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### Original contribution

# H<sub>2</sub>S-induced S-sulfhydration of pyruvate carboxylase contributes to gluconeogenesis in liver cells



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#### ABSTRACT

*Background:* Cystathionine gamma-lyase (CSE)-derived hydrogen sulfide ( $H_2S$ ) possesses diverse roles in the liver, affecting lipoprotein synthesis, insulin sensitivity, and mitochondrial biogenesis.  $H_2S$  S-sulfhydration is now proposed as a major mechanism for  $H_2S$ -mediated signaling. Pyruvate carboxylase (PC) is an important enzyme for gluconeogenesis. S-sulfhydration regulation of PC by  $H_2S$  and its implication in gluconeogenesis in the liver have been unknown.

*Methods:* Gene expressions were analyzed by real-time PCR and western blotting, and protein S-sulfhydration was assessed by both modified biotin switch assay and tag switch assay. Glucose production and PC activity was measured with coupled enzyme assays, respectively.

*Results*: Exogenously applied H<sub>2</sub>S stimulates PC activity and gluconeogenesis in both HepG2 cells and mouse primary liver cells. CSE overexpression enhanced but CSE knockout reduced PC activity and gluconeogenesis in liver cells, and blockage of PC activity abolished H<sub>2</sub>S-induced gluconeogenesis. H<sub>2</sub>S had no effect on the expressions of PC mRNA and protein, while H<sub>2</sub>S *S*-sulfhydrated PC in a dithiothreitol-sensitive way. PC *S*-sulfhydration was significantly strengthened by CSE overexpression but attenuated by CSE knockout, suggesting that H<sub>2</sub>S enhances glucose production through *S*-sulfhydrating PC. Mutation of cysteine 265 in human PC diminished H<sub>2</sub>S-induced PC *S*-sulfhydration and activity. In addition, high-fat diet feeding of mice decreased both CSE expression and PC *S*-sulfhydration in the liver, while glucose deprivation of HepG2 cells stimulated CSE expression.

Conclusions:  $CSE/H_2S$  pathway plays an important role in the regulation of glucose production through S-sulfhydrating PC in the liver.

*General significance:* Tissue-specific regulation of CSE/H<sub>2</sub>S pathway might be a promising therapeutic target of diabetes and other metabolic syndromes.

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#### 1. Introduction

Hydrogen sulfide ( $H_2S$ ) is considered as a novel gasotransmitter that plays a critical role in liver functions, including lipoprotein synthesis, insulin sensitivity, mitochondrial bioenergetics and biogenesis, and detoxification of various metabolites [1–5]. In analogy with protein *S*-nitrosylation, protein *S*-sulfhydration has been proposed as a major mechanism for  $H_2S$ -mediated signaling [6–8].  $H_2S$  can be endogenously produced in a variety of cells, tissues, organs, and systems by

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cystathionine beta-synthase, cystathionine gamma-lyase (CSE) and 3mercaptopyruvate sulfurtransferase (3-MPST). The expressions of these genes are tissue-specific [9,10]. Compared with all other tissues in the body, all these three genes are expressed in liver with a large amount of H<sub>2</sub>S production [1,7,11,12]. Deficiency of CSE gene diminished H<sub>2</sub>S production by more than 90% in mouse liver, suggesting that CSE acts as a major H<sub>2</sub>S-generating enzyme in the liver [2,7]. Altered hepatic H<sub>2</sub>S generation and metabolism have been demonstrated to be involved in the pathogenesis of many liver diseases, such as ischemia/reperfusion injury, hepatic fibrosis and cirrhosis [13–16].

Pyruvate carboxylase (PC; EC6.4.1.1) is a nuclear encoded mitochondrial enzyme that catalyzes pyruvate to form oxaloacetate [17]. PC serves two biosynthetic purposes: it catalyzes the carboxylation of pyruvate to oxaloacetate, which is crucial for replenishing tricarboxylic acid cycle intermediates when they are used for biosynthetic purposes; and it provides oxaloacetate for phosphoenolpyruvate carboxykinase to convert to phosphoenolpyruvate [18]. Phosphoenolpyruvate can be converted into glucose, therefore, PC is considered as an enzyme that

Abbreviations: 3-MPST, 3-mercaptopyruvate sulfurtransferase; CSE, cystathionine gamma-lyase; DMEM, Dulbecco's Modified Eagle's medium; DTT, dithiothreitol; H<sub>2</sub>S, hydrogen sulfide; HFD, high fat diet; MMTS, methyl methanethiosulfonate; KO, knockout; NADH, reduced nicotinamide adenine dinucleotide; NAD<sup>+</sup>, oxidized nicotinamide adenine dinucleotide; PBS, phosphate buffered saline; PC, pyruvate carboxylase; WT, wild type.

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is crucial for intermediary metabolism, controlling fuel partitioning toward gluconeogenesis [18]. Gluconeogenesis is a ubiquitous process, present in animals, plant, fungi, and other microorganisms. In animals, gluconeogenesis takes place mainly in the liver [19]. In the fed state, the liver stores energy as glycogen from glucose. Conversely, when plasma glucose concentration decreases during fasting or under nutrition, the liver produces glucose through glycogenolytic and gluconeogenic pathways [20]. PC is positively regulated by glucagon and glucocorticoid while negatively regulated by insulin [21]. Up to now, H<sub>2</sub>S regulation of PC expression and/or activity as well as its involvement in liver gluconeogenesis is not clear.

In the present study, we performed detailed investigation on H<sub>2</sub>S modification of PC protein by *S*-sulfhydration and the actual *S*-sulfhydration site(s), and explored the functional relevance of PC *S*-sulfhydration in liver glucose production. By using human hepatocellular liver carcinoma cell line (HepG2) and mouse primary hepatocyte isolated from both wild-type (WT) mice and CSE knockout (CSE-KO) mice, we found that H<sub>2</sub>S induces PC activity directly by *S*-sulfhydrating PC protein at cysteine 265, and increased PC activity contributes to H<sub>2</sub>S-stimulated gluconeogenesis. We further demonstrated that high fat diet (HFD) feeding decreases both CSE expression and PC *S*-sulfhydration in mouse liver. This study advances our understanding of H<sub>2</sub>S signal in liver gluconeogenesis by targeting at PC.

#### 2. Materials and methods

#### 2.1. Cell culture and animal preparation

HepG2 and HEK293 cells (American Type Culture Collection, Manassas, VA) were maintained in Dulbecco's Modified Eagle's medium (DMEM, Sigma, Oakville, ON) supplemented with 10% fetal bovine serum (FBS, Clontech, Mountain View, CA) and 1% penicillin–streptomycin solution (Sigma). For overexpression of PC and CSE, recombinant hPC, PC mutant plasmids, or CSE cDNA plasmid [22] was transfected into HEK293 or HepG2 cells using Lipofectamine<sup>™</sup> 2000 reagent as described by the manufacturer's protocol (Invitrogen, Burlington, ON). For high glucose treatment, HepG2 cells and primary liver cells were pre-incubated overnight in DMEM containing 1% FBS and 1 mM glucose, and then subjected to 25 mM glucose for additional 24 h. For the incubation of the cells with NaHS for longer time, the medium was changed every 4 h with newly added NaHS at the required concentration.

CSE-KO mice were generated as previously described [11]. All animal experiments were conducted in compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85-23, revised 1996) and approved by the Animal Care Committee of Lakehead University, Canada. Animals were maintained on standard rodent chow and had free access to food and water. For HFD feeding, mice were initially fed with a standard rodent chow diet (Rodent RQ 22-5, Zelgler Bros Inc., PA) until 6 weeks of age and then switched to either a HFD (TD.02028, Harlan Tekald, Madison, WI) or control diet (TD.05230) for additional 12 weeks. HFD contains 15.5% kcal from protein, 41.9% kcal from carbohydrate, and 42.6% from fat, while the control diet contains 18.7% kcal from protein, 68.6% kcal from carbohydrate, and 12.6% from fat. For the fatty acid, HFD contains 12.5 g/kg cholesterol (100% saturated fatty acid) and the control diet contains 12.8 g/kg soybean oil (16% saturated fatty acid, 23% monounsaturated fatty acid, and 58% polyunsaturated fatty acid). In all HFD feeding experiments, we followed the procedure according to previous study [2].

#### 2.2. Isolation of primary liver cells

Hepatocytes were isolated from 12-week-old male WT and CSE-KO mice as described previously with modification [23]. Briefly, liver organ were perfused through the inferior vena cava with a buffer (140 mM NaCl, 2.6 mM KCl, 0.28 mM Na<sub>2</sub>HPO<sub>4</sub>, 5 mM glucose, and

10 mM HEPES (pH 7.4)). The perfusion was first for 5 min with buffer A (0.5 mM EGTA) and then for 10 min with buffer B (5 mM CaCl<sub>2</sub> and 100 U/ml collagenase type IV) (Worthington, Lakewood, NJ). All solutions were pre-warmed at 37 °C incubator. The isolated hepatocytes were filtered on nylon mesh (100  $\mu$ m pore size), and selected by centrifugation in a 36% Percolliso density gradient. Selected cells were seeded in collagen-coated plates with DMEM containing 10% fetal bovine serum and 5.5 mM glucose.

#### 2.3. PC activity

For the measurement of PC activity, coupled enzyme assay was employed as described previously [24]. Briefly, cells or liver tissues were sonicated in a buffer containing 10 mM HEPES (pH 7.4), 250 mM sucrose, 2.5 mM EDTA, 2 mM cysteine, 0.02% bovine serum albumin, and then centrifuged at 13,000  $\times g$  for 30 min at 4 °C. Collected extract was then added to reaction buffer containing 80 mM Tris/HCl (pH 8.0), 2 mM ATP, 8 mM potassium pyruvate, 21 mM KHCO<sub>3</sub>, 9 mM MgSO<sub>4</sub>, 0.16 mM acetyl CoA, 0.16 mM reduced nicotinamide adenine dinucleotide (NADH), and 5 U/ml malate dehydrogenase. The activity of PC was calculated by the conversion of NADH to NAD<sup>+</sup> with the measurement of the change in absorbance at 340 nm over time at 30 °C. The absorbance at 340 nm was measured in a multicell spectrophotometer (Fisher Scientific, Ottawa, ON) and the PC activity was expressed as nmol/min/mg of total protein. Data was normalized by protein concentration determined by the Bradford method.

PC activity was also measured by using purified PC protein (Sigma, Oakville, ON) as described previously [25]. Briefly, reaction buffer was prepared with combination of 10 ml of substrate solution (135 mM triethanolamine, 7 mM magnesium sulfate, 9 mM Pyruvic acid, and 0.15% bovine serum albumin, pH 8.0), 2.5 ml of malic dehydrogenase enzyme solution (150 units of malic dehydrogenase and 0.3 mM acetyl CoA), and 1.25 ml of beta-NADH solution (2.6 mM beta-nicotinamide adenine dinucleotide, reduced form). For negative control of acetyl CoA, reaction buffer was prepared without acetyl CoA. 0.5 units of purified PC from bovine liver (Sigma) were added in 0.89 ml of the reaction buffer and 50  $\mu$ M of NaHS was then treated in 37 °C for 30 min. After treatment, 1/30 sample volume of ATP/KHCO<sub>3</sub> solution (30 mM adenosine 5'-triphosphate and 450 mM potassium bicarbonate, pH 8.0 in 100 mM triethanolamine buffer) was added. The absorbance at 340 nm was then monitored at 30 °C for 5 min.

#### 2.4. Biotin switch assay of S-sulfhydration

Biotin switch assay was carried-out as described previously with some modifications [7]. Briefly, cells or mouse liver tissues were homogenized in HEN buffer (250 mM HEPES (pH 7.7), 1 mM EDTA, and 0.1 mM neocuproine) supplemented with 100 µM deferoxamine and centrifuged at 13,000  $\times$ g for 30 min at 4 °C. The lysates were added to blocking buffer (HEN buffer adjust to 2.5% SDS and 20 mM methyl methanethiosulfonate (MMTS)) at 50 °C for 20 min with frequent vortexing. The MMTS was then removed by acetone and the proteins were precipitated at -20 °C for 20 min. Proteins were resuspended in HENS buffer (HEN buffer containing 1% SDS) and 4 mM biotin-N-[6-(biotinamido) hexyl]-3'-(2'-pyridyldithio) propinamide (HPDP) in DMSO without ascorbic acid. After incubation for 2 h at 25 °C, biotinylated proteins were purified by streptavidin-agarose beads, which were then washed with HENS buffer. The biotinylated proteins were eluted by SDS-PAGE sample buffer and subjected to Western blotting analysis with anti-PC antibody or anti-His6 antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

#### 2.5. Tag switch assay (maleimide assay) for protein S-sulfhydration

Tag switch assay for PC S-sulfhydration was performed as described previously with modification [8]. Liver tissues and cells were

homogenized in lysis buffer (150 mM NaCl, 0.5% Tween 20, 50 mM Tris, 1 mM EDTA, and 100 µM deferoxamine), and PC was then immunoprecipitated with anti-PC antibody (Santa Cruz Biotechnology, Santa Cruz, CA). To block free thiols and persulfides in PC, immunoprecipitated PC was incubated with 2 µM of maleimide at 4 °C for 2 h. Immunoprecipitated PC was washed with lysis buffer two times. Persulfides were reduced to free thiols with treatment of 1 mM DTT at 4 °C for 1 h. After washing with lysis buffer, reduced free thiols from persulfides will be blocked with Alexa Fluor 350 C5 maleimide (Life Technologies Co., Carlsbad, CA) at 4 °C for 2 h. Immunoprecipitated PC was eluted after washing two times with lysis buffer and final washing with 0.15 M NaCl solution. Fluorescence intensity of Alexa Fluor 350 C5 maleimide which is tagged on S-sulfhydrated PC was measured in a multicell spectrophotometer (Fisher Scientific, Ottawa, ON) in Excitation/Emission of 340/420 nm.

#### 2.6. Western blotting

The cells or mouse liver tissues were harvested and lysed in a cell lysis buffer (Cell signaling, Danvers, MA) including protease inhibitor cocktail (Sigma). The extracts were separated by centrifugation at 14,000 g for 15 min at 4 °C. Equal amount of proteins were boiled in  $1 \times$  SDS sample buffer (62.5 mM Tris–Cl (pH 6.8), 2% SDS, 10% glycerol, 50 mM DTT, and 0.01% bromophenol blue) and run in a 10% SDS-PAGE gel, and then transferred onto pure nitrocellulose blotting membranes (Pall Corporation, Ville St. Laurent, QC). The dilutions of primary antibodies were 1:500 for PC (Santa Cruz Biotechnology), 1:500 for His6 (Santa Cruz biotechnology), 1:1000 for CSE (Proteintech, Chicago, USA), 1:10,000 for  $\beta$ -actin (Sigma), and HRP-conjugated second antibody was diluted as 1:5000 (Sigma). The blots were developed using chemiluminescence (GE Healthcare life sciences, Baie d'Urfe, QC).

#### 2.7. Glucose production in HepG2 and primary hepatocytes

HepG2 cells or primary hepatocytes were planted in 6-well plates at a density of  $1.5 \times 10^5$ /well at 37 °C in a humidified atmosphere (5% CO<sub>2</sub>) for 24 h. Cells were then washed twice with cold phosphate buffered saline (PBS) to remove excess glucose from the media and then incubated for another 3 h in glucose and phenol red-free DMEM containing 2 mM sodium pyruvate and 20 mM sodium lactate (pH 7.4). Medium (300 µl) were collected for glucose measurement using glucose assay kit (Sigma). Glucose concentration was normalized with cellular protein content.

#### 2.8. Site-directed mutagenesis

Human PC cDNA construct was purchased from Origene (Rockville, MD). PC cDNA was cloned into pcDNA3.1/Myc-His tag (Invitrogen). Single mutation at cysteine-265 (hPC-C265) or cysteine-739 (hPC-C739) in PC was conducted using the Quick Change Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) [26]. The oligonucleotides using for mutagenesis were 5'-TGTACGAGCGAGACTcCTCCATCCAGCGGCG-3' (forward) and 5'-GCCGCTGGATGGAGgAGTCTCGCTCGTACAG-3' (reverse) for cysteine-265, and 5'-CGAGCTGGCACCCACATCCTGTcCATCAA GGACAT-3' (forward) and 5'-ATGTCCTTGATGgACAGGATGTGGGTGCC AGCTCG-3' for cysteine-739. The site-directed mutants were confirmed by DNA sequencing at the Paleo-DNA Laboratory of Lakehead University, ON, Canada.

#### 2.9. Immunohistology

Mouse livers were dissected out and fixed in 4% paraformaldehyde for 18 h and then cryoprotected in 30% sucrose/phosphate-buffered saline at 4 °C for 3 days. Samples were embedded in optimal cutting temperature compound (Triangle Biomedical Sciences, Durham, NC). Sections were then cut into 10-µm with Leica CM1850 UV microtomecryostat (Leica Biosystems, Concord, ON) and picked up on poly-Llysine-coated slides. Slides were incubated with 2% BSA at room temperature for 30 min. Primary antibody against CSE was added at 4 °C for overnight. Slides were then washed twice with PBS following incubation with fluorescent secondary antibody (Rabbit anti-mouse IgG, Sigma) for 1 h at room temperature. After 3 times wash with PBS, the stained tissue sections were observed under a fluorescent microscope (Olympus, Richmond Hill, ON).

#### 2.10. Measurement of blood glucose and plasma insulin level

Whole blood glucose concentration was measured in blood obtained from the tail vein of overnight-fasting mice using OneTouch blood glucose strips (LifeScan, Milpitas, CA). Plasma insulin was measured using an enzyme-linked immunosorbent assay (ELISA) kit with mouse insulin as a standard (Mercodia AB, Uppsala, Sweden) according to the manufacturer's procedure. Glucose tolerance tests were performed in overnight-fasting mice on HFD-feeding mice at 12-week. The mice were injected i.p. with glucose (in saline) at 2 g/kg body weight. Blood glucose concentrations were assessed every 30 min for 2 h after injection [27].

#### 2.11. Statistical analysis

Data were presented as means  $\pm$  SEM, representing at least three independent experiments. Student's *t-test* was used to evaluate the difference between two groups. For multiple groups, Two ANOVA was employed followed by post-hoc Tukey test using SigmaPlot 12.0 (Systat Software Inc. San Jose, CA). The significance level was set at p < 0.05.

#### 3. Results

#### 3.1. H<sub>2</sub>S increases PC activity

We first observed that incubation of HepG2 cells with NaHS (a well known H<sub>2</sub>S donor) at as lower as 10 µM significantly increases PC activity by 78.0% (Fig. 1A). The stimulatory role of NaHS on PC activity was time-dependent, as NaHS treatment (50 µM) of HepG2 cells for 30 min, 2 h and 12 h induced PC activity by 35.4%, 69.3%, and 105.6%, respectively (Fig. 1B). We further validated that CSE overexpression in HEK293 cells enhances PC activity in comparison with the control or vehicle transfected-cells (Fig. 1C and D). CSE is a major source of H<sub>2</sub>S production in the liver with cysteine as substrate. Here we found that PC activity in liver tissues from CSE-KO mice is only 58.5% of that from WT mice (Fig. 1E). NaHS (50 µM) incubation with both WT and CSE-KO liver lysates significantly increased PC activity, while cysteine (1 mM) only stimulated PC activity in liver lysates from WT mice but not CSE-KO mice (Fig. 1E and F). We also confirmed that acetyl-CoA is critical for PC function [28]. Acetyl-CoA markedly induced PC activity, which was further enhanced by exogenous applied H<sub>2</sub>S (Supplemental Fig. 1).

#### 3.2. PC mediates H<sub>2</sub>S-stimulated glucose production

To see the functional implication of  $H_2S$ -stimulated PC activity in liver cells, we measured glucose production in HepG2 cells and primary hepatocytes. Incubation of HepG2 cells with 50 µM NaHS induced glucose production by 29.3% compared with the control cells (Fig. 2A). siRNA-mediated PC knockdown completely reversed NaHS-stimulated glucose production (Fig. 2A and B), indicating the involvement of PC in  $H_2S$ -initiated glucose production. PC knockdown also significantly decreased the basal level of glucose production, while PC overexpression increased glucose production in HepG2 cells (Fig. 2A, C, and D). By using primary hepatocytes isolated from both WT and CSE-KO mice, we demonstrated that glucose production is decreased by 55.6% in CSE-KO hepatocytes in comparison with WT hepatocytes (Fig. 2G).



**Fig. 1.**  $H_2S$  stimulates PC activity. (A)  $H_2S$  induced PC activity. HepG2 cells were incubated with NaHS at the indicated concentrations (10–100  $\mu$ M) for 2 h. \*p < 0.05 versus the control. n = 4. (B)  $H_2S$  induced PC activity in a time-dependent manner. HepG2 cells were incubated with NaHS (50  $\mu$ M) for 0.5, 2 and 12 h, respectively. \*p < 0.05 versus the control. n = 4. (C) and (D) CSE overexpression stimulated PC activity in HEK293 cells. HEK293 cells were transfected with CSE cDNA for 24 h to overexpress CSE, the cells were then collected to analyze CSE expression and PC activity, respectively. \*p < 0.05 versus all other groups. n = 4. (E)  $H_2S$  induced PC activity in liver tissues. Liver lysates from both WT and CSE-KO mice were incubated with 50  $\mu$ M NaHS for 30 min. \*p < 0.05 versus WT without NaHS treatment; #p < 0.05 versus CSE-KO without NaHS treatment. (F) Cysteine strengthened PC activity in liver lysates from both WT and CSE-KO mice were incubated with 1 mM cysteine for 30 min. \*p < 0.05 versus WT without cysteine treatment. Eight 12-week-old male WT mice and CSE-KO mice were used for these studies.

#### 3.3. H<sub>2</sub>S S-sulfhydrates PC at cysteine 265

To investigate how H<sub>2</sub>S stimulates PC activity, we studied the effects of H<sub>2</sub>S on PC protein and mRNA expression. Interestingly, treatment of HepG2 cells with different concentration of NaHS (10–100  $\mu$ M) for 0.5–12 h did not affect PC protein and mRNA expression (Fig. 3A–C). CSE deficiency also had no effect on PC protein and mRNA expressions in mouse liver tissues (Fig. 3D and E), suggesting H<sub>2</sub>S may induce PC activity at post-translational level. So next we explored whether H<sub>2</sub>S induces PC activity directly by S-sulfhydrating PC protein with modified biotin switch assay and tag switch assay. Treatment of HepG2 cells with NaHS (10, 50, 100  $\mu$ M) for 2 h significantly increased PC S-sulfhydration compared to the untreated cells, and PC was basically S-sulfhydrated even in the absence of exogenous H<sub>2</sub>S (Fig. 4A and

Supplemental Fig. 4). We further demonstrated that NaHS (50  $\mu$ M) induces PC S-sulfhydration in a time-dependent manner. In comparison with the control cells, the level of S-sulfhydrated PC was increased by 14.1, 38.0, and 76.9% after 30 min, 2 h, and 12 h of NaHS incubation, respectively (Fig. 4B). PC S-sulfhydration was DTT sensitive, and DTT significantly reduced the basal and H<sub>2</sub>S-induced PC S-sulfhydration (Fig. 4C). In line with these data, DTT also markedly attenuated H<sub>2</sub>S-induced PC activity in HepG2 cells (Fig. 4D). The basal level of PC S-sulfhydration was significantly higher in WT liver than that in CSE-KO liver, and the supplement of cysteine further strengthened PC S-sulfhydration in liver lysates from WT mice but not CSE-KO mice (Fig. 4E). In addition, CSE overexpression in HEK293 cells stimulated PC S-sulfhydration by 40.2% compared with the control and vehicle-transfected cells (Fig. 4F).



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**Fig. 2.** PC mediates  $H_2S$ -stimulated glucose production. (A) and (B) knockdown of PC reversed  $H_2S$ -induced glucose production. HepG2 cells were transfected with PC-siRNA or negative siRNA (Neg-siRNA) for 24 h in the presence or absence of NaHS (50  $\mu$ M), and the cells were then collected for analyzing glucose production and PC protein expression. \*p < 0.05 versus the cells transfected with negative siRNA. n = 3. (C) and (D) PC overexpression increased glucose production in HepG2 cells. HepG2 cells were transfected with PC cDNA for 24 h, the cells were then collected for analyzing glucose production and PC protein expression. \*p < 0.05. n = 4. (E) Glucose production was decreased in CSE-KO hepatocytes. Hepatocytes isolated from WT and CSE-KO mice were collected for measuring glucose production. \*p < 0.05. n = 4.

There are total 13 cysteine residues in human PC protein (hPC), and two of them are highly conserved among mammalian and microorganism (Fig. 5A) [29–31]. Through bioinformatic analysis, we found only cysteine 265 in hPC, one of the highly conserved cysteine residues, is located in the surface of PC protein, which forms acid-base motif and is easily targeted by external stimuli (Fig. 5B) [32,33]. To validate whether cysteine 265 is responsible for PC S-sulfhydration, we mutated cysteine 265 to serine in hPC. We also mutated cysteine 739 as an internal control, which is not located in the surface of PC protein and does not form acid-base motif. NaHS significantly induced PC S-sulfhydration in hPC-transfected HEK293 cells, and mutation of cysteine 265 but not cysteine 739 abolished the basal level of PC S-sulfhydration. NaHS further strengthened PC S-sulfhydration in cysteine 739 mutant transfected cells but failed in cysteine 265 mutant transfected cells, pointing to the critical role of cysteine 265 in PC S-sulfhydration (Fig. 5C). Consistently, NaHS induced more PC activity in both hPC and cysteine 739 mutant transfected cells when compared with cysteine 265 mutant transfected cells (Fig. 5D).

By using another slow-releasing  $H_2S$  donor (GYY4137), we confirmed that GYY413 induces more S-sulfhydration of PC protein, stimulates PC activity and glucose production in HepG2 cells (Supplemental Fig. 2) [34]. Although plasma homocysteine level is significantly higher in CSE-KO mice [2,11], homocysteine did not affect PC activity and glucose production in HepG2 cells (Supplemental Fig. 3), suggesting homocysteine is not involved in  $H_2S$ -regulated PC activity.

3.4. Reduced liver CSE expression and PC S-sulfhydration in HFD-feeding mice

We first found that HFD feeding of WT mice for 6 weeks decreases liver PC S-sulfhydration by 37.5% compared with the control dietfeeding WT mice (Fig. 6A). Liver PC S-sulfhydration was hardly detected in control diet-feeding CSE-KO mice, which was not changed by HFD feeding (Fig. 6A). Similar to the change of PC S-sulfhydration, liver PC activity was decreased by 22.1% in HFD-feeding WT mice comparing to control diet-feeding WT mice (Fig. 6B), and PC activity was not altered in CSE-KO liver under either control diet or HFD feeding (Fig. 6B). With immunohistology, we observed that HFD feeding significantly decreases CSE expression in WT mice (Fig. 6C). As expected, CSE expression was undetectable in CSE-KO mice with control diet or HFD feeding (Fig. 6C). We next determined that high glucose (25 mM) treatment of both HepG2 cells and primary hepatocytes reduced CSE expression by 76.9% and 86.0%, respectively (Fig. 6D and E). In parallel of lower CSE expression, PC S-sulfhydration was also significantly reduced by high glucose incubation (Fig. 6D). Differently, compared with the control cells, CSE expression was increased by 42.2% and 49.0% in glucose deprived-HepG2 and primary hepatocytes, respectively (Fig. 6F and



**Fig. 3.**  $H_2S$  has no effect on PC mRNA and protein expression. (A), (B) and (C) PC protein and mRNA expression was not changed by NaHS treatment. HepG2 cells were incubated with different concentrations of NaHS (10–100  $\mu$ M) for 2 h (A) and 50  $\mu$ M of NaHS for the indicated time (0.5–12 h) (B), the cells were then collected and lysed for western blotting analysis of PC protein expression (A and B). (C) HepG2 cells were incubated with different concentration of NaHS (10–100  $\mu$ M) for 2 h, and real-time PCR analysis of PC mRNA expression was then performed. n = 4. (D) and (E) CSE deficiency did not affect PC protein and mRNA expressions in mouse liver tissues. Four WT and four CSE-KO mice were used to prepare liver tissues.

G). We also measured blood glucose and plasma insulin level in both WT and CSE-KO mice with HFD feeding. The results showed that HFD induces higher glucose and insulin level in WT mice in comparison with age-matched CSE-KO mice, and WT mice displayed insulin resistance as evidenced by glucose tolerance test (Supplemental Fig. 5).

#### 4. Discussion

PC is an important enzyme in gluconeogenesis along with fructose-1,6-bisphosphatase, glucose-6-phosphatase, and phosphoenolpyruvate carboxylase [35–37]. PC expression and activity in human is greatest in hepatic cells, where PC catalyzes the first committed step in gluconeogenesis and is well poised to regulate hepatic glucose balance [38]. PC provides the oxaloacetate for both gluconeogenesis and the replenishment of tricarboxylic acid cycle intermediates [39]. Altered liver PC activity has been directly correlated with the pathophysiology of Type 2 diabetes, obesity and other metabolic syndrome [21,40,41]. Given the importance of PC in gluconeogenesis, it is essential to fully understand the fundamental catalytic and regulatory mechanism of PC activity.

In the present study, we demonstrated that H<sub>2</sub>S, a novel gasotransmitter, stimulates PC activity in liver cells, while deficiency of CSE in mouse liver tissues attenuates PC activity. We previously reported that H<sub>2</sub>S plays a critical role in regulating gluconeogenesis in liver [42]. Gluconeogenesis is a metabolic pathway that results in the generation of glucose from non-carbohydrate carbon substrates, which mainly occurs in liver tissue. To investigate whether increased PC activity is involved in H<sub>2</sub>S-induced gluconeogenesis, we used a loss-of-function approach. Consistent with our previous discoveries, exogenously applied H<sub>2</sub>S or CSE overexpression significantly induced gluconeogenesis in liver cells. siRNA-mediated knockdown of PC mRNA blocked H<sub>2</sub>S-strengthened gluconeogenesis, clearly indicating the mediation of PC in H<sub>2</sub>S-induced



**Fig. 4.** H<sub>2</sub>S *S*-sulfhydrates PC protein. (A) H<sub>2</sub>S induced PC *S*-sulfhydration. HepG2 cells were incubated with NaHS at the indicated concentrations (10–100  $\mu$ M) for 2 h, and PC *S*-sulfhydration was then analyzed by biotin switch assay with anti-PC antibody. \*p < 0.05 versus the control. n = 4. (B) H<sub>2</sub>S induced PC *S*-sulfhydration in a time-dependent manner. HepG2 cells were incubated with NaHS (50  $\mu$ M) for 0.5, 2 and 12 h, respectively. \*p < 0.05 versus the control. n = 4. (C) and (D) DTT reversed H<sub>2</sub>S-induced PC *S*-sulfhydration and PC activity. HepG2 cells were incubated with NaHS (100  $\mu$ M) and/or DTT (1 mM) for 30 min, the cells were then collected for analyzing PC *S*-sulfhydration and PC activity, respectively. \*p < 0.05 versus control. n = 4. (E) Cysteine strengthened PC *S*-sulfhydration. Both WT and CSE-KO liver lysates were incubated with L-cysteine (1–10 mM) for 30 min. \*p < 0.05 versus all other groups. n = 4.

glucose generation. Gluconeogenesis has been the target of therapy for diabetes, and inhibition of CSE/H<sub>2</sub>S pathway would decrease PC activity and lower glucose formation [39,42]. Besides with PC, many other enzymes are involved in the process of gluconeogenesis. It has been reported that H<sub>2</sub>S enhances renal and liver gluconeogenesis by activating peroxisome proliferator-activated receptor gamma co-activator 1 alpha, fructose-1,6-bisphosphatase and phosphoenolpyruvate carboxykinase [42,43]. Nevertheless, it is not clear how H<sub>2</sub>S regulates the activities of these gluconeogenesis-related enzymes.

Our next focus is to study how H<sub>2</sub>S affects PC activity. H<sub>2</sub>S-induced activation of PC is not caused by increased PC transcription and translation, because both PC mRNA and protein expressions are not changed by exogenously applied NaHS, CSE overexpression or CSE knockout in liver

cells. H<sub>2</sub>S has been recently demonstrated to post-translational modification of proteins by formation of a persulfide (–SSH) bond to reactive cysteine residues of target proteins, termed as *S*-sulfhydration [7]. Interestedly, we observed that PC is basically *S*-sulfhydrated and PC *S*-sulfhydration was further enhanced by exogenously applied H<sub>2</sub>S. Lack of CSE significantly reduced but overexpression of CSE stimulated PC *S*-sulfhydration. To locate the responsible cysteine residues for H<sub>2</sub>S *S*-sulfhydration of PC, we considered three aspects: the conservativeness of cysteine residues among species, the accessibility by H<sub>2</sub>S, and the potential acid–base motif [32,33,44,45]. The conserved amino acid residue(s) among various species can be often targeted by external stimuli [44,46,47]. Accessibility of H<sub>2</sub>S to the target cysteine residue is critical for forming *S*-sulfhydration [45]. *S*-sulfhydration is also



**Fig. 5.** Cysteine 265 is responsible for H<sub>2</sub>S S-sulfhydration of PC. (A) The domains of human PC and the sites of highly conserved cysteine residues. SA, *Staphylococcus aureus*. (B) The location of cysteine 265 in crystallographic structure of PC. The crystallographic structure of PC was determined in Swiss-PDB viewer 4.0.1 for *Staphylococcus aureus* (PDB-ID: 3BG5). (C) and (D) Mutation of cysteine 265 attenuated H<sub>2</sub>S-induced PC S-sulfhydration and PC activity. HEK-293 cells were transfected with vehicle, hPC, cysteine 265 mutant, or cysteine 739 mutant for 24 h following incubation of NAHS (50  $\mu$ M) for additional 2 h. After that, the cells were collected for biotin switch assay with anti-His6 antibody to differentiate recombinant PC from endogenous PC in HEK293 cells (C) and measuring PC activity (D), respectively. \* p < 0.05 versus the control without NaHS treatment; #p < 0.05 versus the control with NaHS treatment, n = 4.

dependent on the acid dissociation constant (pKa) of cysteine residues. The free thiol group in the cysteine residue located within the acid-base motif can be easily targeted by H<sub>2</sub>S and yield a hydropersulfide moiety, which leads to the formation of protein S-sulfhydration [8,32,33]. In consideration of these factors, we found that cysteine-265 in human PC is highly potential for forming S-sulfhydration. Cysteine-265 in hPC is conserved with cysteine-249 in yeast, the latter has been reported to be the target for nitric oxide to form S-nitrosylation [30]. Cysteine-249 in yeast was also involved in the binding with acetyl-CoA [48]. Acetyl-CoA has also been demonstrated to bind with PC in other amino acid, such as Arginine-469 and Aspartate-471 [28]. In the absence of acetyl-CoA, NaHS hardly induces PC activity, suggesting acetyl-CoA and NaHS work synergistically to strengthen PC function. Further studies need to clarify how H<sub>2</sub>S affects acetyl-CoA binding with PC or how NaHS interacts the same cysteine residue with acetyl-CoA leading to higher PC activity. With mutation study, we validated that cysteine-265 in biotin carboxylase (BC) domain of PC protein is responsible for S-sulfhydration, because mutation of cysteine-265 to serine completely abrogated S-sulfhydration of PC. Cysteine-739 in carboxyl transferase (CT) domain appears not responsible for PC S-sulfhydration.

S-sulfhydration and S-nitrosylation of the same cysteine residues have been studied. S-nitrosylation of GAPDH at cysteine 150 abolished its catalytic activity, but S-sulfhydration of GAPDH at the same cysteine residues augmented its activity [7,49]. S-nitrosylation of p65 at cysteine 38 inhibited NF-kappaB-dependent gene transcription, while Ssulfhydration of the same cysteine 38 decreased cell apoptosis [8,50]. It also has been reported that S-sulfhydration and S-nitrosylation competitively modify the same cysteine residue of eNOS at cysteine 443, resulting in increased NO bioavailability [6]. Probably, the presence of metal ions (Mg<sup>2+</sup> or Ca<sup>2+</sup>), the acid base motif, and orientation of aromatic side chains in the same cysteine residues, etc., affect thiol reactivity to NO and H<sub>2</sub>S, leading to formation of both S-sulfhydration and *S*-nitrosylation. Nevertheless, formation of hydropersulfide is much slower but *S*-nitrosylated cysteines is less stable than *S*-sulfhydrated one [6].

PC consist of four functional domains, the N-terminal BC domain, the CT domain, the allosteric domain and the C-terminal biotin carboxyl carrier protein domain [18]. The regulation of PC activity is extensively reliant on the multifarious interactions of various factors with the functional domains [51]. The crystal structure of PC protein demonstrated the active sites for several critical factors, including MgADP,  $HCO_3^-$ , Mg<sup>2+</sup> and free biotin, are located in BC domain [51]. Acetyl CoA is well known as an allosteric activator of PC, and the main binding site of acetyl CoA is located in the BC domain, where acetyl CoA stabilizes the tetrameric structure of the enzyme, enhances the binding of some substrates, and stimulates the reaction rates [51]. It is predicted that, by change in electrostatic environment, hydrophobicity, contiguity and orientation of aromatic side chains, and proximity of target thiols to transition metals or redox centers, S-sulfhydration can alter protein conformation and the final function and activity of target proteins [52, 53]. In line with acetyl CoA, we deduced that the interaction of  $H_2S$ and PC through cysteine-265 in BC domain may cause a conformational change in PC protein and alter the binding of MgADP, HCO<sub>3</sub><sup>-</sup>, Mg<sup>2+</sup> or free biotin with BC domain, which finally trigger higher pyruvate flux and gluconeogenesis [21,51]. Further kinetic and structural analysis of S-sulfhydrated PC is needed to fully define the roles of the interactions of H<sub>2</sub>S with cysteine-265 and understand the mechanism by which it induces enzyme activation.

Fasting or starvation usually lower endogenous glucose level, in this case, PC activity is induced leading to increased pyruvate flux and higher gluconeogenesis [21]. In our study, we found that glucose deprivation stimulates CSE expression, which would induce H<sub>2</sub>S generation and enhance glucose generation by *S*-sulfhydrating PC activity. In contrast, HFD feeding of mice reduced CSE expression and PC activity, which



**Fig. 6.** Reduced liver CSE expression and PC *S*-sulfhydration in HFD-feeding mice. HFD feeding of WT mice decreased liver PC *S*-sulfhydration and activity. Both WT and CSE-KO mice were feed with HFD for 12 weeks following detection of PC *S*-sulfhydration (A) and activity (B). \*p < 0.05 versus all other groups. n = 4. (C) HFD feeding significantly decreased CSE expression in WT mice. Both WT and CSE-KO mice were feed with HFD for 12 weeks following detection liver CSE expression by immunohistology. Bar: 20 µm. (D) and (E) High glucose inhibited CSE expression and PC *S*-sulfhydration in both HepG2 cells and primary hepatocytes. The cells were incubated with different concentrations of glucose (5.5 and 25 mM) for 24 h following detection of CSE expression in both HepG2 cells and primary hepatocytes. The cells were deprived of glucose for 24 h following detection of CSE expression in both HepG2 cells and primary hepatocytes. The cells were deprived of glucose for 24 h following detection of CSE expression. \*p < 0.05. n = 3.

may inhibits gluconeogenesis but induces glycolysis in liver [54,55]. Similarly, we also proved that high blood glucose decreases CSE expression in the liver cells. In the presence of higher glucose, lower level of H<sub>2</sub>S due to reduced CSE expression would diminish PC *S*-sulfhydration and eliminate PC activity. Lower PC activity will subsequently attenuate the process of gluconeogenesis and decrease glucose production in liver. Following the change of glucose level, CSE expression and H<sub>2</sub>S production can be restored leading to higher PC activity by *S*-sulfhydration. Increased PC activity by *S*-sulfhydration then activates gluconeogenesis. Therefore, CSE/H<sub>2</sub>S pathway would act as a critical factor for regulating gluconeogenesis and maintaining glucose balance in the liver (Fig. 7).

Our previous studies have shown that liver  $H_2S$  production rate is significantly lower in CSE-KO mice than that in WT mice fed with control diet or HFD, and HFD feeding further decreases liver  $H_2S$  production rate in both WT and CSE-KO mice [2]. Liver CBS expression was not changed by HFD in WT mice but was significantly reduced in CSE-KO mice by HFD feeding. In the present study, we further showed that liver 3-MPST protein expression is not affected by HFD in WT mice. The effect of HFD on the expression of H<sub>2</sub>S-generating enzymes has also been reported by several other studies. Similar to our study, Peh et al. showed that HFD feeding of mice leads to reduced H<sub>2</sub>S biosynthesis due to the lower expression of CSE and 3-MST, although liver CBS expression was higher in HFD-feeding mice [54]. Geng et al. also showed HFD induces lower CSE expression and H<sub>2</sub>S production in adipose tissues [56]. In contrast, Hwang et al. demonstrated that the mRNA and protein levels of CBS and CSE in the liver are significantly higher in mice fed a HFD, which then cause a significant elevation in H<sub>2</sub>S production in the liver [57]. The discrepancy of these studies may be due to the different types of mouse genetic background, fat contents, feeding period, and mouse age, etc. Clinical studies on human samples need to be tested in the near future.



Fig. 7. The proposed signaling pathway underlying  $H_2S$  regulation of PC activity by S-sulfhydration in liver gluconeogenesis.

Taken together, our results indicated that CSE/H<sub>2</sub>S pathway plays an important role in the regulation of glucose production through *S*sulfhydrating PC in liver. Tissue-specific regulation of CSE/H<sub>2</sub>S pathway might be a promising therapeutic target of diabetes and other metabolic syndromes.

#### **Conflict of interest**

The authors declare no conflict of interest.

#### **Transparency document**

The Transparency document associated with this article can be found, in the online version.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx. doi.org/10.1016/j.bbagen.2015.08.003.

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