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Original contribution

H_2S-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_2S 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_2S-induced gluconeogenesis. H_2S had no effect on the expressions of PC mRNA and protein, while H_2S *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_2S enhances glucose production through *S*-sulfhydrating PC. Mutation of cysteine 265 in human PC diminished H_2S-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_2S pathway might be a promising therapeutic target of diabetes and other metabolic syndromes.

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 cystathionine beta-synthase, cystathionine gamma-lyase (CSE) and 3-mercaptopyruvate 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_2S production [1,7,11,12]. Deficiency of CSE gene diminished H_2S production by more than 90% in mouse liver, suggesting that CSE acts as a major H_2S-generating enzyme in the liver [2,7]. Altered hepatic H_2S 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_2S, hydrogen sulfide; HFD, high fat diet; MMTS, methyl methanethiosulfonate; KO, knockout; NADH, reduced nicotinamide adenine dinucleotide; NAD^+, 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_2S 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_2S 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_2S induces PC activity directly by *S*-sulfhydrating PC protein at cysteine 265, and increased PC activity contributes to H_2S-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_2S 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™ 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_2HPO_4, 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 $CaCl_2$ 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 μm 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 ×*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 $KHCO_3$, 9 mM $MgSO_4$, 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^+ 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 μM of NaHS was then treated in 37 °C for 30 min. After treatment, 1/30 sample volume of ATP/$KHCO_3$ 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 ×*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