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Hydrogen Sulfide Contributes to Hypoxia-induced Radioresistance on Hepatoma Cells

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Hypoxia/Radioresistance/Hydrogen sulfide/K^+_{ATP} channels/Hepatoma cells.

Growing evidence has demonstrated that, as an endogenous signaling gasotransmitter, hydrogen sulfide (H_2S) plays an important role in regulating numerous biological functions. The role of H_2S in hypoxia-induced radioresistance on hepatoma cells was investigated in the present work. Results showed that, when HepG2 cells were maintained in hypoxia circumstances for 4 h, the cellular radioresistance was extensively increased so that the oxygen enhancement ratio of the survival fraction approached 2.68. Under this hypoxic condition, when the cells were treated with DL-propargylglycine (PPG) and aminooxyacetic acid (AOAA), a specific inhibitor of H_2S synthase of cystathionine-γ-lyase (CSE) and cystathionine-β-synthase (CBS) respectively, radiation responses including cell killing, micronuclei (MN) formation, and caspase-3 activity were significantly enhanced. However, treatment of cells with low concentrations of NaHS (≤ 100 μM) protected cells from these radiation damages. Western bolting assay showed that CSE and CBS were over-expressed in the irradiated hypoxic cells in a dose dependent manner. Moreover, when the hypoxic HepG2 cells were treated with NaHS together with glibenclamide, a specific inhibitor of K^+_{ATP} channels, the role of exogenous H_2S in radioprotection was partly eliminated. This study demonstrated that H_2S contributed to hypoxia-induced radioresistance probably via the opening of K^+_{ATP} channels, which suggests that the endogenous H_2S synthase could be a potential radiotherapeutic target for a hypoxic tumor.

INTRODUCTION

Existence of hypoxia microenvironment is a characteristic of most solid tumors due to transient or chronic insufficiency of blood supply, which allows tumor to be resistant to radiotherapy and chemotherapeutic drugs and results in unfavorable prognosis such as malignant transformation and metastasis. Theoretically, a full hypoxic condition requires three-fold radiation dose to achieve the equivalent cytotoxicity effect under normoxic condition. But radioresistance can also be induced even at a low oxygen condition.[1)] It was reported that the damage effect of radiation began to decline at pO_2 of 25–30 mmHg about one-third of normal pO2 in blood.[2,3)] A well-known hypothesis has been suggested that, under hypoxic condition, radiation-induced DNA damage can not be fixed by oxidization so that the hypoxic tumor cells are able to survive by means of various biological adaptation responses to exogenous stresses.[4,5)] The molecular responses to hypoxic stress have been extensively investigated. For example, hypoxia can trigger an increase of mitochondrial ROS and activates the signaling pathways leading to the stabilization and activation of hypoxia inducible factor (HIF) family proteins.[6,7)] The activation of HIF-1α or HIF-2α can cause reversible cell cycle arrest by up-regulating the expression of the cyclin-dependent kinase inhibitors of p27 and p21 and potentially affording to cellular protection against stressful environments.[8)] In addition, other HIF-1α-independent factors, such as nuclear factor kappa B (NF-κB),[9)] tumour necrosis factor α (TNF-α), interleukin 1β (IL-1β), interleukin 8 (IL-8), and activator protein-1 (AP-1),[10)] were also involved in the hypoxia induced responses in tumor cells.

Over the past decade, solid evidence has identified that hydrogen sulfide (H_2S), as a third endogenous signaling gasotransmitter along with nitric oxide (NO) and carbon monoxide (CO), plays a role in regulating biological effects.[11–13)] H_2S is endogenously synthesized in various mammalian tissues by cystathionine β-synthase (CBS, EC 4.2.1.22) and cystathionine γ-lyase (CSE, EC 4.4.1.1).[14)] Physiological

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concentrations of this gasotransmitter in plasma were between 20 and 160 μM.[15,16] It has been known that H_2S may serve as an O_2 sensor/transducer in the vascular response to hypoxia that can increase H_2S concentration in the pulmonary vessels.[17] Exposure to exogenous H_2S (NaHS) could depress the activity of CSE and thus abolish the generation of endogenous H_2S. The pharmacological blockade of CSE activity is associated with a severely blunted hypoxia-induced response in various types of vessels.[18–20] H_2S can prevent apoptosis by inducing phosphorylation of glycogen synthase kinase-3β at ser-9 and subsequently inhibiting Bax translocation and caspase-3 activation.[21] H_2S also exerts some physiological functions by opening the mitochondrial permeability transition pores and K^+_{ATP} channels.[22] So far there is almost no literature about the role of H_2S in radiation effect except Powers reported in 1960 that H_2S reduced radiation sensitivity of dry bacterial spores.[23] Up to our knowledge, most of previous studies of H_2S focus on its physiological function and toxin effect. In the present work, the potential role of H_2S in hypoxia-induced radioresistance effect was investigated.

MATERIALS AND METHODS

Chemicals

Sodium hydroxyl sulfide (NaHS) was obtained from the Kishida Chem. (Osaka, Japan), sodium dodecylsulfate (SDS) was from the Wako Pure Chem. (Osaka, Japan), and Ac-DEVD-pNA was obtained from Biomolecular Co. (USA). Dulbecco's Modified Eagle Media (DMEM), fetal calf serum (FCS), L-glutamine, penicillin and streptomycin were obtained from Gibco Invitrogen (Hangzhou, China). L-propargylglycine (PPG, an inhibitor of CSE), aminooxyacetic acid (AOAA, an inhibitor of CBS), glibenclamide (an inhibitor of ATP sensitive potassium (K^+_{ATP}) channels), and dimethyl sulphoxide (DMSO) were purchased from Sigma Co. (St. Louis, MO, USA).

NaHS was used as an exogenous donor of H_2S. In aqueous solution, NaHS can be decompounded to Na^+ and HS^-, and HS^- can further associate with H^+ to generate a stable H_2S molecule. Approximately one third of H_2S is in the non-dissociated form.[24] In our experiment, NaHS was dissolved in PBS and diluted to the desired concentrations in DMEM immediately before use.

Cell culture and the generation of hypoxia environment

Human hepatoma HepG2 cells (Shanghai Cell Bank, China) were maintained in the DMEM medium supplemented with 10% FCS, 0.01% sodium pyruvate plus 2 mM L-glutamine, 100 units/ml penicillin, and 100 mg/ml streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

For the hypoxia treatment, cells were maintained for a destined period inside a hypoxia chamber (Model YQX-1, Shanghai, China) that contains a mini-incubator allowing cells to grow at 37°C and an operation window connected with a couple of gloves for cell treatment operation. According to the manufacturer's instruction, pure nitrogen gas (99.99%) was purged though a copper sulfate solution to remove possible toxic gases and then re-filled into the hypoxia chamber a few times until a suitable hypoxic condition was approached. After 1 h of the gas filling, the concentration of dissolved oxygen (DO) in cell culture medium was about 2% that was measured with a microcomputer-based DO meter (Model 9250M/9251M, Jinco Instruments Inc. San Diego, CA). Under this hypoxia environment, plastic Petri-dish is applicable for cell culture.[25] Our pilot experiment showed that the cells could be maintained in this hypoxia chamber up to 6 h without obvious cellular damage.

Cell irradiation and treatments

HepG2 cells were seeded in a 60 mm Petri-dish and cultured for 24 hours under normoxic condition. These cells were then divided into two groups for irradiation under normoxic and hypoxic conditions, respectively. For the normoxia group, cells were irradiated with γ-rays (Cs-137 irradiator, Gammacell-40, MDS Nordian, Canada) of different doses with a dose rate of 0.73 Gy/min at room temperature. For the hypoxia group, cells were further maintained in the hypoxia chamber for 4 h and then the cell dishes were sealed inside a box filled with N_2 gas for irradiation. In some experiments, cells were treated with 5 mM PPG, 1 mM AOAA, or 1 μM glibenclamide at 4 h before irradiation, or treated with NaHS of different concentrations 30 min before irradiation. To keep cells under hypoxic environment during treatments, the drug solutions were pre-purged with N_2 gas and the medium changes were performed inside of the hypoxia chamber.

Colony formation assay

The clonogenic survival assay was performed for both normoxic and hypoxic cells. After irradiation, the cells were reseeded in 60 mm dishes in order to form approximate 100 colonies each dish. After 15 days incubation, the colonies were fixed with 10% formalin, stained with 1% methylene blue, and then counted for the calculation of cell survival fraction and oxygen enhancement ratio (OER).

Micronuclei assay

The frequency of micronuclei (MN) formation was measured using the cytokinesis-block technique developed by Fenech and Morley.[26] Briefly, after drug treatment and irradiation, HepG2 cells were cultured in the medium containing 1 μg/ml cytochalasin-B (Sigma, UK) and incubated at 37°C for 28 h, then rinsed with PBS, fixed with a fixing solution of methanol: acetic acid (9:1, v:v), stained with 10 μg/ml acridine orange for 5 min, and observed with a fluorescence microscope. MN in at least 1000 binucleated cells were counted and the yield of MN (Y_{MN}) was calculated as the

ratio of the number of MN to the number of the binucleated cells.

Western blotting assay

Two hours after irradiation under different conditions, the cells were lysed in SDS buffer (2% SDS, 62.5 mM Tris-HCl and 10% glycerol, pH 6.8) for protein extraction. After denatured at 100°C for 10 min, aliquots of protein samples (20 μg) were separated by electrophoresis on a SDS-polyacrylamide gel (10% separation gel and 4% pycnotic gel, BIO-RAD Laboratories, Inc.), transferred onto a polyvinylidene difluoride (PVDF) membrane (Millipore Corporation), blocked for 1 h with 5% skim milk in 0.05% Tris-buffered saline/Tween (TBST), and incubated with a primary antibody (diluted 1:1000 in blocking buffer for the rabbit anti-CSE (Protein Tech Group, Chicago, USA), 1:1000 for the mouse anti-CBS (Abcam, Cambridge, UK), and 1:1000 for the mouse anti-β-actin (Beyotime Inst. Biotechology, Haimen, China)) at room temperature for 2–3 h or at 4°C for overnight. Then the membrane was triply washed with TBST at room temperature for 10 min and incubated with a secondary antibody (HRP-conjugated anti-mouse IgG (1:2500) or anti-rabbit IgG (1:5000, Beyotime Biotechnology, China) for 1 h. After several washes, the membrane was detected by the enhanced chemiluminescence system (ECL advance, Amersham Biosciences) and the protein image was recorded by the BIO-RAD ChemiDoc XRS and analyzed using the Quantity One software (Bio-Rad, Hercules, CA, USA).

Measurement of caspase-3 activity

The activity of caspase-3 was determined using the APO-ONE homogenous caspase-3/7 assay kit (Promega, Madison, WI). Briefly, after 2 h of irradiation, the cells were centrifuged at 300 xg for 5 min and resuspended in PBS, then 7.5×10^4 cells in 12.5 μl was added to each well of a black-walled 96-well plate and an equal volume of the caspase-3 substrate (Ac-DEVD-pNA) was added. Following to 6 h incubation at 37°C, the released chromophore was measured at 405 nm with a plate-reader. The relative activities of caspase-3 in the drug treated cells and irradiated cells were normalized to that in control cells without any treatment.

Statistical analysis

Statistical analysis was done on the means of data obtained from at least 3 independent experiments. Three replicates were counted for each experimental point to assess the MN formation. All results are presented as mean ± SEM and the significance is determined using the ANOVA test, and the difference between two groups was considered to be statistically significant at $P < 0.05$.

RESULTS

Cell survival and OER

Figure 1 illustrates the dose responses of the survival fraction of irradiated HepG2 cells under different oxygen conditions and drug treatments. The survival curves are fitted with the equation $S = 1–(1–\exp(D/D_0))^N$ where D_0 is the average lethal dose and N is the number of radiation targets. The stimulation results showed that average number of N was 3.39 and the D_0 value was 1.16 and 3.09 under normoxic and hypoxic condition, respectively. Thus the value of OER i.e. the ratio of D_0 (hypoxia) to D_0 (normoxia) was 2.68. This OER value indicates that the nitrogen treatment method here is good enough to generate an effective hypoxic condition.

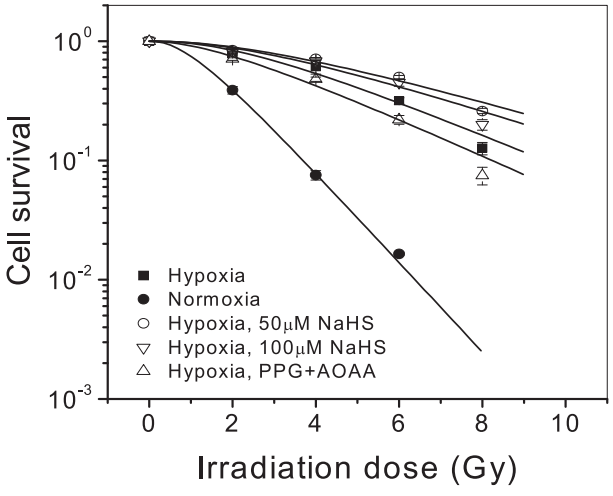

Fig. 1. The survival of HepG2 cells irradiated with γ-rays under normoxic and hypoxic conditions. Under hypoxic condition, the cells were treated with PPG plus AOAA or NaHS of different concentrations before irradiation.

Further experiments showed that, when the hypoxic HepG2 cells were treated with 50 or 100 μM NaHS as a donor of exogenous H_2S before irradiation, the cells became more radioresistive so that its survival was enhanced. In contrast, when the hypoxic cells were pretreated with PPG plus AOAA, the inhibitors of endogenous H_2S synthases, the cell survival was partly reduced but still higher than that of normoxic cells.

Effect of H_2S on radiation-induced micronuclei formation

We further measured radiation-induced DNA damage under hypoxic condition. Results showed that, with 3 Gy radiation, the yield of MN was increased from 0.1 of non-irradiated control to about 0.3 (Fig. 2). When the hypoxic

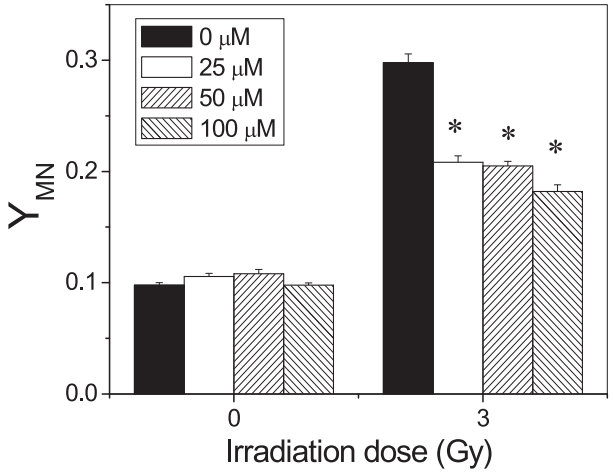

Fig. 2. Induction of MN in the hypoxic HepG2 cells irradiated with 3 Gy γ-rays. The cells were treated with NaHS of different concentrations 30 min before irradiation. *, $P < 0.01$ compared with the irradiated group without NaHS treatment.

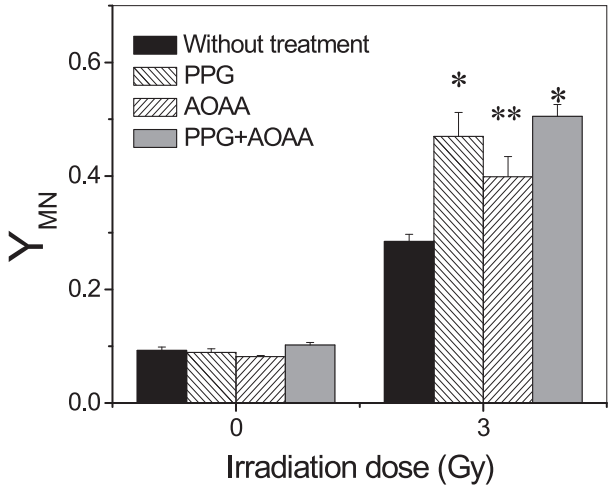

Fig. 3. Induction of MN in the hypoxic HepG2 cells irradiated with 3 Gy γ-rays. The cells were treated with PPG and/or AOAA, the inhibitors of H_2S synthases before irradiation. *, $P < 0.01$ and **, $P < 0.05$ compared with the irradiated group without drug treatment.

cells were treated with 25, 50, or 100 μM NaHS for 30 min before irradiation, the yield of radiation-induced MN was significantly reduced from 0.3 to about 0.2. For the influence of endogenous H_2S on the radiation damage, Fig. 3 illustrates that, when the activity of CSE and CBS in hypoxic HepG2 cells were inhibited by PPG and AOAA, the yield of radiation-induced MN was obviously increased by 65% and 40%, respectively (Fig. 3). When the hypoxic cells were treated with PPG and AOAA in combination before irradiation, the yield of radiation-induced MN could be even much increased by 77%. These data suggest that H_2S plays an

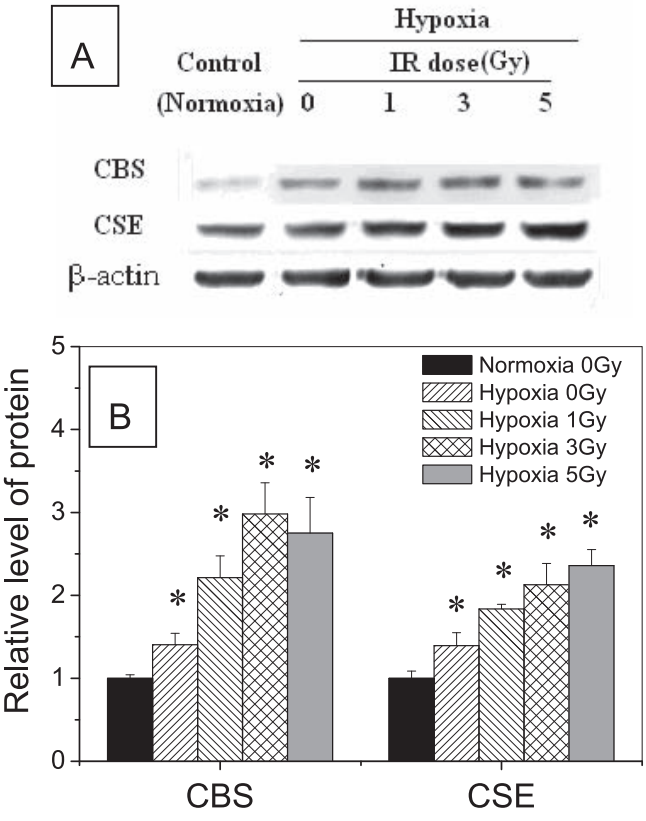

Fig. 4. Expressions of CSE and CBS in the HepG2 cells irradiated with different doses of γ-rays. (A) A representative protein image of the western blotting assay for CSE, CBS and β-actin. (B) The relative levels of CSE and CBS under hypoxic condition. Each CSE/CBS protein was compared with correlative β-actin and then normalized to the expression of corresponding protein under normoxic condition. *, $P < 0.05$ compared with the normoxia control without irradiation.

important role in hypoxia-induced radioresistance, which regulated by the endogenous enzymes of CSE and CBS.

Expressions of radiation-induced CSE and CBS

To get more evidence supporting the above findings, we measured the protein expressions of CSE and CBS in HepG2 cells by western blotting. A representative protein image was shown in Fig. 4A and the semi-quantitative expression of these proteins was shown in Fig. 4B where each protein expression level was normalized to its corresponding β-actin at first and then compared to its normoxic control. It was found that the expressions of CSE and CBS in the hypoxic cells were 1.4-fold of those in the normoxic cells, indicating that the endogenous H_2S generation could be triggered by hypoxia treatment and hence increased cellular intrinsic capacity of radioresistance. Moreover, the expressions of CSE and CBS were also inducible by irradiation and increased along with radiation dose. For example, at 3 Gy of radiation, the expressions of CBS and CSE

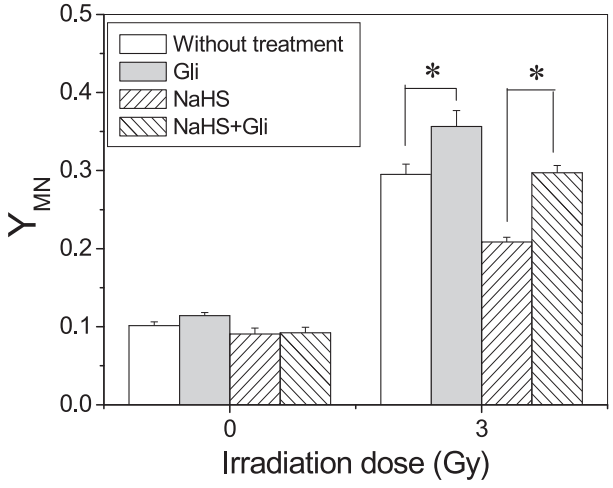

Fig. 5. Influence of glibenclamide on the radioresistance effect of NaHS. Hypoxic HepG2 cells were treated with glibenclamide, 100 μM NaHS, or glibenclamide plus NaHS, respectively. *, $P < 0.05$ between indicated groups.

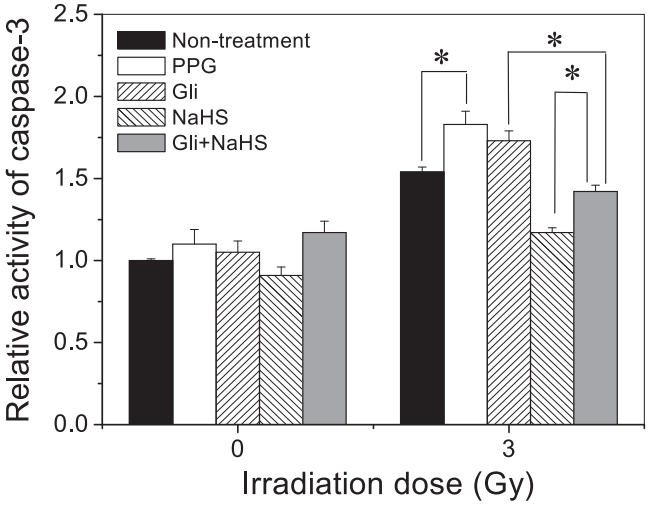

Fig. 6. Relative activity of caspase-3 in HepG2 cells under hypoxic condition. Before irradiation, the cells were treated with PPG, glibenclamide, 100 μM NaHS, and glibenclamide plus NaHS, respectively. *, $P < 0.05$ between indicated groups.

approached to 2.1-fold and 1.5-fold of the nonirradiated control, respectively.

Role of ATP-sensitive potassium channel (K^+_{ATP}) in radioresistance

To investigate the mechanism of H_2S-involved radioresistance on hypoxic HepG2 cells, we treated hypoxic HepG2 cells with glibenclamide to close K^+_{ATP} channels in cellular membrane. Figure 5 illustrates that this treatment increased cell radiosensitivity so that the yield of MN was increased by 35.6% ($P < 0.05$) after 3 Gy irradiation. Moreover, although the MN induction in hypoxic cells was depressed by exogenous H_2S, the yield of MN in the irradiated cells that were pretreated with the combination of glibenclamide and NaHS was significantly higher than that of NaHS-treated cells, i.e. the radioresistance effect of NaHS was eliminated when K^+_{ATP} channels were blocked by glibenclamide.

Influence of H_2S on the activity of caspase-3

Caspase-3 is an important sensor of radiation-induced apoptosis. To explore the role of H_2S in this response, we measured the relative activity of radiation-induced caspase-3 in the hypoxic HepG2 cells under different treatments of PPG, glibenclamide, and NaHS. Figure 6 illustrates that the activity of caspase-3 was promoted by irradiation and it was further increased when cellular endogenous H_2S was prevented by PPG. Meanwhile, when the hypoxic cells were treated with glibenclamide to close the K^+_{ATP} channels, the activity of radiation-induced caspase-3 was also increased. But this increment was extensively depressed when the hypoxic HepG2 cells were treated with 100 μM NaHS. However in the presence of glibenclamide, NaHS could not inhibit the activity of radiation-induced caspase-3. These results reveal that the gasotransmitter H_2S may protect hypoxic HepG2 cells against radiation damage by depressing caspase-dependent apoptosis and this protection function partly relies on the opening of K^+_{ATP} channels.

DISCUSSION

The rapid growth of carcinoma and disproportionate distribution of blood vessel may result in the formation of hypoxia or low oxygen microenvironment in a solid tumor. Hypoxia is postulated to play a role in limiting tumor progression[27,28] and enhancing the capacity of invasion and metastasis.[29] Hypoxic tumor cells are resistant to radiotherapy and major chemotherapeutics so that they can escape from radiation induced cell killing.[30] Investigation for the new mechanism of hypoxia-induced radioresistance on tumor cells is eagerly necessary for developing a new therapy strategy. In this study, we found that both CSE and CBS were not only highly expressed in human hepatoma HepG2 cells under hypoxic condition but also inducible under irradiation. It has been known that hypoxia can increase H_2S concentration in the pulmonary vessels.[18–20] Some hypoxia responses may be related to the increase of endogenous H_2S, such as relaxation of urinary bladder in the trout, hypoxic vasoactive response, and cardiovascular response.[17,18] H_2S has been implicated as a gaseous transmitter fulfilling a potential protective role in defending against the damage induced by ischemic/hypoxic in cardiovascular system[31] and nervous system.[32]

The present study found that H_2S could be an important factor of radioresistance for hypoxic tumor cells. Radiation damage on hypoxic HepG2 cells could be increased when

the endogenous H_2S synthases were inhibited but it was diminished when the cells were treated with NaHS. These results disclose that both endogenous H_2S and exogenous H_2S contribute to the radioresistance on hypoxic hepatoma cells. It was reported that H_2S can be stored *in vivo* as sulfane/sulfur that can release free H_2S after various stimulations.[33] H_2S can be oxidized to thiosulfate or form sulfite finally by sulfurtransferase in mitochondrial. H_2S can also be methylated by the enzyme thiol-s-methyltransferase or be bound to methemoglobin.[14] It was reported that 30–50 μM H_2S could provide protective effect against homocysteine-induced cytotoxicity and reduce the endogenous generation of H_2O_2, superoxide (O_2^-) and $ONOO^-$.[34] We all know that radiation-induced reactive oxygen radicals (ROS) and reactive nitrogen species (RNS) are the main inducers of DNA damage. Under hypoxic condition, the yield of radiation-induced ROS could be lower. In addition, the hypoxia-induced H_2S can be a good free radical scavenger by react with ROS and RNS and thus can efficiently decrease the cellular damage effect of radiation.[35]

It had long been assumed that the concentration of endogenous H_2S in mammal tissues could be very low. Actually, some studies have shown that the concentration of H_2S *in vivo* is much higher than expected and it can approach 20–160 μM in rat, human, and bovine brain tissues.[15,16] Abe and Kimura found that NaHS at concentrations from 10 to 130 μM, similar to the physiological concentrations of H_2S, could selectively enhance N-methyl D-aspartate (NMDA) receptor-mediated responses and played an important role in brain functions, probably acting as a neuromodulator and/or as an intracellular messenger.[36] H_2S also has been reported to inhibit cell proliferation, increase the activity of ERK and p21,[37] induce DNA damage,[38] even can exert pro-apoptotic activity.[39] Here we found that H_2S played a beneficial protection role in the irradiated hypoxic HepG2 cells as seen a decreased MN yield and increased cell survival under NaHS treatment. But this radioresistance effect was restricted in lower concentrations of NaHS (25–100 μM). When the concentration of NaHS exceeded 200 μM, its protective effect was replaced by cytotoxicity (data not shown). This phenomenon is consistent with other reports that H_2S has contrary actions depending on its concentration and the time of application.[38,39]

How does H_2S interact with cells? Recent studies have shown that H_2S can activate K^+_{ATP} channels in both heart[40–42] and vascular tissue[43] and then inhibits Ca^{2+} entering into cells via L-type membrane channels and thereby increases cell viability via the reduction of Ca^{2+} overload during ischemia and early reperfusion.[44] In fact, radiation can induce strong intracellular Ca^{2+} flux in the early stage of DNA damage.[45] Accordingly, we hypothesize that H_2S may exert radioresistance effect on hypoxic tumor cells via activation of K^+_{ATP} channels and inhibition Ca^{2+} flux. This assumption was verified by the experiment results in Fig. 5 where the K^+_{ATP} channels blocker can not only enhance the MN formation in the irradiated hypoxic HepG2 cells but also eliminate the radioresistance effect of exogenous H_2S. In addition, the cell response of caspase-3 activity in the irradiated hypoxic cells could be significantly increased by glibenclamide, the inhibitor of K^+_{ATP}, and was reduced by NaHS. However, when the cells were treated with both glibenclamide and NaHS, the caspase-3 activity became higher than that of NaHS-treated cells but still lower than that of glibenclamide-treated cells, suggesting that the K^+_{ATP} channel is not the sole pathway mediating the radioresistance function of H_2S on hypoxic tumor cells.

In summary, we demonstrate in the present work that the hepatoma cells are radioresistive under hypoxic condition due to the up-expression of CSE and CBS, which catalyze the generation of endogenous H_2S that may play its radioprotective function via stimulating K^+_{ATP} channels. These novel findings have important implication for clinical tumor therapy and suggest that the endogenous H_2S synthases could be a potential radiotherapeutic target for hypoxic tumor treatment.

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