition, H2S exhibits antigrowth potential against a wide variety of human cancer cells (6). In breast cancer cells, H2S decreased the in vivo tumor mass through the inhibition of cellular proliferation, induction of apoptosis and decrease in nuclear factor-κB (NF-κB) levels (7). Furthermore, H2S has been found to inhibit breast cancer-induced osteoclast formation and activity, suppress osteolastogenesis and prevent osteolysis (8). As bone metastases are common in breast cancer, H2S may have inhibitory effects on breast cancer cell invasion and metastasis, thereby suppressing osteoclastogenesis and osteolysis. However, there are few studies concerning the anti-invasive effect of H2S on cancer cells, including breast cancer cells.

The invasion of breast cancer is a multi-stage process that involves abnormal signaling by transforming growth factor-β (TGF-β). TGF-β acts as a tumor suppressor in the early stages of carcinogenesis, but in late-stage breast cancer it promotes invasion and metastatic dissemination (9). Moreover, TGF-β acquires a proinvasive effect in the advanced stages of breast cancer.
through a complex process known as epithelial-mesenchymal transition (EMT) (10). H₂S has been found to attenuate EMT in human alveolar epithelial cells (11). Moreover, H₂S has been reported to inhibit the pathogenesis of pulmonary and hepatic fibrosis, and suppress the migration of human lung fibroblasts, all of which involve the EMT process (12-14). Therefore, it was hypothesized that H₂S may act as a suppressor of EMT in breast cancer and exhibit antitumor and anti-invasive effects.

In the present study, breast cancer cells were incubated with TGF-β1 to induce an EMT phenotype. The effects of NaHS, an H₂S-releasing molecule, on cell viability, cell cycle, apoptosis, invasion and EMT were investigated. The protein expression of CSE and phospho-p38 in breast cancer cells treated with TGF-β1 was also analyzed.

Materials and methods

Cell culture. MCF-7 human breast cancer cells were purchased from the cell bank of the Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). The cells were cultured with high glucose Dulbecco's modified Eagle's medium (Invitrogen Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Sijichun Biotechnology Materials Inc., Hangzhou, China), 100 U/ml penicillin and 100 mg/ml streptomycin at 37°C in a humidified 5% CO₂ incubator. After cultured cells reached 70% confluence, the cells underwent trypsinization and were subcultured with a 1:3 split ratio in new culture flasks. DL-propargylglycine (PPG, Sigma, St. Louis, MO, USA), an inhibitor of CSE, was used to inhibit endogenous H₂S in MCF-7 cells.

Cell viability assay. An MTT (Sigma Chemical Co., St. Louis, MO, USA) assay was performed to determine cell viability. Briefly, cells in suspension at the logarithmic growth phase were added to each well of 96-well culture plates at a density of 1x10⁴ cells/ml, with 100 µl cell suspension in each well. The cells were incubated for 24 h at 37°C in a humidified atmosphere with 5% CO₂. Then, the cells were further cultured with NaHS (500 µmol/l) or TGF-β1 (100 ng/ml) or recombinant human TGF-β1 (100 ng/ml, R&D Systems Inc., Minneapolis, MN, USA) with different concentrations of NaHS (0, 100, 200 and 500 µmol/l; Sigma Chemical Co.). Following treatment for 12, 24, 48 and 72 h, 10 µl of 5 mg/ml MTT solution was added to each well and the plates were incubated at 37°C for 4 h. Following centrifugation at 1,400 x g for 10 min, the formazan pellets were isolated by discarding the supernatant and then dissolved completely in 100 µl dimethyl sulfoxide (DMSO), agitating the plates for 10 min. The optical density (OD) at 570 nm wavelength was measured using an ELISA plate reader (Rico RK201; Shenzhen Rico Technology Co., Ltd., Shenzhen, China) to determine the quantity of pellet.

Cell cycle analysis. MCF-7 cells in the logarithmic growth phase were cultured in serum-free medium for 24 h. They were then incubated with TGF-β1 (100 ng/ml) and/or 500 µmol/l NaHS for 24 h. The cells were harvested by trypsinization and following washes with cold phosphate-buffered saline (PBS), the cells were fixed in cold 70% ethanol. Finally, 1 µl propidium iodide (PI) staining solution (containing 20 mg/ml PI and 1 mg/ml RNase) was added to the samples. The distribution of cells in each phase of the cell cycle was measured and analyzed by flow cytometry (FACScan; Becton-Dickinson, San Francisco, CA, USA). The percentage of cells in the G0/G1, S and G2/M phases were calculated. Results were acquired from 10,000 cells.

Apoptosis assay. MCF-7 cells were randomly divided into four groups: Control, NaHS (500 µmol/l), TGF-β1 (100 ng/ml) and TGF-β1+NaHS. Following incubation for 24 h, at least 2x10⁶ cells were harvested from each group for the apoptosis assay. Subsequent to centrifugation at 626 x g for 5 min and washing with PBS buffer, the pellet was resuspended in 100 µl of 1X binding buffer and incubated with 2.5 µl Annexin V and 5 µl PI (at a final concentration of 10 µg/ml). After incubation in the dark for 30 min, apoptosis was immediately determined by FACScan flow cytometry and the associated data were analyzed using Lysis II software (Becton Dickinson). At least 10,000 events were analyzed for each sample.

Cell migration assay. In vitro invasion capability of MCF-7 cells was measured by Boyden chamber assay with matrigel (BD Bioscience, Bedford, MA, USA) in 24-well tissue culture plates with Transwell® filter membranes (5 µm pore; Costar, Boston, MA, USA). The lower sides of the filters were coated with type I collagen (0.5 mg/ml) and the lower section of the filter contained low-serum media. In each well, 5x10⁴ cells were resuspended in 100 µl DMEM media and seeded in the upper part of a Transwell® plate. The cells were then incubated with NaHS (500 µmol/l) and/or TGF-β1 (100 ng/ml) for 24 h. Subsequent to the removal of the cells on the upper surface of the filter, the cells that had migrated to the lower part were stained with hematoxylin and eosin (Sigma Chemical Co.) and counted under an inverted light microscope (Olympus IX70; Olympus Optical Co., Ltd., Tokyo, Japan; magnification, x200) as the number of migrated cells (invasion index). Each sample was analyzed in triplicate and repeated twice.

Western blot analysis. MCF-7 cells were cultured with NaHS (500 µmol/l) and/or TGF-β1 (100 ng/ml) for 24 h. The cell proteins were then extracted and a bicinchoninic acid protein concentration assay kit (Beijing Biosea Biotechnology Co., Ltd., Beijing, China) was used to determine their concentrations. The cell lysates (50 µg) were resolved in 15% sodium dodecyl sulfate-polyacylamide gels and electrophoretically transferred to a polyvinylidene difluoride membrane (Bio-Rad, Hercules, CA, USA). The membrane was blocked with 5% skimmed milk powder in Tris-buffered saline containing 0.05% Tween-20 at room temperature for 2 h. It was then incubated with primary mouse antibodies against SNAI1 (Snail) protein, CSE or phospho-p38 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), followed by horseradish peroxidase-conjugated rabbit anti-mouse secondary antibody (Santa Cruz Biotechnology, Inc.) at 1:1,000 dilutions for 2 h at room temperature. Enhanced chemiluminescence (Pierce® ECL Plus Western Blotting Substrate; Pierce Biotechnology, Inc., Rockford, IL, USA) was used to visualize the protein blots and β-actin served as an internal control.

Measurement of H₂S concentration in cell culture media. To measure the concentration of H₂S, 500 ml culture media from
each group was mixed with 425 ml distilled water in a microtube containing zinc acetate (1% w/v; 250 ml). N,N-dimethyl-p-phenylenediamine sulphate (20 mM; 133 ml) in 7.2 M HCl was subsequently added, followed by FeCl\(_3\) (30 mM; 133 ml) in 1.2 M HCl. Trichloroacetic acid (10% w/v; 250 ml) was then used to precipitate any protein. The OD value of the resulting solution was measured using a 96-well microplate reader at 670 nm wavelength (Tecan Group Ltd., Männedorf, Switzerland).

Statistical analysis. All quantitative data are presented as the mean ± standard deviation. SPSS version 14.0 (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis. The statistical significance of difference between two groups was determined by Student's t-test (unpaired, two tailed) and P<0.05 was considered to indicate a statistically significant difference.

Results

NaHS inhibits MCF-7 cell growth induced by TGF-β1. The MCF-7 cells were incubated with TGF-β (100 ng/ml), then treated with various concentrations of NaHS (0, 100, 200 and 500 µmol/l) for 12, 24, 48 and 72 h. MCF-7 cells treated with PBS served as a control. The MTT assay revealed that compared with the control, TGF-β treatment promoted cell proliferation and increased cell viability in a time-dependent manner. However, NaHS inhibited cell viability in the MCF-7 cells treated with TGF-β in a dose- and time-dependent manner. NaHS exhibited the most potent effect on cell viability at a 500 µmol/l concentration at all time-points (Fig. 1A). Therefore, in subsequent experiments, a concentration of 500 µmol/l was used for NaHS treatment of the cells.

Cell cycle distribution was analyzed by flow cytometry to investigate the detailed mechanism of the antiproliferative activity of NaHS. The MCF-7 cells were treated with PBS, NaHS, TGF-β1 and TGF-β1+NaHS groups. Annexin V-fluorescein isothiocyanate (FITC) and PI double staining was performed to detect cell apoptosis using flow cytometry 24 h following treatment. NaHS treatment increased the apoptotic rate in MCF-7 cells regardless of whether they were treated with TGF-β1. Representative images from three experiments are shown. (D) Apoptotic rates from the control, NaHS, TGF-β1 and TGF-β1+NaHS groups. The Annexin V\(^{-}\)/PI\(^{-}\) and Annexin V\(^{+}\)/PI\(^{-}\) populations were considered to represent apoptotic cells. A two-tailed, unpaired t-test was performed to compare the differences between two groups. Data are presented as the mean ± standard deviation. *P<0.05, vs. the control group (Con). #P<0.05, vs. the TGF-β1 group.
NaHS inhibited cell proliferation by inducing G0/G1 phase arrest in MCF-7 cells treated with TGF-β1.

To investigate whether a decrease in cell viability was a result of the proapoptotic effect of NaHS, an apoptosis assay was performed in MCF-7 cells by double staining with Annexin V-fluorescein isothiocyanate and PI. It was found that TGF-β1 treatment alone did not change the apoptotic rate of MCF-7 cells. However, treatment with NaHS increased the apoptotic rate of these cells, indicating that NaHS may enhance sensitivity to TGF-β1.

**NaHS decreases cell invasion and EMT induced by TGF-β1.**

To investigate whether NaHS can inhibit the migration of breast cancer cells, the invasive capability of MCF-7 cells was determined by a Boyden chamber invasion assay. The cells were incubated with TGF-β1 to induce an invasive state, and the results revealed that TGF-β1 significantly increased the invasion index of MCF-7 cells (P<0.05). NaHS treatment following TGF-β administration significantly decreased the invasion index of TGF-β1-induced MCF-7 cells (P<0.05, Fig. 2A). However, compared with control cells, NaHS treatment alone made no significant difference to the invasion index.

NaHS increases CSE protein expression and supernatant H2S levels. The expression of CSE protein in TGF-β1-induced MCF-7 cells was significantly increased by NaHS treatment. Moreover,
Discussion

In the present study, NaHS (a bioactive compound releasing H$_2$S) exhibited anticancer effects in TGF-β1-treated MCF-7 breast cancer cells, demonstrated by the inhibition of cell proliferation, cell cycle arrest in G0/G1 phase and induction of apoptosis. NaHS treatment also inhibited tumor invasion and decreased protein expression of an EMT marker, Snail. The underlying mechanisms may be associated with increased endogenous CSE protein expression and decreased p38 MAPK phosphorylation in MCF-7 cells stimulated by TGF-β1 following NaHS administration.

The anticancer effects of exogenous H$_2$S on breast cancer cells indicate a novel therapeutic strategy for breast cancer. NaHS was revealed to reduce cell viability in a dose- and time-dependent manner, and the detailed mechanism lies in G0/G1 cell cycle arrest and induction of apoptosis. These results are in accordance with another study, which revealed that H$_2$S-releasing aspirin decreased tumor mass through inhibition of cell proliferation and induction of G0/G1 arrest in estrogen receptor-negative breast cancer cells (7). In the present study, NaHS, which is an exogenous H$_2$S-releasing molecule and one form of H$_2$S in mammalian tissues, was used, therefore providing additional evidence regarding the anticancer effects of H$_2$S. In a variety of pathological conditions, H$_2$S has been found to exhibit potent antia apoptotic effects including in hypoxia-induced mouse hippocampal neurons (15), high-glucose-induced rat cardiomyocytes (16) and hepatic ischemia/reperfusion injury (17). However, in the present study, NaHS was found to exhibit a potent apoptotic effect on breast cancer cells, particularly in cells treated with TGF-β1. This contradiction may be a result of differences in the reaction to H$_2$S between non-tumor cells and tumor cells as H$_2$S has also been demonstrated to mediate the antisu survival effect of sulforaphane in human prostate cancer cells (18). Therefore, there may be benefits in introducing H$_2$S-releasing therapeutic agents in the treatment of cancer.

In the present study, H$_2$S was found to have an inhibitory effect on invasion and EMT in breast cancer cells. EMT is a complex, multi-step process that involves epithelial cells developing a malignant phenotype, including invasive, migratory and metastatic capabilities (19). MCF-7 cells were incubated with TGF-β1 to induce the EMT state, significantly increasing the invasion index of the cells and increasing the protein expression of an EMT marker, Snail. NaHS was revealed to significantly decrease this invasion index and the expression of Snail protein in the MCF-7 cells induced by TGF-β1. This finding is in accordance with another study, which observed that H$_2$S inhibited EMT in human alveolar epithelial cells, demonstrated by decreased vimentin expression and increased E-cadherin expression (11). EMT is a vital process in driving epithelial cells to acquire a malignant phenotype and invasive properties (20). The results of the present study indicate that NaHS suppressed invasion through inhibition of the EMT process in MCF-7 cells. It was also found that tumor invasion and Snail protein expression remained unchanged following NaHS treatment in control MCF-7 cells without TGF-β1. This indicates that compared with breast cancer cells without metastasis, NaHS may demonstrate an enhanced effect on the metastasis and EMT of breast cancer cells, thereby providing further evidence for the causal link between EMT and invasion in TGF-β1-treated MCF-7 cells.

In the present study, NaHS increased CSE protein expression and supernatant H$_2$S levels. H$_2$S can be generated by CSE with L-cysteine as its substrate. Increased expression of CSE protein following NaHS administration indicates that the CSE protein level was decreased following TGF-β1 treatment alone (Fig. 3A and B). To investigate whether an enhanced CSE protein level produces a greater level of endogenous H$_2$S, the H$_2$S levels in the cell culture media were measured. The H$_2$S level in the cell culture media in cells treated with 100 ng/ml TGF-β1 was significantly decreased in comparison with the control cells (P < 0.05). When compared with the TGF-β1-treated cells, the cells treated with NaHS had a significantly higher H$_2$S level at all concentrations of NaHS (P < 0.05, Fig. 3C). These results indicate that the anticancer effect of NaHS may be mediated by activation of the CSE/H$_2$S pathway.

NaHS decreases p38 mitogen-activated protein kinase (MAPK) phosphorylation in MCF-7 cells stimulated by TGF-β1. To investigate the underlying signaling pathways in NaHS-treated MCF-7 cells, the expression of phospho-p38 MAPK, a signaling protein associated with apoptosis and EMT, was investigated. The MCF-7 cells were pretreated with PPG (an inhibitor of CSE, 100 µmol/l) for 1 h, then administered either TGF-β1 (100 ng/ml), TGF-β1+NaHS (500 µmol/l) or TGF-β1+NaHS+PPG, and incubated for 24 h. Western blot analysis revealed that TGF-β1 significantly increased phospho-p38 protein expression. However, NaHS significantly decreased the phospho-p38 protein levels in MCF-7 cells treated with TGF-β1, which was attenuated by PPG pretreatment (Fig. 4).
Anticancer effect of NaHS not only depend on direct change from NaHS to H₂S in extracellular fluid, but also may be mediated by activation of the CSE/H₂S pathway, thereby acquiring a more durable effect. The elevated H₂S level in the culture media provides further evidence that the CSE/H₂S pathway instigates the anticancer effects of NaHS. In fact, a number of H₂S-releasing drugs may produce their effects through activation of the CSE/H₂S pathway. For example, one H₂S donor, S-propargyl-cysteine, exhibits its anticancer effect by increasing CSE protein expression in gastric cancer cells (21).

In order to investigate the detailed mechanisms underlying the inhibitory effect of H₂S on invasion and EMT in TGF-β1-treated MCF-7 cells, the expression of a signal protein associated with apoptosis and invasion was measured. TGF-β1 treatment alone increased phospho-p38 protein expression, but the addition of NaHS decreased the expression of phospho-p38 in TGF-β1-treated cells. A previous study found that p38 MAPK promotes successful invasion and metastasis in tumor cells (22). p38 MAPK activation has also been demonstrated to mediate TGF-β1-induced EMT in A549 alveolar epithelial cells (23), which is in accordance with the results of the present study. The inhibition of p38 MAPK has been found to reverse the EMT process and may be a potential therapeutic strategy to decrease cancer invasion (24). Therefore, decreased phospho-p38 may be an underlying mechanism for the inhibition of EMT by NaHS. To further confirm the inhibition of phospho-p38 by the CSE/H₂S pathway, MCF-7 cells were pretreated with PPG (an inhibitor of CSE) then incubated with TGF-β1 and NaHS. The decrease in phospho-p38 expression in TGF-β1-treated MCF-7 cells following administration of NaHS was significantly attenuated by PPG. These results suggest a novel mechanism for the anticancer effects of exogenous H₂S via CSE/H₂S-induced inhibition of cell growth, induction of apoptosis, and the inhibition of invasion and EMT.

The detailed association between p38 MAPK and EMT remains unknown. One study demonstrated that the p38/NF-κB/Snail pathway was involved in the caffeic acid-induced inhibition of the migratory capacity of malignant human keratinocytes (25). This suggests that inhibition of NF-κB may mediate the causal association between decreased phospho-p38 expression and reduced EMT by NaHS, which warrants further investigation. The anticancer effect of NaHS and the potential CSE/H₂S pathway also require further verification in animal models.

In conclusion, the results of the present study demonstrate that an H₂S donor, NaHS, exhibits anticancer effects on breast cancer cells, as evidenced by inhibition of proliferation, induction of apoptosis, and the inhibition of invasion and EMT. The underlying mechanisms of the NaHS anticancer effect may be through activation of the CSE/H₂S pathway and decreased phospho-p38. These results suggest that exogenous H₂S may be a potential therapeutic strategy for breast cancer.

References