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Hydrogen sulfide mediates the anti-survival effect of sulforaphane on human prostate cancer cells

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ABSTRACT

Hydrogen sulfide (H_2S) is a novel gasotransmitter that regulates cell proliferation and other cellular functions. Sulforaphane (SFN) is a sulfur-containing compound that exhibits anticancer properties, and young sprouts of broccoli are particularly rich in SFN. There is consistent epidemiological evidence that the consumption of sulfur-containing vegetables, such as garlic and cruciferous vegetables, may help reduce the occurrence of prostate cancer. Here we found that a large amount of H_2S is released when SFN is added into cell culture medium or mixed with mouse liver homogenates, respectively. Both SFN and NaHS (a H_2S donor) decreased the viability of PC-3 cells (a human prostate cancer cell line) in a dose-dependent manner, and supplement of methemoglobin or oxidized glutathione (two H_2S scavengers) reversed SFN-reduced cell viability. We further found both cystathionine gamma-lyase (CSE) and cystathionine beta-synthase are expressed in PC-3 cells and mouse prostate tissues. H_2S production in prostate tissues from CSE knockout mice was only 20% of that from wild-type mice, suggesting CSE is a major H_2S-producing enzyme in prostate. CSE overexpression enhanced H_2S production and inhibited cell viability in PC-3 cells. In addition, both SFN and NaHS activated p38 mitogen-activated protein kinases (MAPK) and c-Jun N-terminal kinase (JNK). Pre-treatment of PC-3 cells with methemoglobin decreased SFN-stimulated MAPK activities. Suppression of both p38 MAPK and JNK reversed H_2S- or SFN-reduced viability of PC-3 cells. Our results demonstrated that H_2S mediates the inhibitory effect of SFN on the proliferation of PC-3 cells, which suggests that H_2S-releasing diet or drug might be beneficial in the treatment of prostate cancer.

Introduction

Prostate cancer is one of the most prevalent malignancies and the second leading cause of cancer deaths in men in North America (Lassi and Dawson, 2011). There is consistent epidemiological evidence that diet and in particular the consumption of cruciferous vegetables have long been associated with a reduced risk in the occurrence of prostate cancer (Zhang et al., 1994). Many of the anticancer effects observed from cruciferous vegetables have been attributed to the sulfide-containing isothiocyanates (ITCs), and sulforaphane (SFN) is postulated to be one of the principle isothiocyanates found in cruciferous vegetables (Keum et al., 2004; Singh et al., 2004; Zhang et al., 2005). The mechanisms of SFN chemoprevention have been well studied and reveal diverse responses depending upon the stage of prostate carcinogenesis, including the induction of apoptosis and cell cycle arrest, inhibition of phase 1 enzymes, induction of phase 2 metabolism enzymes, etc. (Cho et al., 2005; Juge et al., 2007).

Hydrogen sulfide (H_2S) has been traditionally known as a toxic gas with the smell of rotten eggs for centuries. The physiological importance of H_2S surfaced in the mid-1990s. It is clear now that H_2S, joining with other endogenous gasses including nitric oxide and carbon monoxide, is one of gasotransmitters (Wang, 2002, 2011). H_2S at physiologically relevant concentrations hyperpolarizes cell membrane, regulates cell growth, relaxes blood vessels, and modulates neuronal excitability (Calvert et al., 2010; Yang et al., 2008; Yang, 2011; Zhao et al., 2001). Two pyridoxal-5′-phosphate-dependent enzymes, cystathionine beta-synthase (CBS) (EC 4.2.1.22) and cystathionine gamma-lyase (CSE) (EC 4.4.1.1), are responsible for the majority of endogenous production of H_2S in mammalian tissues which use L-cysteine as the main substrate (Wang, 2011; Zhao et al., 2001). The expressions of CBS and CSE have been identified in

Abbreviations: CBS, cystathionine beta-synthase; CSE, cystathionine gamma-lyase; ERK, extracellular signal-regulated kinase; GSH, glutathione; GSSG, oxidized glutathoine; H_2S, hydrogen sulfide; ITCs, isothiocyanates; JNK, c-Jun N-terminal kinase; KO, knockout; MAPK, mitogen-activated protein kinase; NaHS, sodium hydrogen sulfide; SFN, sulforaphane; WT, wild type.

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many human and other mammalian cells, including those from liver, kidney, brain, smooth muscle, pancreas, and lymphocytes (Wang, 2002; Yang et al., 2007; Zhao et al., 2001). Endogenously produced or exogenously applied H_2S regulates cell growth or death in a multitude of settings, and unbalanced cell proliferation and apoptosis due to the altered metabolism and functions of H_2S under different pathological conditions have been documented (Gobbi et al., 2009; Papapetropoulos et al., 2009; Yang, 2011; Yang et al., 2004b).

Recently, H_2S was discovered to mediate the major beneficial effects of garlic on cardiac functions, which suggest that sulfur compounds contained within plants may be transformed chemically or enzymatically in the human body with subsequent formation of H_2S (Benavides et al., 2007). Given cruciferous vegetables such as broccoli tend to release strong smell of H_2S when cooked, stored for a long time, or when rotten, ITCs may release H_2S which would mediate the beneficial effect of ITCs on prostate cancer (Chiao et al., 2002; Giovannucci et al., 2003). It has been shown that intake of SFN enriched food ameliorates both hypertension and atherosclerotic changes in spontaneously hypertensive stroke-prone rats, and H_2S administration generated the same beneficial effects as SFN on these cardiovascular disorders (Wu et al., 2004). It is essential to decipher the signaling and regulatory effects of H_2S and also its mediation on ITCs chemoprevention in prostate cancer.

Here we first investigated the release of H_2S from SFN and the mediation of H_2S on anti-survival effect of SFN on human prostate cancer cells (PC-3). The expressions of CSE and CBS as well as the endogenous production of H_2S in mouse prostate tissues and PC-3 cells were determined. We further explored the signal transduction pathway activated by both SFN and H_2S on stimulating PC-3 cell death.

Methods

Cell culture. Human prostate cancer PC-3 cells were obtained from the American Type Culture Collection (Manassas, VA) and were cultured with F-12K Nutrient Mixture medium (Sigma, Oakville, ON) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. The experiments were performed when the cells reached 70–80% confluence between passages 2 and 6. In all studies, cells were first incubated in the serum-free medium overnight and maintained at a quiescent state (G0 phase), and then 10% serum was added together with different agents.

Cellular viability assays. Cell viabilities were assessed based on conversion of trazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to purple formazan crystals by living cells (Cao et al., 2010; Yang et al., 2004a). Briefly, cells of equal number were plated onto each well of 96-well plates for 24 h. After different treatments, 20 μl (5 mg/ml) MTT was added to each well. The cells were then cultured at 37 °C for 4 h, and absorbance of formazan products at 570 nm was measured in a FLUOstar OPTIMA microplate spectrophotometer (BMG LABTEch, Germany). The cells incubated with control medium were considered 100% viable.

Measurement of H_2S production and concentration. H_2S production rate was measured as described previously (Zhao et al., 2001). Briefly, the cells or tissues were collected and homogenized in 50 mM ice-cold potassium phosphate buffer (pH 6.8). The flasks

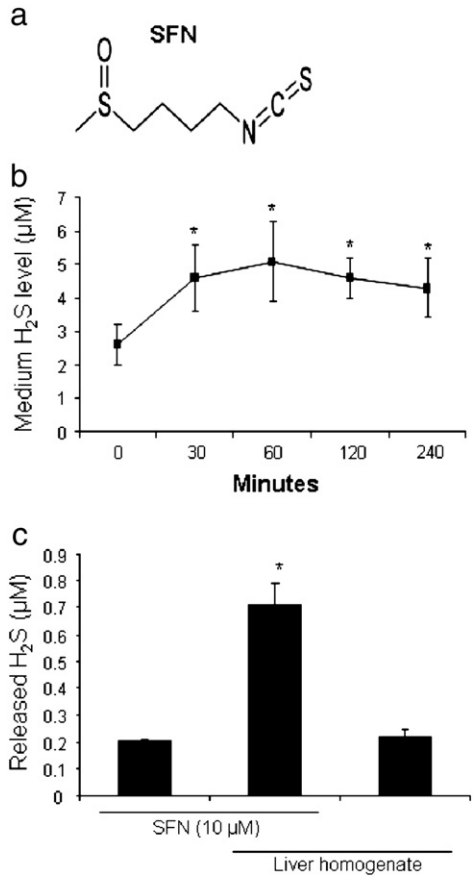


Fig. 1. SFN functions as a H_2S donor. a, Chemical structure of SFN constituting of parent moiety (glucosinolate) and ITCs group (−N=C=S). b, Release of H_2S from SFN when administered into cell culture medium. SFN (10 μM) was added into cell culture medium in the presence of PC-3 cells, and H_2S concentration in the medium was measured at the indicated time point. n=4. * $p<0.05$. c, More H_2S was released from SFN in the presence of liver homogenate. SFN (10 μM) was mixed with mouse liver homogenate at 37 °C for 90 min, and released H_2S was trapped and measured by using methylene blue method. n=3. * $p<0.05$.

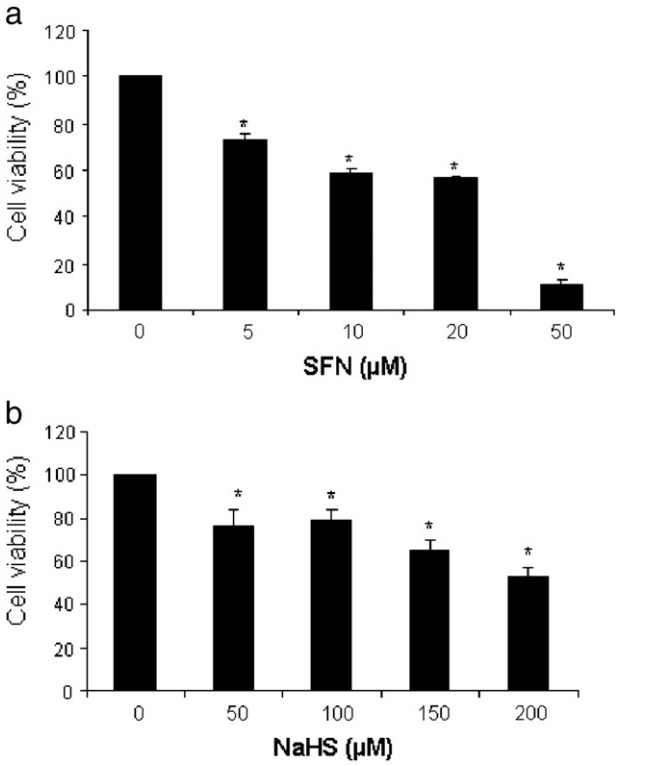


Fig. 2. Both SFN and H_2S reduced PC-3 cell viability. a, SFN decreased PC-3 cell viability. After the cells were incubated with different concentrations of SFN (5–50 μM) for 24 h, cell viability was analyzed by MTT assay. n=4. * $p<0.05$. b, NaHS decreased PC-3 cell viability. After the cells were incubated with different concentrations of NaHS (50–200 μM) for 24 h, cell viability was analyzed by MTT assay. n=4. * $p<0.05$.

containing reaction mixture (100 mM potassium phosphate buffer, 10 mM L-cysteine, 2 mM pyridoxal 5-phosphate, and 10% (w/v) cell/ tissue homogenates) and center wells containing 0.5 ml 1% zinc acetate and a piece of filter paper (2×2 cm) were flushed with N_2 and incubated at 37 °C for 90 min. For measuring H_2S release from SFN, the flasks contained 100 mM potassium phosphate buffer with or without SFN (10 μM) and/or mouse liver homogenate (1 mg/ml) (Distrutti et al., 2006). The reaction was stopped by adding 0.5 ml of 50% trichloroacetic acid, and the flasks were incubated at 37 °C for another 60 min. The contents of the center wells were transferred to test tubes each containing 3.5 ml of water. Then 0.5 ml of 20 mM N,N-dimethyl-p-phenylenediamine sulfate in 7.2 M HCl and 0.5 ml 30 mM $FeCl_3$ in 1.2 M HCl were added. The absorbance of the resulting solution at 670 nm was measured 20 min later with a spectrophotometer.

To measure H_2S concentration in culture medium, 100 μl of culture medium from each treatment were collected and added to microcentrifuge tubes containing zinc acetate (1% w/v, 300 μl) to trap H_2S. After 5 min, the reaction was terminated by adding 300 μl of N,N-dimethyl-p-phenylenediamine sulfate (20 mM in 7.2 M HCl) and 300 μl of $FeCl_3$ (30 mM in 1.2 M HCl) (Yang et al., 2007). After the mixture was kept in the dark for 20 min, 200 μl of trichloroacetic acid (10% w/v) was added to precipitate any protein that might be present in the culture media. Subsequently, the mixture was centrifuged at 10,000×*g* for 10 min. H_2S in the sampled culture media interacts with N,N-dimethyl-p-phenylenediamine sulfate to form methylene blue, and the absorbance of the resulting solution was determined at 670 nm. H_2S concentration was calculated against the calibration curve of standard H_2S solutions.

Western blotting analysis. Cultured cells or prostate tissues were harvested and lysed. Equal amounts of proteins were boiled and separated by SDS-PAGE and electrophoretically transferred to nitrocellulose membrane as described previously (Yang et al., 2004b). The primary antibody dilutions were 1:1000 for CSE and CBS, phosphorylated or total extracellular signal-regulated kinase (ERK), p38 MAPK, or c-Jun N-terminal kinase (JNK), and 1:10,000 for β-actin. Horseradish peroxidase-conjugated secondary antibody was used at 1:5000. The immunoreactions were visualized by ECL and exposed to X-ray film (Kodak Scientific Imaging film).

Vector construction and transfection. The vector pIRES2-EGFP-CSE containing rat CSE gene was constructed as described previously (Yang et al., 2004a). All tranfections were performed with Lipofectamine 2000 (Invitrogen) in an OptiMEM medium (Invitrogen, Carlsbad, CA). The vector pIRES2-EGFP without CSE gene acted as a control. At 48 h after transfection, CSE expression, H_2S production and cell viability were measured, respectively.

Materials. CSE knockout (KO) mice were generated as previously described (Yang et al., 2008). The fourth generation of 8–10 week old male CSE KO offspring and age-matched male wild-type (WT)

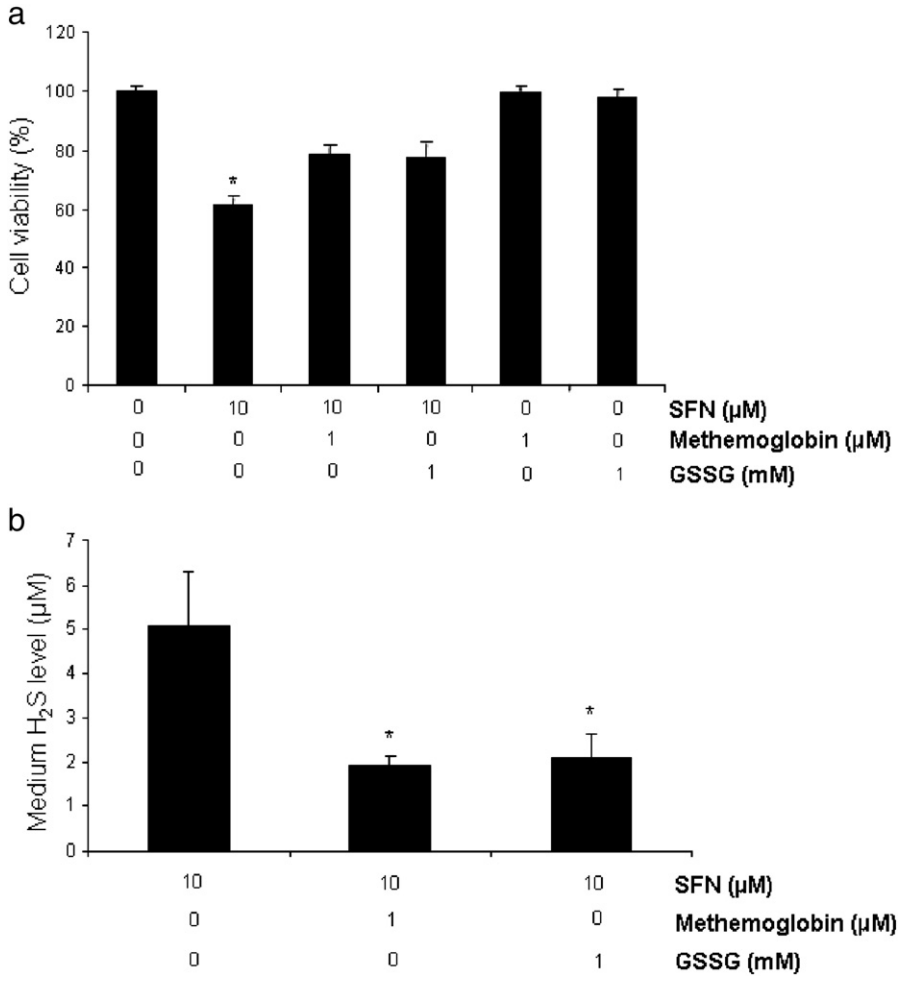


Fig. 3. Mediation of H_2S on the inhibitory effect of SFN on PC-3 cell viability. a, H_2S scavengers (methemoglobin and GSSG) reversed SFN-reduced cell viability. The cells were incubated with 10 μM SFN in the presence or absence of methemoglobin (1 μM) or oxidized glutathione (1 mM) for 24 h. n = 4. * $p<0.05$. b, methemoglobin and GSSG scavenged H_2S released from SFN in cell culture medium. SFN (10 μM) was added to cell culture medium in the presence of methemoglobin (1 μM) or oxidized glutathione (1 mM) for 1 h. n = 4. * $p<0.05$.

littermates on C57BL/6J×129SvEv background were used with an approved protocol by the Animal Care Committee of Lakehead University, Canada. After the mice were anesthetized, the tissues of prostate and liver were dissected and cleaned for protein extraction or H_2S measurement.

The antibodies against CSE and CBS were purchased from Novus Biologicals (Littleton, CO). The anti-MAPK antibodies and MAPK inhibitors were obtained from New England Biolabs (Camarillo, CA). Horseradish peroxidase-conjugated goat anti-rabbit IgG antibody and goat anti-mouse IgG antibody were from Sigma. SFN was purchased from LKT Laboratories (St. Paul, MN) and dissolved at a concentration of 100 mM in dimethyl sulfoxide. All other chemicals were from Sigma or New England Biolabs.

Statistical analysis. Data are presented as means ± SEM, and the data represent at least three independent experiments. Statistical comparisons were made using Student's *t* test or one-way ANOVA followed by a post hoc analysis (Tukey test) where applicable, and significance level was set at $p<0.05$.

Results

SFN functions as a H_2S donor

SFN is an organosulfur compound that exhibits anticancer properties, and more evidence is from anti-prostate cancers (Brooks et al., 2001). Fig. 1a shows the chemical structure of SFN, which constitutes a parent moiety (glucosinolate) and a ITCs group (−N=C=S). The chemopreventive effects of SFN is attributed to ITC group (−N=C=S) rather than the parent moiety glucosinolate (Juge et al., 2007). To investigate whether SFN releases H_2S, SFN (10 μM) was added to the cell culture medium. In the presence of PC-3 cells, H_2S concentration in the cell culture medium was significantly increased 30 min after incubation with SFN and kept stable for at least 4 h (Fig. 1b). We also found more H_2S was released when SFN (10 μM) was mixed with mouse liver homogenate (Fig. 1c). All these data suggests that H_2S can be released from SFN under specific conditions.

H_2S mediates the anti-survival effect of SFN on PC-3 cells

The effects of SFN and H_2S on the viability of PC-3 cells were determined by a colorimetric MTT assay. Both SFN and H_2S exhibited growth inhibitory effects on PC-3 cells in a dose-dependent manner. After treatment for 24 h, the cell viability with 5, 10, 20, and 50 μM SFN was 72.1 ± 3.3%, 58.4 ± 2.6%, 56.7 ± 2.1%, and 10.2 ± 3.1% compared with that in control cells (100%), respectively (Fig. 2a). For H_2S, the cell viability at 24 h was 76.1 ± 6.1%, 79.6 ± 5.3%, 62.5 ± 5.1%, and 51.7 ± 5.2% for 50, 100, 150, and 200 μM NaHS, respectively (Fig. 2b).

SFN acts as a H_2S donor. Therefore, we performed the next experiments about the mediation of H_2S in SFN-inhibited PC-3 cell viability. We treated the cells with 10 μM SFN for 24 h in the presence or absence of H_2S scavengers, methemoglobin (1 μM) or oxidized glutathoine (GSSG, 1 mM), and then performed cell viability assay (Wang, 2002; Yang et al., 2004a). We found that both H_2S scavengers partly but significantly reversed SFN-decreased cell viability ($p<0.05$). Methemoglobin and GSSG alone had no effect on PC-3 cell viability (Fig. 3a). We further provided evidence that methemoglobin and GSSG significantly scavenged SFN-released H_2S by 61.6 ± 7.2% and 58.0 ± 9.4%, respectively (Fig. 3b).

CSE is the predominant H_2S-producing enzyme in prostate tissues and PC-3 cells.

To detect H_2S-generating enzymes in prostate, we first performed Western blotting analysis by using PC-3 cells and prostate tissues. Prostate tissues were collected from 8–10 week male WT mice and CSE KO mice. As shown in Fig. 4a, CSE was clearly expressed in PC-3 cells as well as mouse tissues from WT mice, and CSE protein expression was not found in prostate tissues from CSE KO mice. CBS, another H_2S-generating enzyme, was also expressed in PC-3 cells and prostate tissues. There was no change in CBS protein expression between WT and CSE KO prostate tissues (Fig. 4b). Furthermore, significant higher endogenous H_2S production was detected in prostate tissues from WT mice (4.4 ± 0.3 nmol/g/min), while CSE deficiency in prostate tissues significantly reduced H_2S production by 75.1 ± 5.4% ($p<0.05$, Fig. 4c), suggesting CSE is the major H_2S-producing enzyme in the prostate.

CSE overexpression inhibits PC-3 cell viability

PC-3 cells were transfected with a CSE-containing vector (pIRES2-EGFP-CSE) or an identical empty vector lacking a cDNA insert (pIRES2-EGFP) as a control. The functional expression of CSE was verified by Western blotting analysis and H_2S production rate. Transfection of CSE vector but not of control vector resulted in significant increase in CSE protein expression (Fig. 5a). CSE overexpression also resulted in a

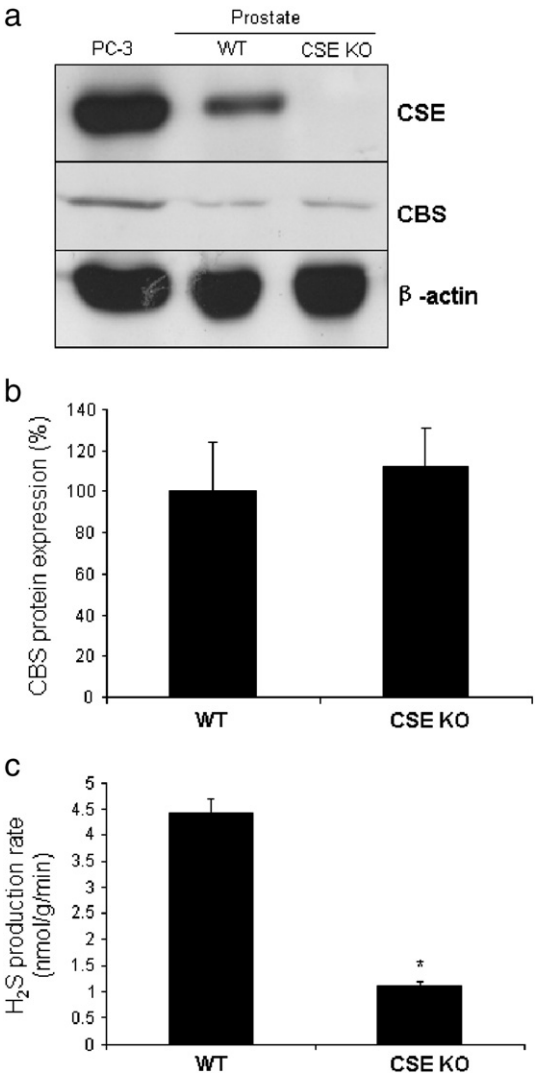


Fig. 4. CSE is the predominant H_2S-producing enzyme in prostate. a, Expressions of CSE and CBS in prostate tissues and PC-3 cells. Results are representative of three individual experiments. b, There was no difference on CBS protein expression between WT and CSE KO prostate tissues. n = 4. c, Significant reduced H_2S production in the prostate tissues from CSE KO mice. Prostate tissues were collected from 8–10 week male WT mice and CSE KO mice. n = 4. * $p<0.05$.

marked increase in the H_2S production rate (6.76 ± 1.61 nmol/g/min) compared with the control vector-transfected cells (1.69 ± 0.53 nmol/g/min, $p<0.05$) (Fig. 5b). To determine whether CSE overexpression affects PC-3 cell growth, cell viability was measured. Compared with control vector-transfected cells (100%), PC-3 cells transfected with CSE vector exhibited a significantly reduced cell viability (76.8 ± 6.2%) at 48 h ($p<0.05$, Fig. 5c). The presence of SFN inhibited more cell viability and induced more H_2S release in CSE-overexpressed cells in comparison with the cells transfected with control vector ($p<0.05$, Figs. 5c, d). We further demonstrated that SFN and H_2S had no effect on the protein expressions of both CSE and CBS (Fig. 5e).

Altered MAPK activation by both SFN and H_2S

MAPK superfamily is highly involved in cellular survival responses regulated by both SFN and H_2S (Juge et al., 2007; Yang, 2011). Here we further found both SFN and H_2S stimulated the phosphorylation of all three MAPK members, ERK, p38 MAPK and JNK in PC-3 cells (Figs. 6a, b). ERK activation appeared during the first 15 min of NaHS treatment followed by a quick decline, while SFN started to induce ERK activation at 30 min and peaked at 1 h followed a slow decline. p38 MAPK phosphorylation was activated by NaHS at the first 15 min and continually increased until after 12 h. SFN-induced p38 MAPK activation occurred at 1 h and peaked at 4 h. For JNK, both NaHS and SFN stimulated phosphorylation after 15 min of incubation and kept stable until 1 h followed a quick dephosphorylation. The protein expression of house keeping gene β-actin and the total amount of MAPK protein remained unchanged with both SFN and H2S treatment.

To investigate the involvement of MAPK in SFN and H_2S-inhibited cell viability, we pretreated the cells with U0126 (inhibitor of ERK), SB203580 (inhibitor of p38 MAPK), and/or SP600125 (inhibitor of JNK), respectively. Co-treatment of the cells with SB203580 (20 μM) and SP600125 (20 μM) significantly reversed the inhibitory roles of both H_2S and SFN on cell viability ($p<0.05$, Figs. 7a, b). The inhibitor alone had no effect on cell viability (data not shown). We further found that pretreatment of PC-3 cells with methemoglocin

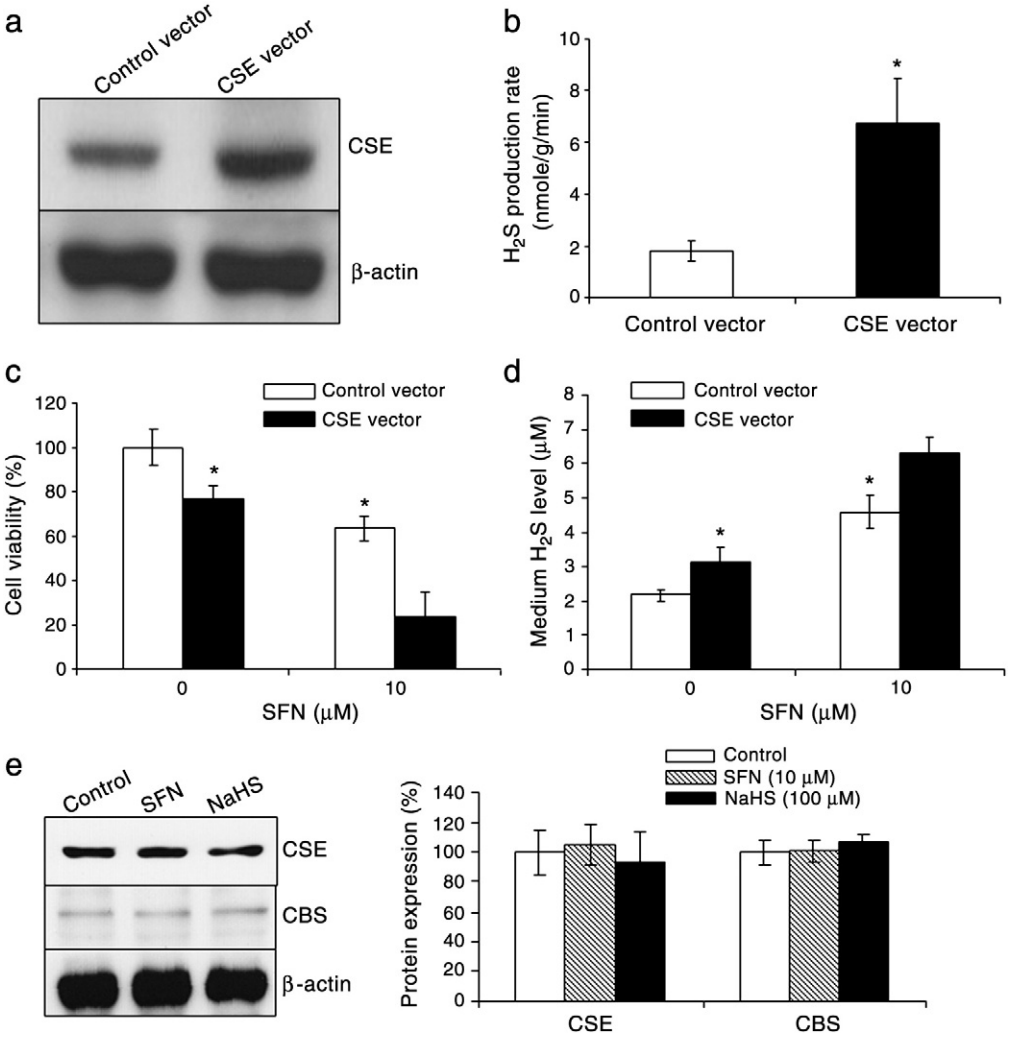


Fig. 5. CSE overexpression reduced PC-3 cell viability. Increased CSE expression (a) and H_2S production (b) in pIRES2-EGFP-CSE vector-transfected cells. After PC-3 cells were transfected with pIRES2-EGFP-CSE vector or control vector for 48 h, CSE protein expression and H_2S production rate were measured, respectively. n = 3. * $p<0.05$. SFN reduced more cell viability (c) and induced more H_2S release (d) in CSE-overexpressed cells. Cell viability was assayed after the cells were transfected with pIRES2-EGFP-CSE vector or control vector for 24 h following another 24 h incubation with SFN (10 μM). Medium H_2S levels were measured after the cells were transfected with pIRES2-EGFP-CSE vector or control vector for 48 h following incubation with SFN (10 μM) for 1 h. n = 4. * $p<0.05$ versus all other groups. e, SFN and H_2S had no effect on the protein expressions of both CSE and CBS. The cells were incubated with SFN (10 μM) or NaHS (100 μM) for 24 h. Results were representative of three individual experiments. * $p<0.05$.

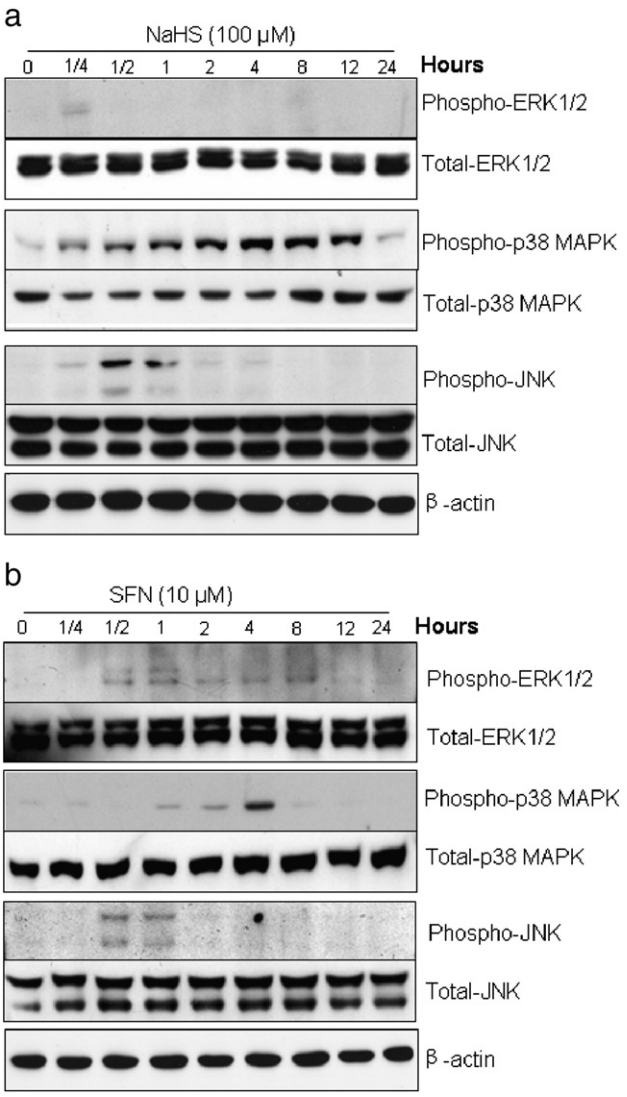


Fig. 6. Activation of MAPKs by both SFN and H_2S. a and b, Time course of the activation of MAPKs induced by SFN and H_2S. After being incubated with 10 μM SFN or 100 μM NaHS for the indicated time, the cells were collected and subjected to Western blot analysis by using different antibodies. The results were representative of three individual experiments.

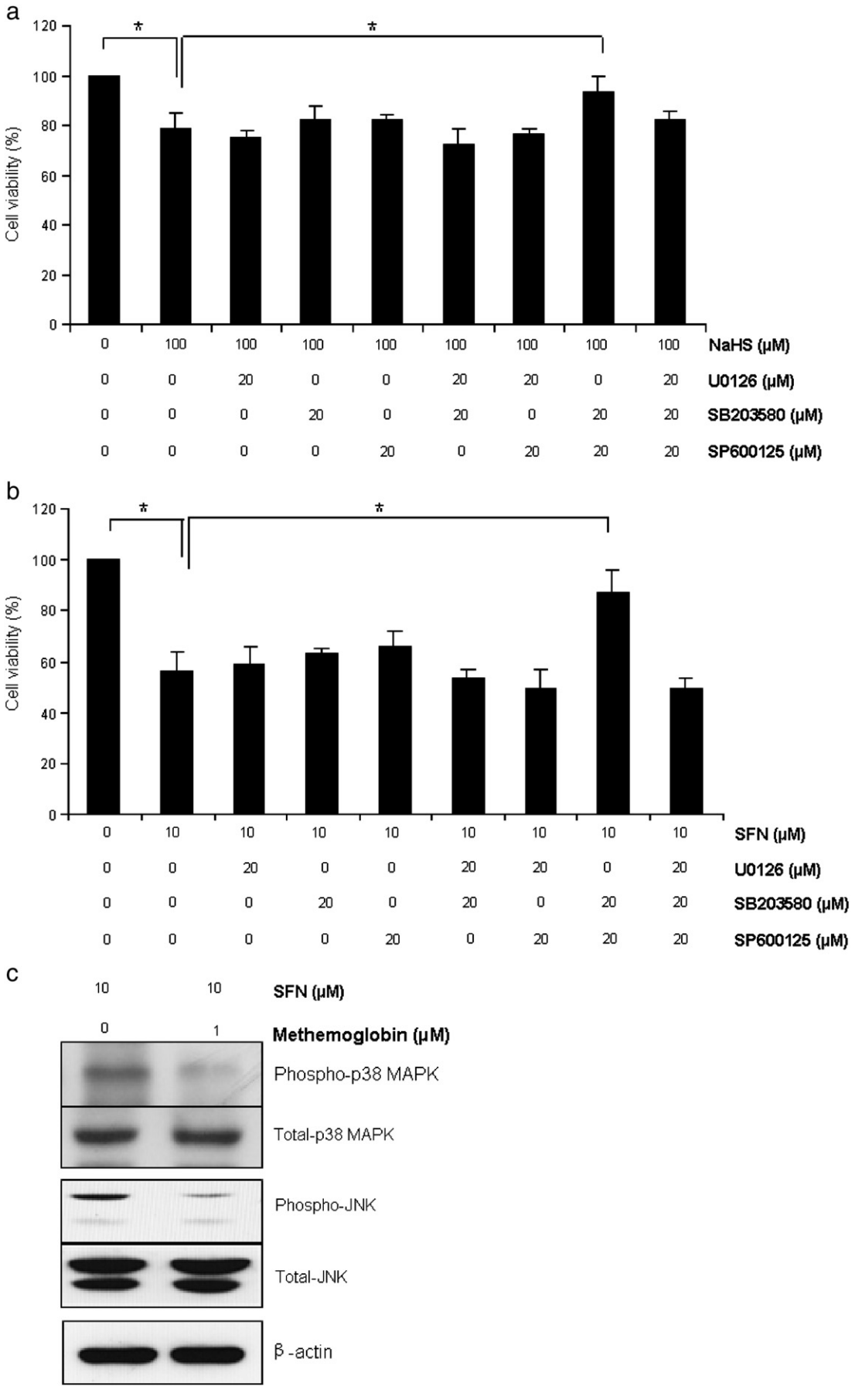
diminished SFN-stimulated activations of p38 MAPK and JNK (Fig. 7c).

Discussion

ITCs are sulfur-containing compounds that are broadly distributed among cruciferous vegetables such as cabbages and broccoli, which are highly effective in preventing or reducing the risk of cancer (Giovannucci et al., 2003; Keum et al., 2004). SFN is so far the most widely studied and characterized ITC. Studies have indicated that SFN and other ITCs can reduce the risk of prostate cancer in animal models, inhibit prostate cancer cell growth, induce apoptosis and retard growth of prostate cancer cell xenografts in vivo (Chiao et al., 2002; Singh et al., 2004). SFN is a hydrolysis product of glucoraphanin catalyzed by enzyme myrosinase. Young sprouts of broccoli and cauliflower are particularly rich in glucoraphanin (Appendino and Bardelli, 2010). When the raw vegetables are damaged or chewed, the plant cells are broken and the enzyme myrosinase hydrolyzes glucoraphanin into SFN (Li et al., 2007). SFN consists of a molecular of ITC (−N=C=S) linked to the parent moiety (glucosinolate), and ITC group but not the parent moiety mostly contributes to the actions of SFN (Juge et al., 2007).

The primary role of H_2S in disparate cell survival and organ functions makes it highly possible that abnormal production and activity of H_2S is involved in human diseases (Wang, 2002, 2011; Yang, 2011). It is true that a growing body of evidence is accumulating regarding the role of this gasotransmitter in different diseases. The physiological levels of plasma H_2S have been reported to be 50 μM in rats, 34 μM in mice, and 44 μM in humans, and the localized tissue levels of H_2S are known to be higher than its circulatory levels (Calvert et al., 2010; Yang, 2011; Yang et al., 2008). There is some controversy regarding the concentration of H_2S in circulation. The concentration of H_2S is undetectable in plasma and whole blood by using a polarographic H_2S sensor (Whitfield et al., 2008). In the present study, we used 100 uM NaHS for most of the experiments. NaHS is the most widely used H_2S donor, but NaHS is unstable associated with high variability in the biological action. NaHS can cause rapid H_2S release, which may lead to high concentration of H_2S accumulation in a short time. In physiological saline, NaHS dissociates from Na^+ and HS^-, and then HS^- associates with H^+ and form H_2S, and about one-third of the H_2S exists as the undissociated form. We had previously demonstrated that a drop of the H_2S concentration around 55% was observed within 30 min, and 76% of H_2S was gone within 12 h after 100 μM NaHS was added into the culture media in the presence of cells (Yang et al., 2010). When 100 μM NaHS is added into the culture media, actually only 33.3 μM H_2S is released in seconds and the concentration of remaining H_2S is below 10 μM within hours. Pharmacologically useful donors should release H_2S slowly and steadily over a period of time (Distrutti et al., 2006). Many novel slow-releasing H_2S donors, such as GYY4137, ATB-429, and ACS-15, display far greater utility and showed preclinical evidence in effectively treating various diseases (Distrutti et al., 2006; Li et al., 2007, 2008). However, most of these novel H_2S donors are from chemical syntheses and not natural products. It has been long known that garlic, a natural sulfide-containing product, improves immune system function and reduces risk of some forms of cancer (Iciek et al., 2009). Just recently, Benavides et al. reported that the sulfide-containing compounds found in garlic are converted into H_2S by molecules that occupy the membranes of red blood cells, and the secret of garlic's apparent health benefits lies in its relation to H_2S (Benavides et al., 2007). In the present study, we observed that SFN functions as a slow-releasing H_2S donor. Firstly, supplement of SFN into cell culture medium enhanced H_2S content. When 10 μM SFN was added into cell culture medium, the concentration of H_2S increased from 2.6 μM to ~5 μM within 1 h and kept stable for at least 4 h (Fig. 1b). Secondly, in the presence of liver homogenate, SFN was shown to liberate more H_2S compared with the buffer only. The mechanism(s) through which SFN liberates H_2S release remains to be identified, although several explanations could be taken into consideration. SFN may release more H_2S under the condition with abundant cellular thiol, but less H_2S under the condition with sparse thiol. SFN is predominantly metabolized via the mercapturic acid pathway which requires SFN first reacts with glutathione (GSH) to form a GSH conjugate. The existence of PC-3 cells or liver homogenates may provide enzymes and GSH for SFN to liberate H_2S (Benavides et al., 2007; Distrutti et al., 2006).

Both SFN and H_2S have tons of similar functions. SFN protected the heart against ischemia-reperfusion injury in rats through an increase in the antioxidant enzyme levels of Mn-superoxide dismutase, catalase and heme oxygenase-1, and this protection was blocked by 5-hydroxydecanoc acid, a mitochondrial K_{ATP} channel blocker (Piao et al., 2010). H_2S is a known K_{ATP} opener in various kinds of cells, and administration of H_2S significantly reduced myocardial infarct size, and this effect was abolished by 5-hydroxydecanoc acid (Sivarajah et al., 2006; Wang, 2002; Yang et al., 2005; Zhao et al., 2001). Feeding spontaneously hypertensive stroke-prone rat with SFN-rich broccoli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Cell viability (%)
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100
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0 100 100 100 100 100 100 100 100 NaHS (μM)
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Cell viability (%)
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*
0 10 10 10 10 10 10 10 10 SFN (μM)
0 0 20 0 0 20 20 0 20 U0126 (μM)
0 0 0 20 0 20 0 20 20 SB203580 (μM)
0 0 0 0 20 0 20 20 20 SP600125 (μM)
c
10 10 SFN (μM)
0 1 Methemoglobin (μM)
Phospho-p38 MAPK
Total-p38 MAPK
Phospho-JNK
Total-JNK
β -actin

sprouts for 14 weeks attenuates the development of hypertension and atherosclerosis, but feeding the rats with sprouts in which most of SFN was destroyed or no sprouts had little effect (Wu et al., 2004). Mice genetically deficient in CSE expression exhibited lower H_2S content and age-dependent increased blood pressure, and exogenous H_2S administration lowered blood pressure of rats (Yang et al., 2008; Zhao et al., 2001). There is also a growing body of evidence demonstrating the anti-atherosclerotic role of H_2S. H_2S reduces atherosclerotic lesions in apolipoprotein E-deficient mice by inhibiting endothelial cell inflammation (Wang et al., 2009). Both H_2S and SFN display pro-survival role in endothelial cells (Papapetropoulos et al., 2009; Shan et al., 2010). In addition, accumulated evidence demonstrated both SFN and H_2S provide cellular protection through their antioxidant actions (Lavu et al., 2010; Mishra et al., 2010; Paolini et al., 2008; Wu et al., 2004). All these suggest that H_2S mediates the beneficial effects of SFN.

Here we further provided evidence that H_2S released from SFN contributes to its anti-survival effect on prostate cancer cells. H_2S plays an important role as a signal molecule in regulating cell survival. We and others have shown that H_2S significantly reduces the growth of colon cancer cells (HT-29 and WiDr cells) (Cao et al., 2010; Leschelle et al., 2005). However, the changes in H_2S levels during the development of prostate cancer are not reported, and the therapeutic effects of H_2S on prostate cancer are unknown. Prostate cancer is a multi-step molecular pathogenesis, induced by genetic and epigenetic changes that disrupt the cellular balance, proliferation, apoptosis, differentiation and senescence (Lassi and Dawson, 2011). The concentration of cysteine (one substrate for producing H_2S) in plasma was significantly decreased as a result of prostate tumor progression in nude mice implanted with human prostate cancer cells, suggesting the dysfunction of H_2S-producing enzymes in the progress of prostate cancer (Al-Awadi et al., 2008). In the present study, we are the first to find the expression of CSE in mouse prostate tissues and prostate cancer cells, which mostly contributes to the endogenously produced H_2S. CSE overexpression generated more H_2S and inhibited PC-3 cell viability. CBS is also expressed in prostate tissues and PC-3 cells, while its contribution to H_2S generation is limited. CSE deficiency did not affect CBS protein expression in mouse prostate tissues (Fig. 4b). Quite differently, CBS but not CSE plays a critical role in butyrate-stimulated H_2S production following cell viability inhibition in colon cancer cells (Cao et al., 2010). PC-3 cell is an androgen-unresponsive metastatic cell line. In another androgen-responsive prostate cancer cell line, downregulation of CBS reduced cell viability under oxidative stress (Prudova et al., 2007). The effect of H_2S on the cell growth of androgen-responsive cell lines needs to be investigated. Involvement of CSE/H_2S system in human prostate cancers and other animal models for prostate cancer will also be further examined.

To gain mechanistic insight into the roles of SFN and H_2S on regulating PC-3 cell growth, we investigated the activation of MAPKs. MAPKs are a group of evolutionarily conserved proline-directed protein serine/threonine kinases that are activated in response to a variety of extracellular stimuli and mediate signal transduction from the cell surface to the nucleus. There are three distinct groups of MAPKs: ERK1/2, p38 MAPK, and JNK (Wang, 2011). SFN has been shown to induce cell apoptosis by stimulating all these three MAPKs in various types of cancer cells (Cho et al., 2005; Juge et al., 2007). In the current study, we further provided evidence that SFN induces phosphorylation of ERK1/2, p38 MAPK, and JNK in PC-3 cells. H_2S may mediate the stimulatory role of SFN on these three MAPKs. Firstly, H_2S also induced the phosphorylation of all three MAPKs. Secondly, compared with SFN, H_2S stimulated all these three MAPKs at the earlier stage of incubation. The phosphorylation of ERK1/2, p38 MAPK, and JNK occurred at 15 min of H_2S treatment. For SFN, the phosphorylation of these three MAPKs happened after 30 min of incubation. Thirdly, the intensity and duration of phosphorylation of p38 MAPK and JNK in H_2S-treated cells were much more compared with those in SFN-incubated cells. In addition, we found that the synchronous phosphorylation of p38 MAPK and JNK were essential for SFN and H_2S-inhibited cell viability, because blockage of both p38 MAPK and JNK but not alone reversed the inhibitory effects of SFN and H_2S on cell viability. More directly, we observed that scavenging of H_2S by methemoglobin reduced SFN-stimulated phosphorylation of both p38 MAPK and JNK. Despite these findings, the supplement of MAPK inhibitors or H_2S scavengers did not completely reverse SFN-decreased cell viability, suggesting that SFN may inhibit cell growth by some other pathways. It is well known that micromole doses of SFN prevent cancer and stimulate cancer cell apoptosis through induction of phase 2 enzymes and enhancing the transcription of tumor suppressor proteins (Cho et al., 2005; Juge et al., 2007). SFN also induces many signaling mediators, such as NF-kappaB, the activator protein 1, phosphoinositide 3-kinase, and protein kinase C, etc. (Li et al., 2011). There have been many reports on the effects of H_2S on the growth patterns of different types of cells by regulating MAPKs (Wang, 2011; Yang, 2011). We first showed that H_2S treatment or CSE overexpression increased the activities of ERK and p38 MAPK, but not JNK, in human aorta smooth muscle cells, suppression of ERK activity, but not of p38 MAPK, inhibited H_2S induced apoptosis of human aorta smooth muscle cells (Yang et al., 2004b). In HEK-293 cells, activation of ERK and p38 MAPK was induced by H_2S, while only ERK was involved in the cell growth change mediated by H_2S (Yang et al., 2004a). We also found that in INS-1E cells, CSE overexpression or H_2S application increased p38 MAPK activity, and inactivation of p38 MAPK inhibited H_2S-induced apoptosis in INS-1E cells (Yang et al., 2007). In contrast, increased endothelial cell proliferation by H_2S is associated with sustained phosphorylation of ERK1/2 (Papapetropoulos et al., 2009). All these suggest that cell-type specific activations of MAPKs by H_2S are required for a variety of cell fate determinations.

In conclusion, our results demonstrated that H_2S mediates the inhibitory effect of SFN on the proliferation of PC-3 cells by activating both p38 MAPK and JNK. H_2S-releasing diet or drug might be beneficial in the treatment of prostate cancer. This study will also advance our understanding of the physiological role of H_2S in prostate cancer, and such an understanding will help reveal pathogenesis of prostate cancer related to abnormal endogenous H_2S metabolism and design new gene therapy for the treatment of prostate cancer.

Conflict of interest

The authors declare that there are no conflicts of interest.

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Fig. 7. Both p38 MAPK and JNK were involved in SFN and H_2S-inhibited PC-3 cell viability. Blockage of both p38 MAPK and JNK reversed SFN (a) and H_2S (b)-inhibited cell viability. PC-3 cells were pretreated with MAPK inhibitor(s) for 30 min before treatment with SFN (10 μM) or NaHS (100 μM) for 24 h. n = 3. * $p<0.05$. c, Scavenging of H_2S by methemoglobin reversed SFN-stimulated phosphorylation of p38 MAPK and JNK. PC-3 cells were treated with SFN (10 μM) in the presence or absence of methemoglobin (1 μM) for 1 h. Results were representative of three individual experiments.

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