ORIGINAL RESEARCH COMMUNICATION

Decreased Gluconeogenesis in the Absence of Cystathionine Gamma-Lyase and the Underlying Mechanisms

Ashley A. Untereiner,^{1–3} Rui Wang,^{1,4} YoungJun Ju,^{1,5} and Lingyun Wu^{1–3}

Abstract

Aims: To investigate the regulation of hepatic glucose production by cystathionine γ -lyase (CSE)-generated hydrogen sulfide (H_2S) in hepatic glucose production under physiological conditions. **Results:** We found that CSE knockout (KO) mice had a reduced rate of gluconeogenesis, which was reversed by administration of NaHS (an H_2S donor) (i.p.). Interestingly, isolated CSE KO hepatocytes exhibited a reduced glycemic response to chemical-induced activation of the cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) and glucocorticoid pathways compared with wild-type (WT) hepatocytes. Treatment with the inhibitors for PKA (KT5720) or glucocorticoid receptor (GR) (RU-486) significantly reduced H_2S -stimulated glucose production from both WT and CSE KO mouse hepatocytes. NaHS treatment upregulated the protein levels of key gluconeogenic transcription factors, such as peroxisome proliferator-activated receptor- γ coactivator-1 α (PGC-1 α) and CCAAT-enhancer-binding protein- β (C/EBP- β). Moreover, exogenous H_2S augmented the S-sulfhydration of the rate-limiting gluconeogenic enzymes and PGC-1 α and increased their activities, which were lower in untreated CSE KO hepatocytes. Finally, knockdown of PGC-1 α , but not C/EBP- β , significantly decreased NaHS-induced glucose production from the primary hepatocytes. **Innovation:** This study demonstrates the stimulatory effect of endogenous H_2S on liver glucose production and reveals three underlying mechanisms; that is, H_2S upregulates the expression levels of PGC-1 α and phosphoenolpyruvate carboxykinase *via* the GR pathway; H_2S upregulates the expression level of PGC-1 α through the activation of the cAMP/PKA pathway as well as PGC-1 α activity *via* S-sulfhydration; and H_2S upregulates the expression and the activities (by S-sulfhydration) of glucose-6-phosphatase and fructose-1,6-bisphosphatase. **Conclusion:** This study may offer clues for the homeostatic regulation of glucose metabolism under physiological conditions and its dysregulation in metabolic syndrome. *Antioxid. Redox Signal.* 24, 129–140.

Introduction

GLUCOSE IS THE MOST EFFICIENT and abundant energy substrate for mammalian cells. This monosaccharide fuels numerous physiological functions, including cell proliferation and growth, protein and nucleic acid synthesis, and fatty acid and cholesterol synthesis. Glucose is converted to adenosine triphosphate, the energy currency of the cell, *via* oxidative phosphorylation mostly and anaerobic glycolysis to

a lesser degree. Thus, glucose homeostasis is critical for maintaining physiological function of our body, whereby its sustained disturbance may lead to metabolic syndrome.

Fasting induces the release of glucagon and glucocorticoid from pancreatic α -cells and the adrenal cortex, respectively, into the bloodstream through which these endocrine hormones act on the liver. The liver is the major glucose-producing organ in which the breakdown of glycogen produces glucose (glycogenolysis) and the *de novo* synthesis of

¹The Cardiovascular and Metabolic Research Unit, Lakehead University, Ontario, Canada.

²Department of Health Sciences, Lakehead University, Ontario, Canada.

³Thunder Bay Regional Research Institute, Ontario, Canada.

⁴Department of Biology, Laurentian University, Ontario, Canada.

⁵Department of Kinesiology, Lakehead University, Ontario, Canada.

Innovation

Disturbed H₂S metabolism plays important roles in the pathogenesis of insulin resistance and diabetes. However, the role of endogenous H₂S availability in gluconeogenesis has been unknown. This article reports a novel discovery that cystathionine γ -lyase-generated endogenous H₂S stimulates hepatic glucose production, which is critical in regulating glucose metabolism. We demonstrate three underlying mechanisms by which H₂S stimulates liver glucose production: (i) H₂S upregulates PGC-1 α and phosphoenolpyruvate carboxykinase expression levels *via* the glucocorticoid receptor pathway. (ii) H₂S upregulates the PGC-1 α expression level through the cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) pathway as well as PGC-1 α activity *via* S-sulfhydration. (iii) H₂S upregulates the expression and the activities of glucose-6-phosphatase and fructose-1,6-bisphosphatase.

glucose (gluconeogenesis) occurs. Both glycogenolysis and gluconeogenesis are activated by glucagon signaling *via* the increasing intracellular cAMP level (an important second messenger), eventually leading to glycogen breakdown and the transcription of gluconeogenic genes. Glucocorticoid, on the other hand, initiates only the gluconeogenic process *via* binding to its cytosolic receptor to activate the transcription of gluconeogenic genes. The glucagon and glucocorticoid signaling pathways synergistically work together to enhance hepatic glucose production.

Among many endogenous substances that regulate glucose production is hydrogen sulfide (H₂S) (27, 35, 40). H₂S is a gasotransmitter with the ability to freely diffuse through cell membranes and elicit physiological responses (17, 31, 36, 41). In the liver, H₂S production is largely catalyzed by cystathionine γ -lyase (CSE) and, to a lesser degree, by cystathionine β -synthase (11). Recently, we showed that liver cells isolated from mice lacking the *cse* gene (CSE KO) have a significantly lower basal glucose level compared with their wild-type (WT) counterparts (40). In subsequent studies, we discovered that NaHS (an H₂S donor) upregulated the expression of peroxisome proliferator-activated receptor- γ coactivator-1 α (PGC-1 α) in rat vascular smooth muscle cells, A-10 cells (27). PGC-1 α is a major transcription factor involved in an array of energy-consuming metabolism pathways, such as cholesterol synthesis, fatty acid oxidation, mitochondria biogenesis, and gluconeogenesis (6). As an important gluconeogenic regulator, PGC-1 α upregulates the expression of gluconeogenic rate-limiting enzymes, such as phosphoenolpyruvate carboxykinase (PEPCK), fructose-1,6-bisphosphatase (FBPase), and glucose-6-phosphatase (G6Pase) (6).

CCAAT-enhancer-binding protein- β (C/EBP- β), another important transcription factor, also regulates glucose homeostasis through the induction of PEPCK and G6Pase (1, 15). Interestingly, CSE (8, 39), PGC-1 α (10, 14, 38), and C/EBP- β (1, 2) are significantly upregulated in livers of streptozotocin-induced diabetic rats (2, 8, 39) and mice (1, 38), *db/db* (10, 14) and *ob/ob* mice (38), as well as in liver-specific insulin receptor knockout (KO) mice (38). These altered gene expression profiles suggest a linkage between the CSE/H₂S system and PGC-1 α and C/EBP- β in the context of gluconeogenesis regulation in the liver. We hypothesized

that endogenous H₂S is an important regulator for liver glucose production. How H₂S enhanced gluconeogenesis and the roles of PGC-1 α and C/EBP- β in H₂S-mediated glucose production were investigated in the present study.

Results

Impaired gluconeogenic system in CSE KO mice

The pyruvate tolerance test (PTT) is an estimation of the rate of gluconeogenesis, reflected by the time the liver takes to generate glucose from pyruvate. The injection of pyruvate to overnight-fasted mice elicited a rapid glycemic response, where the glucose level peaked 20 min after the injection and gradually declined over 2 h (Fig. 1A). The amplitude of glycemic response was significantly lower in CSE KO mice than in WT mice (Fig. 1A). To determine if exogenous H₂S can restore gluconeogenesis in CSE KO mice, the KO mice were given i.p. injections of NaHS or saline 10 min before the pyruvate injection. NaHS pretreatment significantly increased the basal plasma glucose level in CSE KO mice before pyruvate stimulation (Fig. 1B). The pyruvate-stimulated gluconeogenesis in CSE KO mice was rescued by NaHS pretreatment at 39 μ mol/kg and restored to the same level as WT mice (Fig. 1B). NaHS pretreatment at a higher concentration of 63 μ mol/kg resulted in significantly greater pyruvate-stimulated glycemic response than other treatments (Fig. 1B).

To investigate specifically the stimulatory effect of H₂S on glucose production in the liver, we isolated primary liver cells from overnight-fasted WT and CSE KO mice. As expected, the endogenous H₂S level in cultured CSE KO liver cells was about 39% of that in WT liver cells (Fig. 2A). To observe hepatocytes' response to chemical-induced activation of the cAMP/PKA and glucocorticoid pathways, we treated the cultured liver cells with 8-Br-cAMP and dexamethasone (Dex), which are synthetic analogs for cAMP (38) and glucocorticoid (20), respectively. Treatment with 8-Br-cAMP/Dex for 3 and 6 h increased glucose production in WT cells by 87% and 246%, respectively (Fig. 2B). Interestingly, 8-Br-cAMP/Dex treatment for 3 and 6 h elicited a higher percentile increase in glucose production response in CSE KO cells by 215% and 329%, respectively, compared with untreated CSE KO liver cells (Fig. 2B). However, the actual glucose levels in CSE KO cells were 54% and 40% of those in WT cells with 3 and 6 h 8-Br-cAMP/Dex treatment, respectively.

The changes in the levels of gluconeogenic proteins followed the same trend as the glucose production in 8-Br-cAMP/Dex-treated WT cells. For instance, the protein levels of PGC-1 α , PEPCK, FBPase, G6Pase, and C/EBP- β (also known as LAP) were significantly increased in WT liver cells after 3 or 6 h treatment with 8-Br-cAMP/Dex (Fig. 2C–H). A similar increase in the protein level of forkhead box protein O1 [FOXO1; a PGC-1 α coactivator (6)] was also observed in WT hepatocytes with 8-Br-cAMP/Dex treatment (Supplementary Fig. S1A, B; Supplementary Data are available online at www.liebertpub.com/ars). Interestingly, the basal levels of these proteins were significantly higher in CSE KO liver cells than those of WT liver cells (Fig. 2C–E, G, H, and Supplementary Fig. S1), with the exception of FBPase (Fig. 2C, F). CSE KO liver cells exhibited a moderate increase in PGC-1 α expression level by 34% and PEPCK by 48% after being treated with 8-Br-cAMP/Dex for 6 h, respectively (Fig.

FIG. 1. Gluconeogenesis in WT and CSE KO mice. The rate of gluconeogenesis was determined *via* the pyruvate tolerance test on overnight-fasted mice. (A) Mice were injected i.p. with 2 g/kg pyruvate at time 0. $n = 7-11$ for each group. (B) Mice were injected with NaHS or saline 10 min before pyruvate injection. Pyruvate injection was given at time 0. $n = 5-9$ for each group. Blood glucose was measured with a One-Touch Glucometer. * $p < 0.05$ versus respective WT group with or without saline injection; # $p < 0.05$ versus respective CSE KO (39 $\mu\text{mol/kg}$ NaHS) group; † $p < 0.05$ versus respective CSE KO (39 $\mu\text{mol/kg}$ NaHS) group. CSE KO, cystathionine γ -lyase knockout; WT, wild-type.

2D, E). Moreover, the CSE expression level significantly decreased in WT hepatocytes with 8-Br-cAMP/Dex treatment (Fig. 2C, I). This downregulation suggests hormonal control of CSE protein expression by glucagon-stimulated cAMP signaling or/and by glucocorticoid signaling.

It has been reported that under starvation conditions, PGC-1 α and PEPCK are significantly upregulated in mouse liver (15, 38). The *C/EBP- β* gene expression level was also shown to increase in the fasted mouse liver (15) or in the liver of rats that received injections of a chemical analog of cAMP (24). How these key gluconeogenic proteins are regulated by H₂S

under different nutritional states was unknown. In this study, we found a significant increase in PGC-1 α , PEPCK, and *C/EBP- β* expression levels in nonfasted CSE KO livers compared with WT livers (Fig. 3A–D). In agreement with Figure 2I, CSE expression was decreased in fasted WT livers in comparison with the level in nonfasted WT livers (Fig. 3A, E). Furthermore, plasma levels of glucagon and corticosterone (a major glucocorticoid) were significantly elevated in nonfasted and fasted CSE KO mice compared with the respective WT groups (Fig. 3F, G).

NaHS enhances glucose production and protein level and induces S-sulfhydration of major gluconeogenic factors

Based on our previous study (40), it came as no surprise when we found that NaHS treatment significantly increased glucose production in primary cultured WT and CSE KO liver cells (Fig. 4A). To dig further into this mechanism, we discovered that 30 μM NaHS increased the enzymatic activities of total FBPAse and G6Pase in isolated hepatocytes (Fig. 4B, C). Since H₂S-induced S-sulfhydration was shown to enhance enzymatic activity (22), we determined it necessary to measure the sulfhydrated levels of these gluconeogenic proteins. In fact, all three gluconeogenic enzymes, PEPCK, FBPAse, and G6Pase, showed elevated levels of their sulfhydrated forms with NaHS treatment in both WT and CSE KO hepatocytes (Fig. 4D–I).

Bucci *et al.* (3) demonstrated the ability of H₂S to enhance endogenous cAMP in vascular smooth muscle cells. Indeed, we found that this similar phenomenon also occurred in primary hepatocytes treated with NaHS (Fig. 5A). Like its effect on the major gluconeogenic enzymes (Fig. 4D–I), exogenous H₂S also enhanced the S-sulfhydration level of PGC-1 α by approximately onefold in both WT and CSE KO liver cells in comparison with their respective control groups (Fig. 5B, C). Furthermore, NaHS treatment increased the protein expression levels of PGC-1 α , PEPCK, FBPAse, G6Pase, and *C/EBP- β* in WT and CSE KO hepatocytes (Fig. 5D–I). For example, NaHS at 10 and 30 μM increased the levels of PGC-1 α by 35% and 69% in WT cells, respectively (Fig. 5D, E). Contrary to 30 μM NaHS treatment, 5 mM L-cysteine (a major precursor for H₂S biosynthesis) failed to induce glucose production and alter PGC-1 α , FBPAse, and G6Pase protein expression levels in isolated CSE KO hepatocytes (Supplementary Fig. S2).

H₂S induces glucose production through the cAMP/PKA and glucocorticoid pathways

To determine the signaling pathways for H₂S-induced glucose production, we used 5 μM KT5720 to inhibit PKA and 5 μM RU-486 to inhibit glucocorticoid receptor (GR) in primary cultured WT and CSE KO liver cells. Pretreatment with KT5720 or RU-486 for 1 h did not affect cell viability as tested with trypan blue assay (data not shown), but the inhibitors significantly attenuated the gluconeogenic effect of 8-Br-cAMP/Dex (Fig. 6A, B). Pretreatment of WT and CSE KO liver cells with KT5720 or RU-486 also significantly suppressed NaHS-induced glucose production (Fig. 6C, D). The gluconeogenic effects of 8-Br-cAMP/Dex and NaHS on WT and CSE KO liver cells were completely abolished by the cotreatment with both KT5720 and RU-486 (Fig. 6).

FIG. 2. Reduced glycemic response in CSE KO liver cells treated with cAMP/Dex. Primary liver cells were isolated from overnight-fasted 8–12-week-old WT and CSE KO mice. (A) Endogenous H_2S levels in WT and CSE KO liver cells were determined *via in situ* methylene blue assay. $n=6-7$ for each group. (B) Liver cells were treated with 1 mM 8-Br-cAMP and 1 μ M Dex (dexamethasone) for 3 or 6 h. Thereafter, the glucose level in these cells was measured with a glucose assay kit. $n=5-8$ for each group. (C) Representative Western blot results of the changed expression levels of selective proteins in WT and CSE KO liver cells with 8-Br-cAMP/Dex treatment. (D–I) Summary of the changed expression levels of selective proteins in WT and CSE KO liver cells with 8-Br-cAMP/Dex treatment. The densities of selective proteins were normalized to that of β -actin and expressed as a percentage of untreated WT cells. PGC-1 α : $n=6-9$ for each group; PEPCK: $n=7-12$ for each group; FBPase: $n=6-8$ for each group; G6Pase: $n=7-9$ for each group; C/EBP- β : $n=7-8$ for each group; CSE: $n=5-6$ for each group. * $p<0.05$ versus corresponding CT group; # $p<0.05$ versus corresponding WT group; † $p<0.05$. C/EBP- β , CCAAT-enhancer-binding protein- β ; cAMP, cyclic adenosine monophosphate; CT, control; FBPase, fructose-1,6-bisphosphatase; G6Pase, glucose-6-phosphatase; PEPCK, phosphoenolpyruvate carboxykinase; PGC-1 α , peroxisome proliferator-activated receptor- γ coactivator-1 α .

H₂S-induced glucose production is dependent on PGC-1 α in WT and CSE KO primary liver cells

To determine whether H_2S -stimulated hepatic glucose production was mediated by PGC-1 α and/or C/EBP- β , we transfected WT and CSE KO primary liver cells with siPGC-1 α , siC/EBP- β , or both siPGC-1 α and siC/EBP- β for 60 h. These maneuvers significantly knocked down the expression of the respective proteins (Fig. 7A, B). Transfection with siC/EBP- β had no effect on NaHS-induced glucose production in both WT and CSE KO primary liver cells (Fig. 7C). However, glucose production induced by NaHS at 30 μ M was completely abolished by siPGC-1 α transfection in both WT

and CSE KO liver cells (Fig. 7C). In agreement, cotransfection of both PGC-1 α and C/EBP- β also abolished NaHS-induced glucose production in WT and CSE KO liver cells (Fig. 7C).

Discussion

We previously showed that exogenous H_2S increased glucose production in cloned liver carcinoma HepG₂ cells (40). In the present study, we focused on the crucial role of endogenous H_2S in hepatic glucose production under physiological conditions as well as under fasting conditions. CSE KO mice, with diminished hepatic H_2S production, displayed

FIG. 3. Downregulation of CSE in fasted WT livers along with upregulation of gluconeogenic factors in livers and plasma in nonfasted CSE KO mice. Liver extracts were harvested from nonfasted or 24-h fasted WT and CSE KO mice. (A) Representative Western blot results of the changed expression levels of selective proteins in liver tissues from nonfasted and fasted WT and CSE KO mice. (B–E) Summary of the changed expression levels of selective proteins in liver tissues from nonfasted and fasted WT and CSE KO mice. Plasma glucagon (F) and corticosterone (G) levels were analyzed from mice after a 16-h fast. Glucagon: $n=7$ for each group; corticosterone: $n=6$ for each group; PGC-1 α : $n=6-8$ for each group; PEPCCK: $n=6-7$ for each group; C/EBP- β : $n=9$ for each group; CSE: $n=8$ for each group. * $p<0.05$ versus nonfasted WT group; # $p<0.05$ versus nonfasted CSE KO group; † $p<0.05$.

significantly reduced glucose production after pyruvate supplementation. Cultured primary liver cells isolated from CSE KO mice produced significantly lower glucose and exhibited a reduced glycemic response to chemical-induced activation of the cAMP/PKA and glucocorticoid pathways compared with WT liver cells. Endogenous H₂S was also responsible for increased enzymatic activity and sulhydrylation levels of major gluconeogenic factors. Thus, we demonstrated that endogenous H₂S stimulates gluconeogenesis in mouse liver, leading to increased net glucose production. Our results also reveal the critical roles of PKA and GR in H₂S-induced glucose production and that of PGC-1 α in H₂S-induced gluconeogenesis. One very intriguing discovery from our study is the differential responses of endogenous H₂S on the gluconeogenic proteins. In comparison with WT liver cells, we observed higher levels of PGC-1 α , PEPCCK, G6Pase, and C/EBP- β in untreated CSE KO liver cells (Figs. 2 and 5), thus indicating that endogenous H₂S inhibited the gene expression of PGC-1 α , PEPCCK, and G6Pase (Figs. 2 and 5). On the other hand, NaHS treatment upregulated the expression of these gluconeogenic proteins (Fig. 5). In the case of FBPase, both endogenous and exogenous H₂S increased its expression (Figs. 2 and 5). It seems that endogenous and exogenous H₂S plays differential roles in the regulation of this protein.

The stimulatory effect of endogenous H₂S on gluconeogenesis was demonstrated in the present study with the PTT (Fig. 1). PTT measures the net glucose level, a balance between glucose production and its utilization. The systemic utilization of hepatic glucose produced through pyruvate

metabolism is dependent on glucose-stimulated insulin secretion and insulin sensitivity of peripheral tissues. A high endogenous H₂S level in the pancreas of Zucker diabetic rats inhibits insulin synthesis and release (34). By the same token, a low endogenous H₂S level in CSE KO mice may lead to increased insulin release (35). In fact, we previously showed that CSE KO mice have an improved glucose tolerance compared with the WT control (35). That unique metabolic feature of the CSE KO mice was likely due to a higher level of glucose-stimulated insulin release (35), considering that H₂S inhibits insulin release (*via* activating K_{ATP} channels) from pancreatic β -cells (37). Therefore, these previously published results support the notion that more insulin is released in response to glucose stimulation in CSE KO mice, leading to a faster clearance of glucose from the bloodstream. Our previous study also showed no difference in the insulin tolerance test results between CSE KO and WT mice (35), suggesting that endogenous H₂S has no effect on peripheral insulin sensitivity. On the contrary, other studies have reported that NaHS supplementation inhibits insulin-stimulated glucose uptake into both fat (5) and liver (40) cells, implying that exogenous H₂S reduces insulin sensitivity. The discrepancy between Yang *et al.* (35) and the aforementioned studies (5, 40) was due to the use of different preparations, that is, whole animal *in vivo* study (35) versus isolated rat adipocytes (5) and cultured HepG₂ cells (40). It may also reflect the difference in the effects of endogenous H₂S (35) and exogenously applied H₂S donors (5, 40). On another note, our present study shows that H₂S enhances the rate of gluconeogenesis through PGC-1 α signaling and by

FIG. 4. Exogenous H₂S enhanced glucose production and gluconeogenic enzyme activities, as well as S-sulphydrated PEPCK, FBPaase, and G6Paase in primary hepatocytes. Primary liver cells isolated from overnight-fasted WT and CSE KO mice were treated with 30 μ M NaHS for 6 h. (A) Glucose levels in liver cells. $n=6-10$ for each group. (B, C) The enzymatic activities of total FBPaase and G6Paase, respectively, in whole cell lysates. For FBPaase, $n=8$ for each group. For G6Paase, $n=10-12$ for each group. (D-F) Representative Western blot results of the SSH of selective proteins in WT and CSE KO liver cells. (G-I) Summary of changed SSH expression levels of selective proteins in primary hepatocytes. The densities of selective SSH proteins were normalized to total selected protein and expressed as a fold change to that of untreated WT cells. PEPCK: $n=10$ for each group; FBPaase: $n=7-8$ for each group; G6Paase: $n=6-7$ for each group. * $p<0.05$ versus corresponding CT group; # $p<0.05$ versus corresponding WT group. SSH, S-sulphydration.

directly increasing the activities of FBPaase and G6Paase. Taking these current and previously published results into consideration, the decreased glycemic response to pyruvate stimulation in CSE KO mice can be explained by the lack of endogenous H₂S so that two effects of H₂S, that is, the up-regulation of gluconeogenesis in the liver and inhibition of insulin secretion from pancreatic β -cells, cannot be realized. Future studies to compare the glucose levels, with pyruvate added to and insulin removed from the culture medium and vice versa, in cultured WT and CSE KO liver cells will help differentiate H₂S-induced gluconeogenesis from H₂S-inhibited glucose utilization.

To study the liver-specific effect of H₂S on glucose production, we isolated and cultured primary hepatocytes from CSE KO and WT mice. The mice were starved overnight to deplete hepatic glycogen (40) before liver cell isolation. Overnight starvation of rodents suppresses the ability of isolated primary liver cells to synthesize and accumulate glycogen (28). To further prevent the replenishment of glycogen stores, primary liver cells were cultured in glucose (5.5 mM) and insulin- and fetal bovine serum-free medium (28, 29). Under these conditions, we found that CSE KO liver cells produced significantly lesser glucose than WT liver cells with the activation of the cAMP/PKA and glucocorticoid pathways by 8-Br-cAMP/Dex treatment. The expression levels of PGC-1 α , PEPCK, FBPaase, G6Paase, and C/EBP- β proteins were

increased in both WT and CSE KO liver cells with the 8-Br-cAMP/Dex treatment; however, with the exception of FBPaase, no significant difference was observed in these protein levels after 3 h of treatment in CSE KO liver cells. Thus, this diverse pattern of protein upregulation along with lower glucose levels suggests an impaired gluconeogenic system in the CSE KO liver cells. Moreover, the inability of supplemented L-cysteine to increase gluconeogenesis in CSE KO liver cells (Supplementary Fig. S2A-E) supports the notion that CSE is the dominative H₂S-producing enzyme in the liver (11).

During starvation, gluconeogenesis is switched on, meaning that PGC-1 α , PEPCK, FBPaase, and G6Paase are upregulated to increase hepatic glucose output. Interestingly, CSE protein was downregulated upon starvation (Fig. 3) and also in 8-Br-cAMP/Dex-treated WT hepatocytes (Fig. 2), suggesting that these gluconeogenic factors were upregulated in the presence of low endogenous H₂S. We assume that this fasting-induced downregulation of CSE is metabolically advantageous for two reasons: (i) It will prevent a massive outburst of hepatic glucose production into the circulation. H₂S is a potent stimulator of hepatic glucose production *via* activating the cAMP/PKA and glucocorticoid pathways (Fig. 6) as well as increasing the activities of the rate-limiting gluconeogenic enzymes, FBPaase, and G6Paase (Fig. 4). Intermittent bursts of high amount of plasma glucose could lead

FIG. 5. Exogenous H₂S upregulated cAMP levels, induced PGC-1 α SSH, and increased protein levels of key gluconeogenic factors in isolated WT and CSE KO hepatocytes. Primary liver cells isolated from fasted WT and CSE KO mice were treated with 10 or 30 μ M NaHS for 6 h. (A) Endogenous cAMP levels in whole cell lysates. $n = 8-12$ for each group. (B) Representative Western blot result of SSH-PGC-1 α in whole cell lysates. (C) Summary of the changed SSH expression level of PGC-1 α in primary hepatocytes. The density of SSH-PGC-1 α was normalized to total PGC-1 α protein and expressed as a fold change to that of untreated WT cells. $n = 5-7$ for each group. (D) Representative Western blot results of the changed expression levels of selective proteins. (E-I) Summary of the changed expression levels of selective proteins. The densities of selective proteins were normalized to that of β -actin and expressed as a percentage of untreated WT cells. PGC-1 α : $n = 6-9$ for each group; PEPCK: $n = 8-12$ for each group; FBPase: $n = 6-8$ for each group; G6Pase: $n = 7-9$ for each group; C/EBP- β : $n = 7-9$ for each group. * $p < 0.05$ versus corresponding CT group; # $p < 0.05$ versus corresponding WT group.

to elevated levels of reactive oxygen species (ROS) and advanced glycation end products (AGEs) in peripheral tissues (4). The deleterious effects of elevated ROS and AGEs levels have been implicated in the development of hypertension (32) and insulin resistance (4). Additionally, the pancreas would have to respond to the high plasma glucose level by producing more insulin. This would put tremendous strain on the pancreas, which may eventually lead to pancreatic β -cell failure, as seen in the late stages of type 2 diabetes (4). (ii) The hepatocytes would be more sensitive to insulin signaling under fasting conditions, where the downregulation of CSE expression would enable an immediate “turned-off” of gluconeogenesis.

We recently showed that exogenous H₂S blocked insulin-induced phosphorylation of Akt at Ser⁴⁷³ in HepG₂ cells, which abolished the uptake and utilization of glucose (40). Coincident or not, upregulation of CSE has been reported in livers of streptozotocin-induced diabetic rats (8, 39) and in insulin-resistant HepG₂ cells (40). Thus, it is metabolically beneficial to downregulate the CSE/H₂S system in hepatocytes during fasting to preserve glucose homeostasis and possibly prevent the development of metabolic diseases.

We recently reported that hepatic lipolysis was reduced in CSE KO mice (17, 18). Liver tissues from fasted CSE KO mice had lower mRNA levels of *peroxisome proliferator-*

FIG. 6. The roles of cAMP/PKA and GR in NaHS-induced glucose production in WT and CSE KO liver cells. Primary cultured liver cells were pretreated for 1 h with 5 μ M KT 5720 (a PKA inhibitor) or 5 μ M RU-486 (a GR inhibitor) before a 6-h treatment with 8-Br-cAMP/Dex (1 mM/1 μ M) or NaHS (30 μ M). The roles of cAMP/PKA and GR in 8-Br-cAMP/Dex-induced glucose production in WT (A) ($n=4-8$ for each group) and CSE KO (B) ($n=7-9$ for each group) hepatocytes. The roles of cAMP/PKA and GR in NaHS-induced glucose production in WT (C) ($n=6-10$ for each group) and CSE KO (D) ($n=6-10$ for each group) hepatocytes. * $p<0.05$ versus untreated cells; # $p<0.05$ versus cAMP/Dex-treated group; † $p<0.05$ versus 30 μ M NaHS-treated group. GR, glucocorticoid receptor; PKA, protein kinase A.

FIG. 7. NaHS-induced glucose production is dependent on PGC-1 α in primary liver cells from WT and CSE KO mice. Primary liver cells isolated from 8–12-week-old WT and CSE KO mice were transfected with siRNA-A (control), siPGC-1 α , siC/EBP- β , or both siPGC-1 α and siC/EBP- β for 60 h. Lipofectamine RNAiMAX was used as the transfection reagent. (A) Representative Western blot results of the selected proteins in WT and CSE KO liver cells. (B) Summary of the changes in the selected proteins in WT and CSE KO liver cells with different treatments. The densities of the selected proteins were normalized to that of β -actin and expressed as a percentage of the corresponding siRNA-A transfected WT or CSE KO liver cells. PGC-1 α : $n=4-6$ for each group; C/EBP- β : $n=4-6$ for each group. (C) Glucose production in transfected liver cells. NaHS at 30 μ M was used to treat the cells for 6 h and glucose production was measured via a glucose assay kit. $n=7-14$ for each group. * $p<0.05$ versus respective siRNA-A group; # $p<0.05$ versus respective 30 μ M NaHS-treated group; † $p<0.05$ versus WT siRNA-A group. Both siRNAs: siPGC-1 α and siC/EBP- β .

activated receptor (*PPAR*) α and *PPAR* γ , two critical regulators of lipid metabolism (17). Fibroblast growth factor 21 (FGF21), another important mediator of hepatic lipid metabolism, induced by fasting, is upregulated by *PPAR* α (33). With lower *PPAR* α mRNA expression level, FGF21 expression may also be decreased in fasted CSE KO livers. In fact, CSE KO mice fed with a high-fat diet (HFD) for 12 weeks developed severe dyslipidemia, hepatic injury (*i.e.*, higher aspartate aminotransferase, alanine aminotransferase, and alkaline phosphatase levels), and fatty liver in comparison with the HFD-fed WT mice (17). This observation suggests the defect in the clearance of excess dietary fat from CSE KO mouse liver. Clearly, not only is the gluconeogenic system impaired but lipid metabolism as well is impaired in these CSE KO mice. The impact of the impaired lipolysis system on the impaired hepatic gluconeogenic system in CSE KO mice needs to be further clarified.

PGC-1 α and C/EBP- β transcriptions are stimulated by cAMP/PKA in response to fasting-induced glucagon release (10, 23). After its activation by cAMP, PKA phosphorylates the cAMP response element-binding protein (CREB). The latter enters the nucleus and binds to the consensus cAMP response element (CRE) site on the PGC-1 α promoter (10) or to the promoter region of the *c/ebp- β* gene (23), thus increasing their transcriptions. It is possible that exogenous H₂S increases PGC-1 α and C/EBP- β expression through the inhibition of AMP-activated protein kinase (40) and/or through the inhibition of phosphodiesterase (PDE) (3). Intriguingly, H₂S increased not only the PGC-1 α protein level but also its activity *via* S-sulphydration (Fig. 5). Mustafa *et al.* (2010) (22) describes protein sulphydration as a physiological post-transcriptional modification of cysteine residues in the target protein that leads to enhanced protein function. Sirtuin 1 activates PGC-1 α *via* deacetylation (25); however, no deacetylation of PGC-1 α was observed in primary hepatocytes treated with NaHS (data not shown). Thus, we propose a novel mechanism by which PGC-1 α activity is also regulated by S-sulphydration in primary hepatocytes, leading to an enhanced gluconeogenic system.

PGC-1 α is crucial for NaHS-induced glucose production because siPGC-1 α transfection significantly abolished NaHS-stimulated glucose output from both WT and CSE KO primary liver cells (Fig. 7). The present study also demonstrates that both cAMP/PKA and glucocorticoid pathways are involved in NaHS-induced glucose production (Fig. 6C, D). Preincubation with either PKA or GR inhibitor significantly reduced, and the cotreatment with both inhibitors completely abolished, NaHS-stimulated glucose production in both WT and CSE KO liver cells (Fig. 6C, D). PGC-1 α can be activated by cAMP in primary rat (19, 38) and mouse hepatocytes (19) and synergistically by both cAMP and Dex (19, 38). NaHS may increase the endogenous cAMP level (Fig. 5A) through the inhibition of PDE (3), leading to CREB activation and thus *pgc-1 α* transcription (10). C/EBP- β was shown to directly induce *pgc-1 α* transcription *via* binding to its specific promoter region in both hamster kidney cells (30) and in mouse brown fat cells (12). C/EBP- β can be induced by the activation of the cAMP/PKA pathway as well as the GR pathway (1, 20). Therefore, when the cAMP/PKA pathway was inhibited, NaHS could still stimulate PGC-1 α expression *via* GR pathway-mediated C/EBP- β induction. Furthermore, C/EBP- β can directly upregulate *PEPCK* and *G6Pase* gene

expression levels (1, 15). In fact, GR is capable of directly inducing *pepck* transcription (1), thus increasing glucose production. Clearly, the cAMP/PKA and GR pathways work as an interconnected network to regulate PGC-1 α expression. Maximal induction of NaHS-stimulated glucose production requires functional cAMP/PKA and GR pathways, whereby PGC-1 α is the center piece of this signaling web. Therefore, we propose that H₂S may increase gluconeogenesis through three main mechanisms: (i) H₂S upregulates the expression levels of PGC-1 α and PEPCK *via* the GR pathway. (ii) H₂S upregulates the expression level of PGC-1 α through the activation of the cAMP/PKA pathway as well as PGC-1 α activity *via* S-sulphydration. (iii) H₂S upregulates the expression and the activities (by S-sulphydration) of G6Pase and FBPase (Fig. 8).

A balanced and functionally intact CSE/H₂S system is important for the homeostasis of adaptive energy metabolism. A pathophysiologically overactivated CSE/H₂S system in the liver could predispose an individual to metabolic syndrome because H₂S blocks insulin release from pancreatic β -cells (26, 34) and inhibits insulin-stimulated glucose uptake into both fat (5) and liver (40) cells. In this regard, it has been reported that both CSE (8, 39) and PGC-1 α (10, 14, 38) are robustly upregulated in the liver under diabetic conditions. Correspondingly, it was demonstrated that the CSE protein is upregulated in insulin-resistant HepG₂ cells where DL-propargylglycine treatment (a potent inhibitor of CSE activity) improved glucose consumption in these cells (40). In agreement, we previously reported that streptozotocin-induced diabetic CSE KO mice exhibited a delayed onset to diabetic status (hyperglycemic, hypoinsulinemia, and insulin

FIG. 8. Schematic representation of H₂S-induced glucose production and the proposed underlying mechanisms. Endogenous H₂S stimulates liver glucose production *via* three underlying mechanisms: (1) the GR-mediated upregulation of PGC-1 α and PEPCK; (2) the cAMP/PKA-mediated upregulation of PGC-1 α as well as PGC-1 α activity *via* SSH; and (3) the increased expression and activities (*via* SSH) of FBPase and G6Pase. Solid arrows represent either stimulation or the direction of the metabolic reactions. Dotted arrows represent degradation reactions. DHAP, dihydroxyacetone phosphate; F-1,6-P, fructose-1,6-bisphosphate; F-6-P, fructose-6-phosphate; G-6-P, glucose-6-phosphate; G6Pase, glucose-6-phosphatase; GA3P, glyceraldehyde 3-phosphate; H₂S, hydrogen sulfide; P-CREB, phosphorylated cAMP response element-binding protein; PDE, phosphodiesterase; PEP, phosphoenolpyruvate.

resistance) (35). Overall, these findings stress the importance of targeting an overactivated CSE/H₂S system in the insulin resistance liver to prevent or attenuate the progress of metabolic syndrome.

The present study identifies key targets of the CSE/H₂S system in liver gluconeogenesis, deepening insight on hepatic glucose metabolism under physiological conditions and shedding light on the potential impact of endogenous H₂S on the dysregulated gluconeogenesis in metabolic syndrome.

Materials and Methods

Animal and tissue preparation

CSE KO mice were generated and homebred as previously described (36). Eight to twelve-week-old male CSE KO mice and age-matched male WT littermates were used. 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. Mice were maintained on standard rodent chow with free access to food and water. Liver tissues were isolated in ice-cold phosphate-buffered saline (PBS), cleaned, and immediately snap-frozen in liquid nitrogen and stored at -80°C until processing.

Hepatocyte preparation

Before cell isolation, mice (8–12 weeks) were starved for 16 h where glycogen content of hepatocytes was virtually depleted as shown in our previous study (40). Hepatocytes were isolated from CSE KO and WT mice as described elsewhere (40).

Pyruvate tolerance test

Mice were starved overnight and injected i.p. with 2 g/kg pyruvate. The animals received NaHS or saline (i.p.) 10 min before the pyruvate injection. The blood glucose level was measured using blood glucose strips (One-Touch Glucometer; Johnson, local pharmacy).

Hepatocyte glucose production assay

Primary hepatocytes isolated from WT or CSE KO mice were plated onto 12-well tissue culture plates at a density of 5×10^4 cells per well and maintained in low-glucose (5.5 mM) serum- and insulin-free Dulbecco's modified Eagle's medium (DMEM) (containing 1.25 mM pyruvic acid). This maintenance medium prevents the replenishment of glycogen stores in the isolated hepatocytes (28, 29). Cells were treated with either NaHS (10 or 30 μM) for 6 h or with 8-Br-cAMP/Dex (1 mM/1 μM) for 3 or 6 h. For the PKA and glucocorticoid inhibitor studies, cells were pretreated for 1 h with 5 μM KT5720 or 5 μM RU-486. The medium was aspirated and cells were washed with PBS before a 6-h treatment with NaHS (30 μM) or 8-Br-cAMP/Dex (1 mM/1 μM). After treatment, the medium was removed and cells were washed with PBS and incubated for 3 h in glucose-free DMEM. Thereafter, the medium was collected and glucose concentration was determined using a glucose assay kit II (Biovision, Mountain View, CA). The effects of KT5720 and RU-486 on cell viability were assessed by the trypan blue assay (16).

Endogenous H₂S measurement

The endogenous H₂S level was determined by the zinc-agar trap method as described elsewhere (13).

Gene silencing with siRNA

Primary liver cells were transfected with 70 nM siRNA-A (control), siPGC-1 α , siC/EBP- β , or both siPGC-1 α and siC/EBP- β for 60 h. The siRNA complexes were diluted in Opti-MEM media (Invitrogen, Burlington, ON, Canada), and Lipofectamine[®] RNAi/MAX Reagent (Invitrogen) was used as the transfection reagent.

Intracellular cAMP levels

Endogenous cAMP levels in primary mouse hepatocytes were determined by the Cyclic AMP Select EIA Kit (Cayman Chemicals, Burlington, ON, Canada).

Plasma hormone analyses

Blood samples for glucagon measurement were collected in heparin-coated tubes (to prevent interference with the EIA assay) supplemented with aprotinin (20 mg/L) to prevent proteolysis (21). Bloods samples for corticosterone were collected in EDTA-coated tubes. Plasma was collected by centrifuging whole blood at 3000 g for 10 min at 4°C . Plasma glucagon was measured *via* the Glucagon (human/mouse/rat) EIA kit (Biovision) and corticosterone by Corticosterone ELISA (Abcam, Toronto, ON, Canada). Analyses were performed according to the respective manufacturer's protocols.

Immunoblot analysis

Western blotting was performed as described before (7). The primary antibody dilutions were 1:1000 for anti-PGC-1 α , anti-C/EBP- β (Novus biologicals, Oakville, ON, Canada), anti-PEPCK, and anti-CSE (Abnova, Walnut, CA) antibodies; 1:100 for anti-FBPase, anti-G6Pase, and anti-FOXO1 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA); and 1:10,000 for anti- β -actin antibody (Santa Cruz Biotechnology).

Sulfhydration assay

The assay was conducted as described elsewhere (22) with modifications. Briefly, primary liver cells were homogenized in HEN buffer (250 mM Hepes [pH 7.7], 1 mM EDTA, and 0.1 mM neocuproine) supplemented with 100 μM deferoxamine, 1:100 protease inhibitors, and 1% NP-40 and centrifuged at 14,000 rpm for 20 min at 4°C . Blocking buffer (HEN buffer adjusted to 2.5% SDS and 20 mM MMTS) was added to the samples, which were then incubated at 50°C for 20 min with frequent shaking. After the addition of acetone, proteins were precipitated at -20°C for 20 min. Next, acetone was removed and proteins were re-suspended in HENS buffer (HEN buffer adjusted to 1% SDS) with the addition of biotin-HPDP. After incubation at 25°C for 3 h, the biotinylated proteins were precipitated by streptavidin agarose beads and washed 5 $\times$ with HENS buffer. The biotinylated proteins were eluted by 5 $\times$ loading buffer and samples were subjected to Western blot analysis.

Gluconeogenic enzyme assays

FBPase enzyme activity was assayed as described elsewhere (27). G6Pase activity was assayed as described

elsewhere (9) with modifications. Briefly, the assay was conducted at 37°C for 15 min in 100 mM cacodylate buffer (pH 6.5). Hydrolytic activity was initiated with 100 μ l of 100 mM glucose-6-phosphate mixed with 100 μ l cell lysates. After 15 min at 37°C, 1.8 ml of 10% trichloroacetic acid was added and samples were centrifuged at 4000 g for 10 min. Afterward, 1.0 ml of supernatant was added to 5.0 ml of 0.25% ammonium molybdate and 1.0 ml 0.2% 1-amino-2-naphthol-4-sulfonic acid (EMD Millipore Chemicals, Chemicals, Billerica, MA). The amount of liberated phosphate was measured at 700 nm via a Multiskan Spectrum. Ultrapure water (Cayman Chemical) was used to make up the solutions. G6Pase activity was calculated by subtracting the inorganic phosphorus content of cell lysates incubated in the absence of glucose-6-phosphate from that of a complete system incubated in the presence of the substrate. A blank containing all reagents except cell lysates revealed no free inorganic phosphorus.

Chemicals and statistical analysis

All chemicals and enzymes used in this study were obtained from Sigma-Aldrich (Oakville, ON, Canada) unless otherwise stated. siRNA products were obtained from Santa Cruz Biotechnology and antibodies were purchased from Abcam unless otherwise specified. All data sets are presented as mean $\pm$ SEM. For primary liver cell experiments, *n* value designates the number of mice used in the experiments. Results were analyzed using Student's *t*-test or one-way ANOVA, followed by a *post hoc* analysis (Tukey's test) when applicable. Statistical significance was considered at *p* < 0.05.

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Author Disclosure Statement

No competing financial interests exist.

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Address correspondence to:

Dr. Lingyun Wu

Department of Health Sciences

Lakehead University

955 Oliver Road

Thunder Bay, ON, P7B 5E1

Canada

E-mail: lwu@lakeheadu.ca

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Abbreviations Used

AGEs = advanced glycation end products
 cAMP = cyclic adenosine monophosphate
 C/EBP-β = CCAAT-enhancer-binding protein-β
 CRE = cAMP response element
 CREB = cAMP response element-binding protein
 CSE = cystathionine γ-lyase
 DMEM = Dulbecco's modified Eagle's medium
 FBPase = fructose-1,6-bisphosphatase
 FGF21 = fibroblast growth factor 21
 FOXO1 = forkhead box protein O1
 G6Pase = glucose-6-phosphatase
 GR = glucocorticoid receptor
 H₂S = hydrogen sulfide
 HFD = high-fat diet
 NaHS = sodium hydrosulfide
 PBS = phosphate-buffered saline
 PEPCK = phosphoenolpyruvate carboxykinase
 PDE = phosphodiesterase
 PGC-1α = peroxisome proliferator-activated receptor-γ coactivator-1α
 PKA = protein kinase A
 PPAR = peroxisome proliferator-activated receptor
 PTT = pyruvate tolerance test
 ROS = reactive oxygen species
 WT = wild-type
 KO = knockout