Abstract. Hydrogen sulfide (H$_2$S) participates in diverse physiological and pathophysiologic processes of cancer both in vitro and in vivo. We have previously reported the proliferation/anti-apoptosis/angiogenesis/migration effects of exogenous H$_2$S on liver cancer and glioma via amplifying the activation of NF-$\kappa$B and p38 MAPK/ERK1/2-COX-2 pathway. However, the effects of H$_2$S on EC109 esophageal cells remain unclear. The present study demonstrated the effects of exogenous H$_2$S on cancer cell growth via activating HSP90 pathways in EC109 esophageal cells. EC109 esophageal cells were treated with 400 µmol/l NaHS (a donor of H$_2$S) for 24 h. The expression levels of HSP90, bcl-2, caspase-3, bax and MMP-2 were detected by western blot assay. Cell viability was detected by Cell Counting Kit-8 (CCK-8). The migration rate was analyzed using a Transwell migration assay and ImageJ software. NaHS promoted cell proliferation, as evidenced by an increase in cell viability. In addition, NaHS treatment reduced apoptosis, as indicated by the increased bcl-2 expression and decreased cleaved caspase-3 and bax expression. Importantly, exposure of NaHS increased the expression of MMP-2, the migration rate and expression of VEGF. Notably, co-treatment of EC109 cells with NaHS and GA (an inhibitor of HSP90 pathway) largely suppressed the aforementioned NaHS-induced effects. The findings of the present study provided novel evidence that HSP90 pathway was involved in NaHS-induced cancer cell proliferation, anti-apoptosis, angiopoiesis and migration in EC109 esophageal cells.

Introduction

Esophageal cancer (EC), ranked as the sixth leading cause of cancer-related mortality worldwide, is one of the most highly malignant and aggressive cancers (1,2). Esophageal squamous cell carcinoma (ESCC) is the predominant histological subtype, accounting for more than 90% of EC cases in China (3). Although screening technology and multimodality therapies have remarkably improved during the past decade, the prognosis of EC remains dismal and the 5-year overall survival rate is still below 15% (4). Accumulative studies have tried to demonstrate the molecular and biological mechanisms that lead to EC. A series of risk factors for EC have been established, such as epidermal growth factor receptor (EGFR), Her-2, p53 and heat shock proteins (HSPs), which have been found to be associated with the progression of EC (5-8). However, the mechanisms of oncogenesis of EC have not been completely clarified. Therefore, the characterization of molecular markers involved in the pathophysiological process of ESCC is essential.

The HSPs family, as molecular chaperones, are biochemical regulators, which function in mediating cell growth, apoptosis, migration and protein homeostasis (9). HSPs are induced in response not only to cellular stress, but also to other environmental, physical and chemical stresses (10). HSPs are classified into 6 major family members according to molecular weight: HSP100, HSP90, HSP70, HSP60, HSP40 and small HSPs (11). HSP90 is one of the most abundant HSPs and more than 200 types of HSP90 client proteins have been found. A series of previous studies have demonstrated that HSP90 is activated and upregulated in a wide variety of human tumors, such as head and neck squamous cell cancer (HNSCC) (12),...
colonic carcinoma (13) and other adenocarcinomas (14). During the progression of cancers, HSP90 has a key role in the regulation of cell cycle growth, signaling, migration and transcription factors, which may lead to tumorigenesis (15-17). However, there is a paucity of data on the relationship between activation of HSP90 and the malignancy of EC.

Hydrogen sulfide (H₂S), as a specific toxic gas, has been qualified as the third gasotransmitter following nitric oxide (NO) and carbon monoxide (CO) (18-20). Endogenous H₂S is synthesized from L-cysteine by two key enzymes: cystathionine-β-synthase (CBS) and cystathionine-γ-lyase (CSE) which are mainly expressed in the enteric neurons and smooth muscle of the stomach and colon (21,22). Recently, numerous scientific investigations have proven the extensive physiological and pathophysiological properties of H₂S on progress of cancer. Previous findings have demonstrated that H₂S promotes cancer cell growth, proliferation, migration and invasion (23-29), owing to its vascular relaxant and angiogenesis effects. H₂S enhances the supply of nutrients and blood to the tumor cells and tissues (29). Our latest research also showed that exogenous H₂S promoted cancer cell proliferation/anti-apoptosis/angiogenesis/migration effects via amplifying the activation of NF-κB (30) and p38 MAPK/ERK1/2-COX-2 pathways (31). However, research focused on the effect of exogenous H₂S on esophageal EC109 cells and its potential mechanisms is lacking. Hence, we investigated whether exogenous H₂S contributes to cancer progress and explored these potential effects via activation of HSP90 pathways in esophageal EC109 cells.

Materials and methods

Materials. NaHS, a donor of H₂S, was obtained from Sigma Chemicals Co. (St. Louis, MO, USA), stored at 2-4°C and protected from sunlight. GA (a specific inhibitor of HSP90 pathway) was also purchased from Sigma Chemicals Co. The Cell Counting Kit-8 (CCK-8) was supplied by Dojindo Laboratory (Kumamoto, Japan). Fetal bovine serum (FBS) and RPMI-1640 medium were obtained from Gibco-BRL (Grand Island, NY, USA). Anti-MMP2, anti-HSP90, anti-cleaved caspase-3, anti-bcl-2 antibody and anti-bax antibodies were supplied by Cell Signaling Technology (Boston, MA, USA). Horseradish peroxidase (HRP)-conjugated secondary antibody and BCA protein assay kit were obtained from KangChen Bio-tech, Inc. (Shanghai, China). Enhanced chemiluminescence (ECL) solution was purchased from KeyGen Biotech (Nanjing, China). Enzyme-linked immunosorbent assay (ELISA) was supplied by ExCell Bio Co. (Shanghai, China).

Cell culture and treatments. The human esophageal carcinoma cells EC109 (EC109 cells) were supplied by Sun Yat-sen University Experimental Animal Center (Guangzhou, Guangdong, China). The EC109 cells were grown in RPMI-1640 medium supplemented with 10% FBS under an atmosphere of 5% CO₂ and at 37°C with 95% air. The EC109 cells were treated with 500 µmol/l NaHS for 24 h or co-treated with 500 µmol/l NaHS and 20 µmol/l GA for 24 h. The total proteins of the EC109 cells were harvested and lysed with cell lysis solution at 4°C for 30 min. The total proteins were quantified using the BCA protein assay kit. Loading buffer was added to cytosolic extracts, and then after boiling for 6 min, the same amounts of supernatant from each sample were fractionated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). The total proteins were then transferred into polyvinylidene difluoride (PVDF) membranes. The membranes were blocked with 5% fat-free milk for 60 min in fresh blocking buffer [0.1% Tween-20 in Tris-buffered saline (TBS-T)] at room temperature, and incubated with either anti-MMP2 (1:1,000 dilution), anti-HSP90 (1:1,000 dilution), anti-bax (1:1,000 dilution), anti-bcl-2 (1:1,000 dilution) and anti-cleaved caspase-3 antibodies (1:1,000 dilution) in freshly prepared TBS-T with 3% fat-free milk overnight with gentle agitation at 4°C. Membranes were washed for 5 min with TBS-T for 3 times and incubated with HRP-conjugated goat anti-rabbit secondary antibody at a concentration of 1:3,000 dilution (Kangchen Biotech, Shanghai, China), in TBS-T with 3% fat-free milk for 1.5 h at room temperature. Then, membranes were washed 3 times with TBS-T for 5 min. The immunoreactive signals were visualized via the ECL. In order to quantify the protein expression, the X-ray film was scanned and analyzed with ImageJ 1.47i software. The experiment was carried out 3 times.

Measurement of cell viability. The EC109 cells were seeded in 96-well plates at some concentration of 1x10⁴/ml and incubated at 37°C. The CCK-8 assay was employed to assess the cell viability of EC109 cells. After the indicated treatments, 10 µl CCK-8 solution at a 1/10 dilution was added to each well and then the plate was incubated for 1.5 h in the incubator. Absorbance at 450 nm was assayed using a microplate reader (Molecular Devices, Sunnyvale, CA, USA). The means of the optical density (OD) of 3-wells in the indicated groups were used to calculate the percentage of cell viability according to the formula below: Cell viability (%) = (OD treatment group/OD control group) x 100%. The experiment was carried out 5 times.

ELISA for detection of VEGF in culture supernatant. EC109 cells were cultured in 96-well plates. After the different indicated treatments, the level of VEGF in the culture media was tested by ELISA according to the manufacturer's instructions. The experiment was performed at least 5 times.

Transwell migration assay. The EC109 cells were harvested and washed twice with phosphate-buffered saline (PBS). After washing, 1x10⁵ cells were resuspended in 200 µl Dulbecco's modified Eagle's medium (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 groups in the upper chamber were included in the assay: i) control; ii) NaHS, NaHS (500 µmol/l); iii) NaHS + GA (a specific inhibitor of HSP90 pathway), NaHS (500 µmol/l) + GA (20 µmol/l); iv) GA, GA (20 µmol/l). After 24 h at 37°C, cells that migrated to the lower chambers were counted. Triplicate experiments were performed with each group, and the means and standard deviations were calculated.
Statistical analysis. All data are presented as the mean ± SEM. Differences between groups were analyzed by one-way analysis of variance (ANOVA) using SPSS 13.0 (SPSS, Inc., Chicago, IL, USA) software, and followed by LSD post hoc comparison test. Statistical significance was considered at P<0.05.

Results

NaHS promotes cell proliferation in EC109 esophageal cells. In order to test the effect of exogenous H$_2$S on human EC cell proliferation, the dose-response study with varying doses (200, 400, 600, 800 and 1,000 µmol/l) of NaHS (a donor of H$_2$S) for 24 h was performed to calculate the effective doses of NaHS. As shown in Fig. 1A, the doses of NaHS from 200 to 1,000 µmol/l markedly promoted cell proliferation, leading to an increase in cell viability and reaching a peak at 400 µmol/l. Therefore, 400 µmol/l NaHS was used in the subsequent time-response study with different treatment times (12, 24, 36, 48 and 60 h). As shown in Fig. 1B, treatment of EC109 cells with 400 µmol/l NaHS for the indicated times all markedly promoted cell proliferation, reaching the maximal proliferative effect at 24 h. Based on the aforementioned results, EC109 esophageal cells were treated with 400 µmol/l NaHS for 24 h in all subsequent experiments.

NaHS upregulates the expression levels of HSP90 in EC109 esophageal cells. We observed the effects of NaHS on the expression levels of HSP90. As shown in Fig. 2A and B, exposure of EC109 cells for the indicated times (12 and 24 h) to 400 µmol/l NaHS markedly enhanced the expression of HSP90, reaching a peak at 24 h. Therefore, 400 µmol/l NaHS was used in the subsequent time-response study with different treatment times (12, 24, 36, 48 and 60 h). As shown in Fig. 1B, treatment of EC109 cells with 400 µmol/l NaHS for the indicated times all markedly promoted cell proliferation, reaching the maximal proliferative effect at 24 h. Based on the aforementioned results, EC109 esophageal cells were treated with 400 µmol/l NaHS for 24 h in all subsequent experiments.

GA (a specific inhibitor of HSP90 pathway) reduces cell proliferation in EC109 esophageal cells. EC109 cells were treated with different doses of GA (10, 20, 40, 60 and 80 µmol/l) for 24 h. As shown in Fig. 3A, the doses of GA from 10 to 80 µmol/l markedly reduced cell proliferation, dropping to a bottom at 80 µmol/l. Therefore, 400 µmol/l NaHS was used in the subsequent time-response study with different treatment times (12, 24, 36, 48 and 60 h). As shown in Fig. 1B, treatment of EC109 cells with 400 µmol/l NaHS for the indicated times all markedly promoted cell proliferation, reaching the maximal proliferative effect at 24 h. Based on the aforementioned results, EC109 esophageal cells were treated with 400 µmol/l NaHS for 24 h in all subsequent experiments.

GA alleviates NaHS-induced cell proliferation in EC109 cells. As shown in Fig. 3B, exposure of EC109 cells to 400 µmol/l
NaHS for 24 h induced cell proliferation, leading to an increase in cell viability. However, the increased cell viability was repressed by co-treatment with different doses of GA (a specific inhibitor of HSP90 pathway) for 24 h. As shown in Fig. 3A, at the dose of 10 µmol/l, the cell viability did not change. On the contrary, the dose of GA from 20 to 80 µmol/l significantly suppressed the cell proliferation, leading to a decrease in cell viability and reaching the minimum at 20 µmol/l. According to the aforementioned results, EC109 cells were co-treated with 400 µmol/l NaHS and 20 µmol/l GA for 24 h in all following experiments.

**NaHS alleviates the expression level of cleaved caspase-3 and bax, and upregulates the expression levels of bcl-2 in EC109 esophageal cells.** In order to observe the effects of NaHS on the expression levels of cleaved caspase-3, bax and bcl-2 in EC109 cells, EC109 cells were exposed to 400 µmol/l NaHS for different times (1, 3, 6, 9, 12 and 24 h). As shown in Fig. 4A, NaHS significantly enhanced the expression levels of bcl-2 reaching a peak at 12 h, whereas the expression level of caspase-3 and bax was markedly decreased.

**GA inhibits NaHS-induced increased expression levels of bcl-2 and upregulates NaHS-induced decreased caspase-3 expression in EC109 esophageal cells.** As shown in Fig. 4E, EC109 cells were exposed to 400 µmol/l NaHS for 24 h. The expression levels of bcl-2 were significantly increased; on the contrary, the expression level of caspase-3 and bax were
markedly decreased. Notably, co-treatment of EC109 cells with 400 µmol/l NaHS and 20 µmol/l GA for 24 h considerably depressed NaHS-induced increased expression levels of bcl-2; however, expression of caspase-3 and bax was considerably downregulated. Treatment of cells with 20 µmol/l GA for 24 h did not alter the basal expression levels of caspase-3, bax or bcl-2.

NaHS upregulates the expression level of MMP-2. In order to observe the effects of NaHS on the expression levels of MMP-2 in EC109 cells, EC109 cells were exposed to 400 µmol/l NaHS for different times (1, 3, 6, 9, 12 and 24 h). As shown in Fig. 5A, NaHS significantly enhanced the expression levels of MMP-2, which peaked at 24 h. Notably, co-treatment of EC109 cells with 400 µmol/l NaHS and 20 µmol/l GA for 24 h considerably depressed NaHS-induced increased expression levels of MMP-2. Treatment of cells with 20 µmol/l GA for 24 h did not alter the basal expression levels of MMP-2.

The migration rate and Transwell migration assay. As shown in Fig. 5E, NaHS strengthened the migration rate in EC109 esophageal cells while GA depressed NaHS-induced increased migration rate. Treatment of cells with 20 µmol/l GA for 24 h did not alter the migration rate compared with control group.

GA suppresses NaHS-induced upregulated production of VEGF in EC109 esophageal cells. As shown in Fig. 6, the level of VEGF was markedly increased in NaHS-induced EC109 cells compared with the control group (P<0.01). However, the increased level of VEGF was significantly suppressed by co-treatment with GA and NaHS.

H₂S demonstrates proliferation, anti-apoptosis, angiogenesis and migration effects on EC109 esophageal cells via amplifying the activation of HSP90 pathway. We found that NaHS upregulated HSP90 activity resulting in an elevated rate of H₂S production level, which in turn modulated protein expressions of caspase-3, bax, bcl-2, MMP-2 and VEGF. The downregulated caspase-3 and bax directly induced anti-apoptosis, led to decreased apoptosis and increased cell viability of EC109 cells. MMP-2 contributes to cancer cell invasion and migration. The increased production of VEGF stimulates angiogenesis, promoting the supply of nutrients and blood to the tumor. Conversely, the above properties of H₂S were significantly inhibited by the co-condition of 400 µmol/l NaHS and 20 µmol/l GA for 24 h.

Discussion

In the present study, we demonstrated a novel finding of H₂S on esophageal EC109 cells and provided evidence to reveal its potential mechanisms. These findings support our hypothesis that preconditioning with exogenous H₂S mediates...
proliferation, anti-apoptotic, angiopoiisis and migration effects in esophageal cancer. The molecular mechanisms of H$_2$S are not yet fully understood. It is known that H$_2$S is produced in the body mainly by two crucial enzymes, CBS and CSE, which are mainly found in the central nervous system (CNS) (32). A recent study emphasized that H$_2$S played an important role in various physiological and pathological processes of the nervous system as a neuromodulator and neuroprotectant (33). Furthermore, H$_2$S could exert protection to nerve cancer cells, such as PC12 cells (34). However, the performance of H$_2$S on the cancer cells are comparatively complicated and extremely controversial. On the one hand, H$_2$S has shown its anticancer ability based on anti-inflammatory effect, anti-apoptosis and activation of some signal pathways (35,36). On the other hand, H$_2$S can exert totally opposite properties via amplifying the activation of NF-$\kappa$B pathway, and H$_2$S may be involved in esophageal cancer growth. Therefore, we hypothesized that H$_2$S promotes the supply of blood and nutrients to the tumor via angiogenesis effect. Further studies are needed to explore our hypothesis in vivo.

It is well known that tumor invasion and metastasis require increased expressions of MMPs. Among the MMPs, MMP-2 and MMP-9 have been thought to be key enzymes in this process since they degrade type IV collagen, which is one of the important components of extracellular matrix (43). Growing evidence reveals that the upregulated expression of MMPs, particularly the gelatinase (MMP-2 and MMP-9), is closely associated with metastasis potential in several types of carcinomas (44-47). The present study demonstrated that HSP90 activation strongly increases the expression of MMP-2 protein in EC109 cells, which indicated that H$_2$S was involved in EC109 cell invasion and migration.

To investigate the complicated mechanism for NaHS induced pro-proliferative effect, anti-apoptosis, angiogenesis and migration in EC109 cells, we studied the HSP90 pathway, which has been previously demonstrated, linked to cancer progression by regulation of cell proliferation, signaling and apoptosis (15-17). It has been reported that HSP90 can be activated by various stimuli both in normal and in cancer cells (10). Herein, we found that NaHS activated HSP90 pathway in EC109 cells. Notably, GA, an inhibitor of HSP90, blocked NaHS-induced HSP90 pathway activation by decreasing expression levels of bcl-2, MMP-2 and VEGF, and increasing caspase-3 and bax expression. These results suggest that HSP90 activation is necessary in NaHS-induced EC109 cell progression.

In conclusion, H$_2$S-induced cell proliferation, anti-apoptosis, angiogenesis and migration in EC109 esophageal cells. These effects may be mediated by the activation of HSP90 pathway, leading to overexpression levels of MMP-2, bcl-2 and VEGF, downregulation of caspase-3 and bax, increased cell viability, and decreased number of apoptotic cells. In esophageal cancer, the findings provide novel insight into a unified concept and identify H$_2$S as an endogenous tumor-promoting factor and anticancer drug target. The deeper mechanism of H$_2$S in EC109 esophageal cells is still unclear and needs to be further investigated.

References


18. Wang R: Two's company, three's a crowd: Can H2S be the third


