The Roles of H2S on Hepatic Acetyl-CoA and Lipid Metabolism

by

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Abstract

Hydrogen sulfide (H_2S) is a novel gasotransmitter that is endogenously produced in the liver by cystathionine γ-lyase (CSE). The CSE/H_2S system dysfunction has been linked to various liver diseases such as fatty liver diseases. Hepatic acetyl-CoA is a key intermediate from the metabolism of glucose, amino acid, and lipid in most species, but the roles of H_2S in the regulation of hepatic acetyl-CoA and lipid metabolism have not been explored. Here, we found that incubation of human liver carcinoma (HepG2) cells with a mixture of free fatty acids (FFAs) or high glucose reduced CSE expression and H_2S production and promoted intracellular accumulation of acetyl-CoA and lipid. Supply of exogenous NaHS (an H_2S donor) or cysteine (an H_2S precursor) reduced acetyl-CoA content and lipid accumulation, while blockage of CSE activity by DL-propargylglycine promoted intracellular lipid accumulation. Furthermore, H_2S blocked FFAs-induced transcription of *de novo* lipogenesis, inflammation, and fibrosis-related genes. *In vivo*, knockout of CSE gene stimulated more hepatic acetyl-CoA and lipid accumulation in mice induced by high-fat choline-deficient diet. The expression of lipogenesis, inflammation, and fibrosis-related genes were significantly higher in liver tissue from CSE knockout mice when compared with wild-type mice. In conclusion, the CSE/H_2S system is indispensable for maintaining the homeostasis of acetyl-CoA and lipid accumulation and protecting from the development of inflammation and fibrosis in liver under excessive caloric ingestion, and CSE/H_2S system constitutes an interesting target for the prevention and treatment of fatty liver disease.

Keywords: H_2S, cystathionine gamma-lyase, acetyl-CoA, lipid metabolism, high-fat-choline deficiency diet.

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List of abbreviation

3MST:	3-mercaptopyruvate sulfurtransferase
ACC:	Acetyl-CoA carboxylase
AceCS1:	Cytoplasmic acetyl-CoA synthetase 1
ACOX1:	Acyl-CoA oxidase 1
ACSL1:	Long chain acyl-CoA synthetase 1
ALT:	Alanine aminotransferase
ApoB:	Apolipoprotein B
AST:	Aspartate aminotransferase
ATP-CL:	ATP-citrate lyase
CBS:	Cystathionine beta-synthase
CD:	High-fat choline-deficient
COL1A1:	Collagen type I
CpT1A:	Carnitine palmitoyltransferase I
CSE:	Cystathionine gamma-lyase
FAS:	Fatty acid synthase

FFAs:	Free fatty acids
H&E:	Hematoxylin and eosin
HG:	High glucose
IL6:	Interleukin 6
KO:	Knockout
MTT:	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NAFLD:	Non-alcoholic fatty liver disease
PPARα:	Peroxisome proliferator-activated receptor alpha
PPG:	DL-propargylglycine
SMAα:	Smooth muscle actin α
SREBP1:	Sterol regulatory element-binding protein 1
TNFα:	Tumor necrosis factor alpha
VLDL:	Very low-density lipoprotein
WT:	Wild type

1. Introduction

1.1. Gasotransmitters

1.1.1. Gasotransmitters and their criteria

Nitric oxide (NO), carbon monoxide (CO), and hydrogen sulfide (H_2S) are three gases regulating physiological functions at very low concentrations in our body, while they are very toxic in moderate to high levels (Coburn., 2012; Furne et al., 2008; Levitt et al., 2011; Neishi et al., 2005). In terms of their physiological features, NO, CO and, H_2S are collectively known as "gasotransmitters". The same criteria are shared by gasotransmitters except their activities are modulated by different mechanisms as following:

a) NO, CO and H_2S are a little molecule of gases (molecular mass range: 28.01-34.1 $g \cdot mol^{-1}$) that can diffuse freely across cell membranes in mammalian cells (Figueroa et al., 2013; Li et al., 2008; Mathai et al., 2009; Xu et al., 2018).
b) In mammals, including humans, NO, CO and H_2S are endogenously produced via specific enzymes, modulating many signaling pathways (Knowles et al., 1994; Tenhunen et al., 1968; Tenhunen et al., 1969; Yang et al., 2018).
c) NO, and CO generating enzymes are found to be expressed in many organs of human cells like the brain (Blum-Degen et al., 1999; Takahashi et al., 1996), liver (Lavrovsky et al., 2000; Taylor et al., 1999), kidney (Han et al., 2006; Morimoto et al., 2001), vascular endothelial cells (Abraham et al., 1987; Heller et al., 1999; Palmer et al., 1988), etc. Additionally, H_2S found to be endogenously produced in human and mouse liver, spleen,

brain, kidney, lung, adipose tissue, and vascular endothelial cells (Ahmed et al., 2016; Sturman et al., 1999; Van den Born et al., 2016; Yang et al., 2018).

d) NO can modify the protein`s activity through reacting with the thiol group (RSH) and forms S-nitrosothiol (RSNO), this process is called S-nitrosylation (Haldar et al., 2013). CO, on the other hand, interacts with cysteine, lysine, histidine, and arginine residues in target proteins, exerting its biological activity via a process called protein carbonylation (Cattaruzza et al., 2008; Wang et al., 2013; Wong et al., 2008). Although, H_2S in mammalians can directly interact with cysteine residues (RSH) or glutathione to form persulfide (RSSH), in a process called protein S-sulfhydration, increasing the activity of the modified protein (Mustafa et al., 2009; Sen et al., 2012).

e) NO, CO, and H_2S functions can be mimicked by exogenous donors in mammalian cells, such as sodium nitroprusside (NO-donor) (Peralta et al., 2001), CORM (CO-donor) (Takagi et al., 2011), and NaHS (H_2S-donor) (Zhen et al., 2015).

f) Many molecular targets are shared by NO, CO, and H_2S, but their activities are modulated through different mechanisms. In human endothelium cells, NO modulate cellular functions through targeting soluble guanylate cyclase (sGC) in the heme group and increases cGMP production, modulating vasorelaxation effect (Heller et al., 1999). Although, CO has a weak stimulatory property and lowers affinity for sGC compared with NO from a bovine model of mammalian lung. However, CO stimulates sGC when the tissue levels of NO is low (Friebe et al., 1998; Martin et al., 2006; Stone et al., 1994). Contrarily, H_2S does not directly interact with sGC, but suppresses phosphodiesterase enzyme, reducing cGMP degradation, in mouse brain endothelial cells and male Wistar rat aortas (Bucci et al., 2010; Coletta et al., 2012).

1.1.2. **The discovery history of gasotransmitters**

Gasotransmitters were chemical compounds traditionally regarded as industrial air pollutants until the discovery of NO as a physiological molecule in 1987 (Ignarro et al., 1987a and 1987b). Then, Barianga et al. discovered that the microsomal heme oxygenase converts the heme protein into bilirubin and CO (Barianga., 1993). After the discovery of NO and CO as the first two gasotransmitters, the physiological function of H_2S has been recently recognized and H_2S is classified as the third gasotransmitter. In 1996, Abe and Kimura discovered the physiological effect of H_2S as a neuromodulator and they declared that H_2S is produced endogenously from *L*-cysteine in mammalian tissues (Abe et al., 1996). In 2002 Wang discovered the vaso-relaxant effect of H_2S and defined the term "gasotransmitters" (Wang., 2002). Then, in 2008 Yang et al. found that H_2S is essential for vascular functions and maintains body blood pressure (Yang et al., 2008). Since 2009 and till now, thousands of articles were published on the gasotransmitter's role of H_2S in both health and diseases (**Fig. 1**).

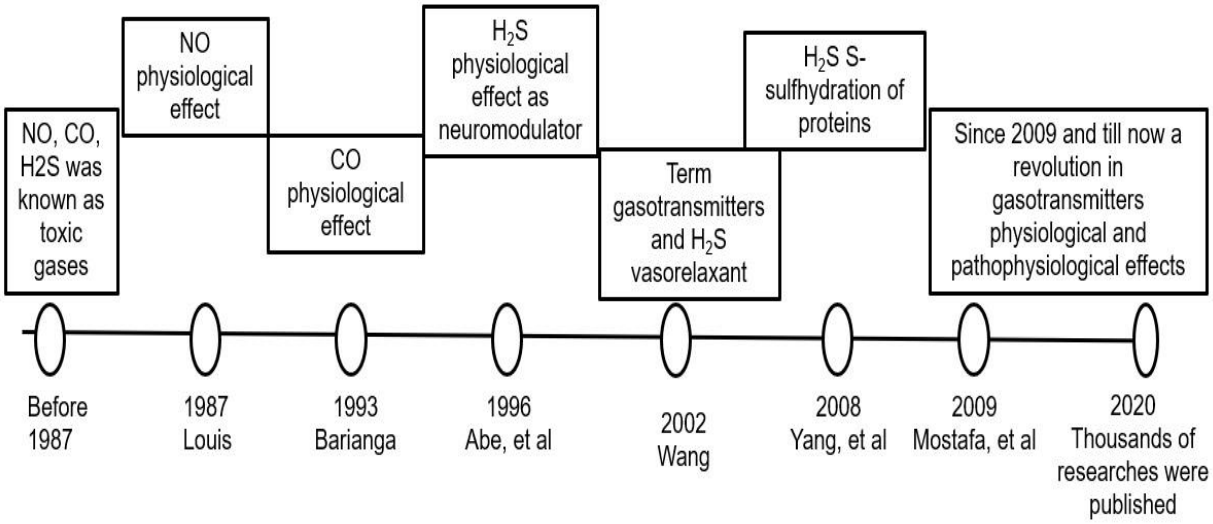


Fig. 1. **The discovery history of gasotransmitters.**

1.2. H_2S in mammalian physiology

1.2.1. Endogenous production of H_2S

Hydrogen sulfide (H_2S) is traditionally known as a toxic environmental hazard with the smell of rotten eggs. The endogenous H_2S production in mammalian cells was widely considered to be nothing more than metabolic waste until the discovery of H_2S as an essential signaling molecule in 1996. Three enzymes, including cystathionine beta-synthase (CBS) (Braunstein et al., 1971; Jhee et al., 2005), cystathionine gamma-lyase (CSE) (Levonen et al., 2000), and 3-mercaptopyruvate sulfurtransferase (3MST) in conjugation with cysteine aminotransferase enzyme (CAT) (Kuo et al., 1983; Spallarossa et al., 2004), have been shown to participate in endogenous H_2S production in various organs (Enokido et al., 2005; Robert et al., 2003; Shibuya et al., 2009a and 2009b; Yang et al., 2018) (**Fig. 2**).

CBS is a homotetramer that localizes in the human chromosome 21 and the mouse chromosome 17, contains 63-kDa subunits, each subunit includes 551 amino acid (Münke et al., 1988; Skovby et al., 1984). CBS was found to catalyze the synthesis of pyruvate, NH_3, and H_2S with pyridoxal-phosphate (Vitamin B6) as cofactor in mammalian cells (Jhee et al., 2005; Kery et al., 1994).

In 1969, Braunstein and colleagues isolated CBS from chicken liver for the first time. They found that CBS catalyzes the conversion of homocysteine and serine to cystathionine and water (Braunstein et al. 1969). In 1996, Abe et al. found that CBS produces H_2S endogenously from *L*-cysteine in the brain of mammalian tissues. Besides, they observed that CBS is highly expressed in the cerebellum and hippocampus compared to the brain stem and cerebral cortex (Abe et al., 1996). Later, CBS was affirmed to be expressed mainly in astrocytes cells of the mouse brain (Enokido et al., 2005). In the lack of CBS, mammalian cells will not be able to metabolize homocysteine via the transsulfuration pathway, leading to systemic toxicity. As a consequence of lack of CBS, Jhee et al. determined that CBS deficiency in the human brain increases homocysteine toxicity and decreases the synthesis of cysteine (Jhee et al., 2005).

CSE was first discovered by Carroll and colleagues in rat livers, catalyzing the cleavage of *L*-cystathionine to *L*-cysteine, α-ketobutyrate, and ammonia (Carroll et al., 1949). Human CSE is located on chromosome 1 at position 31.1 (Levonen et al., 2000). Similar to CBS, CSE is a vitamin B6-dependent enzyme in the synthesis of H_2S from *L*-cysteine (Messerschmidt et al., 2003). From rat livers, CSE was purified and crystallized in 1958 (Matsuo et al., 1958). Then, CSE was found to be expressed in Saccharomyces Cerevisiae (Delavier-Klutchko et al., 1965), Aspergillus niduhns (Paszewski et al., 1973), Sacchuromycopsts lipolyticus (Morzycka et al., 1979), and Streptomyces phaeochromogenes (Nagasawa et al., 1984). In 1999, Clausen et al.

found that CSE is expressed in Nicotiana tabacum plant, and then Pong and others have reported that CSE is expressed in amphibian and mammalian cells (Clausen et al., 1999; Pong et al., 2007). As such, Steegborn et al. demonstrated that CSE catalyzes the cleavage of *L*-cysteine, producing H_2S and NH_3 gases, and pyruvate in human HepG2 cells (Steegborn et al., 1999).

CSE is highly expressed in mammalian liver, kidney, and blood vessels (Ishii et al., 2004; Yang et al., 2006; Yang et al., 2018). Moreover, CSE protein is found to be localized in the cytosol of rat liver and kidney cells (Ogasawara et al. 1994). CSE deficiency in humans can cause cystathioninuria, an excessive amount of cystathionine in urine (Vargas et al., 1999), while multi-mutation of CSE gene in the human may lead to cystathioninemia, increasing the risk of bladder cancer and atherosclerosis (Renga., 2011; Wang J et al., 2003).

In 1956, Chatagner and colleagues reported that CAT enzyme with the cofactor vitamin B6, producing H_2S in rat livers (Chatagner et al., 1956). Following that, Kuo et al. have demonstrated that the CAT enzyme with Vitamin B6, α-ketobutyrate, and 3-MST enzymes generates H_2S (Kuo et al., 1983). Then later, the first crystal structure of the 3-MST protein was identified from E. Coli bacteria (Spallarossa et al., 2004). Recent studies found that 3MST, along with CAT, generate H_2S in mouse neurons (Shibuya et al., 2009a), vascular endothelium (Shibuya et al., 2009b), and retina (Mikami et al., 2011). In the mouse brain, 3-MST is localized in cerebellar Purkinje cells, mitral cells, and hippocampal pyramidal neurons (Shibuya et al., 2009a). Moreover, Nagahara et al. determined that 3-MST is located in rat proximal tubular, hepatocytes, neurons, and cardiomyocytes (Nagahara et al., 1998).

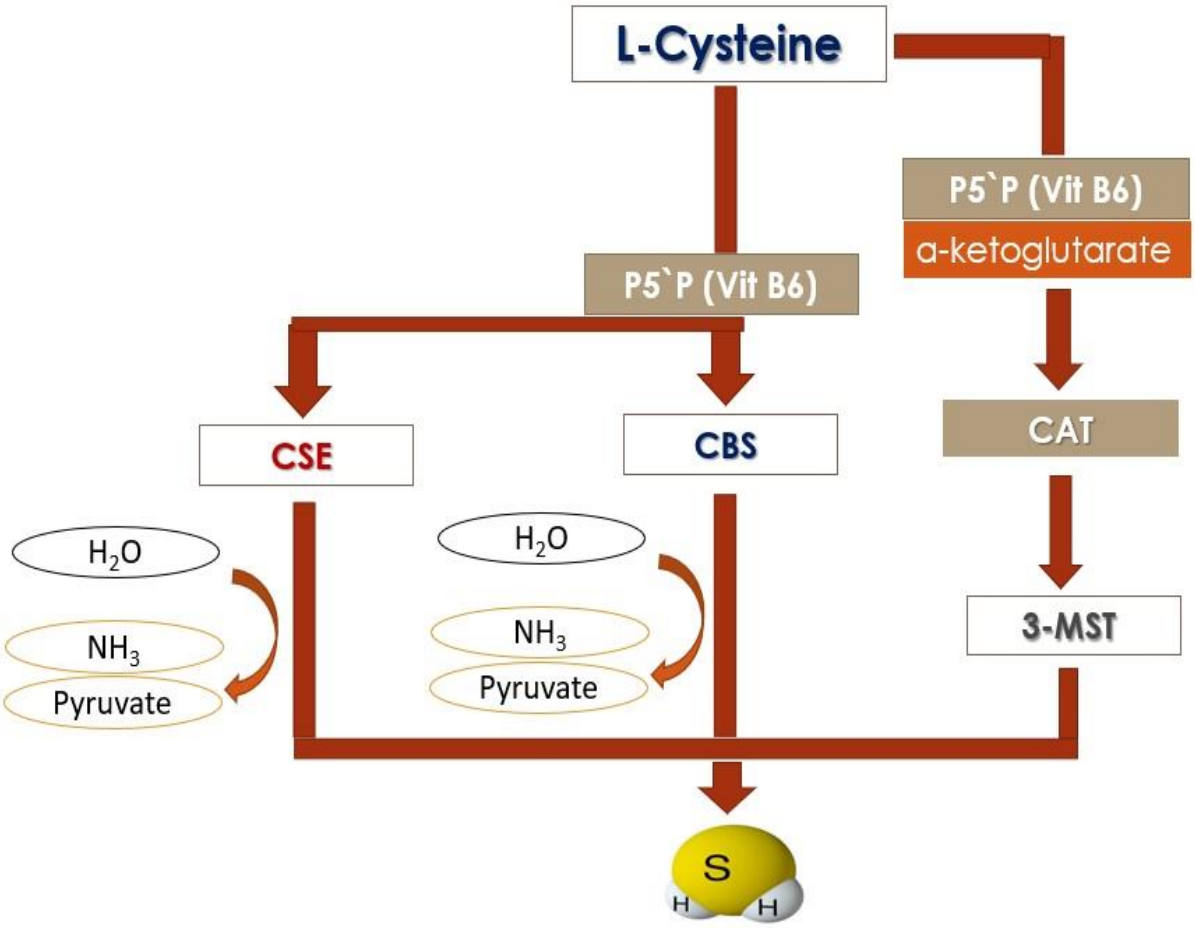


Fig. 2. **Overview of endogenous H_2S production in mammalian tissues.**

The mouse liver has a higher capacity for endogenous H_2S generation than any other organ (Mani et al., 2015; Mustafa et al., 2009). Although all the three enzymes are expressed in liver, CSE has been demonstrated to be the major H_2S-generating enzyme in the liver due to the fact that knockout of CSE in mice abolishes more than 90% of H_2S production (Fiorucci et al., 2006; Sen et al., 2012).

CBS and CSE link the generation of *L*-cysteine from homocysteine and *L*-serine for H_2S production through the reverse-transsulfuration pathway (Carter et al., 2016). The process of

transformation of *L*-cysteine via the intermediate *L*-cystathionine into methionine is called the transsulfuration pathway, but the opposite process termed reverse-transsulfuration. Transsulfuration mainly occurs in bacteria, fungi, and plants (Ferla et al., 2014; Hesse et al., 2003; Saint-Macary et al., 2015), while reverse transsulfuration transpires in mammalian cells (Finkelstein et al., 1984; Mudd et al., 1965). In the reverse transsulfuration pathway, the CBS enzyme is responsible for catalyzing homocysteine and *L*-serine to generate cystathionine, while CSE is accountable for cleaving cystathionine to produce *L*-cysteine, α-ketobutyrate, and ammonia (Flavin et al., 1967; Steegborn et al., 1999) (**Fig. 3**).

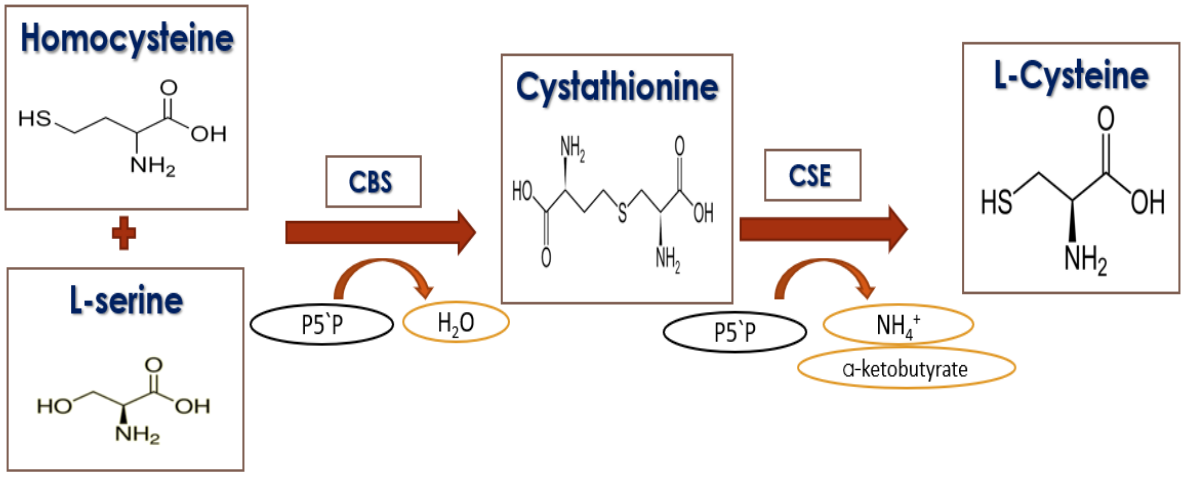


Fig. 3. ***L*-cysteine biosynthesis through the reverse-transsulfuration pathway.**

1.2.2. **H_2S S-sulfhydration regulation of proteins**

The process of post-translational modification of cysteine residues in target proteins by H_2S is termed S-sulfhydration. H_2S can modify proteins through direct or indirect interaction with the thiol group (-SH) of target proteins and forms a hydropersulfide group (-SSH), changing

protein activity (Mustafa et al., 2009). In the last decades, many studies have confirmed the broad physiological importance of protein S-sulfhydration in mammalian cells (**Fig. 4**). In mouse endothelial cells, H_2S activates Poly-(ADP ribose)-polymerase-1 (PARP1) by *S*-sulfhydration of mitogen-activated protein kinase-K (MAPK-K), leading to increased DNA damage repair inside the cells. In rat vascular system, H_2S-mediated S-sulfhydration of Kir6.1 subunit in potassium ATP (K_{ATP}) channels causes hyperpolarization and smooth muscle relaxation (Kang et al., 2015; Zhao et al., 2001). In cellular redox signaling, H_2S-mediates S-sulfhydration of Keap1/Nrf2 (Kelch-like ECH-associated protein-1/ Nuclear factor erythroid 2–related factor 2) promotes Nrf2 translocation to the nucleus and induces the transcription of antioxidant genes (Hourihan et al., 2013; Yang et al., 2013). Also, H_2S S-sulfhydrates the p65 subunit of nuclear factor kappa-B (NF-κB), stimulating its transcriptional activation (Sen et al., 2012). Inside the mitochondria of smooth muscle cells and aorta tissues from mice, H_2S activates transcription factor A mitochondrial (TFAM) via S-sulfhydration of interferon regulatory factor (IRF-1), maintaining cell energy homeostasis (Li et al., 2015). In the endoplasmic reticulum of HEK293 and HeLa cells, H_2S modifies the ER-stress response through the S-sulfhydration of protein tyrosine phosphatase-1B (PTP1B), thus enhancing endoplasmic reticular kinase (PERK) phosphorylation (Krishnan et al., 2011) (**Fig. 4**).

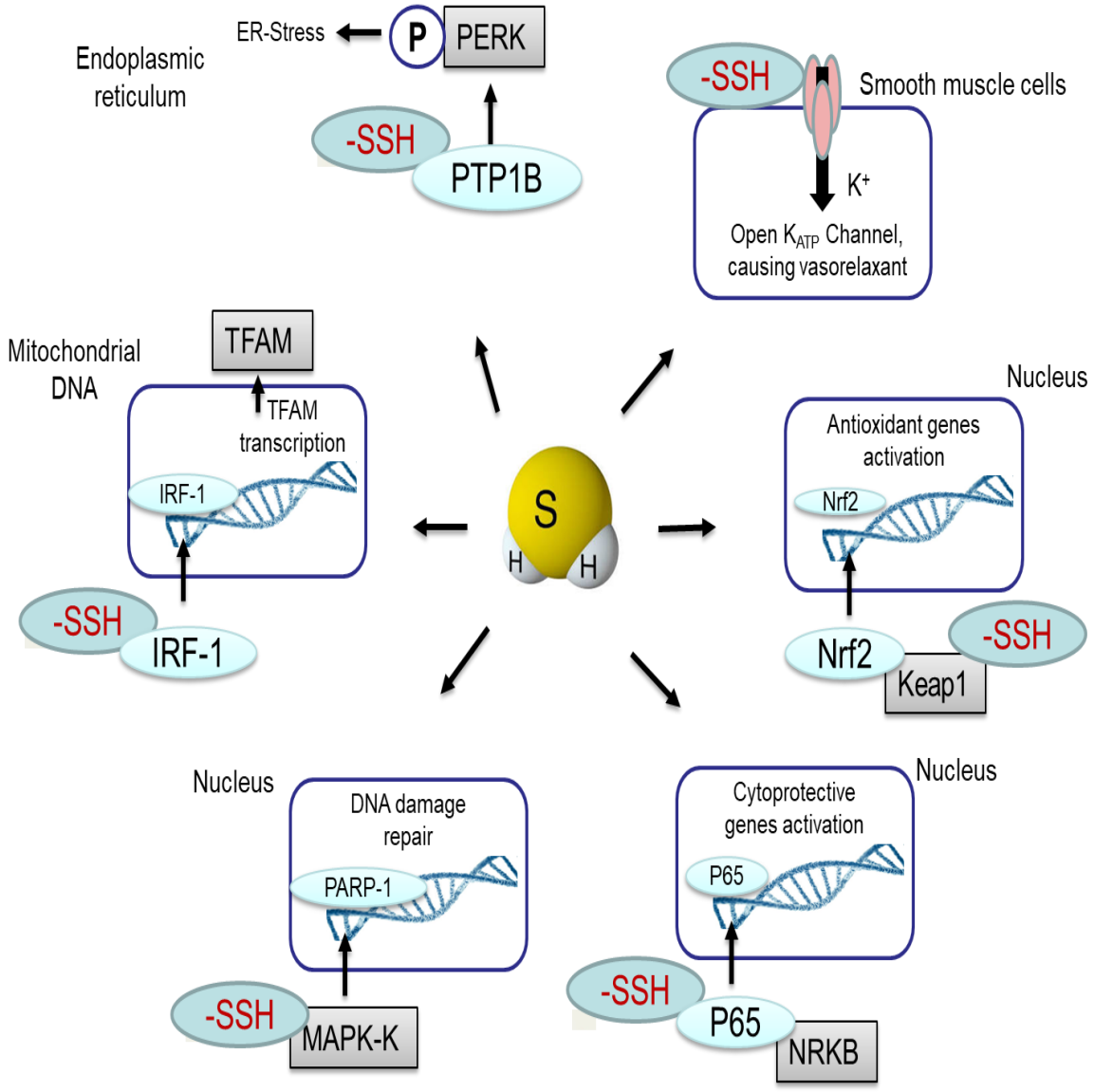


Fig. 4. **H_2S mechanism via protein S-sulfhydration.**

1.2.3. **H_2S metabolism**

In mammalian cells, H_2S is metabolized through oxidation (Bartholomew et al., 1980), methylation (Weisiger et al., 1980), scavenge (Yang et al., 2004), exhalation, or elimination (Insko et al., 2009), to maintain proper physiological levels of H_2S.

Oxidation of H_2S primary occurs in the mitochondria, where H_2S is oxidized to thiosulfate and then to sulfite and sulfate (Caliendo et al., 2010). Previously, Bartholomew has determined thiosulfate can be formed in rat kidneys, lungs, and livers (Bartholomew et al., 1980). In hepatic tissues obtained from male Sprague-Dawley rats, H_2S is oxidized to thiosulfate and sulfate by thiosulfate sulfurtransferase (TSST) (Levitt et al., 1999).

Methylation is another way of H_2S metabolism that occurs in the cytosol. In the intestinal mucosa of Sprague-Dawley rats, Weisiger et al. found that H_2S methylation leads to the formation of methanethiol (CH_3SH) via thiol s-methyltransferase (TSMT) (Weisiger et al., 1980). The generated methanethiol can be further methylated into dimethylsulfate through TSMT (Kolluru et al., 2013). Finally, sulfate and dimethylsulfate from H_2S oxidation and methylation are eliminated by kidney (Lofroth et al., 1995; Nakanishi et al., 2002; Schwartz et al., 1942). Another way of H_2S elimination is through exhalation. In a study on the exhaled amount of H_2S after injecting the rats with Na_2S or diallyl-disulfide (garlic derived natural compound), there was a significant increase in the exhaled H_2S (Insoko et al., 2009).

Methemoglobin and some other molecules in the blood and tissues such as the oxidized glutathione can scavenge H_2S to form sulfhemoglobin, shortening the half-life of the free H_2S (Smith et al., 1966). In HEK-293 cells, treatment with 10 μM of methemoglobin before adding 100 μM H_2S dramatically suppressed the antiproliferative effect of H_2S. Furthermore, methemoglobin reduced H_2S production in CSE overexpressed cells, implying that methemoglobin scavenged the H_2S (Yang et al., 2004).

Sulfide-quinone oxidoreductase has also been reported to regulate H_2S levels in fission yeast (Vande-Weghe et al., 1999). A recent study demonstrated that sulfide-quinone reductase-

like protein, a vertebrate homolog of sulfide-quinone oxidoreductase, contributes to H_2S metabolism in mammalian cells, possibly by coordinating with TSST enzyme in the mitochondria (Ackermann et al., 2014).

1.3. **Liver**

1.3.1. **Liver functions**

As a central metabolic organ, the liver plays an essential role in energy metabolism in response to excess or deficiency of nutrition. Hepatocytes are the central hepatic parenchymal cells, regulating lipid metabolism, energy production, and other biological functions (Vasconcellos et al., 2016). Lipids are the primary source of energy, and they can be synthesized either by *de novo* lipogenesis or derived from the diet. In the intestinal lumen, triglycerides (TG) dietary are emulsified and hydrolyzed by pancreatic lipase enzyme, yielding monoacylglycerol, and fatty acids (FA) (Lowe., 2002). FA and monoacylglycerol are aggregated with cholesterol and taken by the enterocytes. In the enterocytes, TG is resynthesized, then packed in chylomicrons. Chylomicrons are absorbed into the blood and either stored in adipose and muscle tissues or hydrolyzed to TG in the capillaries of the endothelial cell by lipoprotein lipase enzyme. TG are hydrolyzed into FA and transported to the liver (Havel., 1994). FAs are taken up by hepatocytes for oxidation and energy generation, a process of lipolysis. And next FAs are stored in the liver as TGs or exported into the blood as a triglyceride-rich very-low-density lipoprotein (VLDL), process called *de novo* lipogenesis (Bauer., 1996). Under normal diet conditions, the liver stores only tiny amounts of TG, while during a high calorie, liver lipid metabolism is

altered, leading to hepatic lipid accumulation disorder, called non-alcoholic fatty liver disease (NAFLD) **(**Alves-Bezerra et al., 2017; Beyaz et al., 2016) (**Fig. 5**).

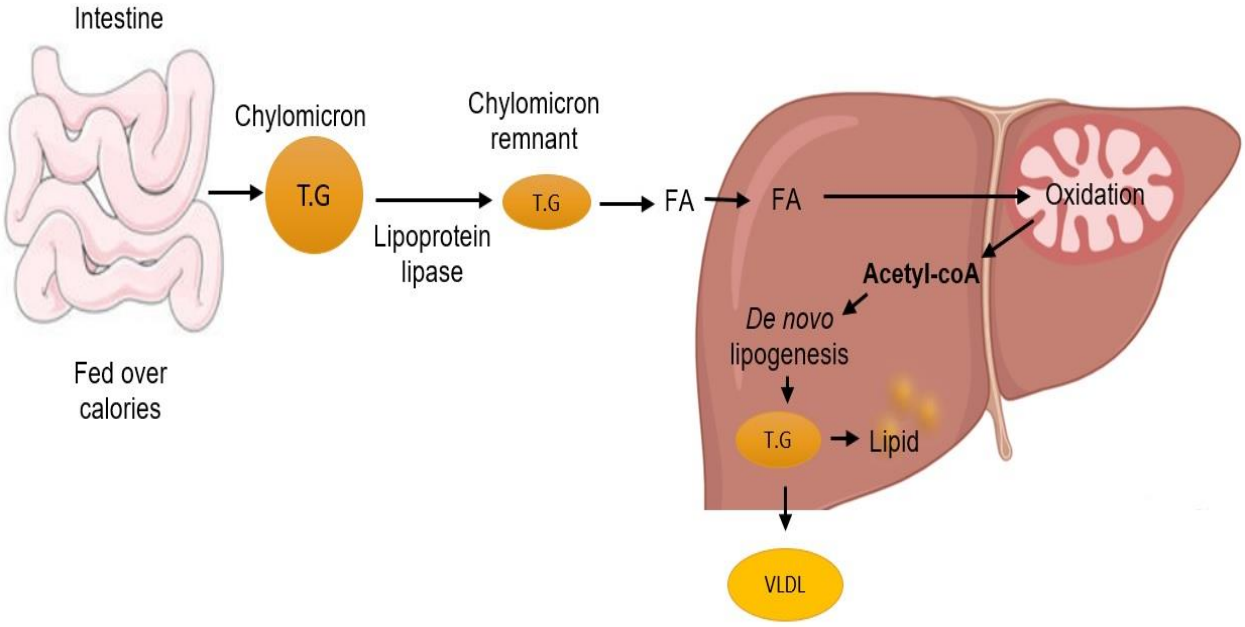


Fig. 5. **Liver lipid metabolism.**

1.3.2. **Hepatic acetyl-CoA and lipid metabolism**

Acetyl Coenzyme A (acetyl-CoA) stands out as a key intermediate from the metabolism of lipid, glucose, and proteins in most species (Cai et al., 2011; Shi et al., 2015). Fatty acids entering the liver from the blood may either be used for TG synthesis and storage in the muscle and fat tissues or be broken down to acetyl-CoA through fatty-acid oxidation (Huang et al., 2014). Alternatively, glucose and proteins can also be broken down into acetyl-CoA.

Mammalian cells monitor the contents of acetyl-CoA as a critical indicator of their metabolic state (Abu-Elheiga et al., 2012; Mehal., 2018). After an excessive meal stress, acetyl-CoA is mostly used for the synthesis of fatty acids, cholesterol, and protein acetylation, while

fasting promotes the use of acetyl-CoA into the mitochondria for ATP production (Klover et al., 2004; Shi et al., 2014).

In the mitochondria of mammalian liver cells, acetyl-CoA is oxidized to CO_2 and water through the Krebs cycle (citric acid cycle) for generating energy. Also, acetyl-CoA can be used to synthesize ketone bodies, which is either oxidized in the liver for energy production or exported outside the liver to the blood (Puchalska et al., 2017; Shi et al., 2015). In starvation, ketone bodies are produced from acetyl-CoA. On the other hand, under conditions of caloric excess, the mitochondrial acetyl-CoA is used to generate citrate, which is then exported to the cytosol and turns back to acetyl-CoA by ATP-citrate lyase enzyme (ATP-CL) to resynthesize fatty acids in human cells and aspergillus nidulans (Foster., 2012; Hynes et al., 2010; Verschueren et al., 2019) (**Fig. 6**).

H_2S can react with acetyl-CoA forming CoA and thioacetate via H_2S S-acetyltransferase (Equation 1) (Brady et al., 1954). But very little information is available on H_2S S-acetyltransferase, including its gene and protein sequence, regulation, and functions.

Equation 1:

$$H_2S + acetylCoA + (H_2S\ \mathrm{S-acetyltransferase}) \rightleftarrows CoA + Acetate$$

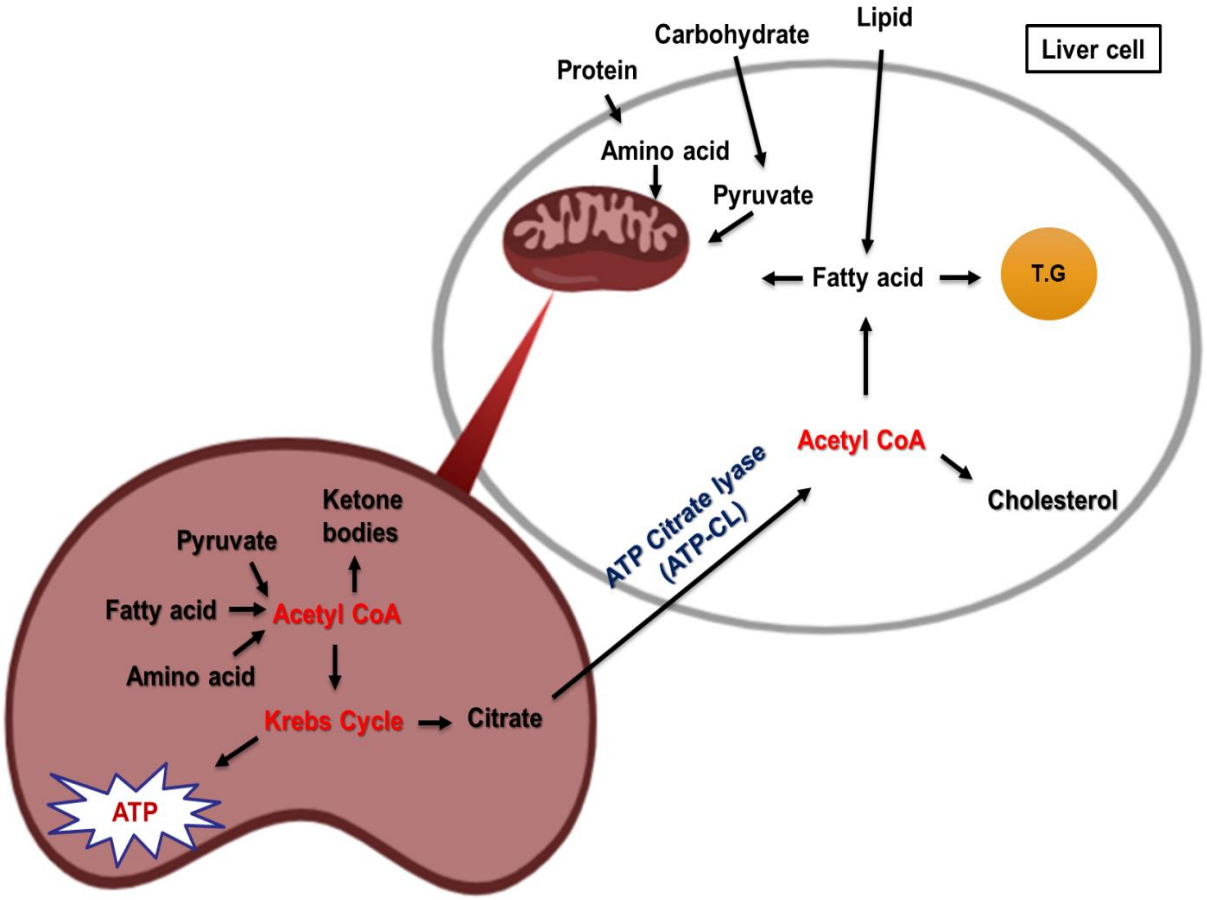


Fig. 6. **The major pathways of acetyl-CoA and lipid metabolism in the liver.**

1.3.3. **Hepatic *de novo* lipogenesis**

Lipogenesis is a *de novo* synthesis of fatty acid from excess glucose, fatty acid, or amino acid. Excess food can be converted to acetyl-CoA, which turns to a fatty acid through enzymatic reactions. However, an increased *de novo* lipogenesis process can contribute substantially to fatty liver disease (Donnelly et al., 2005). Also, it has been reported that inhibition of *de novo* lipogenesis impairs the production and secretion of triglyceride-rich VLDL in rat liver

(Fukuda et al., 1984). ATP-citrate lyase (ACLY) is the first step in *the de novo* lipogenesis enzymatic pathway, which converts citrate from the citric acid cycle to acetyl-CoA in the cytoplasm of human and eukaryotic cells (Hynes et al., 2010; Verschueren et al., 2019). After that, acetyl-CoA carboxylase (ACC) catalyzes the conversion of acetyl-CoA to malonyl CoA (Berg et al., 2002). Fatty acid synthase (FAS) is the key rate-limiting enzyme in palmitate synthesis from malonyl CoA (Jensen-Urstad et al., 2012). Then, palmitate can be easily modified by desaturases and elongases, producing more complex FA (**Fig. 7**).

There are two isoforms of ACC, mitochondrial and cytosolic ACC, forming two different pools of malonyl-CoA. Malonyl-CoA generated in the cytosol of mammalian cells, is utilized as a substrate by FAS, while malonyl-CoA produced by the mitochondrial ACC regulates β-oxidation by inhibiting carnitine palmitoyltransferase-1 (CPT1, an essential enzyme in importing free fatty acids inside the mitochondria) (Kim., 1997). Cytosolic ACC expression is very high in liver cells, where the enzyme activity is increased by a high fat or carbohydrate diet, while it is inhibited during starvation (Ferramosca et al., 2014). Mao et al. have shown that liver-specific cytosolic ACC knockout (KO) mice exhibit more than 70% reduction in hepatic malonyl-CoA and TG contents (Mao et al., 2006). FAS expression is found in all tissues, but accumulated evidence suggested that FAS mRNA and enzymatic activity can be induced by fat diet in rat liver (Semenkovich., 1997). Similar to FAS, ACC is usually higher in NAFLD (Dorn et al., 2010; Kim et al., 2017). These data suggest that ACC and FAS could be the targets in the treatment and prevention of fatty liver diseases.

A previous study has linked the increased mRNA expression of cytosolic ACC and FAS with the activation of carbohydrate-response element-binding protein and Sterol regulatory element-binding protein 1c (ChREBP and SREBP-1c), both of which act as transcription factors

in promoting the expression of glucose and lipid metabolism-related genes (Dentin et al., 2005). Unsurprisingly, the gene expression of both SREBP-1c and ChREBP is often increased in mouse liver with NAFLD (Benhamed et al., 2012; Higuchi et al., 2008).

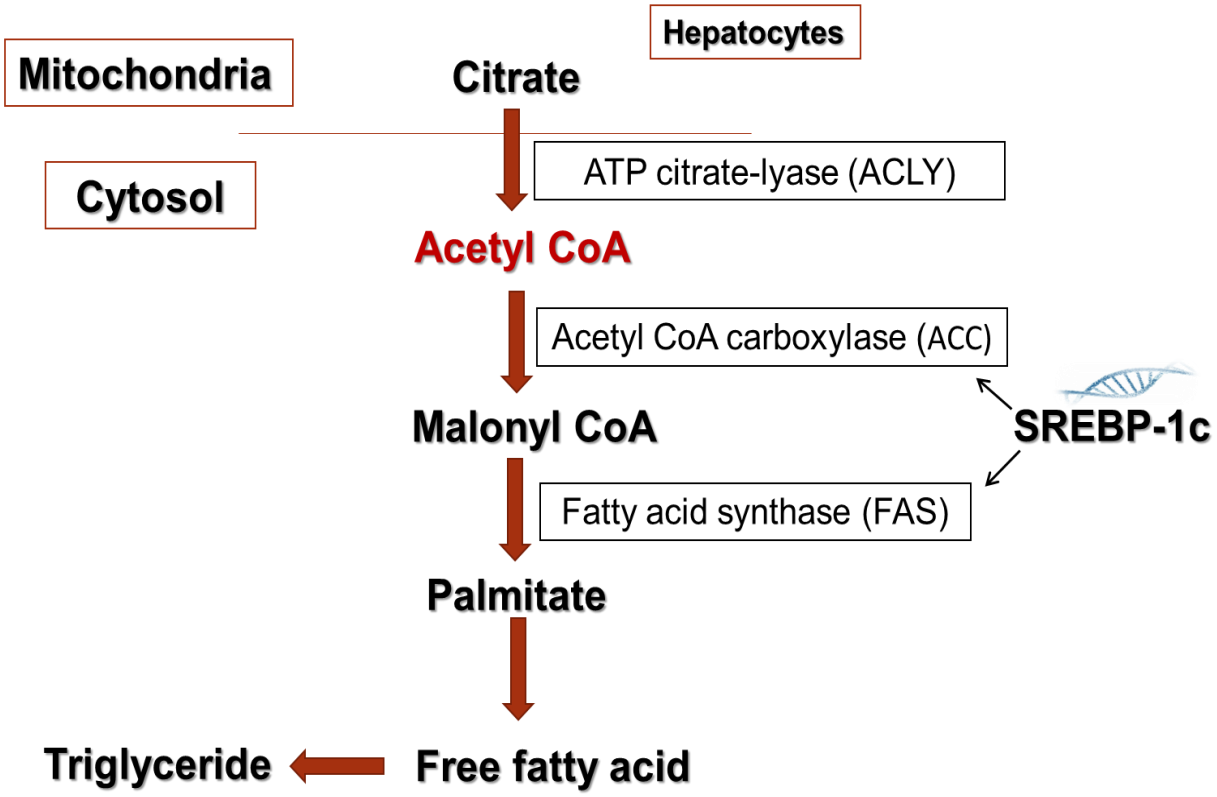


Fig. 7. **Enzymes involved in the *de novo* lipogenesis process.**

1.3.4. **Hepatic triglyceride synthesis**

TG are lipid molecules consisting of one glycerol molecule esterified with three fatty acids molecules. The first step in TG synthesis is catalyzed by the acyl-CoA synthase long-chain (ACSL), which converts FFA into fatty acyl-CoA. Then, fatty acyl-CoA is esterified to

lysophosphatidic acid (LPA) by mitochondrial and endoplasmic reticulum (ER) glycerol-3-phosphate acyltransferase (GPAT) in the mammalian liver (Shindou et al., 2009). In the second step, the produced LPA in this reaction is acylated by acylglycerol-3-phosphate acyltransferase (AGPAT) in the ER of the liver cells, forming phosphatidic acid (PA). Phosphatidic acid phosphatase (PAP) can dephosphorylate PA to form diacylglycerol (DG), which is a precursor for the synthesis of phosphatidylcholine (PC) and TG (Haagsman et al., 1984; Takeuchi et al., 2009). The final step in TG synthesis is catalyzed by diacylglycerol acyltransferase (DGAT) in the ER membrane, forming TG lipid droplets that are directed to the cytosol of hepatocytes in mammalian (Coleman et al., 2004) (**Fig. 8**).

There are four isoforms of GPAT enzyme expressed in mammalian cells. GPAT 1 and 2 are localized in the mitochondrial membrane, while GPAT 3 and 4 are found in the ER membrane (Yu et al., 2018). GPAT 1 and 4 are highly expressed in the liver cells, regulating hepatic glucose and lipid metabolism. GPAT-2 is involved in spermatogenesis and tumor development, while GPAT-3 plays an important role on TG synthesis in fatty cells (Yu et al., 2018). A recent study has observed that a mouse GPAT-1 knockout mutant shows decreased TG and VLDL levels, and protection from hepatic steatosis and insulin resistance (Neschen et al., 2005). Other studies have reported that overexpression of GPAT-1 in rat liver increases the synthesis of LPA, DG, and TG and decreases FA oxidation, causing fatty liver and insulin resistance (Linden et al., 2004; Nagle et al., 2007). Nagle et al. have observed that the upregulation of GPAT-4 increases the intracellular TG level in HepG2 cells, while GPAT-4 knockout in mice results in a reduction in hepatic TG level (Nagle et al., 2008) (**Fig. 8**).

AGPAT enzymes mediate the second step in TG synthesis. There are ten isoforms of AGPAT enzyme, while only AGAT 1 and 2 are highly expressed and localized in the ER

membrane in the mouse liver cells (Agarwal et al., 2011; Takeuchi et al., 2009). However, the role of AGPAT enzyme in the regulation of hepatic lipid metabolism remains unclear.

DGAT catalyzes the last step of TG synthesis, and there are two isoforms detected in the microsomal membrane in the mouse liver. DGAT-1 catalyzes the synthesis of TG that are packaged into VLDL, while DGAT-2 contributes to TG pool synthesis and accumulation in the liver cytosol of mice (Waterman et al., 2002). Both DGAT1 and DGAT 2 act in a sequential and combined way for TG packaging into nascent VLDL particles in HepG2 cells (Wurie et al., 2012). Overexpression of DGAT1 increases intracellular TG contents in rat liver and stimulates TG enriched-VLDL secretion (Liang et al., 2004). DGAT-1 deficiency in mice under a high-fat diet (HFD) has shown a reduction in hepatic TG levels and improved insulin responsiveness (Smith et al., 2000). Targeting DGAT-1 could be a potent therapeutic target for the treatment of hypertriglyceridemia, fatty liver, and obesity (Zhang et al., 2010) (**Fig. 8**)

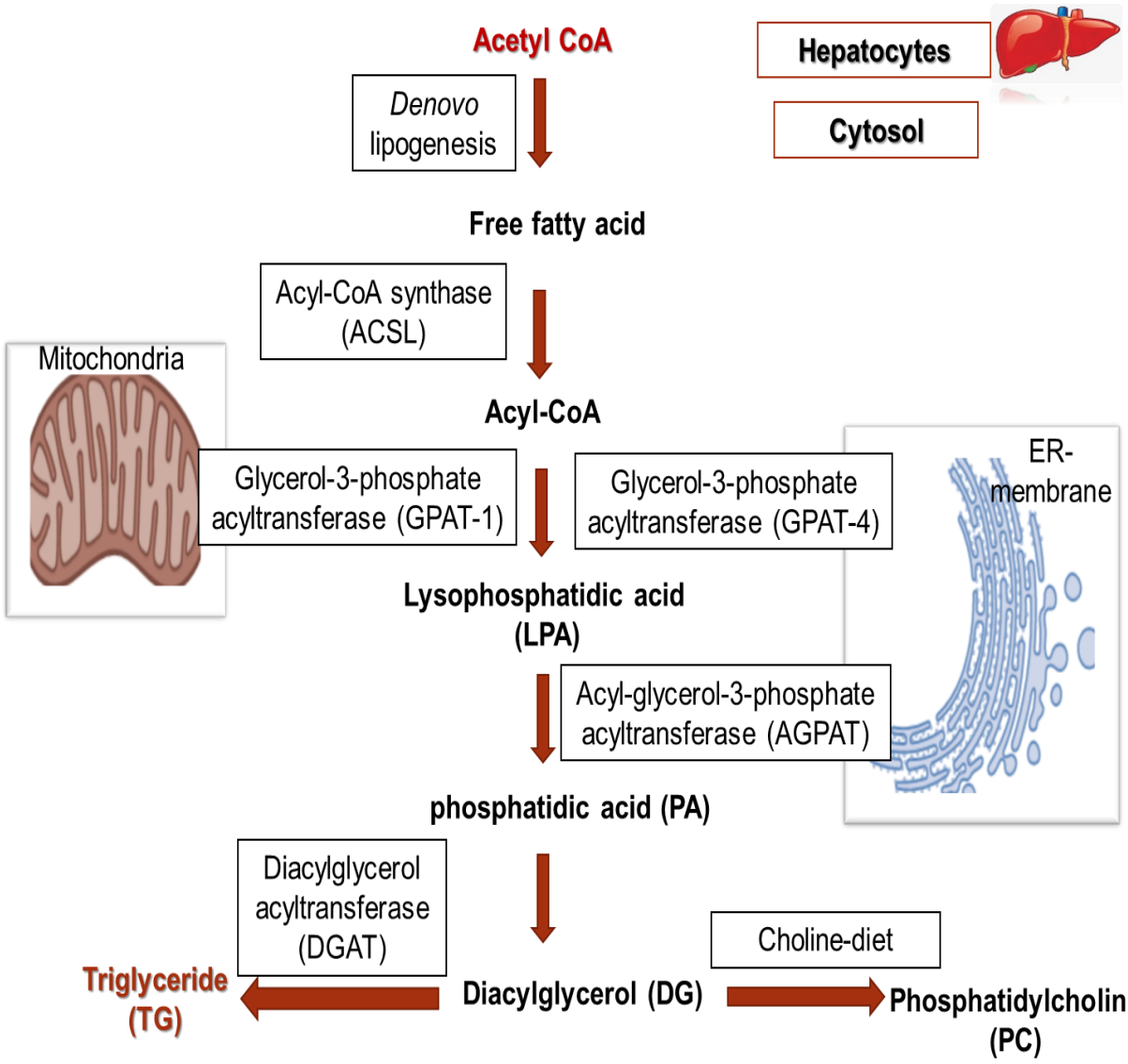


Fig. 8. **Liver triglyceride synthesis.**

1.3.5. **Hepatic VLDL synthesis and secretion**

The synthesized TG is either stored in the liver in the form of lipid droplets or packed in VLDL and exported to the muscle or adipose tissue for oxidation or storage, depending on

nutritional status. VLDL can be formed in two steps. Starting in the lumen of the endoplasmic reticulum, a little amount of TG are incorporated with apolipoprotein-B (apoB) by the microsomal triglyceride transfer protein (MTTP) (Gordon et al., 1995). Then, additional TG is packaged into the nascent apoB and transported to the Golgi apparatus, forming VLDL particles (Cohen et al., 2013). The liver stores a small amount of TG and exports a large amount of VLDL to muscle or fat tissues under normal conditions. Moreover, under a HFD or liver dysfunction, the liver stores a greater amount of TG, leading to NAFLD (Cohen et al., 2013) (**Fig. 9**).

Over secretion of VLDL is linked to many metabolic disorders such as insulin resistance and diabetes, leading to TG over-storage in the liver as a result of increased hepatic *de novo* lipogenesis and FA uptake in men with visceral obesity (Gill et al., 2011; Riches et al., 1998). One study on obese patients with NAFLD has observed a significant increase in hepatic VLDL secretion and increased adipose lipolytic rate (Fabbrini et al., 2010). Fabbrini et al. also have mentioned that hepatic TG enrich-VLDL excretion rate is dramatically increased to the double in nondiabetic obese patients with NAFLD than healthy (Fabbrini et al., 2010). However, the regulation of VLDL excretion leveraged in the management of NAFLD remains unclear and needs more investigation.

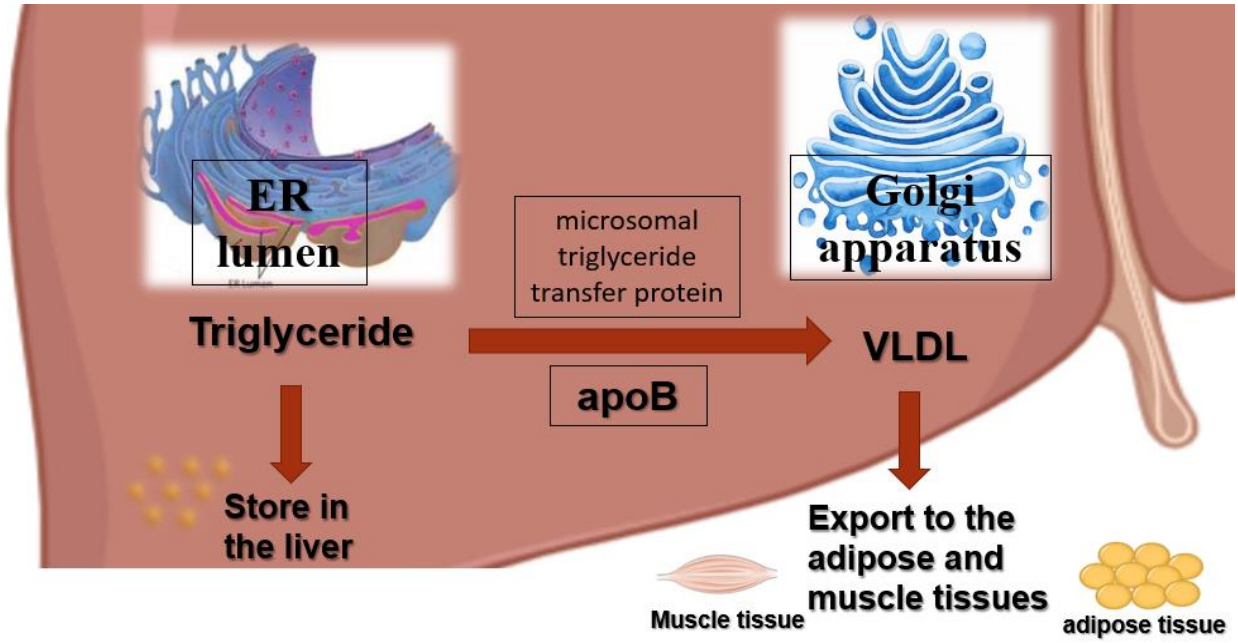


Fig. 9. **VLDL assembly and secretion.**

1.3.6. **Hepatic fatty acid oxidation**

FA can be oxidized in the mitochondria or peroxisomes through β-oxidation to produce acetyl-CoA. β-oxidation of short- (<C4), medium- (C4-C12), and long-chain (C12-C20) of FA occurs in the mitochondria, while the oxidation of very long chain (C20-C26) FA happens in the peroxisomes of mouse livers (Hashimoto et al., 1999).

First, FA is converted to acyl-CoA by the long-chain acyl-CoA synthase enzyme (ASCL), then acyl-CoA is shuttled into the mitochondria via carnitine palmitoyltransferase-1-alpha (CPT1A) for oxidation in mice (Grevengoed et al., 2014; Lee et al., 2011). There are three isoforms of CPT1, but CPT-1-alpha (CPT1A) is the primary isoform expressed in the liver cells.

There are five isoforms of ASCL in mammals with specific tissue distributions. ACSL-1 is the best-characterized isoform, as it is a target gene of peroxisome proliferator-activated receptor alpha (PPARα). PPARα acts a master transcription factor in regulating a set of genes involved in FA oxidation in rat liver (Schoonjans et al., 1995). In the outer membrane of the mitochondria, ACSL-1 converts long chain-FA to acyl CoA, and physically interacts with CPT1 to control the availability of acyl CoA for mitochondrial β-oxidation. Though, this process is still unclear, as liver-specific ACSL1-KO mice show only slightly reduced rates of β-oxidation and TG synthesis, while TG content remains unchanged (Li et al., 2009) (**Fig. 10**).

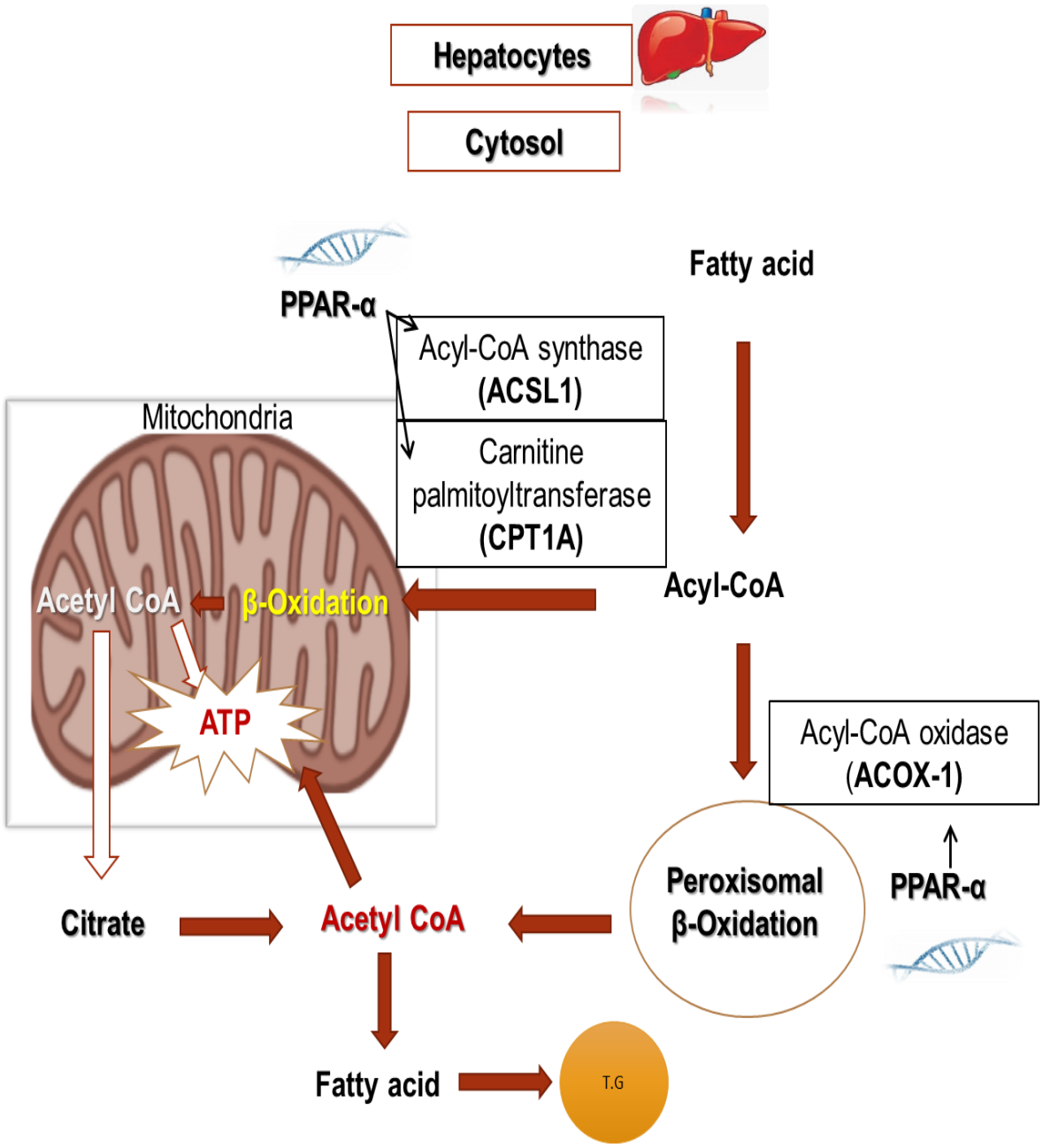


Fig. 10. **Hepatic fatty acid oxidation.**

1.4. H_2S and liver

The liver is the largest organ and is essential for lipid and glucose metabolism. H_2S acts as a novel gasotransmitter and plays a vital role in both health and diseases. Hepatic H_2S is critical for liver functions, including the regulation of lipid metabolism, glucose metabolism, mitochondrial biogenesis, insulin sensitivity, etc. Moreover, failure in hepatic H_2S production can lead to many chronic liver diseases, including NAFLD, fibrosis, cirrhosis, and hepatocellular carcinoma (HCC) (Mani et al., 2014).

1.4.1. **Role of H_2S in liver lipid metabolism**

CSE, CBS, and 3-MST enzymes are all expressed in the liver, but CSE was demonstrated to be the main H_2S-generating enzyme in mouse livers (Fiorucci et al., 2006; Sen et al., 2012). The dysfunctions of the CSE/H_2S system are linked to liver damage induced by HFD in mice (Yang et al., 2019). A recent study has demonstrated that NaHS (an H_2S donor) reduces lipid accumulation in the liver of HFD-induced obese mice (Wu et al., 2015). A study on CSE-KO mice fed with atherogenic diet revealed a rise in LDL, total cholesterol level, and early development of atherosclerosis when compared to the wildtype (WT) mice, and the administration of NaHS to these animals decreased plasma LDL and cholesterol levels (Mani et al., 2013).

Some compounds used to treat liver diseases were shown to be targets of the CSE/H_2S pathway. Metformin, the most common drug for the treatment of type 2 diabetes, enhances the H_2S levels and reverses fatty liver and steatosis in mice (Wiliński et al., 2013). Statins, the drugs used to lower high cholesterol, have been recently shown to increase hepatic H_2S levels of mice

(Wiliński et al., 2011). In folk medicine, garlic is very vital for human health, due to the higher level of polysulfides. Polysulfides can be converted to H_2S in mammalian cells via a nonenzymatic reaction (Munchberg et al., 2007). A garlic derivative called diallyl-trisulfide releases H_2S and protects against NAFLD through decreasing lipid accumulation in Sprague–Dawley rats (Benavides et al., 2007; Lai et al., 2014). These studies determined that the CSE/H_2S system plays an essential role in liver lipid metabolism, but the underlying mechanism is still unclear and needs more investigations.

1.4.2. **Role of H_2S in liver glucose metabolism**

The role of H_2S in liver glucose metabolism has been brought to light more recently. A study on a rat model of streptozotocin-induced diabetes has found that the liver H_2S level was higher in the diabetic than the non-diabetic rats (Yusuf et al., 2005). In another study on mice treated with metformin (an insulin sensitizer for type-2 diabetes) has shown that metformin significantly increased the hepatic H_2S production rate (Willinski et al., 2013). Moreover, methylglyoxal (an intermediate of glucose metabolism) has been shown to interact with hepatic H_2S, affecting the development of insulin resistance in rats (Chang et al., 2010).

Zhang et al. have shown that H_2S significantly diminished insulin-stimulated glucose uptake and glycogen storage and improved gluconeogenesis and glycogenolysis in both HepG2 cells and mice (Zhang et al., 2013). H_2S increases liver gluconeogenesis through the phosphorylation of adenosine monophosphate protein kinase (AMPK**)** and phosphoenolpyruvate carboxykinase (PEPCK), contributing to the higher level of glucose (Zhang et al., 2013). Other studies showed that H_2S induces the activities of the glucocorticoid receptor (GR), pyruvate

carboxylase (PC), glucose-6-phosphatase, and fructose-1,6-bisphosphatase, which are essential proteins involved in glucose production in HepG2 cells and mouse livers (Ju et al., 2015; Untereiner et al., 2015; Yang., 2016) (**Fig. 11**).

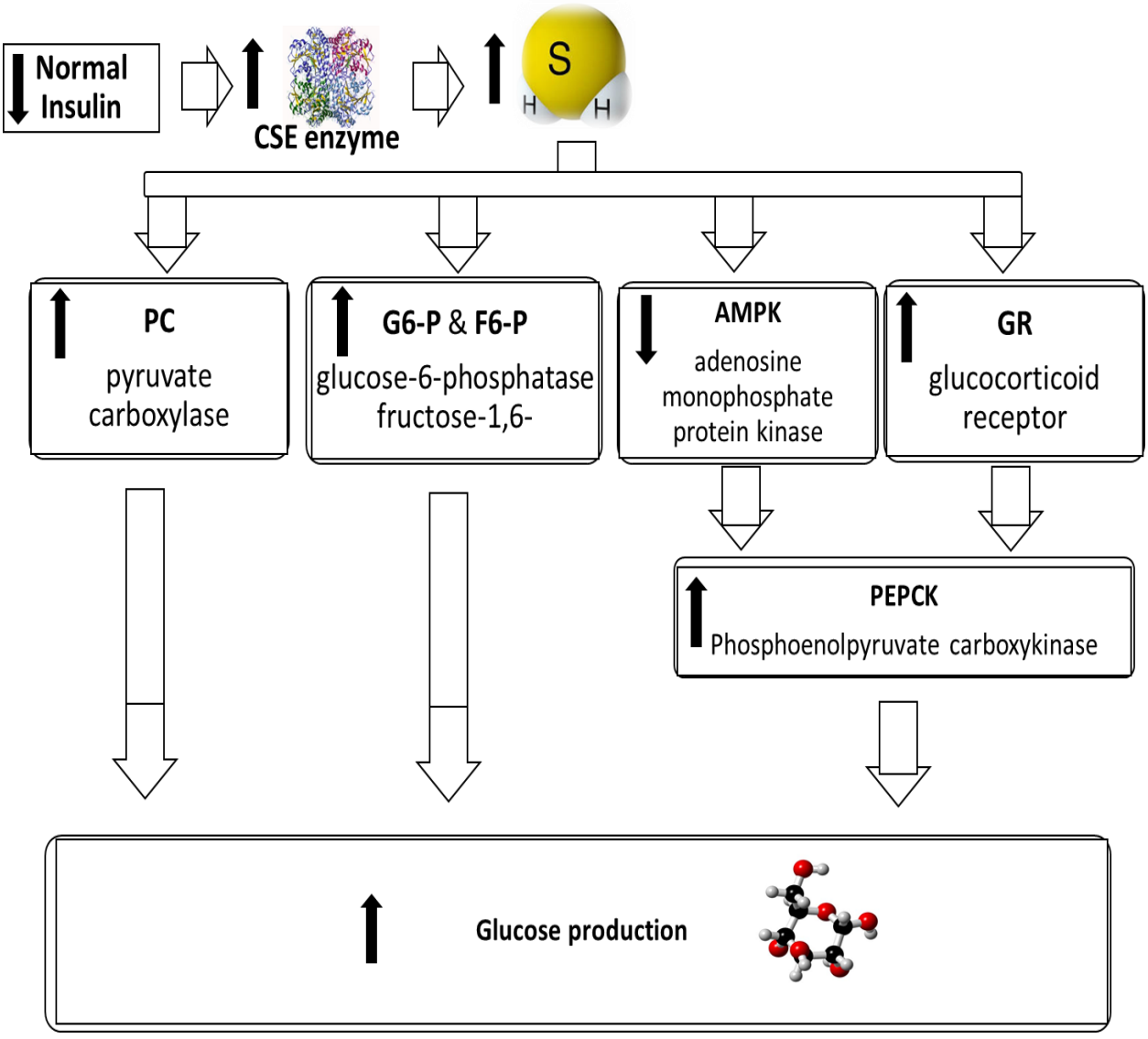


Fig. 11. **The mechanism for H_2S-mediated hepatic glucose metabolism.**

1.4.3. **Role of H_2S in mitochondrial biogenesis**

H_2S regulates mitochondrial biogenesis through direct S-sulfhydration of the peroxisome proliferator-activated receptor-coactivator-1 alpha (PGC-1α) in mouse livers (Untereiner et al., 2016). The S-sulfhydrated PGC-1α stimulates the expression of nuclear respiratory factor-1 and 2 (NRF-1 and -2) in the nucleus of the liver cells. Then, NRF-1 and 2 ameliorate the expression of the mitochondrial transcription factors (Tfam), which increases the mitochondrial DNA contents and stimulates mitochondrial fusion (Fernandez-Marcos et al., 2011; Untereiner et al., 2016) (**Fig. 12**).

The hepatocytes from CSE-KO mice had less mitochondrial DNA content, which was reversed by the supplement of exogenous H_2S (Untereiner et al., 2016). Furthermore, Li et al. have demonstrated that CSE deficiency decreases mitochondrial DNA replication, mitochondrial contents, and Tfam gene expression in both aorta tissues and smooth muscle cells, which can be reversed by H_2S via S-sulfhydration of the transcription repressor interferon regulatory factor 1 (IRF-1) followed by lower level of DNA methyltransferase 3a (DNAmt3a) and higher mitochondrial biogenesis in mice (Li et al., 2015) (**Fig. 12**). These studies indicate that the CSE/H_2S system plays an important role in the maintenance of mitochondrial biogenesis homeostasis and provides a novel therapeutic avenue for many metabolic diseases.

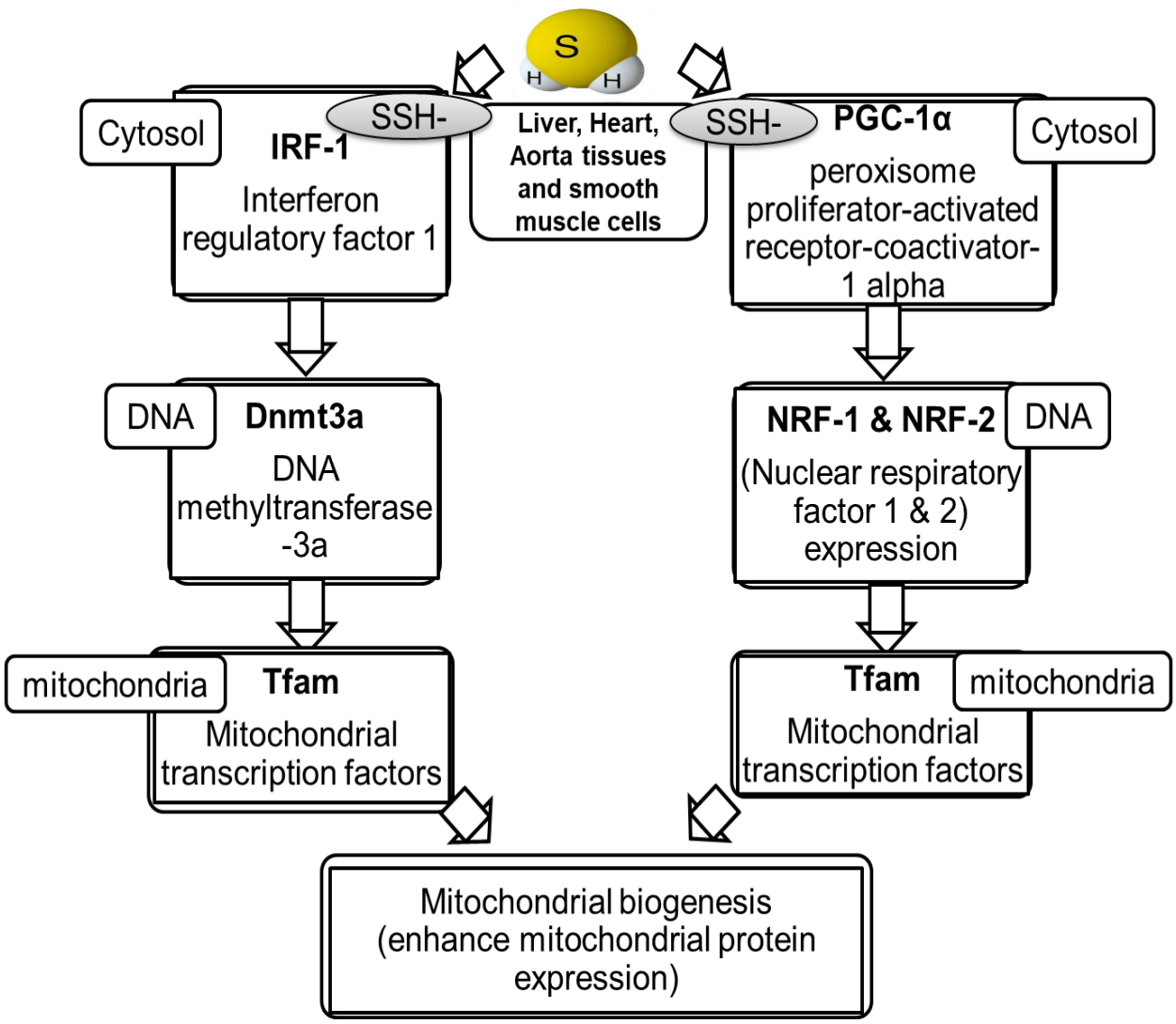


Fig. 12. **Role of H_2S in mitochondrial biogenesis.**

1.4.4. **H_2S in liver injury**

1.4.4.1. **Roles of H_2S in non-alcoholic fatty liver disease**

NAFLD is the most prevalent liver disease which is caused by abnormal accumulation of lipid inside the hepatocytes. NAFLD may lead to liver fibrosis, cirrhosis, and potentially HCC

(Sutti et al., 2015; Tobita et al., 2018). The development of NAFLD is also a high risk for many metabolic syndromes, such as obesity, diabetes, and cardiovascular diseases (Li et al., 2017; Wu et al., 2015). Recently, tons of studies have demonstrated the protective role of H_2S in NAFLD. Peh et al. reported that HFD downregulates CSE protein expression in mouse liver after 8 to 16 weeks of high-fat treatment (Peh et al., 2014). Mani and others showed that deficiency of CSE induces fatty liver in CSE-KO mice fed with HFD for 4 months when compared with wild type (WT) mice (Mani et al., 2015).

A methionine and choline–deficient diet (MCD) reduced the hepatic phosphatidylcholine synthesis and TG secretion, leading to the development of NAFLD in mice (Kulinski et al., 2004; Lombardi et al., 1968; Luo et al., 2014; Wang et al., 2017). Phosphatidylcholine is essential for the outer phospholipid component of lipoproteins. A previous study mentioned that mice fed on MCD diet have lower fasting insulin and higher glucose levels, indicating an increased liver insulin sensitivity (Rinella et al., 2004). Liver damage was often observed in MCD-fed mice, evidenced by increased plasma level of alanine aminotransferase (ALT) and tumor necrosis factor-α (TNFα), suggesting the development of liver inflammation and cirrhosis (Dolganiuc et al., 2009). A recent study on rat liver demonstrated that hepatic H_2S was lower in MCD-induced NAFLD (Luo et al., 2014). Actually, abnormal hepatic H_2S production has been demonstrated to be correlated with the pathogenesis of several other hepatic disorders, such as liver fibrosis, cirrhosis, and carcinoma (Fiorucci et al., 2006; Mani et al., 2014; Tan et al., 2011). These studies indicate the potential roles of H_2S against NAFLD and other liver diseases, but the mechanism underlying this effect remains unclear.

1.4.4.2. **Roles of H_2S in liver fibrosis**

Hepatic fibrosis is a scarring process that reflects the liver's response to chronic inflammation, injury, and damage in conjunction with the excessive accumulation of collagen type I (Ni et al., 2018). A variety of factors such as HFD, ischemia-reperfusion (I/R), carbon tetrachloride (CCl_4), lipopolysaccharides, or HClO contribute to hepatic fibrosis and liver damage in most species (Bekpinar et al., 2014; Ci et al., 2017; Jiao et al., 2018; Yang Y et al., 2019). Once liver fibrosis has developed, the risk of developing liver cirrhosis or HCC will be higher (Bosetti et al., 2007). It is essential to develop a novel treatment to attenuate or prevent hepatic fibrosis, as there are limited available treatments for this disease (Lim et al., 2008).

An early study on CBS-KO mouse liver showed an enlargement in hepatocytes with prominent nucleoli and cytoplasmic lipid droplets (Watanabe et al., 1995). Another study reported that CBS deficiency promotes the development of fatty liver and fibrosis and increases the expression of proinflammatory cytokines in mouse liver, suggesting that H_2S is involved in hepatic fibrosis (Robert et al., 2005). Many studies have reported that inflammation is the first stage in liver fibrosis development, causing parenchymal cells apoptosis, fibroblasts proliferation, and ultimately leading to irreversible fibrotic injury (Bataller et al., 2005). Recent studies have shown that treatment with H_2S downregulates pro-inflammatory cytokines including TNF-α and interleukin-6 (IL-6), inhibiting the progression of fibrosis in Tamm-Horsfall protein 1 cells and rat livers (Rios et al., 2015; Tan et al., 2011). Another study has determined that exogenous NaHS (H_2S donor) attenuates CCl_4-induced liver fibrosis in rat liver through reducing transforming growth factor-β1 (TGF-β1) protein expression and extracellular matrix sedimentation (Shen et al., 2012).

Collagen type I α1 (COL1A1) and smooth muscle actin alpha (SMAα) are reliable liver fibroblastic markers (Bataller et al., 2005; Liu et al., 2013). Administration of NaHS decreased the number of collagenous fibers and downregulated SMAα by 50% in cirrhotic rats induced by CCl_4 (Tan et al., 2011). Therefore, H_2S can be a therapeutical target for the treatment of liver fibrosis.

1.4.4.3. **Roles of H_2S in liver cirrhosis**

Hepatic cirrhosis is a late stage of chronic scarring (fibrosis), mainly caused by NAFLD. The complications of liver cirrhosis cause severe liver failure, increasing the risk of HCC (Farrell et al., 2006). H_2S deficiency has been shown to promote liver cirrhosis in the mouse livers (Fiorucci et al., 2006; Song et al., 2015). Other studies have demonstrated that CCl_4 attenuates hepatic CSE expression and H_2S levels in rat liver, leading to liver cirrhosis and portal hypertension (Taguchi et al., 1995; Tan et al., 2011). Sequential studies in rat liver demonstrated that H_2S protects from portal hypertension, I/R injury, and CCl_4-induced liver damage and cirrhosis (Kang et al., 2009; Tan et al., 2011). Liver damage is associated with an elevation in plasma ALT and aspartate aminotransferase (AST). Exogenous NaHS significantly reversed CCl_4-induced plasma levels of ALT and AST in rats (Tan et al., 2011).

2. **Hypothesis and objectives**

NAFLD is the most prevalent liver disease which is caused by abnormal accumulation of lipid inside the hepatocytes (Sutti S et al., 2015; Tobita H et al., 2018). Recent studies indicate the potential roles of H_2S against NAFLD, but the mechanism underlying this effect remains unclear (Ci L et al., 2017; Sun L et al., 2015; Wang B et al., 2017; Yang Y et al., 2019). Here we **hypothesized** that H_2S is essential for the maintenance of acetyl-CoA homeostasis and inhibits lipid accumulation in liver cells under excessive nutritional status. To this end, by using both *in vitro* cell culture model and *in vivo* animal model, three objectives were proposed: 1) investigate the role of H_2S in hepatic acetyl-CoA homeostasis; 2) study the role of H_2S in hepatic lipid metabolism as well as the underlying mechanism; 3) determine the role of H_2S in hepatic lipid inflammation/fibrosis. This project would provide a potential mechanism underlying the roles of H_2S against NAFLD.

3. **Materials and Methods**

3.1. **Cell culture**

Human HepG2 cells (American Type Culture Collection, Manassas, VA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich, Oakville, ON) supplemented with 10% Fetal Bovine Serum (FBS, Clontech, Mountain View, CA), 100 mg/ml streptomycin and 100 U/ml penicillin (Sigma-Aldrich, Oakville, ON) at 37 °C in a humidified atmosphere of 5% CO_2 (Greasley et al., 2019). To passage the cells, the culture medium was removed from HepG2 cells, and then the cells were washed once with PBS. After the PBS was removed, the cells were treated with 1 mL of trypsin-EDTA solution (Corning, Corning, NY) per plate. The plates were then put into an incubator at 37 °C, and were examined at 5 minute intervals until all cells became detached from the bottom of the plates, at which time the plates were removed from the incubator. Next, 2 mL of culture medium was added to each plate to stop the action of the trypsin. The detached cells were then pipetted into a 15 mL centrifuge tube, and centrifuged for 3 minutes at 2,000 × g at 4 °C. Following centrifugation the supernatant was aspirated from the cell pellet, and the cells were resuspended in fresh culture medium. The volume of medium used for resuspension was determined based on the desired number of plates of cells. HepG2 cells with 75-80% confluence were used for various treatments. For high glucose (HG) treatment, HepG2 cells cultured in standard medium containing 5.5 mM glucose were washed once and changed to medium containing 25 mM glucose for three days. For the treatment with free fatty acids (FFAs), the cells in standard medium were switched to medium containing 5 µL/mL of a chemically defined lipid mixture, which includes non-animal derived fatty acids (2 µg/mL arachidonic and 10 µg/mL each linoleic, linolenic, myristic, oleic, palmitic and stearic), 0.22 mg/mL cholesterol, 2.2 mg/mL Tween-80, 70 µg/mL tocopherol acetate and 100 mg/mL pluronic F-68 (Sigma-Aldrich) (Gunn et al., 2017).

3.2. **Cell survival assay**

The effect of FFAs, HG, and/or NaHS treatment on HepG2 cell survival was measured with 3-((4,5)-dimethylthiazol-(2)-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described before (Stokes et al., 2018). Briefly, 25,000 cells per well were seeded in a 96-well plate to form 75-80% confluence. After various treatments, 0.5 mg/mL of MTT solution in a serum-free medium was added for an additional 4 hours at 37 °C. The MTT formazan was dissolved in 100 µL of dimethyl sulfoxide. Then the absorbance hwas detected at 570 nm using FLUOstar OPTIMA microplate spectrophotometer (BMG Labtech, Germany). The control cells without any treatment were considered as 100% survival. To further confirm the effects of cell viability, cell number count was determined using a hemocytometer.

3.3. **Oil Red O staining**

Oil Red O staining was used to observe and quantify intracellular lipid accumulation (Sun et al., 2015; Yang et al., 2018). After various treatments, HepG2 cells were fixed and stained with Oil Red O using the Hepatic Lipid Accumulation/Steatosis assay kit (Abcam). The stained lipids were then observed by light microscopy (Olympus, Tokyo, Japan). In another study, the cells were subject to lipid extraction with isopropanol, and the absorbance was measured at 520 nm with a microplate spectrophotometer.

3.4. **Acetyl-CoA measurement**

The contents of acetyl-CoA in HepG2 cells and mouse liver tissues were measured by a coupled enzyme assay with an acetyl-CoA assay kit (Abcam, Toronto, ON), according to the manufacturer's instructions (Schroeder et al., 2005). Acetyl-CoA values were expressed as nanomole per milligram (nmol/mg) of protein.

3.5. **Animal feeding**

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 Committees of Lakehead University and Laurentian University, Canada. Both WT and CSE-KO mice were fed with a standard rodent chow diet until eight weeks of age and then shifted to either a high-fat choline-deficient (CD) diet (catalog no. A06071302, Research Diets, New Brunswick, NJ) or control diet for a further four weeks (Yang et al., 2008; Mani et al., 2015). The food intake and mouse weight were measured weekly. At the end of the experiment, the mice were starved overnight and anesthetized. Liver tissues were removed and weighed and immediately frozen and stored at -80°C for subsequent studies.

3.6. **Histological examinations and biochemical analyses**

The mouse livers were fixed in 4% paraformaldehyde and then embedded with paraffin. Tissues were cut into 5 μm sections and dyed with hematoxylin/eosin (H&E). The tissues were also imbedded in Tissue-Tek OCT media and kept at -80^{o}C for two weeks. The frozen tissue was

then sectioned into 5 μm using a cryo-microtome (Leica Biosystems, Concord, ON) and placed on slides for Oil Red O staining. Histopathological changes were studied using light microscopy (Olympus, Japan). Plasma was collected by centrifugation at 2,000 x g for 15 min at 4°C, and plasma ALT, AST and lipid profiles were determined using standard enzymatic colorimetric techniques (Roche Modular-P800, Roche-Diagnostics, Indianapolis, IN).

3.7. **Western blotting**

HepG2 cells or mouse liver tissues were collected and solubilized in tris-EDTA/sucrose buffer in the presence of protease inhibitors (Sigma-Aldrich). To extract the supernatant containing cellular proteins, the buffer solution, centrifuged at 14,000 x g for 15 minutes at 4°C. Perform a Bradford protein assay for each sample to determine their protein concentration, as described previously (Ernst et al., 2010). Once concentrations are known, correct the concentration of each sample according to the lowest concentration across all samples. An equal amount of proteins (50 μg/well) were mixed with loading buffer (0.5M Tris-HCL pH 6.8, 10% SDS, 0.05% bromophenol blue, 2-b-mercaptoethanol and glycerol), and boiled for 5 min followed by running in an SDS/PAGE gel and transferred onto a nitrocellulose membrane (Pall Corporation, Pensacola, FL). The membranes were blocked at 25^{o}C for 2 hours with phospho-buffered saline solution (PBS; NaCl, KCl, $NaHPO_4$, KH_2PO_4, pH 7.4), including 3% non-fat milk and 0.1% tween 20, followed by incubation with primary antibody overnight at 4°C. Then, membranes were washed with PBS-T (phospho-buffered saline and 0.1% tween-20) 3 times and each time for 5 min on a shaker at room temperature. After washing, the membranes were then incubated with horseradish peroxidase-conjugated anti-rabbit or anti-mouse secondary antibody (1:5000, Sigma-Aldrich) for 1.5 hours followed by rinsing the membranes with PBS-T for 5 min

with three repeats. Next, the chemiluminescent signals detected with ECL (GE Healthcare, Amersham, UK) and exposed to X-ray film (Kodak Scientific Imaging film, Kodak, Rochester, NY). The dilutions of primary antibodies were used as follows: β-actin (1:20000, Sigma-Aldrich), CSE (1:1000, Abnova, Taipei), fatty acid synthase (FAS, 1:1000, Cell Signaling Technology, Danvers, MA), long-chain acyl-CoA synthetase 1 (ACSL1, 1:1000, Cell Signaling Technology), ATP-citrate lyase (ATP-CL, 1:1000, Cell Signaling Technology), cytoplasmic acetyl-CoA synthetase 1 (AceCS1, 1:1000, Cell Signaling Technology), and acetyl-CoA carboxylase (ACC, 1:1000, Cell Signaling Technology). To semi-quantification of the protein expression, the X-ray films were scanned and analyzed with ImageJ software 1.47i (NIH). Bands were normalized against the β-actin band. Each experiment was carried out three times.

3.8. **Real-time PCR**

Total RNA from HepG2 cells or mouse livers were extracted using TRIzol reagent and then centrifuged at 12,000 ×g for 15 min. The supernatant top layer carried to a new tube and precipitated with isopropanol. Total RNA was reverse transcribed into cDNA by using ReverAid™ First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA). To ensure that there is no DNA contamination, negative control in the absence of reverse transcriptase was prepared. The quantification of mRNA expression was performed with an iCycler iQ5 (Bio-Rad, Mississauga, ON, CA) apparatus associated with the iCycler optical system software (v. 3.1), using SYBR Green PCR Master Mix (Bio-Rad). The PCR program was as follows: 1 cycle at 94 °C for 5 minutes, 35 cycles at 94 °C for 20 seconds then 62 °C for 30 seconds then 72 °C for 30 seconds, 1 cycle at 72 °C for 5 minutes, and cool to 4 °C. All samples were run in triplicates, and relative gene expression was calculated as $2^{-\Delta\Delta CT}$ formula and

presented as a percentage over control samples. The primers set used for real-time PCR analysis in HepG2 cells and mouse liver tissues were provided in the following tables 1 and 2.

Table 1: Primers sets used for real-time PCR analysis in HepG2 cells.

GAPDH (NM_002046.5)	5'-GCGGGGCTCTCCAGAACATCAT-3' (forward) 5'-CCAGCCCCAGCGTCAAAGGTG-3' (reverse)
SREBP1 (NM_001005291.3)	5'-GTGGCGGCTGCATTGAGAGTGAAG-3' (forward) 5'-AGGTACCCGAGGGCATCCGAGAAT-3' (reverse)
ACOX1 (NM_004035.7)	5'-CCCGAAAGCCTAACCGAAGCATA-3' (forward) 5'-CATCATAGCGGCCAAGCACAGAG-3' (reverse)
ApoB (NM_000384.3)	5'-TGACCGGGGACACCAGATTAGA-3' (forward) 5'-CAGGCGACCAGTGGGCGAGGAT-3' (reverse)
CpT1A (NM_001876.4)	5'-CCCGGGGGAATGTCAAGAGGTT-3' (forward) 5'-CGGTCATGGCGAGGCGATACA-3' (reverse)
PPARα (NM_001001928.3)	5'-TGCCCCCTCTCCCCACTCG-3' (forward) 5'-AGCCCTTGCAGCCTTCACACG-3' (reverse)
IL6 (NM_000600.4)	5'-AAAGAGGCACTGGCAGAAAACAAC-3' (forward) 5'-TTAAAGCTGCGCAGAATGAGATGA-3' (reverse)
TNFα (NM_000594.3)	5'-GGGCTCCAGGCGGTGCTTGTTC-3' (forward) 5'-GCGGCTGATGGTGTGGGTGAGG-3' (reverse)
SMAα (NM_001141945.2)	5'- CCACTGCCGCATCCTCATCCT-3' (forward) 5'- CCCGGCTTCATCGTATTCCTGTT-3' (reverse)
COL1A1 (NM_000088.4)	5'- ACCTCCGGCTCCTGCTCCTCTTAG -3' (forward) 5'- GCGCCGGGGCAGTTCTTGGTCT-3' (reverse)

Table 2: Primer sets used for real-time PCR analysis in mouse liver tissues.

GAPDH (NM_008084.3)	5'-TCTCCTGCGACTTCAACAGC-3' (forward) 5'-GGTGCACGAACTTTATTGATGGT-3' (reverse)
SREBP1 (BC_056922.1)	5'-TGCGCAAGGCCATCGACTACATCC-3' (forward) 5'-CCGCTGGGCTTTGACCTGGCTATC-3' (reverse)
ACOX1 (NM_015729.3)	5'-CGCCGCCACCTTCAATCCAGAG-3' (forward) 5'-TCCAGGCCGGCATGAAGAAAC-3' (reverse)
ApoB (NM_009693.2)	5'-CCGGGATCAAGGCTGGTGTAA-3' (forward) 5'-GTGGAGCTGGCGTTGGAGTAAG-3' (reverse)
IL6 (NM_031168.2)	5'-ACCAGAGGAAATTTTCAATAGGC-3' (forward) 5'-TGGGAGTGGTATCCTCTGTGA-3' (reverse)
TNFα (NM_013693.3)	5'-CCCACACCGTCAGCCGATTT-3' (forward) 5'-GTCTAAGTACTTGGGCAGATT-3' (reverse)
COL1A1	5'-GGTCCCCCTGGCTCTGCTGGTT-3' (forward)

(NM_007742.4)	5’-TTCGGGGCTGCGGATGTTCTCAAT-3’ (reverse)
SMAα (NM_007392.3)	5’-CTACTGCCGAGCGTGAGATTGTCC-3’ (forward) 5’-CTGGGTGCGAGGGCTGTGAT-3’ (reverse)

3.9. **H_2S production measurement**

The H_2S production rate of liver tissues was determined with a methyl blue method (Yang et al., 2008; Yang et al., 2018). Briefly, the live tissues were weighed and homogenized in ice-cold 50 mM potassium phosphate buffer (pH 6.8). The flasks contained the reaction mixture, including 100 mM potassium phosphate buffer, 10 mM *L*-cysteine, 2 mM pyridoxal 5’-phosphate, 10% wt/vol liver tissue homogenate. A 1.5 ml Eppendorf tube containing a 0.5 mL trapping solution (1% zinc acetate) with a piece of filter paper (2 cm × 2.5 cm) was left inside each flask, which was flushed with N_2 and incubated at 37^{o}C for 1.5 h. The reaction was stopped by adding 0.5 mL of 50 % trichloroacetic acid to the flask. Afterward, the contents of center wells were then transferred to test tubes containing 3.5 mL water, and then 0.5 mL of 20 mM N, N-diethyl-p-phenylenediamine in 7.2 M HCl and 0.5 mL of 30 mM $FeCl_3$ in 1.2 M HCl was added into the test tubes. The methylene blue generated from the reaction was determined at 670 nm using a FLUOstar OPTIMA microplate spectrophotometer (BMG Lab-Tech). The H_2S produced from each reaction was represented with a standard curve of NaHS and expressed in nanomole per gram per minute (nmol/g/min).

A lead sulfur method was also used to analyze the H_2S generation from HepG2 cells (Hine et al., 2015). After various treatments, the cells were collected and sonicated in ice-cold PBS buffer without a protease inhibitor. In the well of 96-well plate, 80 µL of cell lysates with an equal amount of protein (100 µg) were mixed with 10 µL cysteine (10 mM) and 10 µL pyridoxal 5’-phosphate (2 mM), and a lead acetate paper (Sigma-Aldrich) was placed over the

well. The reaction was incubated for 2 hours at 37 °C in the dark. The intensity of the darkening on the lead acetate paper was analyzed with Image J software and relative to the capacity of H_2S gas released from NaHS standard solution.

3.10. **Statistical Analysis**

The data were expressed as means ± standard error. Statistical analysis was determined using one-way ANOVA followed by a posthoc analysis (Tukey test) where applicable. Values of $p<0.05$ were considered to be statistically significant.

4. **Results**

4.1. **FFAs or high glucose reduces CSE expression and H_2S generation**

The human hepatoma cell line HepG2 is often used for liver function and mechanistic studies (Gunn et al., 2017; Lee et al., 2016). To mimic the effect of excessive nutrition *in vitro*, HepG2 cells were incubated with FFAs or HG for 72 hours. We first found that the protein expression of CSE was significantly lower in FFAs or HG-treated cells in comparison with the control (**Fig. 13*A***). CSE is a major H_2S-generating enzyme in liver cells (Fiorucci et al., 2006; Sen et al., 2012). We further investigated the effect of FFAs or HG on endogenous H_2S production with lead acetate paper. As presented in **Fig. 13*B***, the H_2S production rate was significantly decreased by 55% when HepG2 were incubated with FFAs or HG. These data suggest that CSE/H_2S signaling is downregulated in HepG2 cells under excessive nutrition conditions.

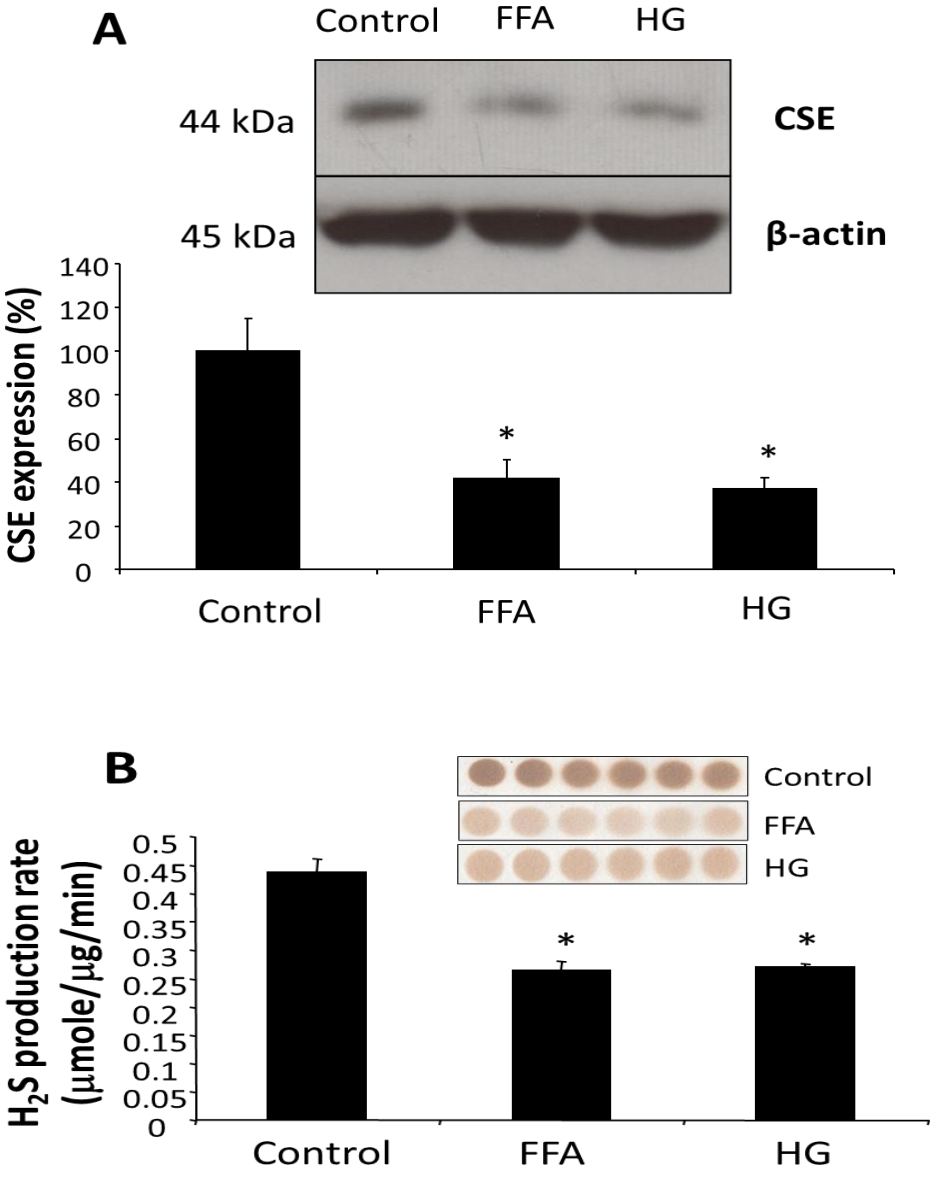


Fig. 13. Free fatty acids (FFAs) or high glucose (HG) reduces CSE protein expression and H_2S production. After incubation with FFAs (5 μL/mL) or glucose (25 mM) for 72 hours, HepG2 cells were collected for the detection of CSE protein expressions by Western blotting (***A***) and H_2S production by

lead acetate paper (***B***). The intensity was quantified using image J software. *, $p < 0.05$ vs. control cells. The data were from four independent experiments.

4.2. **H_2S inhibits FFAs or HG-induced intracellular lipid accumulation**

To explore whether H_2S influences lipid accumulation in HepG2 cells, we detected lipids by staining cells with Oil Red O. Exposure of HepG2 cells to FFAs or HG led to a significant increase in intracellular lipids, while co-incubation with NaHS (30 μM) prevented the lipid deposition (**Fig. 14*A*-14*C***). We also examined the effect of *L*-cysteine (an H_2S precursor) or DL-propargylglycine (PPG, a CSE inhibitor) on FFAs-induced lipid accumulation in HepG2 cells (**Fig. 14*D***) (Bekpinar et al., 2014; Carter et al., 2016). The addition of cysteine (1 mM) produced a similar effect as H_2S on attenuating FFAs-induced lipid accumulation, while the supply of PPG (10 mM) enhanced the stimulatory role of FFAs on lipid accumulation. Lipid accumulation was not affected by the treatment of NaHS, cysteine, or PPG alone. The altered lipid accumulation was not due to the change of cell survival since incubation of the cells with FFAs, HG, and/or NaHS did not affect cell viability and cell number (**Fig. 15*A*-15*D***).

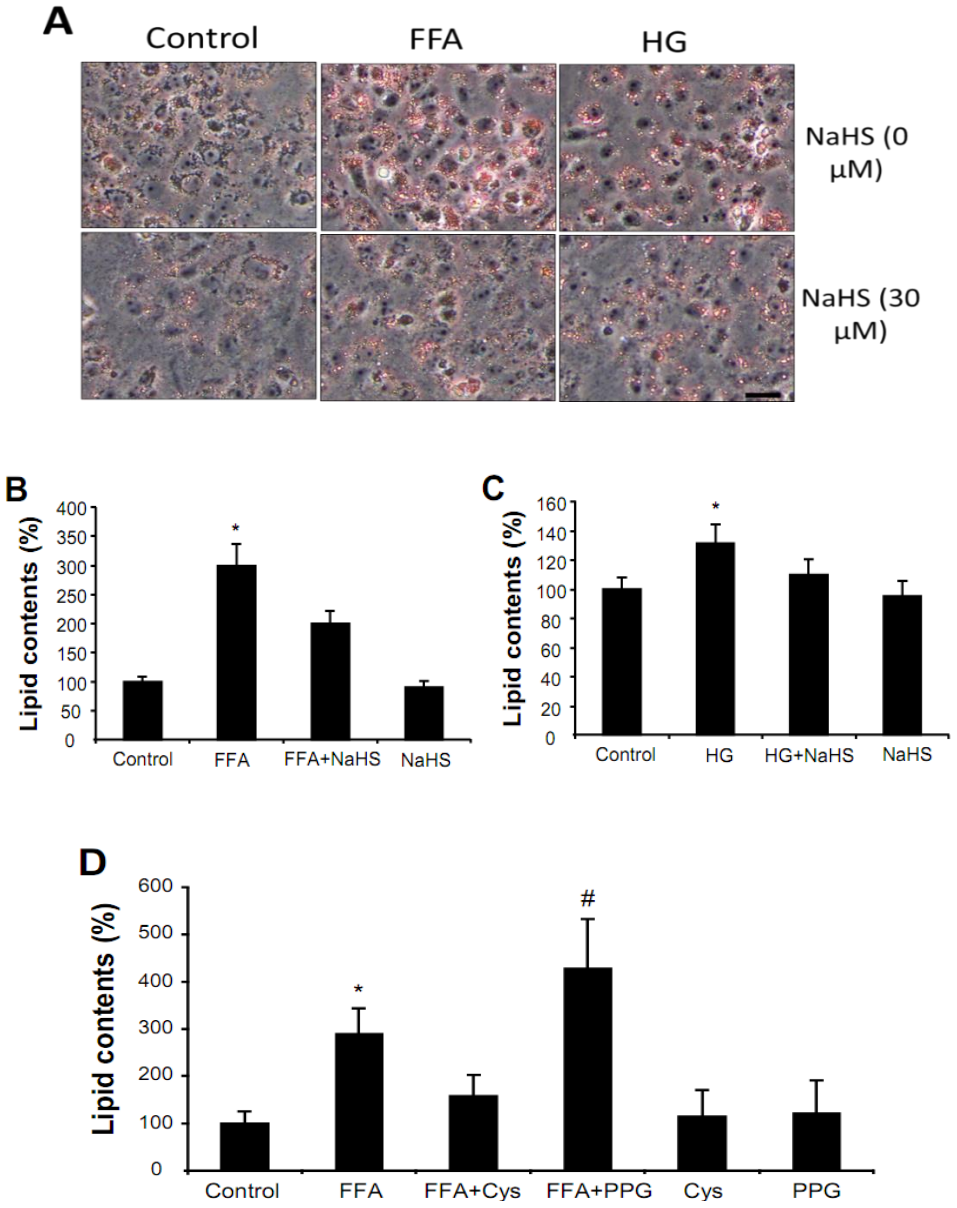


Fig. 14. H_2S reverses FFAs or HG-induced lipid accumulation. After the cells were incubated with FFAs (5 μL/mL) or glucose (25 mM) in the presence or absence of NaHS (30 μM), cysteine (1 mM), or PPG (10 mM) for 72 hours, the cells were subject to Oil Red O staining for observation of lipid accumulation (***A***), and the quantitative analysis result of lipid accumulation was shown in ***B***, ***C***, and ***D***. Scale bar in ***A***: 20 μm. *, $p < 0.05$ vs. control cells; #, $p < 0.05$ vs. FFA group or HG group. The data were from four independent experiments.

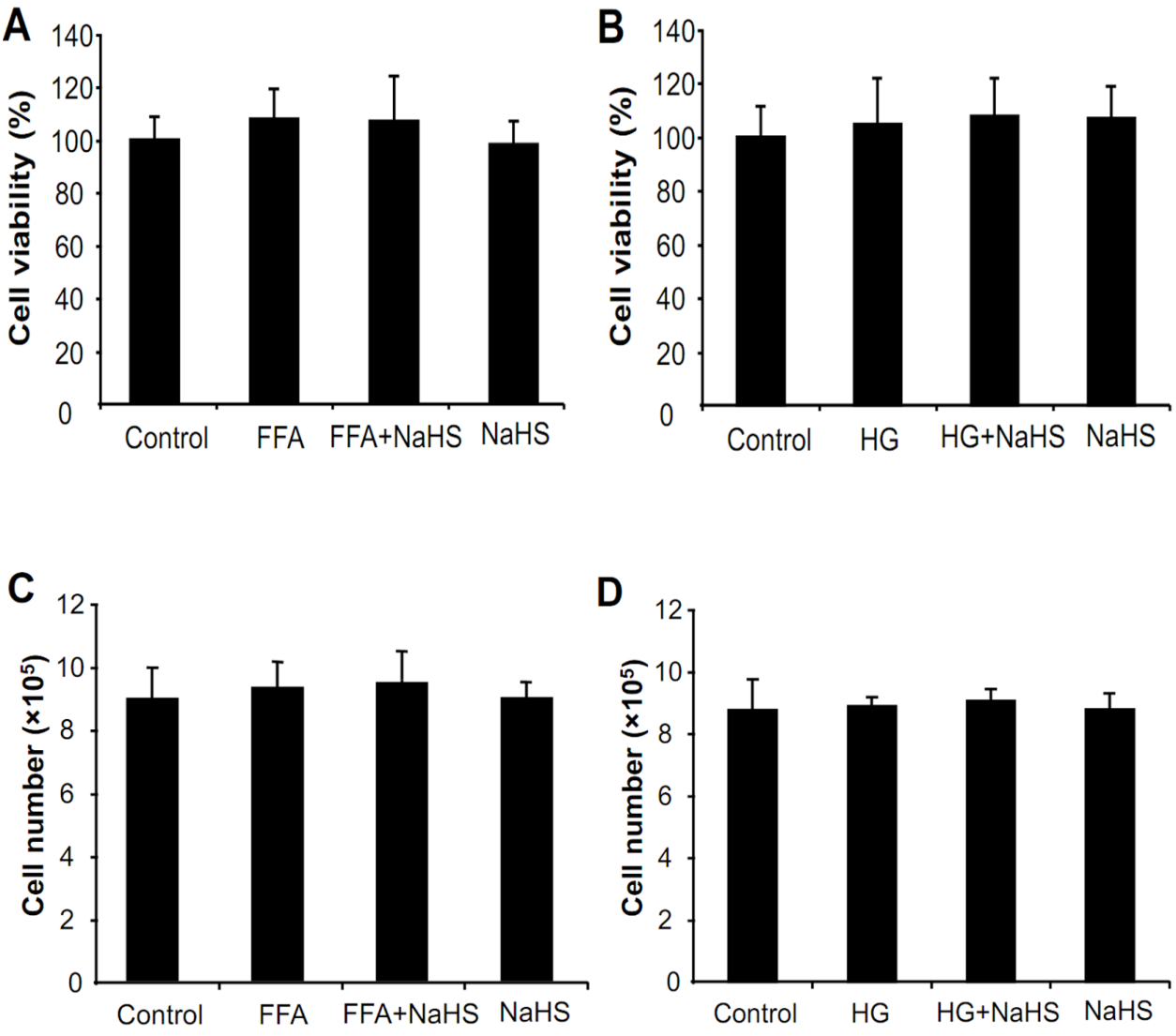


Fig. 15. FFAs or HG does not affect the cell growth of HepG2 cells. After the cells were incubated with FFAs (5 μL/mL) or glucose (25 mM) in the presence or absence of NaHS (30 μM) for 72 hours, the cells were subject to cell viability determination by MTT assay (***A*** and ***B***) or cell number counting by a hemocytometer (***C*** and ***D***). The experiments were repeated at least 3 times.

4.3. **H_2S attenuates FFAs or HG-induced acetyl-CoA contents and lipogenesis-related genes**

Acetyl-CoA is a key molecule participating in the metabolism of lipid and glucose (Cai et al., 2011; Shi et al., 2015). We found that FFAs or HG incubation significantly induces the accumulation of acetyl-CoA, while exogenously applied NaHS limites this increase in acetyl-CoA by 24.3% and 18% in the presence of FFAs and HG, respectively (**Fig. 16*A*** and **16*B***). NaHS alone slightly reduced acetyl-CoA level but without statistical significance when compared with the control cells. To explore the regulatory mechanism of H_2S on acetyl-CoA contents, we then investigated the alteration of acetyl-CoA metabolism-related proteins by Western blotting. As described in **Fig. 17*A***, the protein expression of FAS, ACSL1, ATP-CL, AceCS1 and ACC in HepG2 cells were significantly upregulated by FFA treatment, while exogenously applied NaHS prevented the stimulatory role of FFAs on the expression of these protein. The mRNA expression of sterol-regulatory-element-binding-protein-1 (SREBP1), a critical transcription factor for lipogenesis-related genes, was increased by 7 times in the presence of FFAs, and co-incubation with NaHS attenuated the increase in SREBP1 mRNA expression by 55% (**Fig. 17*B***). In contrast, the FFAs-stimulated transcription of several lipolysis-related genes, including acyl-CoA oxidase-1 (ACOX1), CpT1A and PPARα, was not altered by NaHS treatment. The mRNA expression of apolipoprotein-B (ApoB), a molecule responsible for lipid transportation, was remarkably induced by FFAs, an effect which was potentiated by NaHS. We also observed that FFAs promoted the transcription of inflammation-related genes (interleukin-6 (IL6) and tumor necrosis factor-alpha (TNFα)) and fibrosis-related genes (smooth muscle actin-α (SMAα) and collagen type-I (COL1A1)), which were all clearly abolished by NaHS co-treatment. The mRNA expression of all these genes was not changed by NaHS alone.

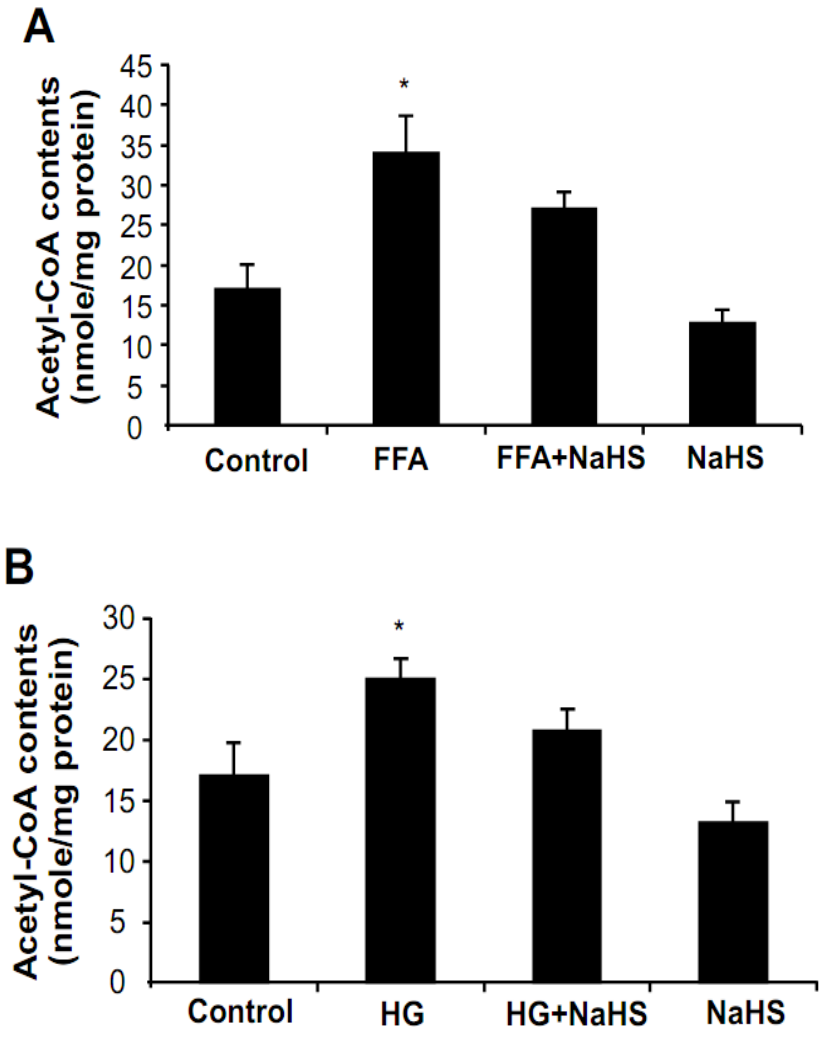


Fig. 16. H_2S attenuates FFAs or HG-induced Acetyl-CoA contents. After the cells were incubated with 5 μL/mL FFAs (***A***) or 25 mM glucose (***B***) in the presence or absence of NaHS (30 μM) for 72 hours, the cells were subject to acetyl-CoA measurement by a coupled enzyme assay. *, $p < 0.05$ vs. all other groups. The data were from three independent experiments.

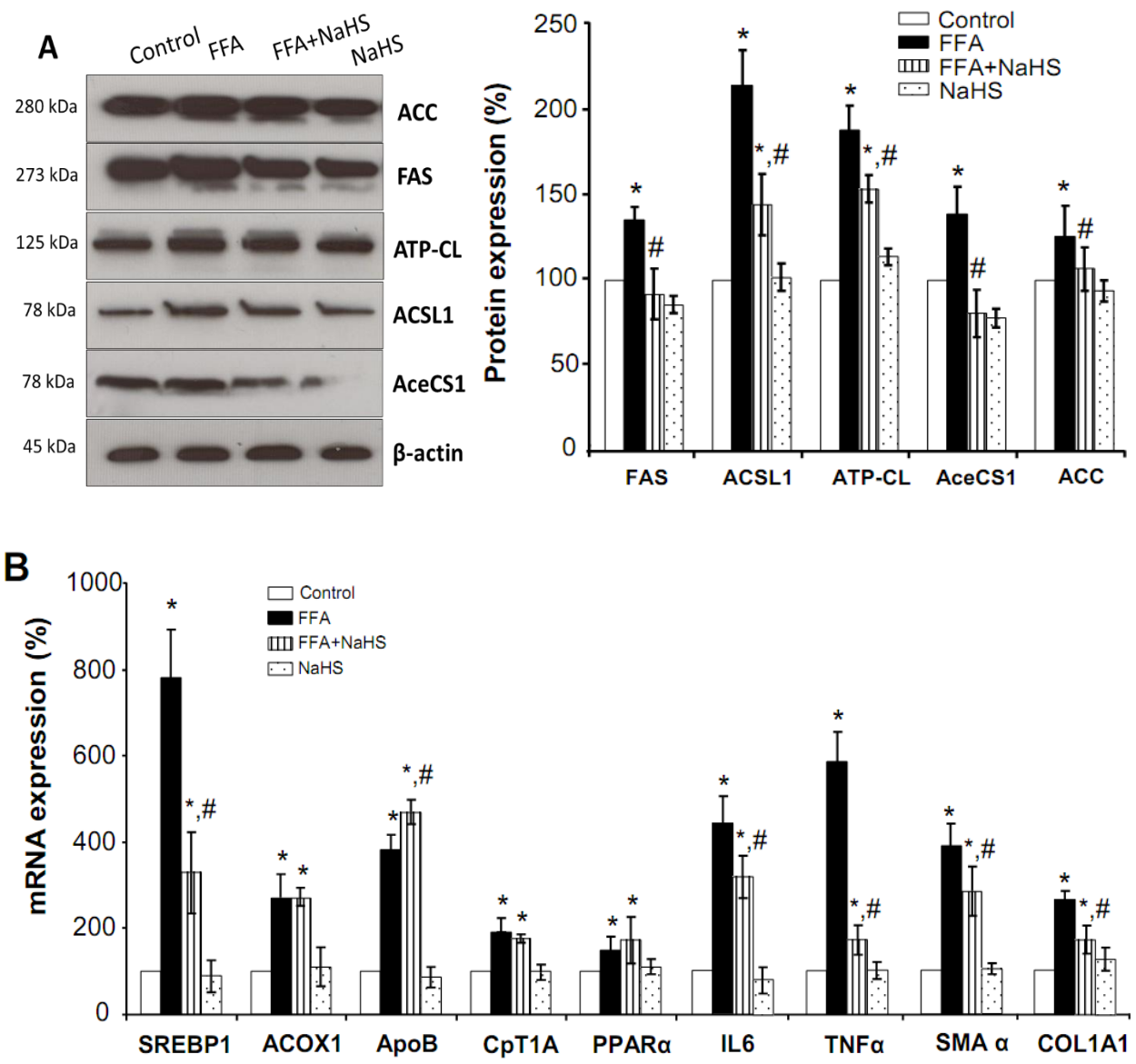


Fig. 17. H_2S suppresses the expressions of lipogenesis, inflammation, and fibrosis-related genes.

After the cells were incubated with 5 μL/mL FFAs with or without 30 μM NaHS for 72 hours, the cells were subject to Western blotting analysis of FAS, ACSL1, ATP-CL, AceCS1, and ACC protein expression (***A***) or real-time PCR analysis of the mRNA expressions of SREBP1, ACOX1, ApoB, CpT1A, PPARα, IL6, TNFα, SMAα, and COL1A1 (***B***). *, $p < 0.05$ vs. control; #, $p < 0.05$ vs. FFA group. The data were from three independent experiments.

4.4. **CSE deficiency deteriorates CD-diet-induced fatty liver**

We next determined the effects of endogenous H_2S on hepatic steatosis using a mouse model of CD diet-induced NAFLD (Sutti et al., 2015; Tobita et al., 2018). Feeding CD-diet for 4 weeks did not alter the bodyweight of WT mice but resulted in a significant decrease in CSE-KO mice (**Fig. 18*A***). CD-diet feeding led to lower food intake (**Fig. 18*B***) and higher glucose level (**Fig. 18*C***) in both mice, and the liver/body weight ratio was not changed by either CSE deficiency or CD-diet feeding (**Fig. 18*D***). CD-diet induced a substantial increase in plasma ALT level in CSE-KO mice but not in WT mice, while the AST level was slightly higher in CD-diet-fed CSE-KO mice without statistical significance when compared with all other groups (**Fig. 18*E***). Compared with the individual group with the control diet, CD-diet increased the hepatic acetyl-CoA levels in both groups with more significance in CSE-KO mice (**Fig. 18*F***). To visualize the change of lipid accumulation in mice, liver tissues were stained with H&E and Oil Red O (**Fig. 19*A***). Histological examination of liver sections revealed some hepatocyte swelling, and larger vacuoles were prominent in the livers after 4-weeks' CD-diet feeding (**Fig. 19*A***). However, the hepatic lipid deposition and liver TG were significantly greater in CSE-KO mice than WT mice. The livers of the normal control group showed no significant pathological changes irrespective of CSE deficiency, except higher levels of liver TG in CSE-KO mice. Moreover, CSE deficiency promoted an increase in CD-diet-induced liver TG levels, as well as plasma cholesterol and LDL levels in mice (**Fig. 19*B*** and **Fig. 19*C***). We further noticed that hepatic expression of CSE protein and endogenous H_2S production were significantly down-regulated by CD-diet, while CSE protein was not detected and H_2S generation was inhibited by 94% in CSE-KO liver (**Fig. 19*D*** and **19*E***). The hepatic protein expression of ACC, FAS, and

ACSL were significantly higher in CD-diet-fed CSE-KO mice when compared with CD-diet-fed WT mice (**Fig. 19*D***). CD-diet significantly induced the liver mRNA expression of many genes related to lipogenesis (SREBP1), lipolysis (ACOX1), lipid transportation (ApoB), inflammation (IL6 and TNFα), and fibrosis (SMAα and COL1A1) in both mice (**Fig. 19*F***). The knockout of CSE further enhanced the stimulatory role of CD-diet on the transcription of SREBP1, IL6, TNFα, COL1A1, and SMAα but not ACOX1. In contrast, the mRNA expression of ApoB was lower in CSE-KO liver when compared with WT liver when fed with CD-diet. These results indicate that CSE/H_2S is essential for inhibiting lipid synthesis and protecting from inflammation and fibrosis in mouse liver under CD-diet feeding.

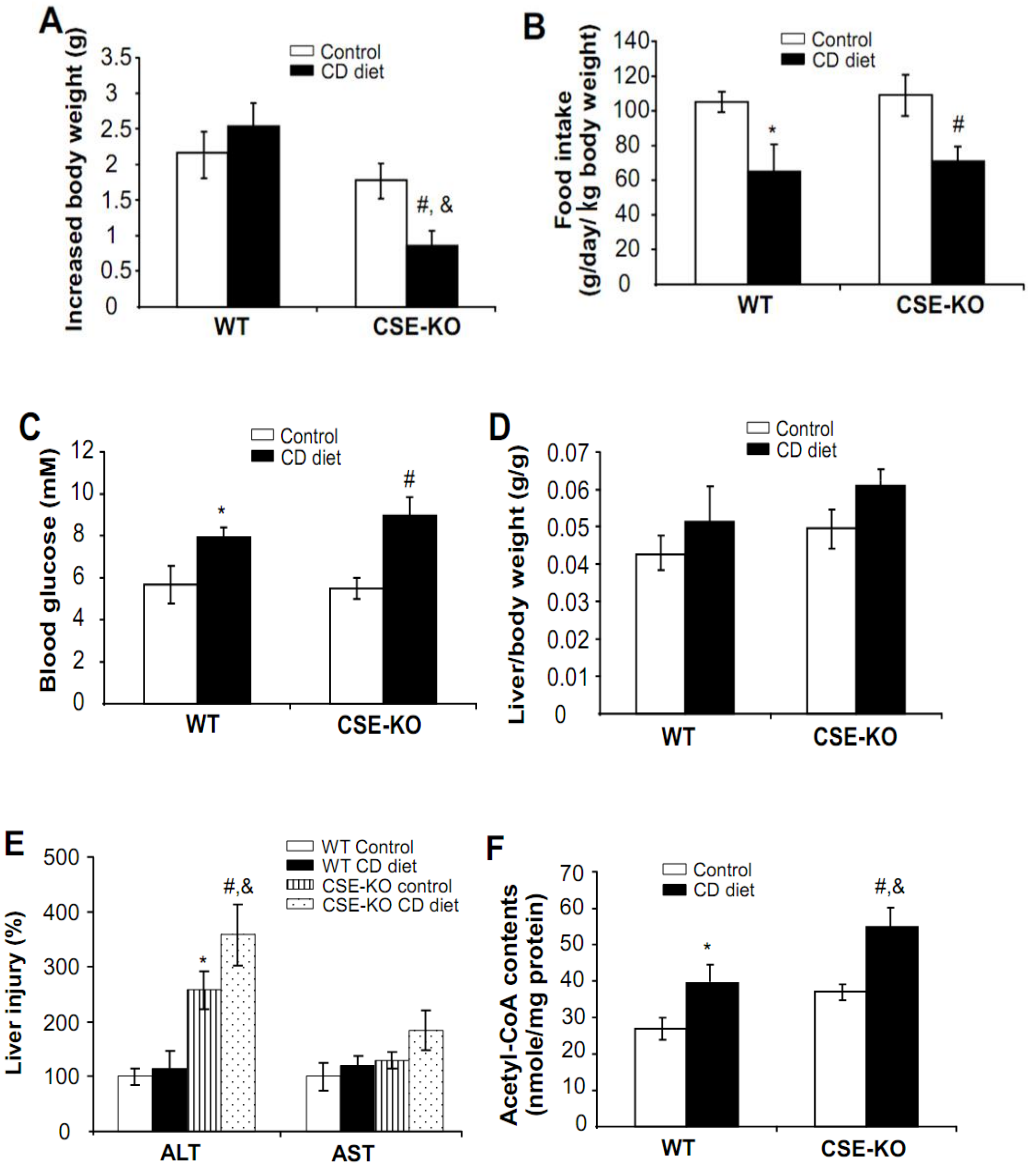
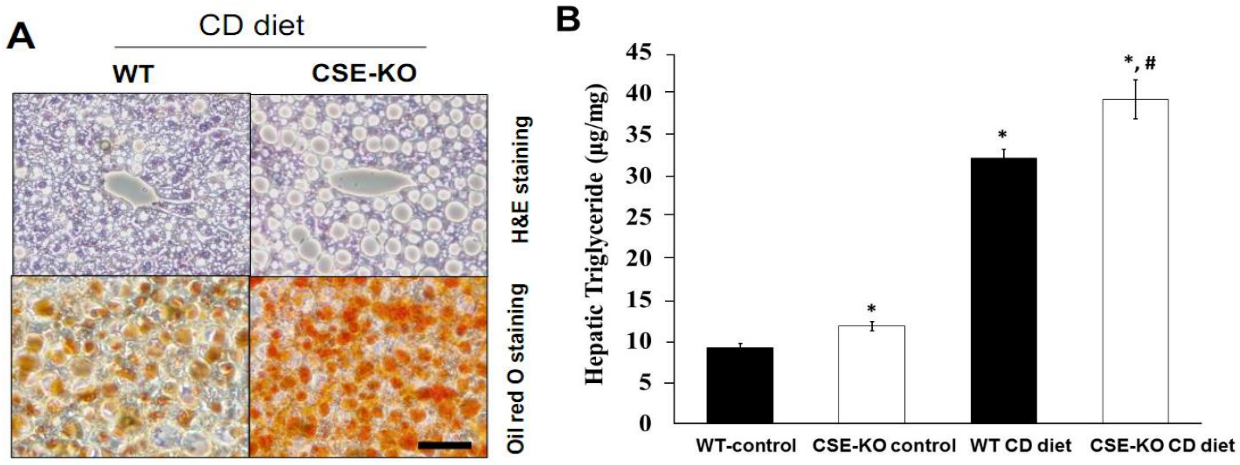
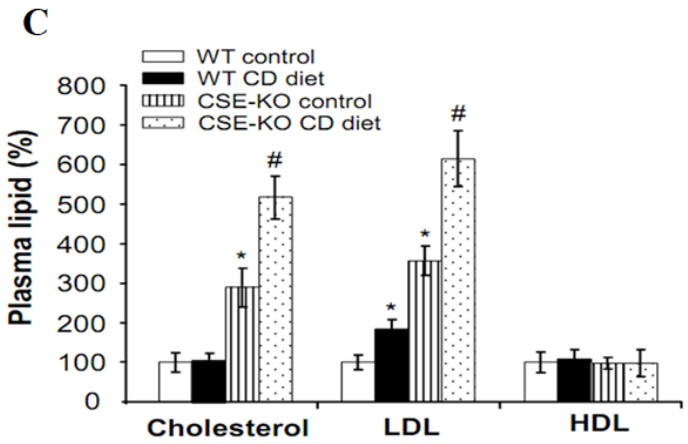
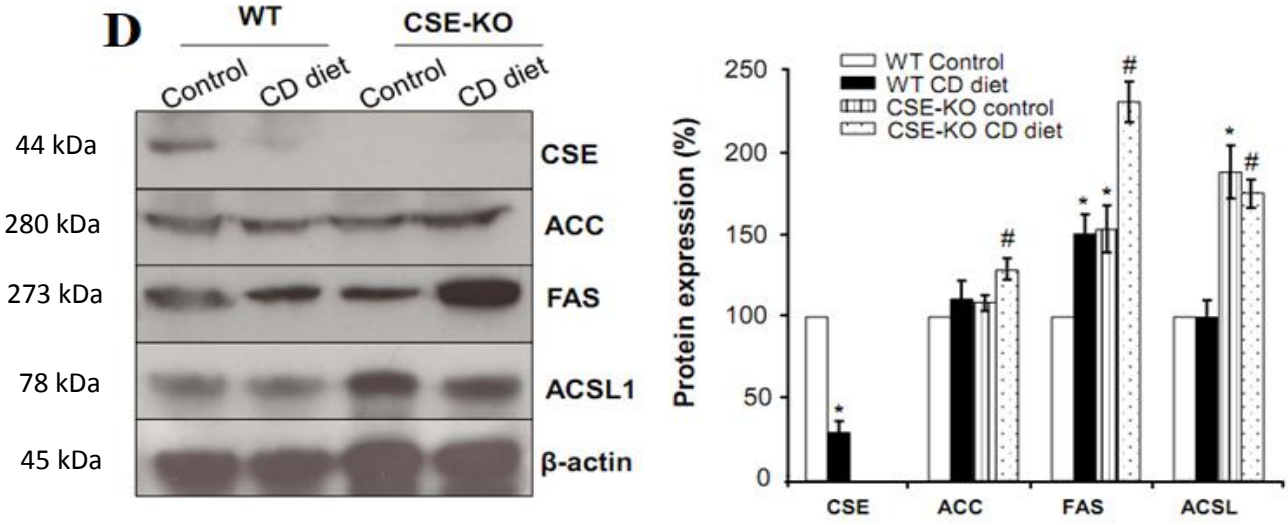


Fig. 18. CSE deficiency aggravated CD-diet-induced liver damage and acetyl-CoA contents in mice. Both 12-week male WT and CSE-KO mice were fed with CD diet or control diet for 4 weeks, afterward, the body weight (***A***), food intake (***B***), blood glucose (***C***), liver to body weight (***D***), plasma ALT and AST level (***E***), and liver acetyl-CoA contents were analyzed. *, $p < 0.05$ vs. WT control mice; #, &, $p < 0.05$ vs. WT-CD group. 5-13 mice were used in each group.


A
CD diet
WT
CSE-KO
H&E staining
Oil red O staining
B
Hepatic Triglyceride (μg/mg)
45
40
35
30
25
20
15
10
5
0
*
*
*, #
WT-control
CSE-KO control
WT CD diet
CSE-KO CD diet


C
WT control
WT CD diet
CSE-KO control
CSE-KO CD diet
Plasma lipid (%)
800
700
600
500
400
300
200
100
0
Cholesterol
LDL
HDL


D
WT
CSE-KO
Control
CD diet
Control
CD diet
44 kDa
280 kDa
273 kDa
78 kDa
45 kDa
CSE
ACC
FAS
ACSL1
β-actin
Protein expression (%)
250
200
150
100
50
0
WT Control
WT CD diet
CSE-KO control
CSE-KO CD diet
CSE
ACC
FAS
ACSL

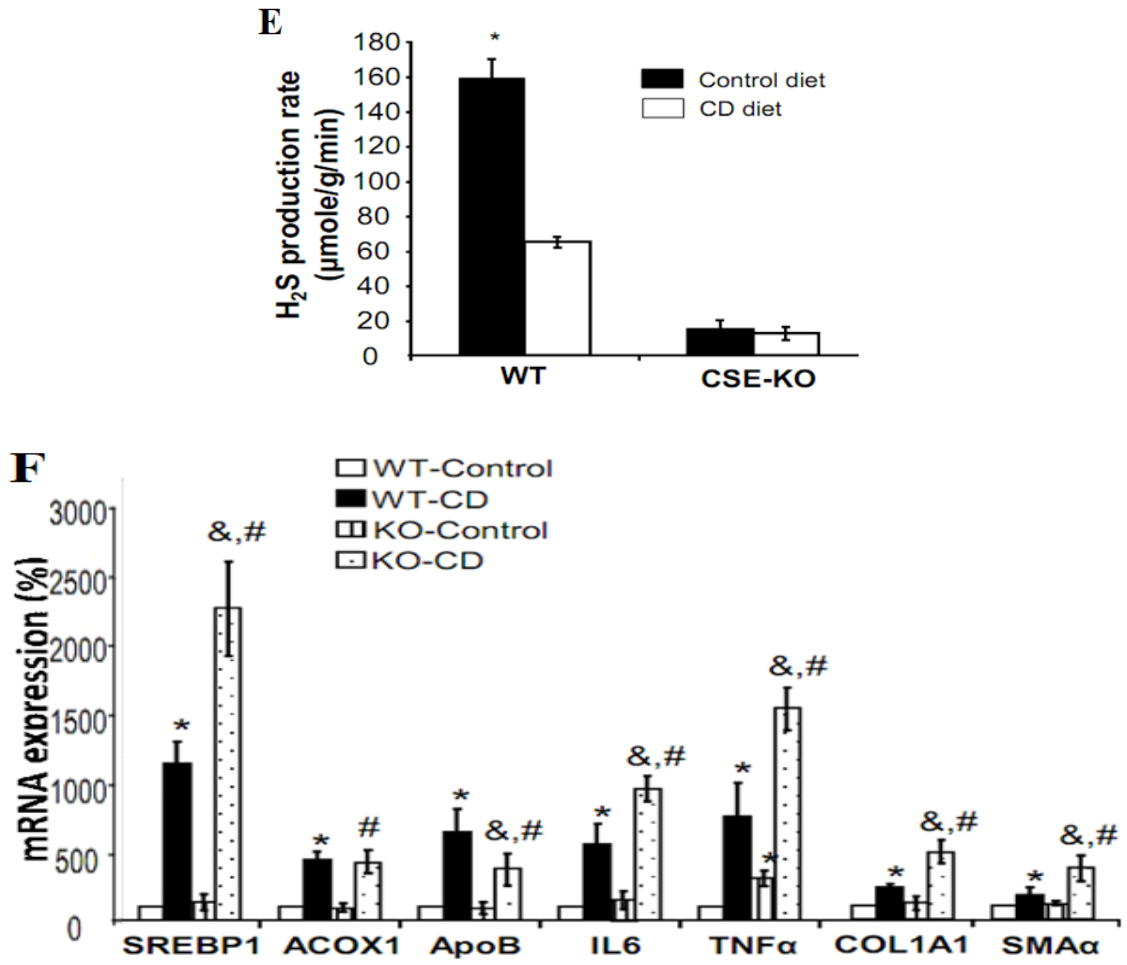


Fig. 19. CSE deficiency deteriorates CD-diet-induced fatty liver. ***A***, Representative photomicrographs of H&E and Oil red O staining of liver sections under CD diet. Scale bar: 100 μm. ***B,*** Liver TG level. *, $p < 0.05$ vs. WT control mice; #, $p < 0.05$ vs. all other groups. ***C***, Plasma lipid profile. *, $p < 0.05$ vs. WT control mice; #, $p < 0.05$ vs. all other groups. ***D***, Increased expression of lipogenesis-related proteins in liver tissues by CD diet. *, $p < 0.05$ vs. WT control mice; #, $p < 0.05$ vs. WT-CD group. ***E***, Reduced liver H_2S production by CD diet. *, $p < 0.05$ vs. all other groups. ***F***, Induced mRNA expressions of lipogenesis, inflammation, and fibrosis-related genes in liver tissues by CD diet. *, $p < 0.05$ vs. WT control mice; #, &, $p < 0.05$ vs. WT-CD mice.

5. **Discussion**

The liver is a central organ governing body energy metabolism. Excessive caloric intake often leads to increased fatty acid biosynthesis, β-oxidation, and lipid secretion in the liver. Various animal models are used to elucidate the pathophysiological mechanisms of liver diseases. The HFD and methionine and/or choline-deficient diet are the two most widely used dietary models for NAFLD (Luo et al., 2019). With these models, the endogenous formation of H_2S has been shown to be impaired in liver tissues (Luo et al., 2014; Wang et al., 2017). However, the specific role of H_2S on lipid metabolism and particularly the molecular mechanism by which this gasotransmitter influences acetyl-CoA remains undiscovered.

Acetyl-CoA is a key molecule involved in the metabolism of lipids, glucose, and proteins (Cai et al., 2011; Shi et al., 2015). Under normal dietary conditions, acetyl-CoA is oxidized through the citric acid cycle to generate energy. While after excessive meal stress of HG or HFD, acetyl-CoA is mostly used for the synthesis of fatty acids (Shi et al., 2015). Here we showed that CSE/H_2S system was negatively correlated with intracellular acetyl-CoA contents and lipid accumulation induced by FFAs/HG/CD-diet, indicating that H_2S may attenuate lipid accumulation by targeting acetyl-CoA metabolism. To investigate the possible mechanisms underlying the altered acetyl-CoA contents by H_2S, we analyzed the effects of H_2S on acetyl-CoA metabolism-related enzymes. ATP-CL and AceCS1 help the synthesis of acetyl-CoA in the cytosol (Alves-Bezerra et al., 2017; Zhao et al., 2018). FAS and ACC are important enzymes for converting acetyl-CoA to palmitate, and ACSL1 catalyzes the ligation of the fatty acid to CoA to form fatty acyl-CoA (Fiorucci et al., 2006; Mehal W., 2018). All these proteins are critical for *de novo* lipogenesis. Wu et al. reported that FAS may be a therapeutic target for treating hepatic steatosis and hyperlipidemia in mouse model (Wu et al., 2011)**.** Further, Mao et al. found

that liver-specific ACC-KO mice showed a significant reduction in malonyl-CoA levels and liver TG contents (Mao et al., 2006). Furthermore, Parkes et al. determined that overexpression of ASCL1 increases lipid accumulation in HepG2 cells and mouse livers (Parkes et al., 2006). Kuriyama et al. mentioned that ASCL1 expression increased in obese and hyper-triglyceridemic rats with fatty livers (Kuriyama et al., 1998). The Abrogation of the ATPCL enzyme in the liver of leptin-receptor- deficient mice protects against liver steatosis and improves glucose metabolism (Wang Q et al., 2009). Our data demonstrated that CSE/H_2S system interfered with the expression of these proteins under FFAs incubation or CD-diet feeding. It is possible that H_2S maintains the level of acetyl-CoA by targeting these proteins. H_2S may also affect acetyl-CoA metabolism by an enzyme, termed as H_2S *S*-acetyltransferase (EC 2.3.1.10), which has been reported to convert acetyl-CoA and H_2S into CoA and thioacetate (Brady et al., 1954). When the endogenous H_2S level is lower, H_2S *S*-acetyltransferase would not catalyze acetyl-CoA. We further found that CSE/H_2S system inhibited the expression of SERBP1 but had no effect on PPARα. As both transcription factors involved in lipid metabolism, SREBP1 primarily enhances the transcription of fatty acid synthesis-related genes, while PPARα can effectively activate the expression of genes involved in fatty acid β-oxidation (Huang et al., 2014). CPT-1 facilitates the entry of fatty acids into the mitochondria for ACOX1-mediated β-oxidation, while both genes of CPT1 and ACOX1 were not changed by CSE/H_2S system (Chen et al., 2017). These data suggest that the inhibitory role of H_2S on lipid accumulation could be mostly due to lower *de novo* lipid synthesis over higher β-oxidation. Although the molecular mechanisms which connect these processes have yet to be adequately studied, the importance of H_2S to liver health suggests potential drug development for therapeutic treatment. The present study was designed to investigate the role of CSE/H_2S in hepatic acetyl-CoA and lipid metabolism using HepG2 cells

under various stress conditions of FFA or HG or mouse liver isolated from both WT mice and CSE-KO-mice under CD-diet.

The liver is a major organ producing endogenous H_2S (Mustafa et al., 2009; Sen et al., 2012). The three H_2S-producing enzymes, including CBS, CSE, and 3MST, are all expressed in liver tissues, while they make different contributions to endogenous H_2S production. Mani et al. demonstrated that HFD feeding significantly reduced CSE expression in WT mice, but not CBS expression, decreasing H_2S production in the liver of WT-mice. Besides, CSE-KO mice fed-HFD exhibited lower CBS expression, which further lowered H_2S production in CSE-KO mice, but the mechanisms by which HFD reduces CBS expression still unknown (Mani et al., 2015). It has been reported that the expression of CSE is more abundant than CBS, and the absence of CSE diminishes the majority of H_2S production in mouse liver (Fiorucci et al., 2006; Mani et al., 2014). This evidence suggests that CSE acts as the main H_2S-producing enzyme in hepatic tissues. Here we further validated that H_2S production was reduced by more than 90% in CSE-KO liver when compared with WT liver. Incubation of the cells with FFAs or feeding WT mice with CD-diet significantly inhibited H_2S production by attenuating CSE expression. This is consistent with a previous study showing that glucose deprivation stimulates CSE expression (Ju et al., 2015). In response to starvation or consumption of excess calories, CSE expression and H_2S production can be precisely regulated for cellular energy maintenance. A deeper understanding is needed regarding the mechanisms whereby nutritional status regulates CSE/H_2S signals within the liver.

A methionine and choline-deficient diet was often used to induce non-alcoholic steatohepatitis. Deficiency of both methionine and choline inhibits the synthesis of phosphatidylcholine and blocks hepatocyte secretion of lipids in most species (Luo et al., 2014;

Wang et al., 2017). Methionine is a sulfur-containing amino acid in mammals and acts as the precursor of cysteine and H_2S generation in the transsulfuration pathway (Distrutti et al., 2008). To determine the special role of H_2S on lipid deposition in hepatocytes and avoid the disturbance of methionine on CSE/H_2S system, we used a high-fat choline-deficient-0.1% methionine diet (CD). In the present study, we determined that the deficiency of CSE in the liver diminishes liver ability to metabolize the TG leads to lipid accumulation in the liver. We observed extreme lipid accumulation in CSE-KO liver tissues under CD-diet feeding, indicating that the lack of CSE negatively impacts lipid metabolism. Both WT and CSE-KO mice were equal in terms of growth models under a regular rodent diet (Yang et al., 2008). In this study, the body weight of WT mice was not significantly changed by the CD diet, while CD-diet feeding caused bodyweight loss in CSE-KO mice. We reported previously that H_2S is essential for adipogenesis in white adipose tissues, which points to the possibility that the lower body weight in CSE-KO mice would be due to less fat tissues (Yang et al., 2018). H_2S may perform a double role in regulating lipid metabolism in liver and fat tissues. The evidence from the present study and also other reports suggest that H_2S is able to decrease lipid synthesis in liver tissue but stimulate lipid formation in fat tissues (Mani et al., 2015; Wu et al., 2015; Yang et al., 2018). By doing so, it is hypothesized that H_2S may also affect lipid secretion from hepatocytes. ApoB is the main lipoprotein responsible for the assembly and transportation of VLDL and LDL (Lee et al., 2016). We noticed that H_2S exacerbated the stimulatory role of FFAs-induced ApoB mRNA expression, while CSE deficiency attenuated the CD-diet-triggered increase of ApoB transcription. It can be inferred that H_2S may partially suppress hepatic lipid accumulation by promoting their secretion via ApoB. Consistent with our finding, it has been reported that H_2S promoted lipid efflux by HepG2 cells through inhibiting the expression of proprotein convertase subtilisin/kexin type 9

and LDL receptor degradation (Xiao et al., 2019). The higher level of plasma lipid levels from CSE-KO mice could be due to the extreme hepatic lipid accumulation and the alteration of other lipid transportation systems, which deserves further studies.

Previous studies mentioned that normal diet-fed CBS-KO mice had increased liver TG level (Hamelet et al., 2007; Namekata et al., 2004). Similarly, we found that CSE-KO mice-fed normal diet show a significant increase in hepatic TG content compared to WT-mice. Moreover, CSE-KO mice exhibited significantly higher levels of hepatic TG compared to the WT-mice fed CD-diet. In combination, both CSE and CBS enzymes competed in the same reverse-transsulfuration pathway and deficiency of any affect's liver lipid metabolism. Hepatic TG content, plasma LDL, cholesterol, TG and nonesterified fatty acid levels were markedly elevated in CBS-KO mice (Namekata et al., 2004). Interestingly, we found that CSE-KO under CD-diet and control diet significantly increased plasma LDL and cholesterol levels than WT-mice. Furthermore, hepatic TG levels in CD-diet and control-diet-fed CSE-KO mice were significantly higher than WT mice. Additionally, apoB was significantly higher in WT-mice fed-CD-diet than CSE-KO mice. Lower levels of apoB can cause dyslipidemia as a consequence of impaired clearance of LDL and increase hepatic TG content which explains the increase in liver TG and plasma LDL levels in CSE-KO mice. This observation supports the involvement of hepatic TG in fatty liver development in CSE-KO mice.

WT-mice can catabolize and tolerate extreme nutrition consumption of lipid and CD-diet, proved by the normal levels of plasma AST and ALT in WT-mice when fed with control or CD-diet. However, CSE-KO mice fed with control and CD-diet obtained more plasma AST and ALT levels. Obviously, CSE-KO mice show slight differences in liver function that WT-mice under normal diet, suggesting that in the long term CSE-KO mice may develop fatty liver and

cardiovascular diseases (Mani et al., 2013). Once the normal diet shifted to CD-diet, CSE-KO mice showed a significant increase in the plasma levels of AST and ALT. Both AST and ALT are predominantly expressed in the liver, but their plasma elevates are indicative of liver injury and damage. These results illustrate that significant increased in plasma ALT and AST indicates abnormal liver lipid metabolism and liver dysfunction in CSE-KO mice.

Initial metabolic disturbance can cause inflammation, which further drives the development of NAFLD (Dumeus et al., 2015; Sutti et al., 2015). Intraperitoneal injection of inflammatory cytokine to mice dramatically stimulated the accumulation of fat and induced-fatty liver (Endo et al., 2007). In the present study, real-time PCR analysis of gene expression revealed that FFAs or CD diet induced more transcription of the genes encoding hepatic IL6 and TNFα, two pro-inflammatory cytokines, this was attenuated by exogenously applied NaHS treatment but deteriorated by CSE deficiency. Apart from inflammation, fibrosis is believed to be another essential mediator in the progression of NFALD in rat model (Li et al., 2017). During steatohepatitis progression, the increase in the level of pro-inflammatory cytokines could induce SMAα and COL1A1, two markers for myofibroblasts in rats (Beyaz et al., 2016; Luo et al., 2014). We also observed that the CSE/H_2S system was negatively correlated with the expression of SMAα and COL1A1. In a carbon tetrachloride-induced liver injury model, CSE deficiency increased pro-inflammatory cytokines in the liver and exacerbated acute hepatitis and fibrosis (Ci et al., 2017). Ci et al. reported that upregulation in pro-inflammatory cytokines including TNFα in CSE-deficiency mice after injecting carbon tetrachloride was followed by a significant rise in SMAα and histopathologic changes in the liver. However, pre-treatment with H_2S donor dramatically attenuated most of these imbalances (Ci et al., 2017). Many, natural sulfur-containing agents have been shown to inhibit the pro-inflammatory and pro-fibrogenic properties

through the production of H_2S (Anandasadagopan et al., 2017; Zhang et al., 2017). Anandasadagopan et al. reported that level of free radical-induced liver damage and the expression of TNF-α was increased during hepatotoxicity induced by Cr (VI), subsequent administration of S-allyl cysteine (a constituent of garlic (Allium sativum) and a sulfur-containing compound) significantly reduced the expression of TNF-α and protected the liver from the Cr (VI)-induced free radical damage through production of H_2S in rats (Anandasadagopan et al., 2017). Zhang et al. found that diallyl trisulfide (a primary organosulfur compound in garlic) significantly decreased fibrosis and attenuated oxidative stress in rat fibrotic liver (Zhang et al., 2017). These data indicated that the CSE/H_2S system can ameliorate fatty liver by controlling inflammation and fibrosis.

6. **Conclusion**

In conclusion, we proved that H_2S inhibits hepatic acetyl-CoA and lipid accumulation, and lower levels of H_2S due to CSE deficiency promoted liver steatosis in mice under excessive nutritional conditions. As shown in **Fig. 20**, hepatic CSE/H_2S signal is downregulated upon exposure to FFAs or HG, and targeting the CSE/H_2S system can mitigate the fatty liver most likely through suppressing hepatic acetyl-CoA and fatty acid synthesis.

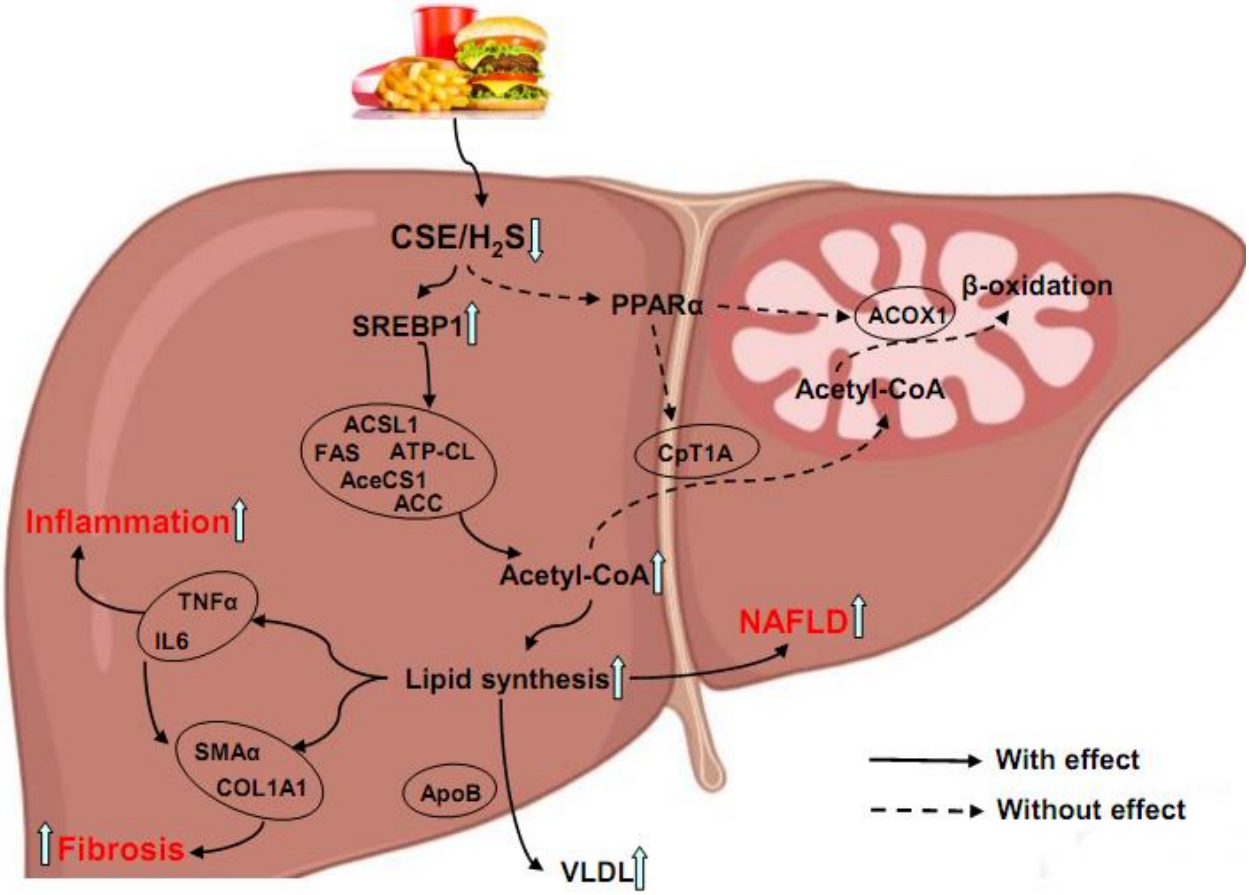


Fig. 20. The proposed mechanism underlying the protective effect of CSE/H_2S signal against hepatic steatosis and liver damage.

7. **Limitation of this study and future work**

H_2S acts as a critical regulator of metabolism and is involved in many diseases, including fatty liver diseases and cancer. The involvement of H_2S in liver health has been brought to light more recently, but the underlying mechanisms are not clear. The present study has the potential to reveal the novel mechanism for CSE/H_2S regulation of hepatic lipid metabolism.

One limitation of this study is that a fast releasing H_2S donor NaHS was used. When added to cells, one-third of H_2S is released from NaHS within seconds. This indicates that the real concentration of H_2S inside the cells will be much lower than expected. To overcome this limitation, a slow and stable H_2S-producing donor, such as GYY4137 (Yu et al., 2010) can be investigated as a potential alternative to NaHS. Also, future studies need to confirm how H_2S can modify the *de novo* lipogenesis process through cysteine post-translational modification, protein S-sulfhydration (Mustafa et al., 2009). In addition, the altered expression of *de novo* lipogenesis-related proteins by H_2S need to be explored. By altering lipid metabolism, it is also interesting to study the roles of H_2S in the development of hepatocellular carcinoma. H_2S S-acetyltransferase is an important enzyme that reacts with acetyl-CoA. Therefore, much more work is needed to figure out its gene sequence, protein function, and the regulation under normal diet, excessive calories and fasting conditions.

Future studies also need to investigate the effect of exogenous H_2S-donors in preventing the development of fatty liver diseases in mice. Moreover, many signaling pathways contribute to liver lipid metabolism (Nguyen et al., 2008). Thus, more further studies are needed to investigate if CSE/H_2S can alter any of these pathways, how and by which mechanism?

8. **References**

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