Exogenous hydrogen sulfide exerts proliferation, anti-apoptosis, migration effects and accelerates cell cycle progression in multiple myeloma cells via activating the Akt pathway

DONG ZHENG1*, ZIANG CHEN1*, JINGFU CHEN2, XIAOMIN ZHUANG3, JIANQIANG FENG4 and JUAN LI1

Departments of 1Hematology, 2Cardiovasology and Cardiac Care Unit (CCU), Huangpu Division, 3Endocrinology, The First Affiliated Hospital, Sun Yat-sen University; 4Department of Physiology, Zhongshan School of Medicine, Sun Yat-sen University, Guangzhou, Guangdong 510080, P.R. China

Received January 16, 2016; Accepted February 24, 2016

DOI: 10.3892/or.2016.5014

Abstract. Hydrogen sulfide (H2S), regarded as the third gaseous transmitter, mediates and induces various biological effects. The present study investigated the effects of H2S on multiple myeloma cell progression via amplifying the activation of Akt pathway in multiple myeloma cells. The level of H2S produced in multiple myeloma (MM) patients and healthy subjects was measured using enzyme-linked immunosorbent assay (ELISA). MM cells were treated with 500 µmol/l NaHS (a donor of H2S) for 24 h. The expression levels of phosphorylated-Akt (p-Akt), Bcl-2 and caspase-3 were measured by western blot assay. Cell viability was detected by Cell Counting Kit 8 (CCK-8). The cell cycle was analyzed by flow cytometry. Our results show that the concentration of H2S was higher in MM patients and that it increased in parallel with disease progression. Treating MM cells with 500 µmol/l NaHS for 24 h markedly increased the expression level of Bcl-2 and the activation of p-Akt, however, the expression level of caspase-3 was decreased, cell viability was increased, and cell cycle progression was accelerated in MM cells. NaHS also induced migration in MM cells in transwell migration assay. Furthermore, co-treatment of MM cells with 500 µmol/l NaHS and 50 µmol/l LY294002 for 24 h significantly overset these effects. In conclusion, our findings demonstrate that the Akt pathway contributes to NaHS-induced cell proliferation, migration and acceleration of cell cycle progression in MM cells.

Introduction

Multiple myeloma (MM) is a malignant disorder that is characterized by the proliferation of a single clone of plasma cells, which are derived from B cells in the bone marrow (1). The incidence of MM varies globally from 1 per 100,000 people in China to approximately 4 per 100,000 people in most developed countries (2). Advances in therapies for MM, such as high-dose therapy followed by autologous stem cell transplantation (ASCT), have been shown to improve response rates, event-free survival, and overall survival (3). However, the molecular mechanisms by which the myeloma microenvironment influences myeloma cell survival and its responsiveness to therapy remain unclear. Previous investigations have demonstrated alterations in the phosphatidylinositol 3-kinase (PI3K)/Akt signaling cascades in MM cells and implicated the pathway in clonal expansion (4,5).

Akt is a serine (Ser)/threonine (Thr) protein kinase that resides within the cytosol in a catalytically inactive state in quiescent or serum-starved cells (6). Activated Akt phosphorylates downstream target molecules, including Bcl-2, caspase-9, and Bad, which promote induction of its anti-apoptotic effects (5). The PI3K/Akt pathway is one of many signaling pathways that play oncogenic roles in a wide spectrum of human cancers (7). Several studies have strongly suggested that Akt signaling mediates MM cell resistance to conventional therapeutics, and biologically based treatments targeting Akt may induce anti-MM activity in the bone marrow microenvironment (8). However, the specific role of the activation of Akt pathway in the oncogenic processes involved in multiple myeloma is not completely understood.

Hydrogen sulfide (H2S), a toxic gas that smells like rotten eggs, forms with nitric oxide (NO) and carbon monoxide (CO) a group of biologically active gases that are termed gasotransmitters or gasomediators (9,10). H2S is endogenously generated from L-cysteine by pyridoxal-5'-phosphate-dependent enzymes, including cystathionine β-synthase (CBS) and/or cystathionine γ-lyase (CSE), in mammalian cells (11). Work over the last decade has recognized the importance of endogenously produced H2S in a variety of biological functions in the nervous, cardiovascular, and immune systems (10,12).
There is currently no information available regarding the effect of exogenous H$_2$S on multiple myeloma or its related mechanisms. The present study is aimed at investigating whether H$_2$S contributes to cancer progress and at exploring whether its effects involve the amplification of the Akt pathway in multiple myeloma cells.

**Materials and methods**

**Patients.** Twenty MM patients (11 males and 9 females) were included in this study. Their median age was 57 years (range: 37-70 years). According to international staging system (ISS), 4 were classified as stage I, 7 as stage II and 9 as stage III. Concerning the types of monoclonal proteins present in the patients, 12 had IgG, 5 had IgA and 3 had light chain disease. Fifteen age- and gender-matched healthy subjects were the control group. This work was performed in accordance with the guidelines of the Declaration of Helsinki. This study was cleared by our Institutional Ethics Review Board for human studies, and all subjects signed an informed consent document. After informed consent was provided, peripheral venous blood was collected in sterile tubes using EDTA as anticoagulant and centrifuged at 1000 g for 10 min within 30 min of collection. Plasma was extracted in the supernatant and stored at -70°C for H$_2$S determination.

**Reagents.** Sodium hydrosulfide (NaHS) and LY294002 were obtained from Sigma-Aldrich (St. Louis, MO, USA). Freshly made NaHS solution was used as the H$_2$S donor. The Cell Counting Kit 8 (CCK-8) was supplied by Dojindo Laboratories (Kumamoto, Japan). Fetal bovine serum (FBS) and DMEM medium were obtained from Gibco BRL (Grand Island, NY, USA). Anti- phosphorylated-Akt (p-Akt) antibody, and Anti-total Akt (t-Akt) antibody, anti-Bcl-2 antibody and anti-caspase-3 antibody were purchased from Cell Signaling Laboratories (Danvers, MA, USA).

**Cell culture.** The human myeloma cell line NCI-H929 was supplied by the Sun Yat-sen University Cancer Center (Guangzhou, Guangdong, China) and maintained in DMEM medium supplemented with 10% FBS, 100 µg/ml streptomycin and 100 IU/ml penicillin at 37°C in a 5% CO$_2$ incubator. The NCI-H929 cells were treated with 500 µmol/l NaHS for 24 h or co-treated with 500 µmol/l NaHS and 50 µmol/l LY294002 for 24 h.

**Cell proliferation assay.** NCI-H929 cells were cultured in 96-well tissue culture plates (2x10$^5$ cells per well) in DMEM supplemented with 10% FBS for 24 h. Then, the cells were exposed to different concentrations of NaHS (500 and 1,000 µmol/l) for 24 h. Cell proliferation was measured by CCK-8 assay kit. Briefly, 10 µl CCK-8 solution was added to each well, and the plates were incubated for an additional 2 h. Absorbance was measured using a spectrophotometer at a wave length of 450 nm. The means of the optical density (OD) of three wells in the indicated groups were used to calculate the percentage of cells that were viable according to the formula: cell viability (%) = (OD treatment group/OD control group) x100%. The experiments were performed three times.

**Cell cycle analysis by flow cytometry.** Cells were plated in 6-well plates at a density of 2x10$^6$ cells per well and grown in serum-free medium. Then, the cells were exposed to different concentrations of NaHS or co-treated with 500 µmol/l NaHS and 50 µmol/l LY294002. After 24 h, the cell cycle analysis was performed. The cells were harvested and fixed in 70% ethanol at 4°C overnight. The fixed cells were washed twice with PBS, treated with RNase A (50 µg/ml) for 30 min at room temperature, and then stained with propidium iodide. The stained cells were examined to analyze for the cell cycle using a Beckman Coulter XL instrument (Beckman Coulter, Brea, CA, USA).

**Western blot analysis.** As described above, the cells were harvested and lysed using RIPA lysis buffer supplemented with protease inhibitors. Total proteins were extracted and quantified using a bicinchoninic acid (BCA) protein assay kit. Loading buffer was added to the cytosolic extracts and the solution was then boiled for 5 min. The same amount of supernatant was obtained from each sample and fractionated using 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and the total proteins were then transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked in 5% fat-free milk in fresh blocking buffer [0.1% Tween-20 in Tris-buffered saline (TBS-T)] for 60 min at room temperature. They were then incubated in either anti-t-Akt antibody (1:1,000), anti-p-Akt antibody (1:1,000), anti-Bcl-2 antibody (1:1,000), or anti-caspase-3 antibody (1:1,000) in freshly prepared TBS-T containing 3% free-fat milk overnight with gentle agitation at 4°C. The membranes were washed three times for 5 min each with TBS-T and then incubated with HRP-conjugated goat anti-rabbit secondary antibody (1:3,000) in TBS-T containing 3% fat-free milk for 1.5 h at room temperature. Then, the membranes were washed three times for 5 min each in TBS-T. The immunoreactive proteins were visualized using ECL reagent. To quantify protein expression levels, the X-ray film was scanned and analyzed using ImageJ 1.47i software. The experiments were performed 3 times.

**ELISA assay.** The concentrations of H$_2$S in plasma samples were determined using ELISA (Quantikine R&D System, Minneapolis, MN, USA) according to the manufacturer's instructions. Briefly, these assays involved the application of the quantitative sandwich enzyme immunoassay technique. Monoclonal antibodies that were specific for each assay were pre-coated onto microplates. Standard controls and samples (100 µl of plasma) were pipetted into the wells in duplicate. After H$_2$S was bound and the plates were washed, an enzyme-linked polyclonal antibody that was specific for H$_2$S was added to each well. After the plates were thoroughly washed, a substrate solution was added to the wells, and the color developed in proportion to the amount of H$_2$S that was bound during the first step. The optical density of each well was determined using a microplate reader at 450 nm. The value for the blank was subtracted from both the standard controls and the samples. A standard curve was created by plotting the logarithm of the mean absorbance of each standard versus the logarithm of the known H$_2$S concentration. Concentrations are shown as picograms per milliliter. The experiments were repeated 3 times.
Transwell migration assay. Human myeloma cells were harvested and washed twice with PBS. After the cells were washed, 1x10^5 cells were resuspended in 200 µl DMEM and added to the upper chamber of the transwell membrane (Transwell Permeable Support with a 5.0-µm polycarbonate membrane, 6.5-mm insert, and 24-well plate; Corning Costar, Tewksbury, MA, USA), and 600 µl of 10% FBS-DMEM was added to each bottom chamber. Four upper chamber conditions were included in the assay: 1) control, 2) NaHS (500 µmol/l), 3) NaHS (500 µmol/l) + LY294002 (50 µmol/l), and 4) LY294002 (50 µmol/l). After 24 h of incubation at 37˚C, the cells that had migrated to the lower chambers were counted. Triplicate experiments were performed for each group, and the means and standard deviations were calculated.

Statistical analysis. The results are expressed as mean ± SD. Differences between groups were analyzed using one-way ANOVA followed by LSD post-hoc comparison tests in SPSS 13.0 (SPSS, Chicago, IL, USA) software. Significance was established at the P<0.05 level.

Results

Hydrogen sulfide concentration in the serum of MM patients is significantly higher than in healthy controls. All measured parameters were significantly higher in MM patients than in healthy controls. Furthermore, they increased in parallel with disease progression, with higher values observed in more advanced ISS stages. Higher serum levels of hydrogen sulfide were observed in MM patients than in controls, and these levels increased as the disease advanced. Serum H₂S concentrations in patients with MM were significantly elevated compared to the controls (P<0.01) (Fig. 1A). Furthermore, statistically significant differences were found in the levels of H₂S between the stage I-II and stage III MM patient groups (P<0.01) (Fig. 1B).

NaHS promotes cell proliferation in multiple myeloma cells. To test the effect of exogenous H₂S on human myeloma cell proliferation, a dose-response study was performed using varying doses (250, 500, 750 and 1,000 µmol/l) of NaHS (a donor of H₂S) for 24 h to calculate the most effective dose of NaHS (Fig. 2A). The doses of NaHS ranging between 250 and 1,000 µmol/l markedly promoted cell proliferation, leading to an increase in cell viability that reached a peak at 500 µmol/l. Therefore, 500 µmol/l NaHS was used in the subsequent time-response study in which we analyzed the impact of different treatment times (12, 24, 36 and 72 h). As shown in Fig. 2B, treatment of myeloma cells with 500 µmol/l NaHS for all of the indicated times markedly promoted cell proliferation, which reached a maximal proliferative effect at 24 h. Based on the above results, myeloma cells were treated with 500 µmol/l NaHS for 24 h in all of the following experiments.

NaHS accelerates cell cycle progression in multiple myeloma cells. To investigate the effect of exogenous H₂S on cell cycle progression in human myeloma cells, effects on the cell cycle were analyzed using flow cytometry. The results showed that cell cycle progress was markedly altered when the cells were treated with NaHS. The results indicated that in myeloma cells, NaHS reduced cell cycle arrest in the G0/G1 phase and increased the proportion of cells in the S and G2/M phases (Fig. 3A).
To compare the experimental results more intuitively, we drew histograms. The percentage of cells in the different cell cycle phases after exposure to NaHS was compared to the corresponding percentage in the untreated controls at the same time point. The results revealed a dose-dependent trend in which the proportion of cells in the G0/G1 phase decreased and the proportion of cells in the S phase and G2/M phases increased as the NaHS concentration increased. The most obvious change was observed at the 24 h time point when cells were treated with 500 µmol/l NaHS (Fig. 3B).

NaHS amplifies the activation of Akt in multiple myeloma cells. We analyzed the effects of NaHS on the level of Akt phosphorylation. Multiple myeloma cells were exposed to 500 µmol/l NaHS for the indicated times (3, 6, 9, 12 and 24 h), and the expression level of p-Akt was significantly upregulated, reaching a peak at 24 h (Fig. 4A and B).

NaHS reduces the expression of caspase-3 and upregulates the level of Bcl-2 in multiple myeloma cells. To analyze the effect of NaHS on the expression of caspase-3 and Bcl-2 in multiple myeloma cells, multiple myeloma cells were exposed to 500 µmol/l NaHS for different periods of time (3, 6, 9, 12 and 24 h). As shown in Fig. 4, NaHS significantly enhanced the expression of Bcl-2, which reached a peak at 9 h, and reduced the expression of caspase-3.

LY294002 suppresses NaHS-induced increased cell viability in multiple myeloma cells. Exposing multiple myeloma cells to 500 µmol/l NaHS for 24 h significantly induced cell proliferation and increased cell viability. However, the increased cell viability was repressed by co-treatment with different doses of LY294002 (a specific inhibitor of the Akt pathway) for 24 h. As shown in Fig. 5, increasing the dose of LY294002 from 1 to 10 µmol/l did not change cell viability. However, increasing the dose of LY294002 from 50 to 200 µmol/l significantly suppressed cell proliferation, with the decrease in cell viability reaching a minimum at 50 µmol/l. In accordance with the above results, multiple myeloma cells were co-treated with 500 µmol/l NaHS and 50 µmol/l LY294002 for 24 h in all subsequent experiments.

LY294002 reduces the NaHS-induced acceleration of cell cycle progression in multiple myeloma cells. As shown in Fig. 6A-D, multiple myeloma cells were exposed to 500 µmol/l NaHS for 24 h, the S-phase and G2/M-phase cells were significantly increased. However, the G0/G1-phase population of cells was markedly decreased. Co-treatment of multiple myeloma cells with 500 µmol/l NaHS and 50 µmol/l LY294002 for 24 h substantially depressed the NaHS-induced increase in the proportions of S-phase and G2/M-phase cells and significantly decreased the number of G0/G1-phase cells. Treating the cells with 50 µmol/l LY294002 for 24 h did not alter cell cycle progression.

LY294002 inhibits the NaHS-induced increase in the expression of Bcl-2 and upregulates the NaHS-induced decrease in caspase-3 expression in multiple myeloma cells. As shown...
in Fig. 7, multiple myeloma cells were exposed to 500 µmol/l NaHS for 24 h, and the expression of Bcl-2 was significantly increased. However, the expression of caspase-3 was markedly decreased. Notably, co-treatment of multiple myeloma cells with 500 µmol/l NaHS and 50 µmol/l LY294002 for 24 h considerably depressed the NaHS-induced increase in the expression of Bcl-2 while significantly upregulating caspase-3 expression. Treating the cells with 50 µmol/l LY294002 for 24 h did not alter the basal expression levels of Bcl-2 and caspase-3.

Figure 4. NaHS amplifies the activation of Akt via phosphorylation in myeloma cells. Myeloma cells were exposed to 500 µmol/l NaHS for the indicated times (3, 6, 9, 12 and 24 h). NaHS reduced the expression of caspase-3 and upregulated the expression of p-Akt and Bcl-2 in myeloma cells. The expression of caspase-3 and Bcl-2 was measured using western blot analysis (B, D and F). The data shown in (A, C and E) were quantified using densitometric analysis in ImageJ 1.47. The data are shown as the mean ± SEM (N=3). *P<0.05, **P<0.01 compared to the control group. Con, the control group; NaHS, a donor of H₂S.

LY294002 inhibits NaHS-induced migration in MM cells. In transwell migration assays, the migration rates of MM cells (NCI-H929) toward conditioned medium that was collected from NaHS was higher than the spontaneous migration rate, indicating that NaHS induced migration in MM cells. This process was inhibited by the Akt inhibitor.

LY294002 (Fig. 8). These results show that LY294002 inhibits NaHS-induced migration in MM cells and indicates that the Akt pathway may play an important role in the process of NaHS-induced migration.

Discussion

H₂S, the third gaseous transmitter following NO and CO, modulates a range of cellular and molecular mechanisms. Endogenous H₂S in mammalian tissues is mainly synthesized via the metabolism of L-cysteine by the catalysis of two key enzymes, CBS and CSE (13,14). Experiments have shown that plasma or blood H₂S concentrations range between 30 and 300 µM in humans, depending on the method used for measurement and the age of the donor (15). The role of H₂S, as a physiological molecule with pleiotropic functions, is becoming increasingly apparent. Specifically, H₂S can elicit cardio-protective, inflammation-preventing, anti-proliferative and anti-thrombotic effects (16,17). There is an apparent paradox in the effects of H₂S on cancer. Many reports have shown that inhibiting H₂S biosynthesis exerts anticancer effects, while other studies have shown that H₂S donors of various types exert anticancer actions both in vitro and in vivo (18). In recent years, an increasing number of studies have shown that hydrogen sulfide (H₂S) can mediate pathophysiological processes during cancer cell growth, proliferation, migration, and invasion (17,19,20). Nevertheless, it was shown that H₂S exerted potential anticancer effects on gastric cancer cells (21), oral cancer cell lines (22), PLC/PRF/5 hepatoma cells (23), and colon cancer cells (24). Furthermore, an increasing amount of evidence has shown that H₂S is involved in pathophysiological processes in tumors. The biological effects of H₂S that are relevant to cancer biology include the regulation of vascular...
functions (vasorelaxation and the stimulation of angiogenesis) (19) and the regulation of intracellular signaling and cell death (during which it acts as a direct and indirect antioxidant and inhibits oxidative damage and cell death in response to diverse stimuli) (25,26).

Based on the results of previous studies, we first collected samples from patients to determine the levels of H\textsubscript{2}S that are present in the serum. We found some interesting results in this analysis. In our study, serum H\textsubscript{2}S levels were significantly higher in the multiple myeloma patient group than in the control group. Moreover, they also increased in parallel with disease progression, with higher levels of H\textsubscript{2}S observed in patients with advanced ISS stages. We hypothesized that this gasotransmitter may be involved in the progression of multiple myeloma. To verify this hypothesis, we treated multiple myeloma cells with NaHS (a donor of H\textsubscript{2}S) LY294002, a specific inhibitor of the Akt pathway.

Figure 6. LY294002 reduces the NaHS-induced acceleration in cell cycle progression in multiple myeloma cells. After the indicated treatments (A–D), the cell cycle was analyzed using flow cytometry. (A) Control group. (B) Multiple myeloma cells were exposed to 500 µmol/l NaHS for 24 h. (C) Multiple myeloma cells were co-treated with 500 µmol/l NaHS and 50 µmol/l LY294002 for 24 h. (D) Multiple myeloma cells were treated with 50 µmol/l LY294002 for 24 h. (E) The histogram shows the percentage of myeloma cells in the stages of the cell cycle for each of the above treatments. The data are presented as the means ± SEM (n=3). *P<0.01 compared to the control group; †P<0.01 compared to the NaHS group. Con, the control group; NaHS, a donor of H\textsubscript{2}S; LY294002, a specific inhibitor of the Akt pathway.

Figure 7. LY294002 inhibits the NaHS-induced increase in the expression of bcl-2 and decrease in the expression of caspase-3 in multiple myeloma cells. Multiple myeloma cells were co-conditioned with 500 µmol/l NaHS and 50 µmol/l LY294002 for 24 h. (B and D) The expressions of caspase-3 and Bcl-2 were measured using western blot analysis. (A and C) Data were quantified using densitometric analysis in ImageJ 1.47. The data are presented as the mean ±SEM (n=3). *P<0.05 compared to the control group; †P<0.01 compared to the control group; ††P<0.05 compared to the NaHS group; †††P<0.01 compared to the NaHS group. Con, the control group; NaHS, a donor of H\textsubscript{2}S.
the control group; NaHS, a donor of H2S. Compared to the control group, myeloma cells. The data are presented as the mean ± SEM (n=3). **P<0.01 at 37˚C. LY294002 suppressed the NaHS-induced increase in migration in migration of MM cells were analyzed in 96-well migration assays after 24 h. Figure 8. The effects of NaHS, NaHS + LY294002, and LY294002 on the phosphorylation of Akt, which may have acti-

leading to increased cell viability, indicating that H2S might contribute to multiple myeloma growth. Then, the cells were co-treated with the indicated doses of NaHS for 24 h to analyze its effects on the cell cycle. The results showed that the proportion of G0/G1-phase cells was significantly decreased, while the proportions of S-phase cells and G2/M phase cells were significantly increased. NaHS was therefore found to accelerate cell cycle progression. Treatment of multiple myeloma cells with 500 µmol/l NaHS for 24 hours markedly diminished cell apoptosis and decreased the expression of caspase-3, an apoptotic factor. At the same time, the expression of Bcl-2, a protein that protects against apoptosis, was increased. The Bcl-2 protein is a component of a complex signaling system that controls apoptosis, and its overexpression can prevent apoptosis in cells, potentially leading to the continued division of mutated cells lines and eventually to cancer. Additionally, the overexpression of Bcl-2 can contribute to metastasis in certain cancers (27). These results demonstrate that the Bcl-2 pathway is implicated in the NaHS-induced effects on MM cell proliferation, apoptosis, and migration. All of these data indicate that H2S, when present at a relative high level, might evoke proliferative and anti-apoptotic effects.

Many MM cell lines express high baseline levels of p-Akt. Moreover, Akt is activated in the cells of MM patients (4). The PI3K/Akt pathways play a crucial role during MM cell growth, survival and drug resistance (28). We sought to explore the mechanism underlying NaHS-induced pro-proliferative, anti-apoptotic, and pro-migration effects in multiple myeloma cells by decreasing the expression of Bcl-2, increasing the expression of caspase-3 and reducing the NaHS-induced acceleration of cell cycle progression in multiple myeloma cells. Our data show that NaHS markedly increased cell migration in MM cells. However, the increase in the level of migration was significantly suppressed by co-treating cells with LY 249002. These results suggest that the activation of Akt is necessary for NaHS-induced cell progression in multiple myeloma and the phosphorylation of Akt is required for NaHS-induced cell proliferation. This result is consistent with the results of previous reports that showed that NaHS promotes cancer cell proliferation by up-regulating p-Akt (29).

In summary, H2S induced cell proliferation, inhibited apoptosis, accelerated cell cycle progression, and increased migration in multiple myeloma cells. These effects might be mediated by the activation of the Akt pathway, which leads to the overexpression of Bcl-2, the down-regulation of caspase-3, an increase in cell viability and migration rates, the acceleration of cell cycle progression and a decrease in the number of apoptotic cells. In myeloma cells, H2S is an endogenous tumor-promoting factor that plays a deleterious role in multiple myeloma cells. The contribution of H2S to myeloma cell growth remains to be further investigated. Further investigations into the biological effects of H2S on multiapoptotic and pro-migration effects in multiple myeloma cells may advance our knowledge of this novel gaseous transmitter and lead to a better understanding of multiple myeloma development.

References


