

# Exogenous hydrogen sulfide promotes C6 glioma cell growth through activation of the p38 MAPK/ERK1/2-COX-2 pathways

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**Abstract.** Hydrogen sulfide (H<sub>2</sub>S) participates in multifarious physiological and pathophysiological progresses of cancer both *in vitro* and *in vivo*. We have previously demonstrated that exogenous H<sub>2</sub>S promoted liver cancer cells proliferation/anti-apoptosis/angiogenesis/migration effects via amplifying the activation of NF- $\kappa$ B pathway. However, the effects of H<sub>2</sub>S on cancer cell proliferation and apoptosis are controversial and remain unclear in C6 glioma cells. The present study investigated the effects of exogenous H<sub>2</sub>S on cancer cells growth via activating p38 MAPK/ERK1/2-COX-2 pathways in C6 glioma cells. C6 glioma cells were treated with 400  $\mu$ mol/l NaHS (a donor of H<sub>2</sub>S) for 24 h. The expression levels of phosphorylated (p)-p38 MAPK, total (t)-p38 MAPK, p-ERK1/2, t-ERK1/2, cyclooxygenase-2 (COX-2) and caspase-3 were measured by western blotting assay. Cell viability was detected by Cell Counting Kit-8 (CCK-8). Apoptotic cells were observed by Hoechst 33258 staining assay. Cell proliferation was directly detected under fully automatic inverted microscope. Exposure of C6 glioma cells to NaHS resulted in cell proliferation, as evidenced by

an increase in cell viability. In addition, NaHS treatment reduced apoptosis, as indicated by the decreased apoptotic percentage and the cleaved caspase-3 expression. Importantly, exposure of the cells to NaHS increased the expression levels of p-p38 MAPK, p-ERK1/2 and COX-2. Notably, co-treatment of C6 glioma cells with 400  $\mu$ mol/l NaHS and AOAA (an inhibitor of CBS) largely suppressed the above NaHS-induced effects. Combined treatment with NaHS and SB203580 (an inhibitor of p38 MAPK) or PD-98059 (an inhibitor of ERK1/2) resulted in the synergistic reduction of COX-2 expression and increase of caspase-3 expression, a decreased number of apoptotic cells, along with decreased cell viability. Combined treatment with NS-398 (an inhibitor of COX-2) and NaHS also resulted in the synergistic increase of caspase-3, a decreased in the number of apoptotic cells and the decrease in cell viability. The findings of the present study provide novel evidence that p38 MAPK/ERK1/2-COX-2 pathways are involved in NaHS-induced cancer cell proliferation and anti-apoptosis in C6 glioma cells.

## Introduction

Glioma is the most common malignant tumor and accounts for ~10% of tumors in the central nervous system (CNS) (1,2). Glioma accounts for 80% of malignant brain tumors (3). It is alarming that the prognosis of glioma, particularly high-grade (III-IV) glioma, is generally poor with standard chemotherapy and radiation therapy, and patients can only survive for an average of 1 year after diagnosis as a result of chemotherapy resistance (4). It is regrettable that the etiology of this disease still remains largely elusive. Elucidation of the molecular mechanisms of glioma is expected to lead to the identification of new therapeutic targets and to contribute to better clinical treatment and management of this devastating disease.

Mitogen-activated protein kinase (MAPK), which is a serine based protein kinase, and a highly conserved group of protein kinases that responds to miscellaneous changes of the cellular, intracellular or/and extracellular environment

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(DNA damage, hyperosmosis, oxidative stress, potentially harmful stimuli) (5-9), playing significant roles in signal transduction and taking part in a diverse range of physiological and pathological processes by modulating gene transcription in the nucleus, including cell proliferation, mitosis, differentiation, apoptosis, cell survival and gene expression (6). In mammals, MAPK consists of at least 11 members. The most studied MAPKs are the p38 MAPK, extracellular signal-regulated protein kinases 1/2 (ERK1/2) and c-Jun N-terminal kinases (JNK) (6). It has been reported that p38 MAPK and ERK1/2 pathways are important for glioma development (10-12). Previous studies have demonstrated that MAPKs (p38 MAPK and ERK1/2) also activate other signaling cascades, such as cyclooxygenase-2 (COX-2) (13,14), in a variety of cancer cell types. COX-2 expression is closely associated with tumor cell growth and is a crucial molecule in the development of malignant tumors, and angiogenesis (15,16), anti-apoptosis (17), invasiveness (18) and proliferation (19). In addition, a previous study demonstrated that COX-2 was activated dependent on the ERK1/2 pathway in glioma (20). However, evidence that the p38 MAPK/ERK1/2-mediated activation of the COX-2 pathway may be involved in growth of C6 glioma is lacking.

Hydrogen sulfide ( $H_2S$ ) has been qualified as the third gasotransmitter following nitric oxide (NO) and carbon monoxide (CO) (21-23). It can be endogenously produced mainly by cystathionine  $\beta$ -synthase (CBS) in the CNS. In recent years, more and more attention is paid to  $H_2S$  for its extensive physiological and pathophysiological properties on cancer progresses. Some findings from *in vivo* and *in vitro* studies showed that  $H_2S$  is beneficial for cancer cell growth, proliferation, migration and invasion (24-30), owing to its vascular relaxant and angiogenesis effects.  $H_2S$  promotes the supply of nutrients and blood to the tumor cells and tissues (24). Our latest research also demonstrated that exogenous  $H_2S$  promoted cancer cell proliferation/anti-apoptosis/angiogenesis/migration effects via amplifying the activation of NF- $\kappa$ B pathway (31). Furthermore, to the best of our knowledge, no study exists focused on the effect of exogenous  $H_2S$  on C6 glioma cells and its potential mechanisms. Based on recent studies (24-31), we investigated whether exogenous  $H_2S$  contributes to cancer progress and explored these potential effects via amplification of p38 MAPK/ERK1/2-COX-2 pathways in C6 glioma cells.

## Materials and methods

**Materials.** NaHS, a donor of  $H_2S$ , was obtained from Sigma Chemicals Co. (St. Louis, MO, USA), stored at 2-4°C and protected from sunlight. Hoechst 33258, AOAA (an inhibitor of CBS), SB203580 (an inhibitor of p38 MAPK), PD-98059 (an inhibitor of ERK1/2) and NS-398 (an inhibitor of COX-2) were also purchased from Sigma Chemicals Co. The Cell Counting Kit-8 (CCK-8) was supplied by Dojindo Laboratories (Kumamoto, Japan). Fetal bovine serum (FBS) and 1640 medium were obtained from Gibco-BRL (Grand Island, NY, USA). Anti-p38 MAPK, anti-ERK1/2, anti-p-p38 MAPK, anti-p-ERK1/2, anti-caspase-3 and anti-COX-2 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 Biotech, Inc. (Shanghai, China). Enhanced chemiluminescence (ECL) solution was purchased from KeyGen Biotech.

**Cell culture and treatments.** The C6 glioma cells were supplied by Sun Yat-sen University Experimental Animal Center (Guangzhou, Guangdong, China). The cells were grown in 1640 medium supplemented with 10% FBS under an atmosphere of 5%  $CO_2$  and at 37°C with 95% air. The cells were treatment with 400  $\mu$ mol/l NaHS for 24 h or co-treatment with 400  $\mu$ mol/l NaHS and 30  $\mu$ mol/l SB 203580 or 20  $\mu$ mol/l PD-98059 or 10  $\mu$ mol/l NS-398 for 24 h.

**Hoechst 33258 nuclear staining for evaluation of apoptosis.** Apoptotic cell death was tested by the Hoechst 33258 staining followed by photofluorography. Firstly, C6 glioma cells were plated in 35 mm dishes at a density of  $1 \times 10^6$  cells/well. Following the above indicated treatments, the C6 glioma cells were fixed with 4% paraformaldehyde in 0.1 mol/l phosphate-buffered saline (PBS; pH 7.4) for 10 min at 4°C. Then, the slides were washed three times with PBS. After staining followed by 5 mg/ml Hoechst 33258 for 15 min, the PLC cells were washed three times with PBS. Lastly, the cells were visualized under a fluorescence microscope (Bx50-FLA; Olympus, Tokyo, Japan). Viable C6 glioma cells displayed a uniform blue fluorescence throughout the nucleus and normal nuclear size. However apoptotic C6 glioma cells showed condensed, distorted or fractured nuclei. The experiment was carried out three times.

**Western blot analysis.** The cells were harvested and lysed with cell lysis solution at 4°C for 30 min. The total proteins were quantified through using the BCA protein assay kit. Loading buffer was added to cytosolic extracts, and then boiling for 6 min, the same amounts of supernatant from each sample were fractionated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and the total proteins were 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-p38 MAPK (1:1,000 dilution), anti-ERK1/2 (1:1,000 dilution), anti-p-p38 MAPK (1:1,000 dilution), anti-p-ERK1/2 (1:1,000 dilution), anti-caspase-3 (1:1,000 dilution) and anti-COX-2 antibodies (1:1,000 dilution) in freshly prepared TBS-T with 3% free-fat milk throughout the night with gentle agitation at 4°C. Membranes were washed for 5 min with TBS-T three times and incubated with HRP-conjugated goat anti-rabbit secondary antibody at a concentration of 1:3,000 dilution; (KangChen Biotech, Inc.), in TBS-T with 3% fat-free milk for 1.5 h at room temperature. Then membranes were washed three times with TBS-T for 5 min. The immunoreactive signals were visualized using the ECL detection. In order to quantify the protein expression, X-ray film was scanned and analyzed with ImageJ 1.47i software. The experiment was carried out three times.

**Measurement of cell viability.** The cells were seeded into 96-well plates at concentration of  $1 \times 10^4$ /ml, and were

incubated at 37°C, the CCK-8 assay was employed to assess the cell viability of PLC cells. After the indicated treatments, 10  $\mu$ l CCK-8 solution at a 1/10 dilution was added to each well and the plate was incubated for 1.5 h in the incubator. Absorbance at 450 nm was evaluated using a microplate reader (Molecular Devices, Sunnyvale, CA, USA). The means of the optical density (OD) of three wells in the indicated groups were used to calculate the percentage of cell viability according to the formula: Cell viability (%) = (OD treatment group/OD control group)  $\times$  100%. The experiment was carried out three times.

**Cell proliferation.** The cells were seeded into 24-well plates at concentration of  $1 \times 10^4$ /ml and were incubated at 37°C. After the indicated treatments, the cell number were directly detected under a fully automatic inverted microscope.

**Statistical analysis.** All data are presented as the mean  $\pm$  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 as  $P < 0.05$ .

## Results

**NaHS promotes cell proliferation in C6 glioma cells.** In order to test the effect of exogenous  $H_2S$  in C6 glioma cell viability, firstly, a dose-response study with varying doses (100, 200, 400, 800 and 1,600  $\mu$ mol/l) of NaHS (a donor of  $H_2S$ ) for 24 h was performed to calculate the effective doses of NaHS. As shown in Fig. 1A, the doses of NaHS from 100 to 1,600  $\mu$ mol/l markedly promoted cells proliferation, leading to an increase in cell viability and reaching a peaking at 400  $\mu$ mol/l. Therefore, 400  $\mu$ mol/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 C6 glioma cells with 400  $\mu$ mol/l NaHS for the indicated times all markedly promoted cells viability, reaching the maximal effect at 24 h. Based on the above results, C6 glioma cells were treated with 400  $\mu$ mol/l NaHS for 24 h in all subsequent experiments.

Notably, the above increased cell viability was repressed by co-treatment with 400  $\mu$ mol/l NaHS and different doses of AOAA (a specific inhibitor of CBS) for 24 h. As shown in Fig. 1C, at the doses of AOAA from 0.1 to 2 mmol/l significantly suppressed cell proliferation, leading to a decrease in cell viability and reaching the minimum at 1 mmol/l. According to the above results, C6 glioma cells were co-treated with 400  $\mu$ mol/l NaHS and 2 mmol/l AOAA for 24 h in all the following experiment.

**AOAA alleviates NaHS-induced cell proliferation in C6 glioma cells.** As shown in Fig. 1, NaHS notably promotes cells proliferation in C6 glioma cells. In order to validate this phenomenon, the number of the cells was detected under the microscope. The cells were treated with different doses of NaHS (100, 200, 400, 800 and 1,600  $\mu$ mol/l). As shown in Fig. 2, the doses of NaHS from 100 to 1,600  $\mu$ mol/l markedly promoted cell proliferation, leading to an increase in cell number and reaching a peaking at 400  $\mu$ mol/l. Thus, we

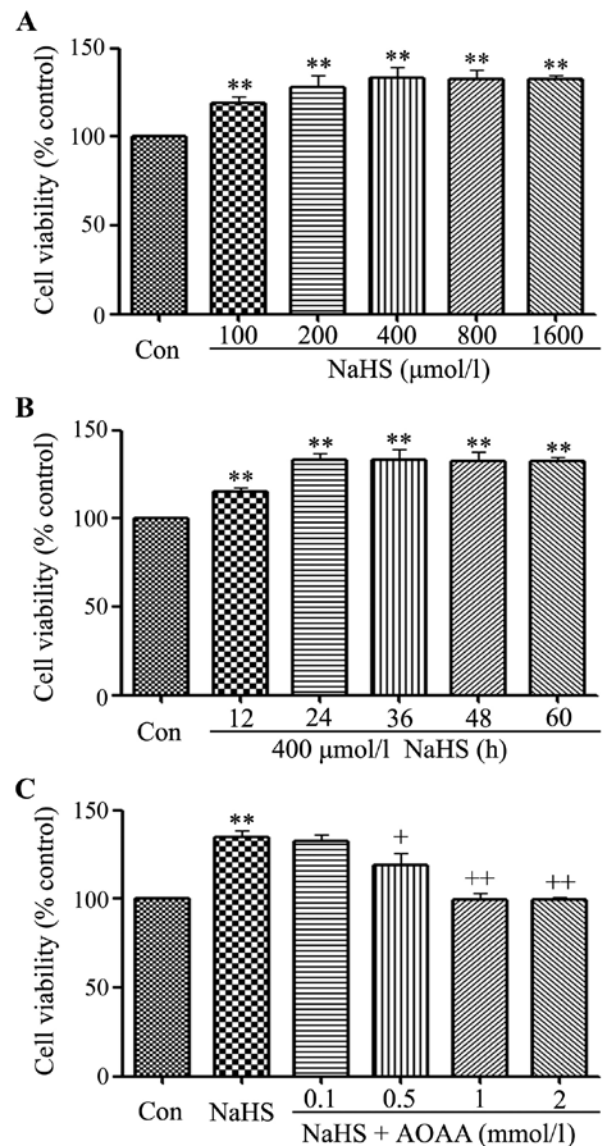


Figure 1. NaHS promotes cell proliferation in C6 glioma cells. Cell viability was tested using the Cell Counting Kit-8 (CCK-8). (A) C6 glioma cells were treated with different doses of NaHS (100, 200, 400, 800 and 1,600  $\mu$ mol/l) for 24 h. (B) Cells were treated with 400  $\mu$ mol/l NaHS for the indicated times (12, 24, 36, 48, 60 and 72 h). (C) Cells were co-treated with 400  $\mu$ mol/l NaHS and different doses of AOAA (0.1, 0.5, 1 and 2 mmol/l) for 24 h. Data are the mean  $\pm$  SEM (n=3). \*\* $p < 0.01$  compared with the control group, + $p < 0.05$ , ++ $p < 0.01$  compared with the NaHS group. Con, the control group; NaHS, a donor of  $H_2S$ .

showed that AOAA alleviates NaHS-induced increased cell proliferation in C6 glioma cells. As shown in Fig. 2G, H and I, at the doses of AOAA from 0.1 to 1 mmol/l significantly alleviated NaHS-induced increase of cell proliferation in C6 glioma cells, leading to a decrease in cell number and reaching the minimum at 1 mmol/l.

**AOAA ameliorates the phosphorylation of p38 MAPK and ERK1/2 is induced by NaHS in C6 glioma cells.** We observed the effects of NaHS on MAPK (including p38 MAPK and ERK1/2) phosphorylation. First, we further performed the time-study on the expression of p-p38 MAPK, after C6 glioma cells were exposed to 400  $\mu$ mol/l NaHS for the indicated times

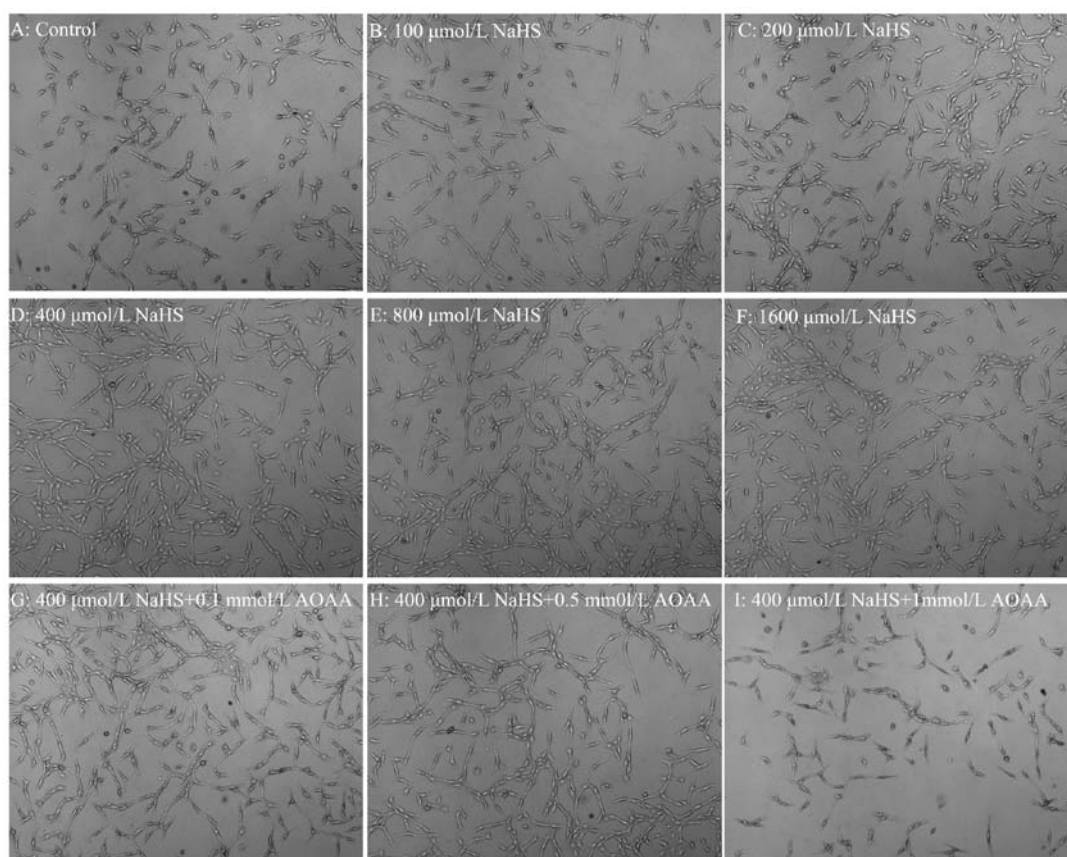


Figure 2. AOAA alleviates NaHS-induced cell proliferation in C6 glioma cells. Cell number was detected using a fully automatic inverted microscope. (A-F) C6 glioma cells were treated with different doses of NaHS (0, 100, 200, 400, 800 and 1,600  $\mu\text{mol/l}$ ) for 24 h, respectively. (G-I) The cells were co-treated with 400  $\mu\text{mol/l}$  NaHS and different doses of AOAA (0.1, 0.5 and 1 mmol/l) for 24 h, respectively. Con, the control group; NaHS, a donor of  $\text{H}_2\text{S}$ .

(3, 6, 9, 12 and 24 h), the expression levels of p-p38 MAPK were significantly upregulated, reaching a peak at 3 h, and the t-p38 MAPK expression was unchanged.

Similarly, exposure of cells to 400  $\mu\text{mol/l}$  NaHS also increased the expression levels of p-ERK1/2, as shown in the time-response study (Fig. 3E and F).

To observe effects of AOAA on the activation of MAPK induced by NaHS, C6 glioma cells were co-treated with 400  $\mu\text{mol/l}$  NaHS and 2 mmol/l AOAA for 24 h. As shown in Fig. 3C, D, G and F, the increased phosphorylation of p38 MAPK and ERK1/2 was reduced.

*SB203580 and PD98059 alleviates the NaHS-induced increase of cell viability in C6 glioma cells.* As shown in Fig. 4, exposure of C6 glioma cells to 400  $\mu\text{mol/l}$  NaHS for 24 h obviously induced cell proliferation, leading to an increase in cell viability. However, the increased cell viability was repressed by co-treatment with 400  $\mu\text{mol/l}$  NaHS and different doses of SB203580 (a specific inhibitor of p38 MAPK pathway) or PD-98059 (a specific inhibitor of ERK1/2 pathway) for 24 h.

As shown in Fig. 4A, at 5  $\mu\text{mol/l}$  SB203580 did not alter the cell viability. On the contrary, the dose of PDTC (10, 20 and 30  $\mu\text{mol/l}$ ) significantly suppressed the cell proliferation, leading to a decrease in cell viability and reaching the minimum at 30  $\mu\text{mol/l}$ . According to the above results, C6 glioma cells were co-treated with 400  $\mu\text{mol/l}$  NaHS and 30  $\mu\text{mol/l}$  SB203580 for 24 h in all the following experiments.

Similarly, exposure of cells to 400  $\mu\text{mol/l}$  NaHS and different doses of PD-98059 (1, 5, 10, 20 and 40  $\mu\text{mol/l}$ ) also suppressed cell proliferation, leading to a decrease in cell viability and reaching the minimum at 20  $\mu\text{mol/l}$ , as shown in Fig. 4B. According to the above results, C6 glioma cells were co-treated with 400  $\mu\text{mol/l}$  NaHS and 20  $\mu\text{mol/l}$  PD-98059 for 24 h in all the following experiments.

*SB203580 and PD98059 increase the NaHS-induced cell proliferation in C6 glioma cells.* The cell number was detected using a fully automatic inverted microscope (Fig. 5-a-d). C6 glioma cells were co-treated with 400  $\mu\text{mol/l}$  NaHS and 30  $\mu\text{mol/l}$  SB203580 or 20  $\mu\text{mol/l}$  PD-98059 for 24 h, respectively. As observed in Fig. 2, 400  $\mu\text{mol/l}$  NaHS markedly promoted cells proliferation, leading to an increase in cell number. However, exposure of cells to 400  $\mu\text{mol/l}$  NaHS and 30  $\mu\text{mol/l}$  SB203580 or 20  $\mu\text{mol/l}$  PD-98059 both significantly alleviated NaHS-induced cell proliferation in C6 glioma cells.

Apoptotic cell death was tested by Hoechst 33258 staining followed by photofluorography. As shown in the Fig. 5B-a-e, exposure of C6 glioma cells to 400  $\mu\text{mol/l}$  NaHS for 24 h reduced the typical characteristics of apoptosis, as evidenced by the condensation of chromatin, the shrinkage of nuclei and the formation of apoptotic bodies. On the contrary, exposure of cells to 400  $\mu\text{mol/l}$  NaHS and 30  $\mu\text{mol/l}$  SB203580 or 20  $\mu\text{mol/l}$  PD-98059 significantly induced the typical characteristics of apoptosis.

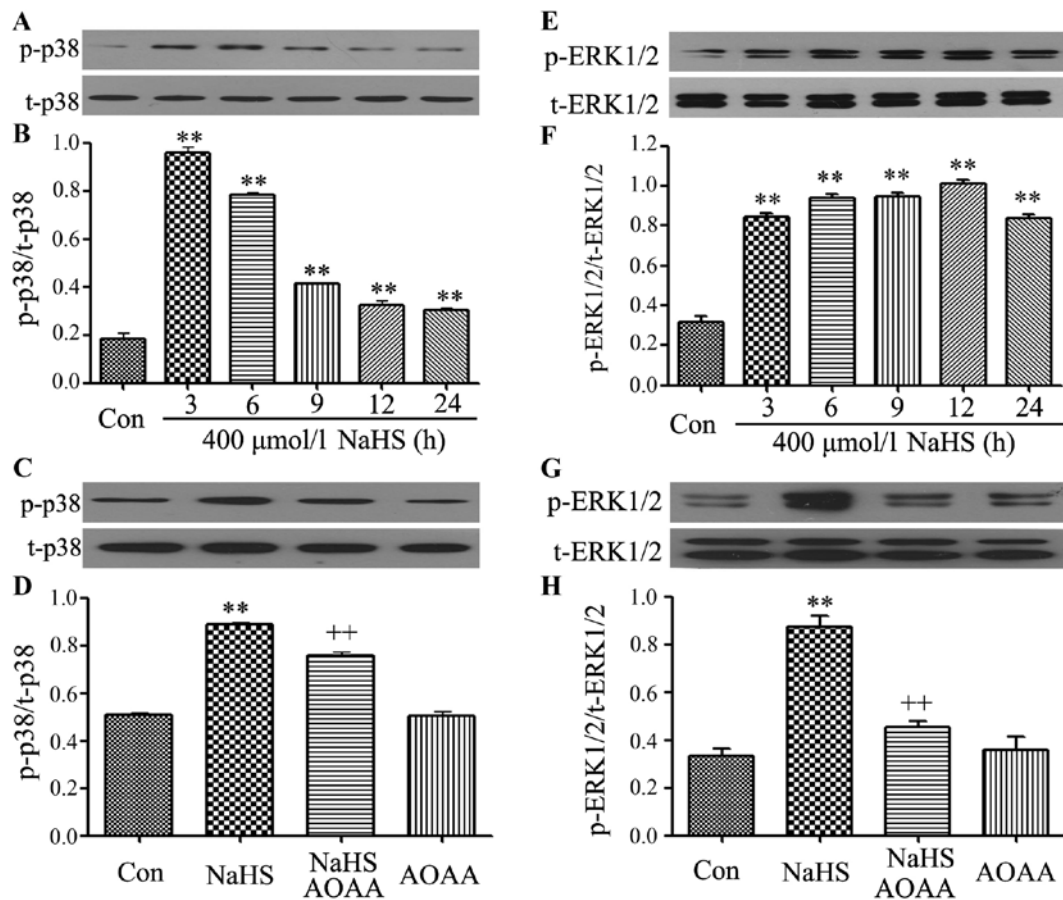


Figure 3. AOAA ameliorates the phosphorylation of p38 MAPK and ERK1/2 induced by NaHS in C6 glioma cells. (A, B, E and F) C6 glioma cells were treated with 400  $\mu\text{mol/l}$  NaHS for the indicated times (3, 6, 9, 12 and 24 h). (C, D, G and F) C6 glioma cells were co-treated with 400  $\mu\text{mol/l}$  NaHS and 2 mmol/l AOAA for 24 h. The expression levels of p38 MAPK, and ERK1/2 were detected by western blotting. (A, C, E and G) The representative image shows the changes in the expression levels of p38 MAPK (A and C) and ERK1/2 (E and G) in the indicated groups. (B, D, F and H) Densitometric analysis for the results in A, C, E and G, respectively. Data are presented as mean  $\pm$  SEM (n=3). \*\*p<0.01 compared with the control group; ++p<0.01 compared with the NaHS-treated group.

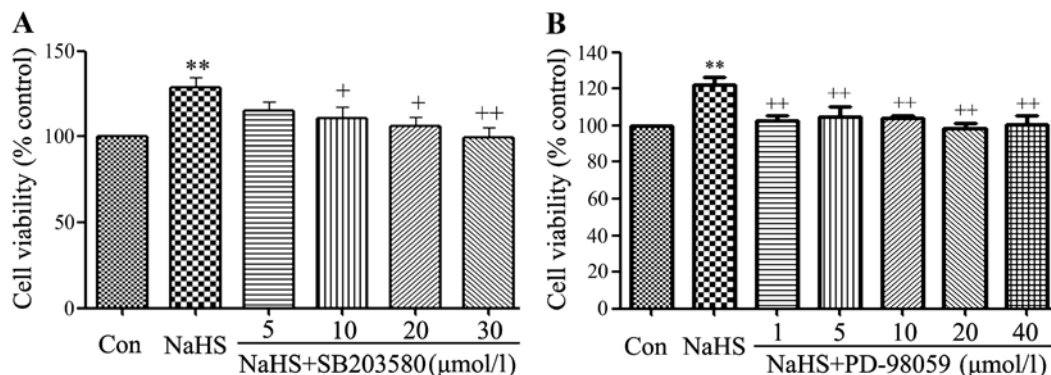


Figure 4. SB203580 and PD98059 alleviate NaHS-induced cell proliferation in C6 glioma cells. C6 glioma cells were co-conditioned with 400  $\mu\text{mol/l}$  NaHS and different doses of SB203580 (5, 10, 20 and 30  $\mu\text{mol/l}$ ) or PD-98059 (1, 5, 10, 20 and 40  $\mu\text{mol/l}$ ) for 24 h. Data are the mean  $\pm$  SEM (n=3). \*\*p<0.01 compared with the control group. \*p<0.05, ++p<0.01 compared with the NaHS group. Con, the control group; NaHS, a donor of  $\text{H}_2\text{S}$ ; SB203580, a specific inhibitor of p38 MAPK pathway; PD-98059, a specific inhibitor of ERK1/2 pathway.

On the contrary, NaHS alone markedly upregulated the expression level of cleaved caspase-3. As illustrated in Fig. 5F and G, exposure of cells to indicated doses of NaHS (100, 200, 400, 800 and 1,600  $\mu\text{mol/l}$ ) for 24 h markedly downregulated the expression level of cleaved caspase-3. Whereas, exposure of cells to 400  $\mu\text{mol/l}$  NaHS and

30  $\mu\text{mol/l}$  SB203580 (Fig. 5C-c and -d) or 20  $\mu\text{mol/l}$  PD-98059 (Fig. 5C-e and -f) significantly upregulated the expression level of cleaved caspase-3.

*SB203580, PD98059 and AOAA inhibit the NaHS-induced increase of expression of COX-2 in C6 glioma cells.* In order



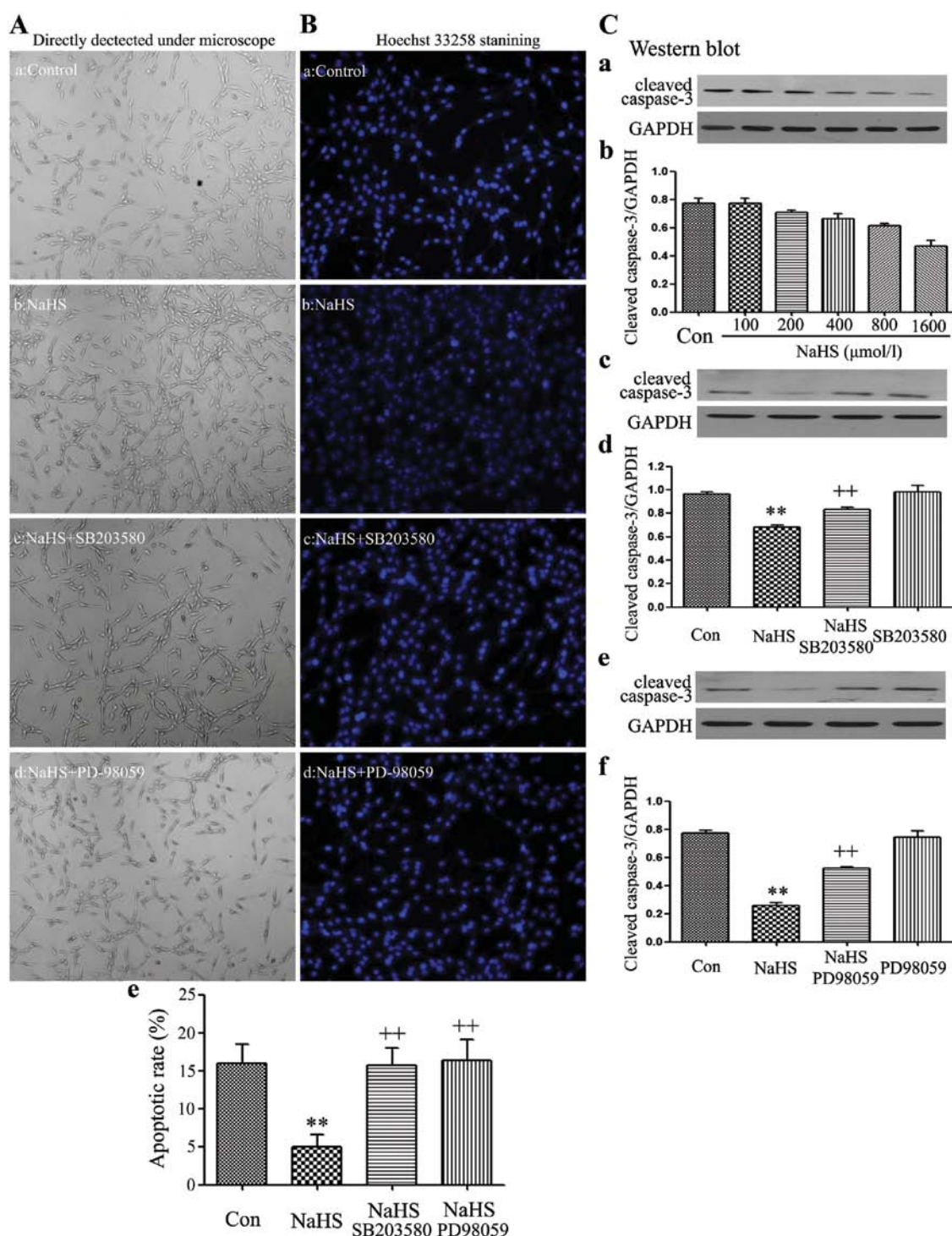


Figure 5. SB203580 and PD98059 increase NaHS-induced cell proliferation in C6 glioma cells. (A-a-d) Cell number was detected using a fully automatic inverted microscope. (B-a-d) Hoechst 33258 nuclear staining followed by photofluography was carried out to test cell apoptosis. (A-a and B-a) Control group. (A-b and B-b) Cells exposed to 400  $\mu\text{mol/l}$  NaHS for 24 h. (A-c and B-c) Cells were co-treated with 400  $\mu\text{mol/l}$  NaHS and 30  $\mu\text{mol/l}$  SB203580 for 24 h. (A-d and B-d) Cells were co-treated with 400  $\mu\text{mol/l}$  NaHS and 30  $\mu\text{mol/l}$  SB203580 for 24 h. (B-e) The apoptotic rate was analyzed using a cell counter and ImageJ 1.47i software. The expression levels of cleaved caspase-3 were measured by western blotting assay. (C-a, -c and -e) Quantified by densitometric analysis with ImageJ 1.47i software (C-b, -d and -f). Data are shown as the means  $\pm$  SEM ( $n=3$ ). \*\* $p<0.05$ , \*\* $p<0.01$  compared with the NaHS group. Con, the control group; NaHS, a donor of  $\text{H}_2\text{S}$ ; SB203580, a specific inhibitor of p38 MAPK pathway; PD-98059, a specific inhibitor of ERK1/2 pathway.

to observe the effects of NaHS on the expression levels of COX-2 and MMP-2 in C6 glioma cells, cells were exposed to 400  $\mu\text{mol/l}$  NaHS for different times (3, 6, 9, 12 and 24 h). As shown in Fig. 6A and B, NaHS significantly enhanced the expression levels of COX-2 reaching a peak at 12 h.

Notably, co-treatment of C6 glioma cells with 400  $\mu\text{mol/l}$  NaHS and 30  $\mu\text{mol/l}$  SB203580 (Fig. 6E and F) or 20  $\mu\text{mol/l}$  PD-98059 (Fig. 6G and H) or 2 mmol/l AOA (Fig. 6C and D) for 24 h considerably depressed the NaHS-induced increase of expression of COX-2. Treatment of cells with 30  $\mu\text{mol/l}$ ,

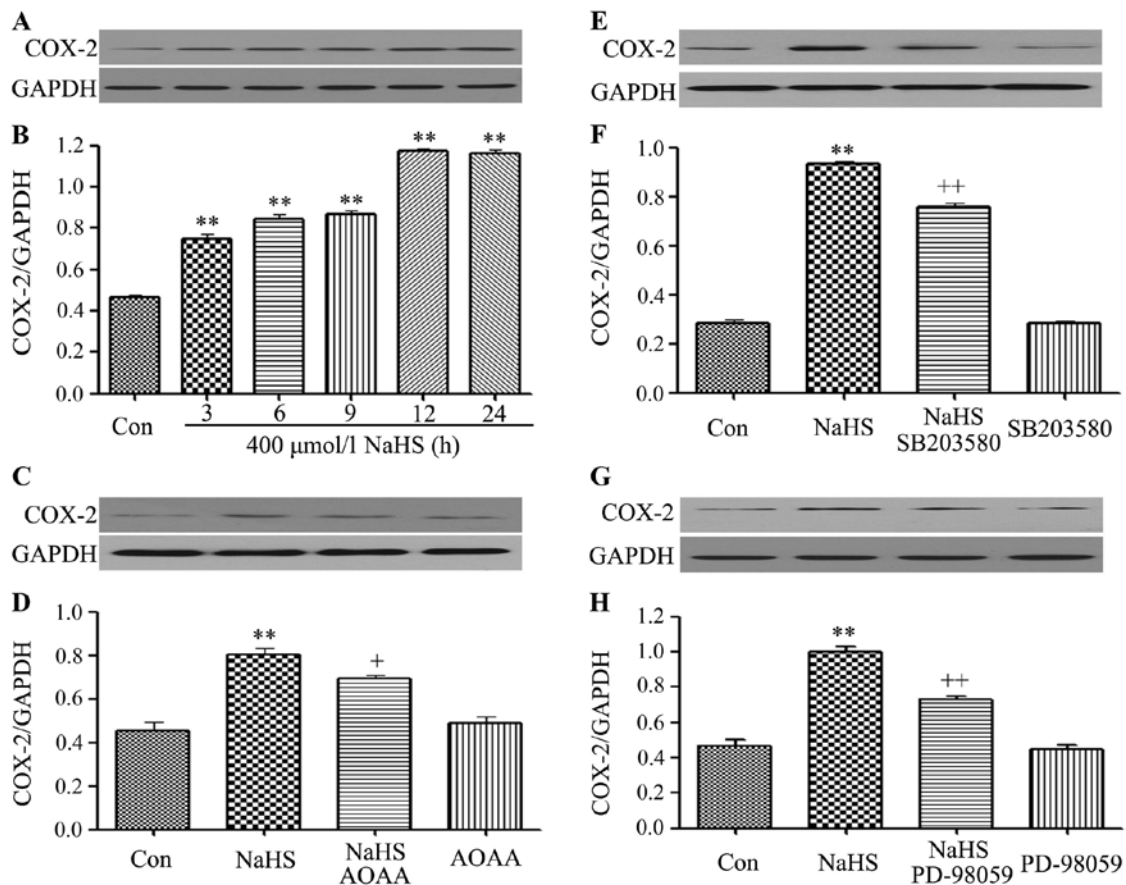


Figure 6. SB203580, PD98059 and AOA inhibit the NaHS-induced increase of COX-2 expression in C6 glioma cells. (A and B) C6 glioma cells were exposed to 400  $\mu$ mol/l NaHS for different times (3, 6, 9, 12 and 24 h). (C and D) Cells were co-treated with 400  $\mu$ mol/l NaHS and 2 mmol/l AOA for 24 h. (E and F) Cells were co-treated with 400  $\mu$ mol/l NaHS and 30  $\mu$ mol/l SB203580 for 24 h. (G and H) Cells were co-treated with 400  $\mu$ mol/l NaHS and 20  $\mu$ mol/l PD-98059 for 24 h. The expression level of COX-2 was measured by western blot analysis. (B, D, F and H) The data in (A, C, E and G) was quantified by densitometric analysis with ImageJ 1.47i software. Data are shown as the mean  $\pm$  SEM (n=3). \*\*p<0.01 vs. with the control group; +p<0.05, ++p<0.01 vs. the NaHS group. Con, the control group; NaHS, a donor of H<sub>2</sub>S; SB203580, a specific inhibitor of p38 MAPK pathway; PD-98059, a specific inhibitor of ERK1/2 pathway.

SB203580 alone, or single treatment of 20  $\mu$ mol/l PD-98059 or 2 mmol/l AOA, respectively, for 24 h did not alter the basal expression level of COX-2.

*NS-398 alleviates the NaHS-induced increase of cell viability in C6 glioma cells.* As shown in Fig. 7, exposure of C6 glioma cells to 400  $\mu$ mol/l NaHS for 24 h obviously induced cell proliferation, leading to an increase in cell viability. However, the increased cell viability was repressed by co-treatment with different doses of NS-398 (a specific inhibitor of COX-2 pathway) for 24 h.

As shown in Fig. 4, 0.01  $\mu$ mol/l NS-398 did not alter cell viability. On the contrary, the dose of NS-398 from 0.1 to 10  $\mu$ mol/l significantly suppressed the cell proliferation, leading to a decrease in cell viability and reaching the minimum at 10  $\mu$ mol/l. According to the above results, C6 glioma cells were co-treated with 400  $\mu$ mol/l NaHS and 10  $\mu$ mol/l NS-398 for 24 h in the following experiments.

*NS-398 increases the NaHS-induced cell proliferation in C6 glioma cells.* Cell number was detected using a fully automatic inverted microscope (Fig. 5-a-d). C6 glioma cells were co-treated with 400  $\mu$ mol/l NaHS and 10  $\mu$ mol/l NS-398 for 24 h. As observed in Fig. 8A-b, alone 400  $\mu$ mol/l NaHS markedly promoted cell proliferation, leading to an increase in the

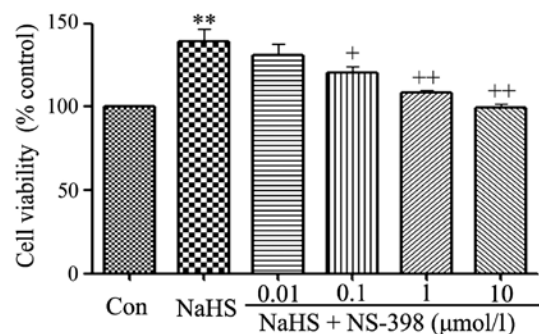


Figure 7. NS-398 alleviates NaHS-induced cell proliferation in C6 glioma cells. C6 glioma cells were co-conditioned with 400  $\mu$ mol/l NaHS and different doses of NS-398 (0.01, 0.1, 1 and 10  $\mu$ mol/l) for 24 h. Data are the mean  $\pm$  SEM (n=3). \*\*p<0.01 compared with the control group, +p<0.05, ++p<0.01 compared with the NaHS group. Con, the control group; NaHS, a donor of H<sub>2</sub>S; NS-398, a specific inhibitor of COX-2 pathway.

cell number. However, exposure of cells to 400  $\mu$ mol/l NaHS and 10  $\mu$ mol/l NS-398 significantly alleviated the NaHS-induced cell proliferation in C6 glioma cells.

Apoptotic cell death was evaluated by the Hoechst 33258 staining followed by photofluorography. As shown in the Fig. 8B-a-d, exposure of C6 glioma cells to 400  $\mu$ mol/l

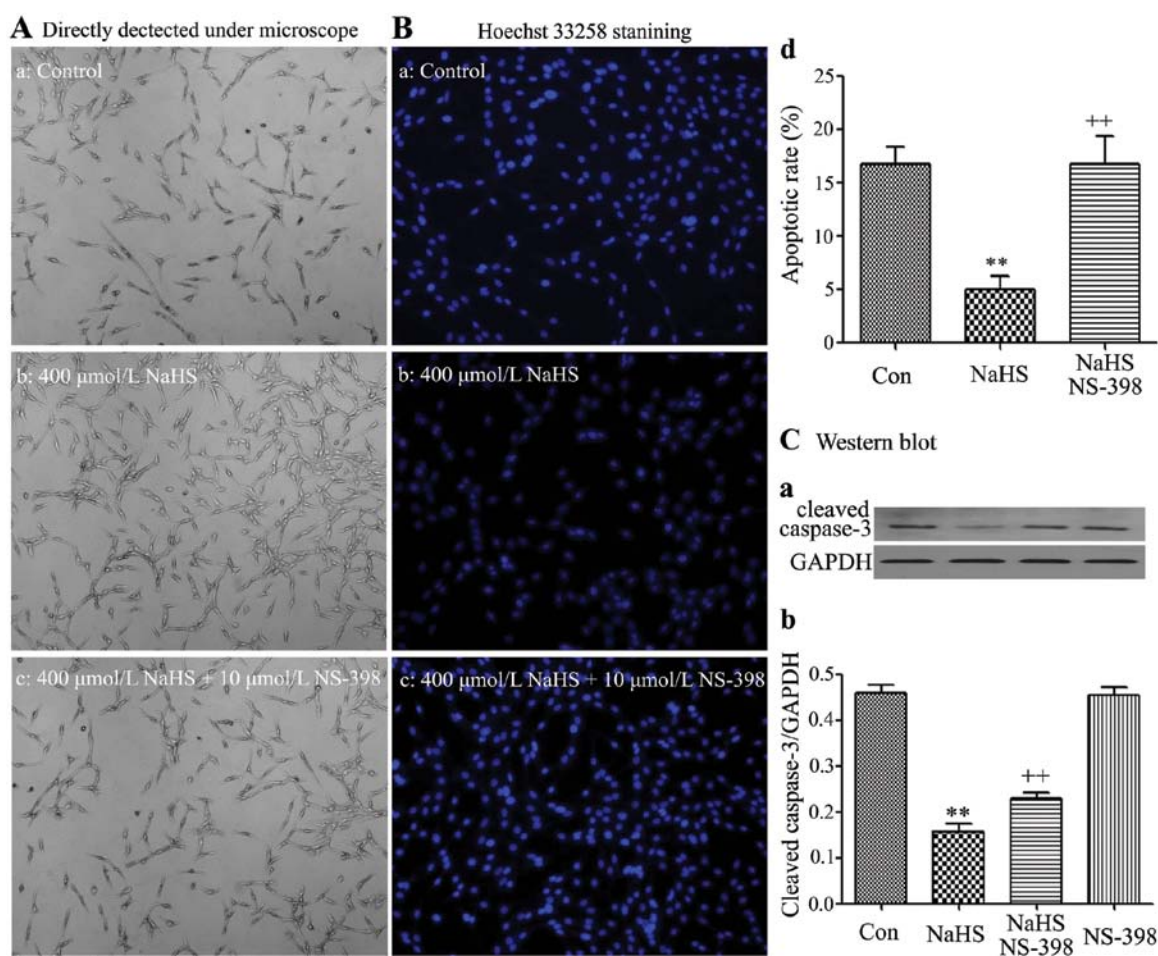


Figure 8. NS-398 increases the NaHS-induced cell proliferation in C6 glioma cells. (A-a-c) Cell number was detected using a fully automatic inverted microscope. (B-a-d) Hoechst 33258 nuclear staining followed by photofluography was carried out to test cell apoptosis. (A-a and B-a) Control group. (A-b and B-b) Cells exposed to 400  $\mu\text{mol/L}$  NaHS for 24 h. (A-c and B-c) Cells were co-treated with 400  $\mu\text{mol/L}$  NaHS and 10  $\mu\text{mol/L}$  NS-398 for 24 h. (B-d) The apoptotic rate was analyzed using a cell counter and ImageJ 1.47i software. The expression levels of cleaved caspase-3 were measured by western blotting assay. (C-a) Quantified by densitometric analysis with ImageJ 1.47i software (C-b). Data are shown as the means  $\pm$  SEM (n=3). \* $p$ <0.01 compared with the control group. \*\* $p$ <0.01 compared with the NaHS group. Con, the control group; NaHS, a donor of  $\text{H}_2\text{S}$ ; NS-398, a specific inhibitor of COX-2 pathway.

NaHS for 24 h reduced the typical characteristics of apoptosis, as evidenced by the condensation of chromatin, the shrinkage of nuclei and the formation of apoptotic bodies. On the contrary, exposure of cells to 400  $\mu\text{mol/L}$  NaHS and 10  $\mu\text{mol/L}$  NS-398 significantly induced the typical characteristics of apoptosis.

On the contrary, NaHS alone markedly downregulated the expression level of cleaved caspase-3. As illustrated in Fig. 8C-a and -b, exposure of cells to 400  $\mu\text{mol/L}$  NaHS for 24 h markedly downregulated the expression level of cleaved caspase-3. Whereas, exposure of cells to 400  $\mu\text{mol/L}$  NaHS and 10  $\mu\text{mol/L}$  NS-398 significantly upregulated the expression level of cleaved caspase-3.

## Discussion

In the present study, we demonstrated a novel finding of tumor development by hydrogen sulfide ( $\text{H}_2\text{S}$ ) on C6 glioma cells and also provided data to reveal its potential mechanisms. This conclusion is supported by several lines of evidence as follows: i) addition of NaHS (a donor of  $\text{H}_2\text{S}$ ; 100-1,600  $\mu\text{mol/L}$ ) promoted cell proliferation, leading to an increase in cell

viability and an increase in cell number; ii) NaHS-induced a decrease in cell apoptosis, by decreasing expression level of caspase-3 and cell apoptosis; iii) AOAA inhibited the NaHS-induced proliferation and anti-apoptosis; iv) NaHS activated the p38 MAPK/ERK1/2 pathways and promoted the expression level of COX-2, and those effects were abolished by AOAA; v) SB203580 and PD98059 downregulated the NaHS-induced increased expression level of COX-2, respectively; vi) co-treatment of C6 glioma cells with NaHS SB203580, PD98059 or NS-398 suppressed the NaHS-induced proliferation and anti-apoptosis.

$\text{H}_2\text{S}$  is produced in the body mainly by three pyridoxal-59-phosphate-dependent enzymes, 3-mercaptopyruvate sulfurtransferase (3-MST), cystathionine- $\beta$ -synthase (CBS) and cystathionine- $\gamma$ -lyase (CSE). However, CBS is mainly found in the central nervous system (CNS) (32,33). A recent study suggested that  $\text{H}_2\text{S}$  plays an important role in various physiological and pathological processes of the nervous system as a neuromodulator and neuroprotectant (34). Jiang *et al* found that  $\text{H}_2\text{S}$  protected the brain against injuries and exerted neuroprotection by modulating the underlying signaling pathways (35-37). In addition,  $\text{H}_2\text{S}$  exerted protection to nerve



cancer cells, such as PC12 cells (38). It can be concluded that H<sub>2</sub>S may be involved in development of the nervous system cancer cells. In order to confirm this hypothesis, C6 glioma cells were exposed to NaHS (a donor of H<sub>2</sub>S). Unexpectedly, we found two interesting results. Firstly, different doses of NaHS (100-1,600  $\mu$ mol/l) promoted cell proliferation in the range of physiological doses of H<sub>2</sub>S (0.2-1 mmol/l) the optimal concentration of NaHS that induced maximal effect of proliferation was 400  $\mu$ mol/l, leading to an increase in cell viability and an increase in cell number. Secondly, treatment of cells with 400  $\mu$ mol/l NaHS for 24 h markedly diminished cell apoptosis, and decreased the expression of caspase-3, one of the apoptotic factors, and the above NaHS-induced effects were inhibited by AOAA. These results demonstrate that H<sub>2</sub>S induces C6 glioma cell proliferation via exerting its dual cytoprotective and anti-apoptosis effects, and H<sub>2</sub>S may be involved in glioma growth under physiological conditions. Along with a previous study that H<sub>2</sub>S-protected PC12 cells from formaldehyde induced apoptosis (38), the results suggest that H<sub>2</sub>S exerts a cytoprotective effect for C6 glioma cells. The effects of H<sub>2</sub>S on cell proliferation and anti-apoptosis are complicated and unclear. In the present study, we investigated whether H<sub>2</sub>S promoted the proliferation ability and anti-apoptosis of C6 glioma cells *in vitro* by activation of the p38 MAPK/ERK1/2-COX-2 signaling pathways.

It has been reported that COX-2 pathway is closely associated with tumor cell growth and is a crucial molecule in the development of malignant tumors, and angiogenesis (15,16), anti-apoptosis (17), invasiveness (18) and proliferation (19). In the present study, we found that NS-398 (an inhibitor of COX-2) increased caspase-3 expression, which is consistent with previous research results (17,31). In addition, treatment of cells with 400  $\mu$ mol/l NaHS for 24 h promoted the expression level of COX-2, along with the NaHS-induced pro-proliferative effect and anti-apoptosis. Combined treatment with a COX-2 inhibitor (NS-398) and 400  $\mu$ mol/l NaHS resulted in a decrease in cell viability, a decrease in cell number, an increase in cell apoptosis and in increased expression level of cleaved caspase-3, indicating that NS-398 increases NaHS-induced cell proliferation in C6 glioma cells. Those results suggest that COX-2 pathway is necessary in NaHS-induced C6 glioma cell proliferation and anti-apoptosis.

The COX-2 pathway and its inhibitors are regulated by multiple signaling cascades, including the p38 signaling pathway as well as ERK1/2 and NF- $\kappa$ B-mediated pathways (15,20,31). To confirm whether NaHS-mediated activation of COX-2 was regulated by p38 MAPK or ERK1/2, C6 glioma cells were co-treated with 400  $\mu$ mol/l NaHS and SB203580 (an inhibitor of p38 MAPK) or PD98059 (an inhibitor of ERK1/2), the expression level of COX-2 was downregulated. This result suggested that COX-2 was downstream of p38 MAPK or ERK1/2 pathways. In addition, treatment C6 glioma cells with 400  $\mu$ mol/l NaHS activated p38 MAPK and ERK1/2 pathway, which is supported by NaHS-induced upregulation of p-p38 MAPK and ERK1/2 expression levels. Co-treatment of C6 glioma cells with NaHS and SB203580, PD98059 or NS-398 suppressed NaHS-induced proliferation and anti-apoptosis. Therefore, we concluded that p38 MAPK/ERK1/2-COX-2 pathways are involved in NaHS-induced cancer cell proliferation and anti-apoptotic in C6 glioma cells.

NaHS exert its proliferation and anti-apoptosis via activating p38 MAPK/ERK1/2-COX-2 pathways in C6 glioma cells, however, whether NaHS exerts its migration via activating p38 MAPK/ERK1/2-COX-2 pathways is still unclear, and needs further research *in vivo* and *in vitro*. The effects of H<sub>2</sub>S on the cancer are not completely coincident. Our research (31) and other studies (24-30) suggest that H<sub>2</sub>S is beneficial for cancer cells growth, proliferation, migration and invasion. On the contrary, some other researchers found that H<sub>2</sub>S exerted potential anticancer efficiency in SGC-7901 gastric cancer cells (40), oral cancer cell lines (41), colon cancer cells (42), and in several different human cancer cell lines (HeLa, HCT-116, Hep G2, HL-60, MCF-7, MV4-11 and U2OS) (43). As the above is contradictory further research must be carried out to clarify these issues.

In summary, H<sub>2</sub>S-induced cells proliferation and anti-apoptosis in C6 glioma cells. This effect may be mediated by the activation of p38 MAPK/ERK1/2-COX-2 pathways, leading to downregulation of caspase-3, increased cell viability, increased in cell number and decreased number of apoptotic cells. In C6 glioma, the findings provide a novel insight into a unified concept and identify CBS-derived H<sub>2</sub>S as an endogenous tumor-promoting factor and anticancer drug target. Furthermore, the migration and invasion of H<sub>2</sub>S in C6 glioma cells is still unclear and needs to be further investigated.

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