Exogenous hydrogen sulfide exerts proliferation/anti-apoptosis/angiogenesis/migration effects via amplifying the activation of NF-κB pathway in PLC/PRF/5 hepatoma cells

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Abstract. Hydrogen sulfide (H_2S) takes part in a diverse range of intracellular pathways and hss physical and pathological properties *in vitro* and *in vivo*. However, the effects of H_2S on cancer are controversial and remain unclear. The present study investigates the effects of H_2S on liver cancer progression via activating NF-κB pathway in PLC/PRF/5 hepatoma cells. PLC/PRF/5 hepatoma cells were pretreated with 500 μmol/l NaHS (a donor of H_2S) for 24 h. The expression levels of CSE, CBS, phosphosphorylate (p)-NF-κB p65, caspase-3, COX-2, p-IκB and MMP-2 were measured by western blot assay. Cell viability was detected by cell counter kit 8 (CCK-8). Apoptotic cells were observed by Hoechst 33258 staining assay. The production level of H_2S in cell culture medium was measured by using the sulfur-sensitive electrode method. The production of vascular endothelial growth factor (VEGF) was tested by enzyme-linked immunosorbent assay (ELISA). Our results showed that the production of H_2S was dramatically increased in the PLC/PRF/5 hepatoma cells, compared with human LO2 hepatocyte cells group, along with the overexpression levels of CSE and CBS. Treatment of PLC/PRF/5 hepatoma cells with 500 μmol/l NaHS (a donor of H_2S) for 24 h markedly increased the expression levels of CSE, CBS, p-IκB and NF-κB activation, leading to COX-2 and MMP-2 overexpression, and decreased caspase-3 production, as well as increased cell viability and decreased number of apoptotic cells. Otherwise, the production level of H_2S and VEGF were also significantly increased. Furthermore, co-treatment of PLC/PRF/5 hepatoma cells with 500 μmol/l NaHS and 200 μmol/l PDTC for 24 h significantly overturned these indexes. The findings of the present study provide evidence that the NF-κB is involved in the NaHS-induced cell proliferation, anti-apoptisis, angiogenesis, and migration in PLC/PRF/5 hepatoma cells, and that the PDTC against the NaHS-induced effects were by inhibition of the NF-κB pathway.

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Introduction

The incidence and mortality of primary liver cancer (PLC) are increasing year by year (1). PLC is one of the most common malignant tumors in China, the occurrence of liver cancer is approximately one million people all around the world each year and most of them occur in mainland of China, accounting for 54% of all cases worldwide (2). PLC contains mainly three subcategories: hepatocellular carcinoma (HCC), intrahepatic bile duct carcinoma (IHBD) and hybrid liver cancer. HCC is the major form of PLC and is the sixth most common neoplasm in the world (3). Over the past several decades, a series of risk factors for HCC have been established (4-7), including hepatitis B virus (HBV) or hepatitis C virus (HCV) infection, aflatoxin exposure, and tobacco smoking. However, the mechanisms of the oncogenesis of HCC have not been completely described. Recently, several studies have strongly suggested that nuclear factor-κB (NF-κB) may play a pivotal role in this pathophysiological process (8-11).

NF-κB, which contains five subunits [RelA (p65), C-Rel, NF-κB1 (P50/P105), RelB and NF-κB2 (P52/P100)], is a major transcription regulator of the cell functions, including adhesion, immune response, cell invasion, cell differentiation, cell proliferation and apoptosis (12). In the resting cells, NF-κB

is a dimer and is inactivated retentively in the cytoplasm via binding to specific inhibitor of NF-κB: IκB family. However, NF-κB can be activated by various stimuli (13-18). First, IκB is phosphorylated, and NF-κB is activated and released from its IκB-bound complex. In addition, NF-κB translocates into the nucleus from cytoplasm. The activated NF-κB (p65) binds to κB sequences and alters the expression of various target genes (13). The above physiologic process also occurred in different tumor tissues or in cancer cells (14-18), such as breast cells, and especially in liver cancer cells (19), indicating that NF-κB is involved in oncogenesis process of liver cancer.

Hydrogen sulfide (H_2S), is an unusual virulent gas, and has been qualified as the third gasotransmitter following nitric oxide (NO) and carbon monoxide (CO) (20-22). H_2S can be endogenously produced mainly by 3-mercaptopyruvate sulfurtransferase (3-MST), cystathionineb-synthase (CBS) or cystathionine-γ-lyase (CSE) (23,24). The expression of CSE and CBS is distinctly tissue-specific. CBS is mainly found in the central nervous system (CNS), and CSE mostly in the cardiovascular system. Interestingly, both CBS and CSE have been simultaneously identified in some systems, including kidney, liver, intestine and brain (25). In recent years, more attention is paid to H_2S for its extensive physiological and pathophysiological properties. Accumulating studies have demonstrated that H_2S can exert cardioprotection (26-31), angiogenesis (32-34), antioxidant (35), pro- and anti-inflammatory (36,37) and other wide range of physiological functions (38-41). Furthermore, increasing evidence has shown that H_2S is involved in the pathophysiological process of tumors (42-52). Notably, the effects of H_2S on cancer are still controversial. On the one hand, some findings from *in vivo* and *in vitro* studies showed that H_2S is beneficial for cancer cell growth, proliferation, migration, and invasion (42-48), owing to its angiogenesis and vascular relaxant effects, H_2S promotes the supply of nutrients and blood to the tumor cells and tissues (42). Nevertheless, on the other hand it was found that H_2S exerted its potential anticancer effects on SGC-7901 gastric cancer cells (49), oral cancer cell lines (50), colon cancer cell (51) and several different human cancer cell lines (HeLa, HCT-116, Hep G2, HL-60, MCF-7, MV4-11 and U2OS) (52). Thus, the effects of H_2S on cancer are complicated and still unclear. Furthermore, to our knowledge, no study has been focused on the effect of exogenous H_2S on liver cancer cells and its mechanisms. Based on recent studies (42-48), we investigated whether exogenous H_2S can contribute to cancer progress and explored these potential effects via amplification of NF-κB pathway in PLC/PRF/5 hepatoma cells.

Materials and methods

Materials. NaHS, a donor of H_2S, was obtained from Sigma Chemical Co. (St. Louis, MO, USA), stored at 2-4˚C and protected from sunlight. Hoechst 33258 and PDTC were also purchased from Sigma Chemical Co. The cell counter kit-8 (CCK-8) was supplied by Dojindo Lab (Kumamoto, Japan). Fetal bovine serum (FBS) and RPMI-1640 medium were obtained from Gibco BRL (Grand Island, NY, USA). Anti-MMP2 antibody, anti-COX-2 antibody, anti-p-IκB antibody, anti-NF-κB p65 antibody and anti-p-NF-κB p65 antibody 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). A sulfur-sensitive electrode was obtained from Fuji Electric (ELIT 8225, ISEIonometer, EA Instruments Ltd., UK).

Cell culture and treatments. The human hepatoma cells PLC (PLC) and the Lo2 cells (LO2) were supplied by Sun Yat-sen University Experimental Animal Center (Guangzhou, Guangdong, China). The PLC cells and LO2 cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum under an atmosphere of 5% CO_2 and at 37˚C with 95% air. The PLC cells were treatment with 500 μmol/l NaHS for 24 h or co-treatment with 500 μmol/l NaHS and 200 μmol/l PDTC for 24 h.

Western blot analysis. After the indicated treatments, 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, then boiled 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-MMP2 antibody (1:1,000 dilution), anti-COX-2 antibody (1:1,000 dilution), anti-p-IκB antibody (1:1,000 dilution), anti-NF-κB p65 antibody (1:1,000 dilution), and anti-p-NF-κB p65 antibody (1:1,000 dilution) in freshly prepared TBS-T with 3% free-fat milk overnight 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 Bio-tech), 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 via using the ECL (enhanced chemiluminescence) detection. 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 three times.

Measurement of cell viability. The PLC cells were seeded in 96-well plates at concentration of $1x10^4$/ml, and incubated at 37˚C, the CCK-8 assay was employed to assess the cell viability of PLC 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 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) x 100%. The experiment was carried out in three times.

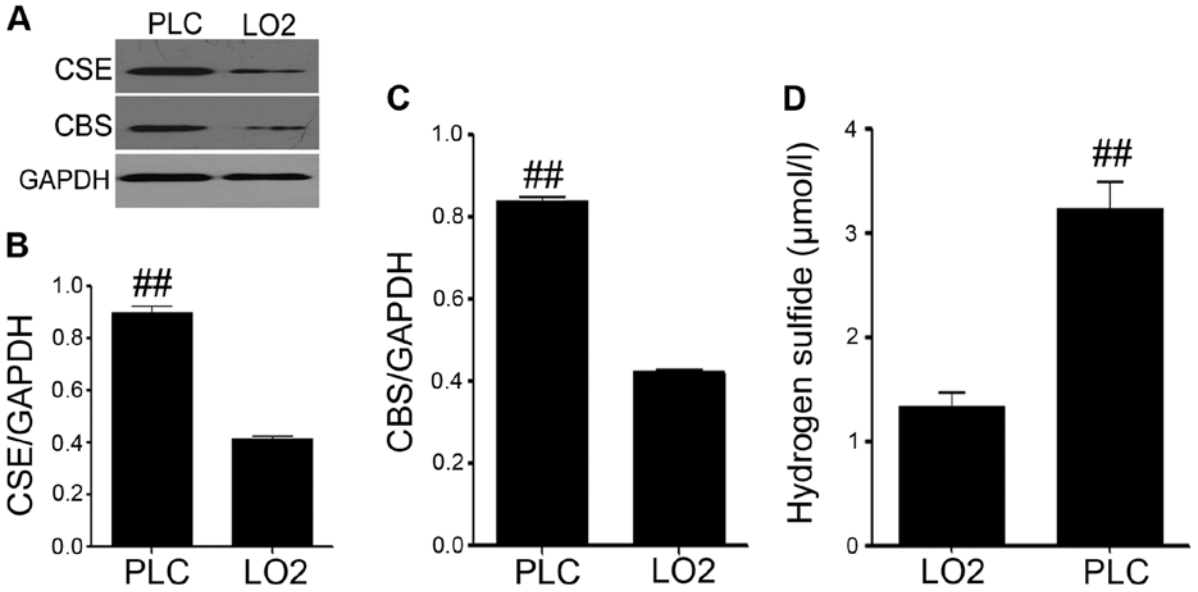

Figure 1. H_2S, CBS and CSE are overexpressed in PLC/PRF/5 hepatoma cells. (A-C) The expression levels of both CSE and CBS were detected by western blot assay and quantified by densitometric analysis with ImageJ 1.47i software (B and C). The production of H_2S in the cell culture medium was explored by sulfur-sensitive electrode (D). Data are shown as the mean ± SE (n=3). $^{\#\#}$P<0.01, compared with the PLC group. CSE, cystathionine-γ-lyase; CBS, cystationine-β-synthase; PLC, PLC/PRF/5 hepatoma cells; LO2, human normal liver cell line.

Hoechst 33258 nuclear staining for evaluation of apoptosis. Apoptotic cell death was tested by the Hoechst 33258 staining followed by photofluorography. PLC cells were plated in 35-mm dishes at a density of $1x10^6$ cells/well. After the above indicated treatments, the PLC cells were fixed with 4% paraformaldehyde in 0.1 mol/l phosphate-buffered saline (PBS, pH 7.4) for 10 min at 4˚C. In addition, 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. The PLC cells were visualized under a fluorescence microscope (Bx50-FLA; Olympus, Tokyo, Japan). Viable PLC cells displayed a uniform blue fluorescence throughout the nucleus and normal nuclear size. However, apoptotic PLC cells showed condensed, distorted or fractured nuclei. The experiment was carried out three times.

Measurement of cell culture medium H_2S levels. A sulfur-sensitive electrode (ELIT 8225, ISEIonometer, EA Instruments Ltd.) was used to measure the production levels of H_2S in the cell culture medium. Cell culture medium samples were obtained from all subjects and mixed with an equal volume of antioxidant solution. The total volume covered the electrode; usually >0.8 ml. The electrode was activated in the deionized water for ≥2 h. The modified sulfide electrode and a reference electrode were dipped into the above mixture. The electrode was flushed with the deionized water after sample determination, and the activity state was maintained through dipping the electrode in deionized water. A standard curve was generated using a standard S2-solution, and then the H_2S concentration was calculated according to this standard curve.

ELISA for detection of VEGF in culture supernatant. PLC/PRF/5 hepatoma cells were cultured in 96-well plates. After the different indicated treatments, the level of VEGF in the culture media was tested by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instruction. The experiment was performed at least five times.

Statistical analysis. All data are presented as the mean ± SEM. Differences between groups were analyzed by one-way analysis of variance (ANOVA) by using SPSS 13.0 (SPSS, Chicago, IL, USA) software, and followed by LSD *post hoc* comparison test. Statistical significance was set at P<0.05.

Results

The expression levels of CBS and CSE, and endogenous H_2S production in PLC/PRF/5 hepatoma cells. To determine the role of hydrogen sulfide (H_2S) in the oncogenesis process of liver cancer, the expression levels of CSE and CBS were detected by western blot assay both in PLC/PRF/5 hepatoma cells (PLC) and in the human hepatic cell lines LO2 (LO2). As shown in Fig. 1A-C, both the expression of CSE (Fig. 1A and B) and CBS (Fig. 1A and C) were significantly upregulated, compared with the LO2 group. In addition, the sulfur-sensitive electrode was used to explore the production level of H_2S in the cell culture medium. We found that the production of hydrogen sulfide in PLC/PRF/5 hepatoma cells was distinctly increased, compared with the LO2 group (Fig. 1D). So we hypothesized that endogenous H_2S might take part in the development of liver cancer.

NaHS promotes cells proliferation in PLC/PRF/5 hepatoma cells. In order to test the effect of exogenous H_2S in human liver cancer cell proliferation, the dose-response study with varying doses (10, 50, 100, 200, 300, 400, 500, 600,

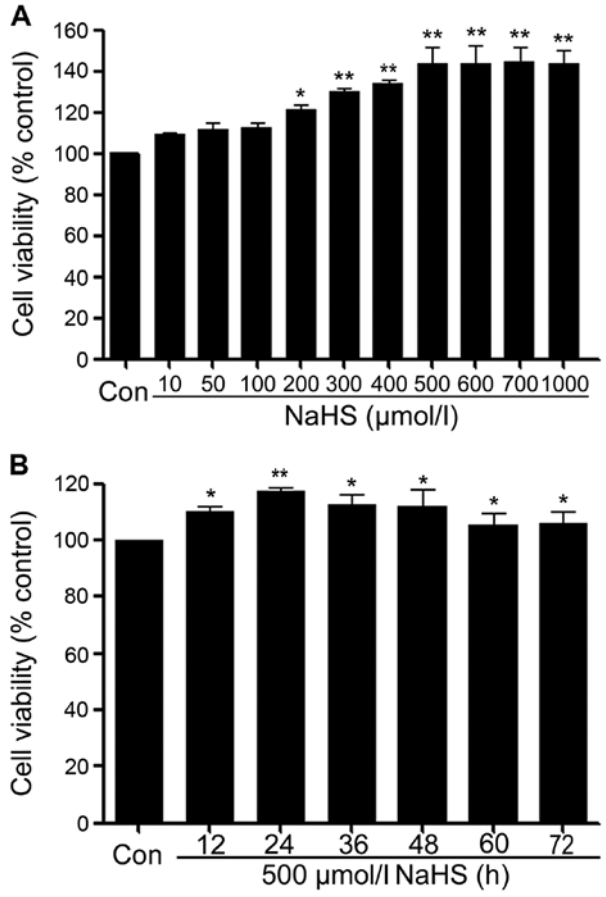

Figure 2. NaHS promotes cells proliferation in PLC/PRF/5 hepatoma cells. Cell viability was tested by using the cell counter kit (CCK-8). (A) PLC cells were treated with different doses of NaHS (10, 50, 100, 200, 300, 400, 500, 600, 700 and 1,000 μmol/l) for 24 h. (B) Cells were treated with 500 μmol/l NaHS for the indicated times (12, 24, 36, 48, 60 and 72 h). Data are the mean ± SEM (n=3). *p<0.05, **p<0.01 compared with the control group. Con, the control group; NaHS, a donor of H_2S.

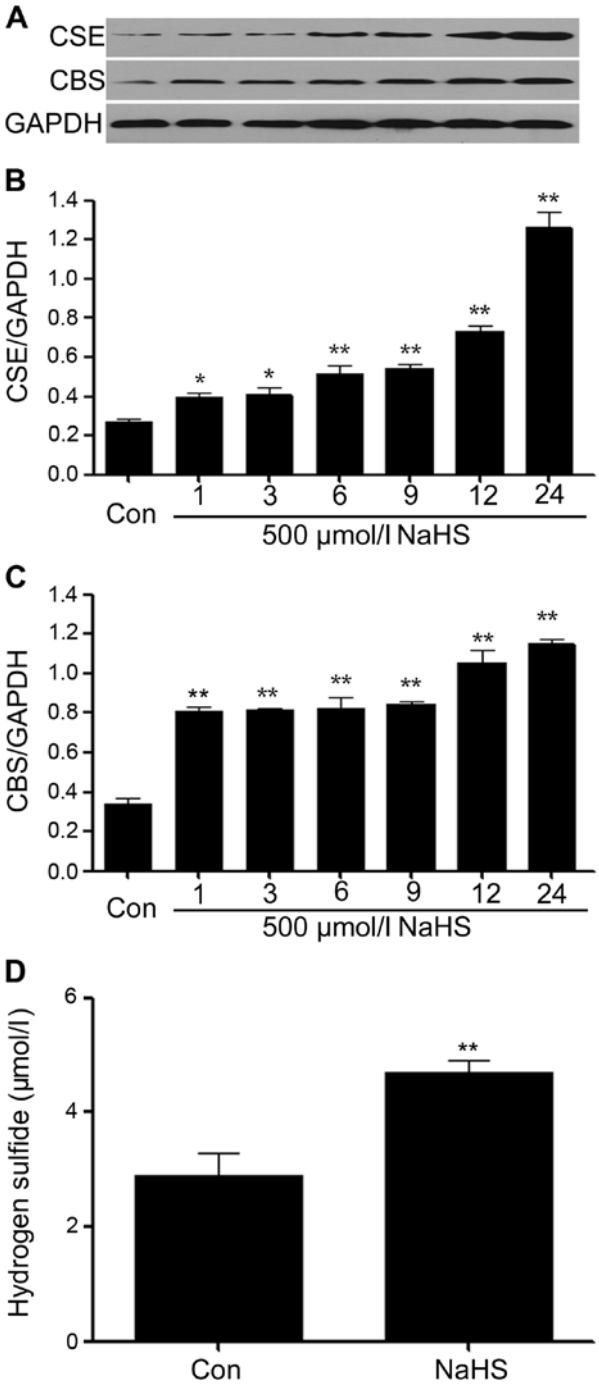

Figure 3. NaHS upregulates the expression levels of CSE and CBS and H_2S production in PLC/PRF/5 hepatoma cells. (A-C) PLC/PRF/5 hepatoma cells were exposed to 500 μmol/l NaHS for the indicated times (1, 3, 6, 9, 12 and 24 h). The expression levels of CSE and CBS were measured by western blot analysis. The production of H_2S in the cell culture medium was explored by sulfur-sensitive electrode. The data in (A) was quantified by densitometric analysis with ImageJ 1.47i software. (D) The production level of H_2S was tested by a sulfur-sensitive electrode (ELIT 8225). Data are shown as the mean ± SEM (N=3). *P<0.05, **P<0.01 versus the control group; Con, the control group; NaHS, a donor of H_2S.

700 and 1,000 μmol/l) of NaHS (a donor of H_2S) for 24 h was performed to calculate the effective doses of NaHS. As shown in Fig. 2A, low concentrations of NaHS (10, 50 and 100 μmol/l) did not alter cell viability. Whereas, 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 peaking at 500 μmol/l. Therefore, 500 μmol/l NaHS was used in the subsequent time-response study with different treatment times (12, 24, 36, 48, 60 and 72 h). As shown in Fig. 2B, treatment of PLC cells with 500 μmol/l NaHS for the indicated times all dramatically promoted cell proliferation, reaching the maximal proliferative effect at 24 h. Based on the above results, PLC/PRF/5 hepatoma cells were treated with 500 μmol/l NaHS for 24 h in all subsequent experiments.

NaHS increases the expression levels of CSE and CBS and production of H_2S in PLC/PRF/5 hepatoma cells. We observed the effects of NaHS on the expression levels of CSE and CBS. As shown in Fig. 3A-C, exposure of PLC/PRF/5 hepatoma cells for the indicated time (1, 3, 6, 9, 12 and 24 h) to 500 μmol/l NaHS markedly enhanced the expression levels of CSE and CBS, reaching a peak at 24 h. In addition, at the same time, we found that the production of H_2S was significantly increased in the cell culture medium, compared with the control group.

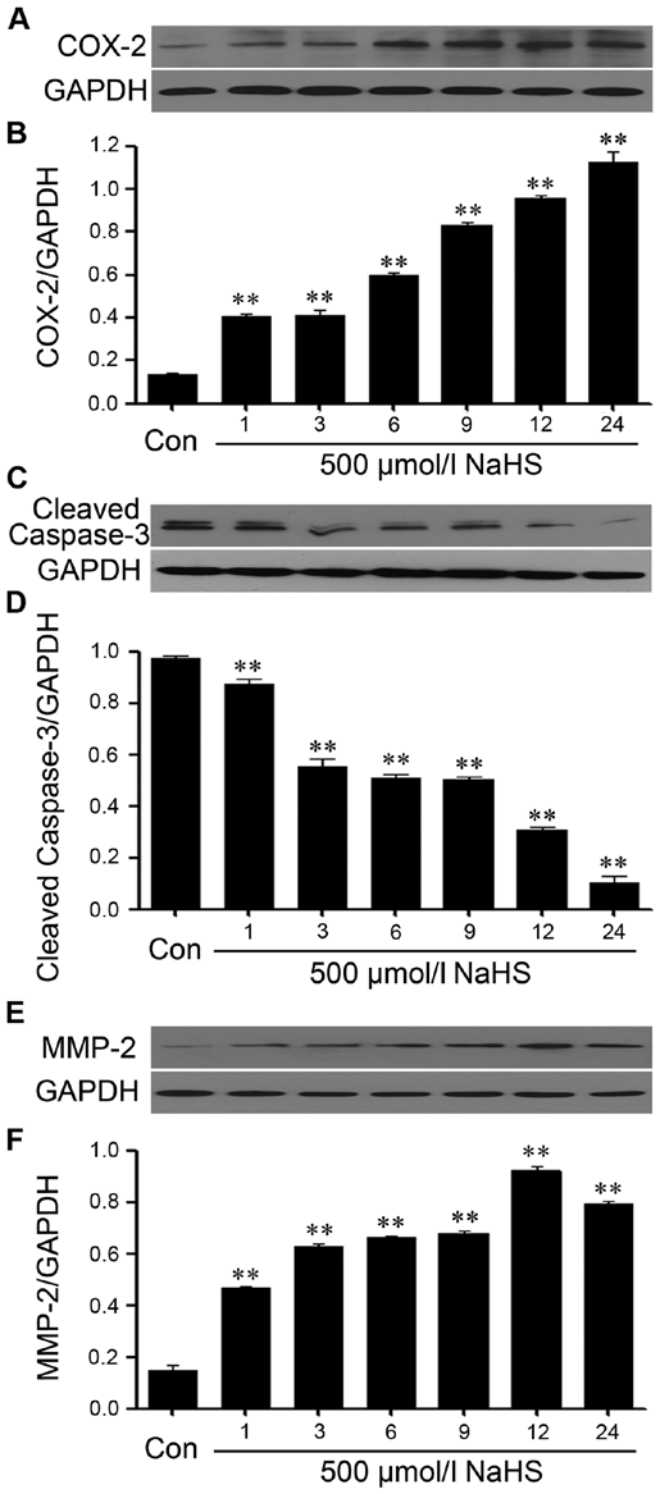

Figure 4. NaHS alleviates the expression level of caspase-3 and upregulates the expression levels of COX-2 and MMP-2 in PLC/PRF/5 hepatoma cells. PLC/PRF/5 hepatoma cells were exposed to 500 μmol/l NaHS for different times (1, 3, 6, 9, 12 and 24 h). The expression levels of caspase-3, COX-2 and MMP-2 were measured by western blot analysis. (B, D and F) The data in (A, C and E) were quantified by densitometric analysis with ImageJ 1.47i software. Data are shown as the mean ± SEM (N=3). **P<0.01 versus the control group; Con, the control group; NaHS, a donor of H_2S.

NaHS alleviates the expression level of caspase-3 and upregulates the expression levels of COX-2 and MMP-2 in PLC/PRF/5 hepatoma cells. In order to observe the effects of NaHS on the expression levels of caspase-3, COX-2 and MMP-2 in PLC/PRF/5 hepatoma cells, exposure of PLC/PRF/5 hepatoma cells to 500 μmol/l NaHS for different times (1, 3, 6, 9, 12 and 24 h). As shown in Fig. 4, NaHS significantly enhanced the expression levels of COX-2 and MMP-2, and reaching a peak at 12 h, whereas, the expression level of caspase-3 was markedly decreased.

NaHS amplifies the activation of NF-κB and p-IκBα in PLC/PRF/5 hepatoma cells. We observed the effects of NaHS on NF-κB p65 phosphorylation and IκBα phosphorylation in PLC/PRF/5 hepatoma cells. PLC/PRF/5 hepatoma cells were exposed to 500 μmol/l NaHS for the indicated times (3, 6, 9, 12 and 24 h), the expression levels of p-NF-κB p65 were significantly upregulated, reaching a peak at 12 h (Fig. 5A and B), and the t-NF-κB p65 expression was unchanged. Interestingly, the expression levels of p-IκBα were also markedly increased from 3 h, reaching the maximum at 24 h. Subsequently, we explored the effect of NaHS on the nuclear translocation of NF-κB p65 subunit. NaHS treatment significantly increased the nuclear translocation (Fig. 5E and F), with ameliorating amounts of NF-κB p65 in the cytosol (Fig. 5G and H). These results suggested that NaHS amplifies the activation and nuclear translocation of NF-κB in PLC/PRF/5 hepatoma cells.

PDTC alleviates NaHS-induced increased cell viability in PLC/PRF/5 hepatoma cells. As shown in Fig. 6, exposure of PLC/PRF/5 hepatoma cells to 500 μmol/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 PDTC (a specific inhibitor of NF-κB pathway) for 24 h.

As shown in Fig. 6, at the dose of PDTC from 5 to 100 μmol/l did not change in the cell viability. On the contrary, the dose of PDTC from 150 to 250 μmol/l significantly suppressed the cells proliferation, leading to a decrease in cell viability and reaching the minimum at 200 μmol/l. According to the above results, PLC/PRF/5 hepatoma cells were co-treated with 500 μmol/l NaHS and 200 μmol/l PDTC for 24 h in all following experiments.

PDTC increases NaHS-induced decreased cell apoptosis in PLC/PRF/5 hepatoma cells. We observed the effect of PDTC against NaHS-induced decreased cell apoptosis in PLC/PRF/5 hepatoma cells. It was showed that exposure of cells to 500 μmol/l NaHS for 24 h markedly enhanced proliferation, as evidenced by a decrease in apoptotic cells (Fig. 7B). In addition, the above proliferation was partly inhibited by co-treating PLC/PRF/5 hepatoma cells with 500 μmol/l NaHS and 100 μmol/l PDTC for 24 h or almost completely inhibited by co-treating PLC/PRF/5 hepatoma cells with 500 μmol/l NaHS and 200 μmol/l PDTC for 24 h.

PDTC inhibits NaHS-induced increased expression levels of MMP-2 and COX-2 and upregulates NaHS-induced decreased caspase-3 expression in PLC/PRF/5 hepatoma cells. As shown in Fig. 8, PLC/PRF/5 hepatoma cells were exposed to 500 μmol/l NaHS for 24 h, the expression levels of MMP-2 and COX-2 were significantly increased, on the

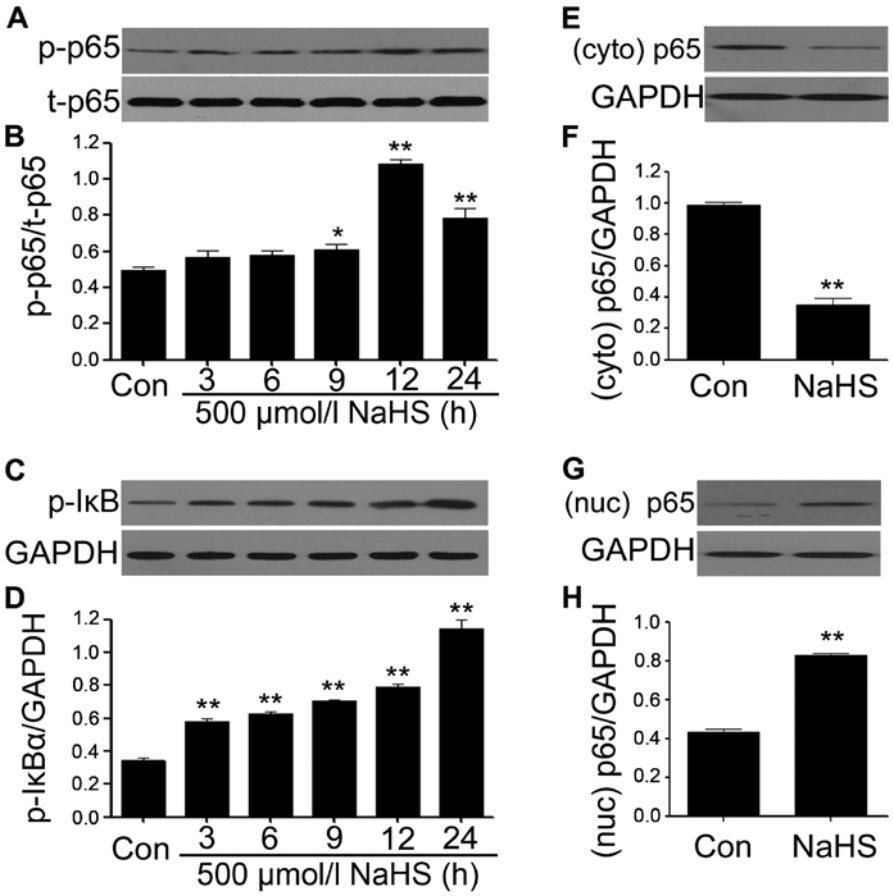

Figure 5. NaHS amplifies the activation of NF-κB in PLC/PRF/5 hepatoma cells. (A-D) PLC/PRF/5 hepatoma cells were exposed to 500 μmol/l NaH for the indicated times (3, 6, 9, 12 and 24 h). To test the effects of NaHS on NF-κB p65 phosphorylation, the cells were exposed to 500 μmol/l NaHS for 24 h. Cytoplasm (E and F) and nuclear (G and H) extracts were extracted. The expression of p65 was analyzed 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 ± SEM (N=3). *P<0.05, **P<0.01 versus with the control group; Con, the control group; Nuc, nuclear, Cyto, cytoplasm.

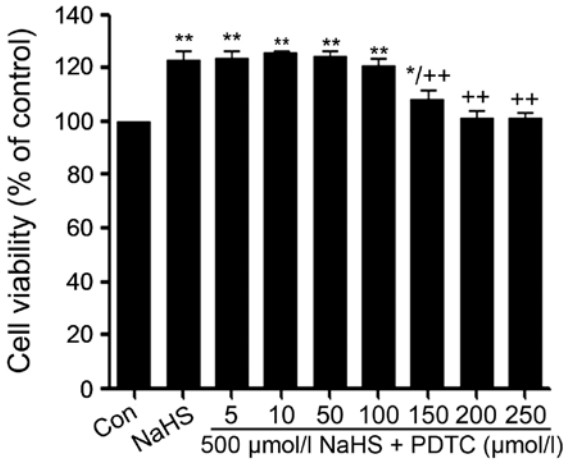

Figure 6. PDTC alleviates NaHS-induced cell proliferation in human liver cancer cell line PLC/PRF/5 hepatoma cells. PLC/PRF/5 hepatoma cells were co-conditioned with 500 μmol/l NaHS and different doses of PDTC (0, 5, 10, 50, 100, 200 and 250 μmol/l) for 24 h. Data are the mean ± SEM (n=3). *p<0.05, **p<0.01 compared with the control group. $^{++}$p<0.01 compared with the NaHS group. Con, the control group; NaHS, a donor of H_2S; PDTC, pyrrolidine dithiocarbamate, a specific inhibitor of NF-κB pathway.

contrary, the expression level of caspase-3 was markedly decreased. Notably, co-treatment of PLC/PRF/5 hepatoma cells with 500 μmol/l NaHS and 200 μmol/l PDTC for 24 h considerably depressed NaHS-induced increased expression levels of MMP-2 and COX-2, however, caspase-3 expression was obviously downregulated. Treatment of cells with 200 μmol/l PDTC for 24 h did not alter the basal expression levels of MMP-2, COX-2 and caspase-3.

PDTC suppresses NaHS-induced upregulated production of VEGF in PLC/PRF/5 hepatoma cells. As shown in Fig. 9, the level of VEGF was markedly increased in NaHS-induced PLC/PRF/5 hepatoma cells, compared with the control group (P<0.01). However, the increased level of VEGF was significantly suppressed by co-treatment cells with PDTC and NaHS.

H_2S demonstrates pro-proliferation, anti-apoptosis, angiogenesis, invasion and migration effects on PLC/PRF/5 hepatoma cells via amplifying the activation of NF-κB pathway. We found that NaHS upregulated both CSE and CBS activity resulting in an elevated rate of H_2S production level, which in turn modulates protein expressions of caspase-3, MMP-2 and VEGF. The downregulated caspase-3 directly induced anti-apoptosis, led to decreased apoptosis and increased cell viability of PLC/PRF/5 hepatoma cells. MMP-2 contributes to cancer cell invasion and migration. The increased production

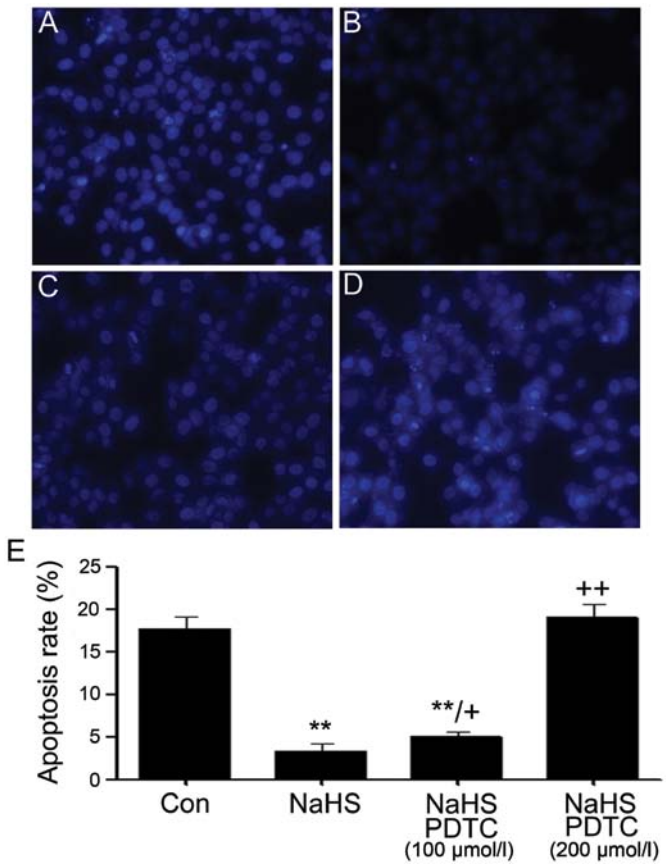

Figure 7. PDTC diminishes NaHS-induced increased cells apoptosis in PLC/PRF/5 hepatoma cells. After the indicated treatments (A-D), cellular apoptosis was assessed by Hoechst 33258 staining followed by photofluorography. (A) Control group. (B) PLC/PRF/5 hepatoma cells exposed to 500 μmol/l NaHS for 24 h. (C) PLC/PRF/5 hepatoma cells cells were co-treated with 500 μmol/l NaHS and 100 μmol/l PDTC for 24 h. (D) PLC/PRF/5 hepatoma cells were co-treated with 500 μmol/l NaHS and 200 μmol/l PDTC for 24 h. (E) The apoptosis rate was analyzed with a cell counter of the ImageJ 1.47i software. Data are shown as means ± SEM (n=3). **p<0.01 versus with the control group; $^{+}$<0.05, $^{++}$p<0.01 versus with the NaHS group. Con, the control group; NaHS, a donor of H_2S.

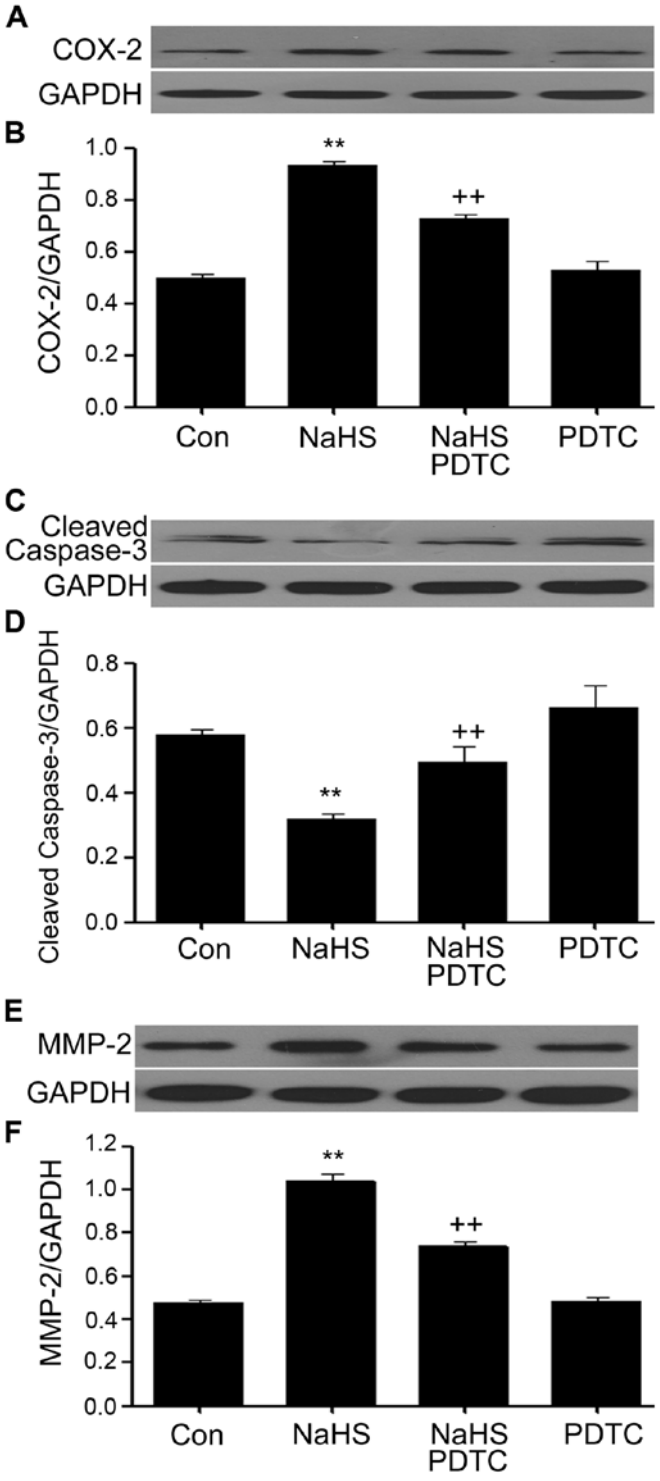

Figure 8. PDTC inhibits NaHS-induced increased MMP-2 and COX-2 expression and upregulates NaHS-induced decreased caspase-3 in PLC/PRF/5 hepatoma cells. PLC/PRF/5 hepatoma cells were co-conditioned with 500 μmol/l NaHS and 200 μmol/l PDTC for 24 h. The expression of MMP-2, COX-2 and caspase-3 were measured by western blot assay (A, C and E). (B, D and F) Densitometric analysis for the data (MMP-2, COX-2 and caspase-3) (A, C and E). Data are presented as the means ± SEM (n=3). **p<0.01 vs. the control group; $^{++}$p<0.01 vs. the NaHS group. Con, the control group; NaHS, a donor of H_2S.

of VEGF stimulates angiogenesis, promoting the supply of nutrients and blood to the tumor. Conversely, the above properties of H_2S were significantly inhibited by the co-condition of 500 μmol/l NaHS and 200 μmol/l PDTC for 24 h.

Discussion

In this study, we demonstrated a novel finding in tumor development of H_2S on PLC/PRF/5 hepatoma cells and also provide data to revel its potential mechanisms. Herein we report that: i) the production of H_2S was dramatically increased in the PLC/PRF/5 hepatoma cells compared with the human LO2 hepatocyte cells group, along with overexpression levels of CSE and CBS, ii) NaHS upregulated the expression levels of CBS and CSE, as well as the production of H_2S, iii) NaHS caused an increase in cell viability, iv) NaHS induced a decrease in cell apoptosis, due to the increased expression level of caspase-3, v) NaHS activated the NF-κB pathway and promoted NF-κB nuclear translocation, vi) NaHS induced increased expression levels of COX-2, MMP-2 and VEGF, vii) co-treatment of PLC/PRF/5 hepatoma cells with NaHS and PDTC (an inhibitor of NF-κB) was able to largely suppress the above NaHS-induced effects.

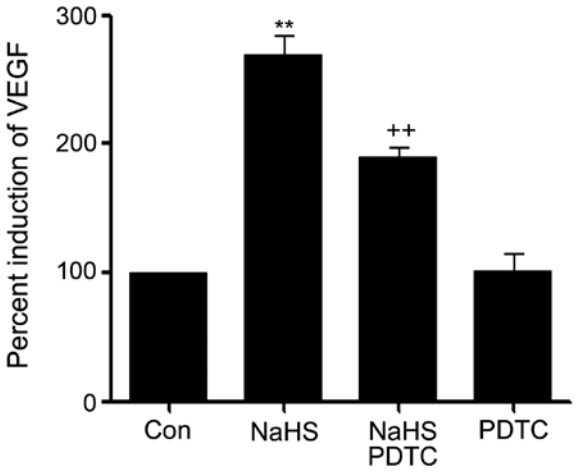

Figure 9. PDTC suppresses NaHS-induced upregulated production of VEGF in PLC/PRF/5 hepatoma cells. PLC/PRF/5 hepatoma cells were co-conditioned with 500 μmol/l NaHS and 200 μmol/l PDTC for 24 h. The production of vascular endothelial growth factor (VEGF) was tested by enzyme-linked immunosorbent assay (ELISA). Data are presented as the means ± SEM (n=3). **p<0.01 vs. the control group; $^{++}$p<0.01 vs. the NaHS group. Con, the control group; NaHS, a donor of H_2S.

H_2S, as the third gaseous transmitter following NO and CO, modulates an array of cellular and molecular mechanism, physiological and pathophysiological processes. It can be endogenously catalyzed by CBS or CSE or both and contributes to cardioprotection (26-31,53), angiogenesis (32-34), antioxidant (35), pro- and anti-inflammatory (36,37) and other wide range of physiological functions (38-41) in a variety of animal or human non-tumor cells. However, the effects of H_2S on the cancer cells are comparatively complicated and extremely controversial. Accumulating evidence has demonstrated that H_2S may possess anticancer functions by reason of its anti-inflammatory effect (54), activation of MAPKs pathway (p38 MAPK, ERK1/2 and JNK) (49), and pro-apoptosis performance (49). Significantly, it has been reported that H_2S can exert totally opposite properties, in the main mechanisms related to AKT/ERK pathways (47), and angiogenesis (42,49). NaHS, a donor of H_2S, is actively being investigated on account of the above effects of H_2S. In the present study, our results suggested that both CSE and CBS were expressed in PLC/PRF/5 hepatoma cells and significantly increased compared with the human hepatic cell line

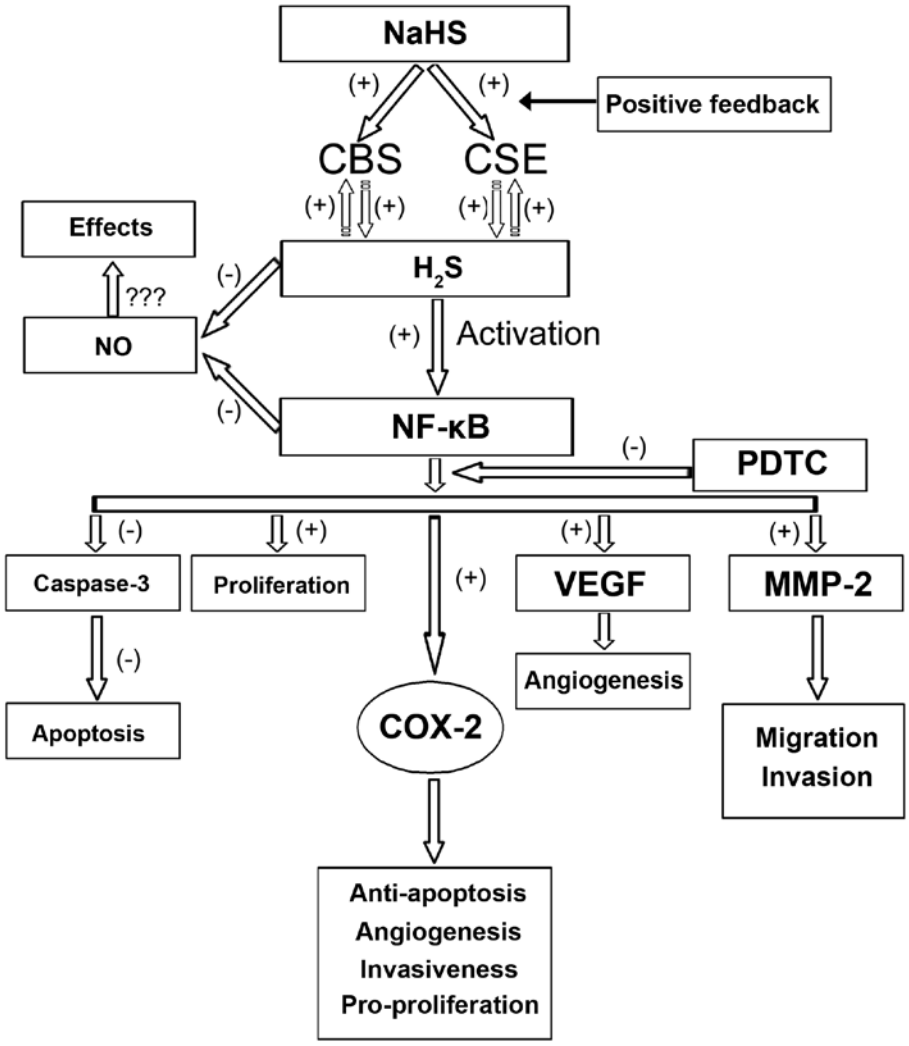

Figure 10. H_2S demonstrated pro-proliferation/anti-apoptosis/angiogenesis/invasion and migration effects on PLC/PRF/5 hepatoma cells via amplifying the activation of NF-κB pathway. NaHS upregulated both CSE and CBS activity resulting in an elevated H_2S production level, which in turn modulates protein expression of caspase-3, MMP-2 and VEGF. Conversely, the above properties of H_2S were significantly inhibited by the co-condition of 500 μmol/l NaHS and 200 μmol/l PDTC for 24 h.

LO2, suggesting H_2S might be closely linked to liver cancer progression. Previous studies have demonstrated that H_2S can exert anti-apoptosis and pro-proliferation on various diversified cells (26-31,53,55,56). In this study, we treated PLC/PRF/5 hepatoma cells with NaHS and found interesting results. Our findings demonstrated that NaHS improved PLC/PRF/5 hepatoma cells proliferation at the concentrations ranging from 100 to 1,000 μmol/l and the optimal concentration of NaHS that induced maximal effect of proliferation was 500 μmol/l, leading to increased cell viability, which indicates that H_2S might participate in the PLC cancer growth. Treatment of PLC/PRF/5 hepatoma cells with 500 μmol/l NaHS for 24 h markedly diminished cell apoptosis, and decreased the expression level of caspase-3, an apoptotic factor. The above result was consistent with previous reports (13-19). These results demonstrate that H_2S induces cell proliferation via exerting its dual cytoprotective and anti-apoptosis effects. A large number of experiments have shown that H_2S can contribute to VEGF production (42,57-61). In agreement we found that H_2S notably increased the production of VEGF in PLC/PRF/5 hepatoma cells, compared with the control group. VEGF is one of the most potent and pivotal angiogenic factors and is crucial for the persistent proliferation and metastasis of tumor cells (62). Therefore, we hypothesized that H_2S promotes the supply of blood and nutrients to the tumor via angiogenesis effect. Further studies are needed to explore our hypothesis *in vivo*. Fourthly, we studied the MMP-2 and found for the first time that treatment with 500 μmol/l NaHS significantly upregulated the expression level of MMP-2, and reached a peak at 12 h. The upregulated expression of MMPs, particularly the gelatinase (MMP-2 and MMP-9), is high associated with metastasis potential in several types of carcinomas (63-66). It indicated that H_2S was involved in PLC/PRF/5 hepatoma cell invasion and migration. In our observational study, treatment with 500 μmol/l NaHS for 24 h obviously promoted protein COX-2 secretion in PLC/PRF/5 hepatoma cells. Importantly, accumulating evidence has demonstrated that cyclooxygenase (COX)-2 is overexpressed in several types of cancers cells (73-75) including hepatocellular carcinoma (HCC) (76). COX-2 expression in cells and animal models is closely associated with tumor cell growth and is a crucial molecule in the development of malignant tumors, including promotion of angiogenesis (77), anti-apoptotic effects (78), invasiveness of tumor cells (74), and tumor cell proliferation (79), demonstrating that COX-2 pathway is implicated in NaHS-induced PLC/PRF/5 hepatoma cell proliferation, anti-apoptosis, angiogenesis and migration.

To investigate the complicated mechanism for NaHS-induced pro-proliferative effect, anti-apoptosis, angiogenesis and migration in PLC/PRF/5 hepatoma cells, we studied the NF-κB pathway, which has been demonstrated previously linked to cancer progression by mean of cell invasion, cell differentiation, cell proliferation, and apoptosis (12). It has been reported that NF-κB can be activated by various stimuli both in normal cells (13) and in cancer cells (13-19). Herein, we found that NaHS not only activated NF-κB in PLC/PRF/5 hepatoma cells, leading to increased phosphorylation of NF-κB p65 and IκBα, but also increased NF-κB nuclear translocation. Interestingly, PDTC, an inhibitor of NF-κB, blocked NaHS-induced NF-κB activation, along with NaHS-induced pro-proliferative effect, anti-apoptosis, angiogenesis and migration in PLC/PRF/5 hepatoma cells, because of decreased expression levels of MMP-2, VEGF, and COX-2, and increased caspase-3 expression. These results suggest that NF-κB activation is necessary in NaHS-induced PLC/PRF/5 hepatoma cell progression.

NO, which is another important gaseous transmitter, can also exert a wide variety of biological properties. It is hard to prove that treatment of PLC/PRF/5 hepatoma cells with 500 μmol/l NaHS for 24 h significantly reduced NO production in culture medium (data not shown). Thefore, the interaction of H_2S and NO in PLC/PRF/5 hepatoma cells is still unclear and need to be further investigated.

A novel finding of our present study is the interaction between H_2S and its catalyzing enzyme (CSE and CBS) in PLC/PRF/5 hepatoma cells. On the one hand, naturally, H_2S can be catalyzed by CBS or CSE and treatment of PLC/PRF/5 hepatoma cells with NaHS significantly increased its production compared with the control group. Unexpectedly, treatment of PLC/PRF/5 hepatoma cells with NaHS significantly upregulated both CSE and CBS expression. This finding means that there may be positive regulation mechanism between H_2S and its catalyzing enzyme (CSE and CBS) in PLC/PRF/5 hepatoma cells. The positive regulation mechanism might play a crucial role in NaHS-induced liver cancer cell progression. However, this mechanism remains to be further elucidated.

In conclusion, H_2S induced cells proliferation, anti-apoptosis, angiogenesis, and migration in PLC/PRF/5 hepatoma cells. These effects might be mediated by the activation of NF-κB pathway, leading to overexpression levels of MMP-2, COX-2 and VEGF, down-expression of caspase-3, increased cell viability, and decreased number of apoptotic cells. In PLC, the findings provide novel insight into a unified concept and identify CBS- and CSE-derived H_2S as an endogenous tumor-promoting factor and anticancer drug target. The interaction of H_2S and NO in PLC/PRF/5 hepatoma cells is still unclear and needs to be further investigated.

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