Abstract. Hydrogen sulfide (H₂S) is an endogenous gaseous signaling molecule that plays important roles in the cardiovascular system. In our previous studies, we demonstrated that H₂S regulates lipid metabolism. In the present study, we aimed to explore the mechanisms through which H₂S regulates lipid metabolism in HepG2 cells in vitro. Treatment of the HepG2 cells with H₂S inhibited the expression of proprotein convertase subtilisin/kexin type 9 (PCSK9) and increased the level of low-density lipoprotein receptor (LDLR) in a time- and dose-dependent manner. The knockdown of PCSK9 by siRNA effectively increased the levels of LDLR and 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate-labeled LDL (diI-LDL) uptake in the H₂S-treated HepG2 cells. Furthermore, the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt)-sterol regulatory element-binding proteins 2 (SREBP-2) signaling pathway was confirmed to be involved in H₂S-regulated PCSK9 expression. Notably, the HepG2 cells were incubated with 30% serum and diI-LDL for 24 h, and the results revealed that H₂S increased lipid uptake, but caused no increase in lipid accumulation. On the whole, the findings of this study demonstrate that H₂S is involved in the regulation of lipid metabolism in HepG2 cells through the regulation of the expression of PCSK9 via the PI3K/Akt-SREBP-2 signaling pathway. To the very best of our knowledge, this study is the first to report that H₂S can regulate the expression of PCSK9.

Introduction

Hydrogen sulfide (H₂S), an endogenous gaseous signaling molecule, performs important regulatory functions, such as vascular toning, leukocyte adhesion and smooth muscle cell proliferation, in the cardiovascular system. Recently, Li et al demonstrated that H₂S reduces atherosclerosis by regulating lipid metabolism (1). Exogenous H₂S can mitigate fatty liver and reduce triglyceride and total cholesterol levels in obese mice (2). Cytathiohine β-synthase (a key enzyme for the biosynthesis of H₂S) knockout mice have been reported to exhibit higher low-density lipoprotein cholesterol (LDL-C) and lower plasma levels of high-density lipoprotein cholesterol than normal mice (3). Additionally, patients with atherosclerosis exhibit reduced serum H₂S concentrations (4). However, the precise mechanisms through which H₂S regulates lipid metabolism remain unclear.

It is well established that proprotein convertase subtilisin/kexin type 9 (PCSK9) plays an important role in lipid metabolism and atherosclerosis (5). PCSK9 alters LDL-C concentrations by promoting hepatic low-density lipoprotein receptor (LDLR) degradation and decreasing the hepatic clearance of plasma LDL-C levels (6). Numerous factors regulate PCSK9 expression. For example, statin treatment leads to an increased transcription of PCSK9 (7), in contrast to berberine treatment, which decreases PCSK9 expression (8). However, to date, whether H₂S is involved in the regulation of hepatic PCSK9 expression remains unknown.

In the present study, we thus aimed to examine the effects of H₂S on the expression of PCSK9 in HepG2 cells and to elucidate the mechanisms through which H₂S regulates lipid metabolism.

Materials and methods

Reagents. Sodium hydrosulfide (NaHS) and Oil Red O were purchased from Sigma-Aldrich (St. Louis, MO, USA). Specific
monoclonal anti-PCS9 (cat. no. 55206), anti-LDLR (cat. no. 66414), anti-SREBP-2 (cat. no. 557037) and anti-SREBP-1-c (cat. no. 66875) antibodies were purchased from Proteintech (Rosemont, IL, USA). Specific monoclonal anti-protein kinase B (Akt) antibody (cat. no. BM4400) was purchased from Wuhan Boshide Bioengineering Co. (Wuhan, China). Specific monoclonal anti-phosphorylated Akt antibody (cat. no. YM3621) was purchased from ImmunoWay Biotechnology (Plano, TX, USA). Anti-β-actin antibody (cat. no. LCA01) was purchased from Aijia Biological Technology Co., Ltd. (Changsha, China). Cy3-conjugated goat anti-rabbit IgG secondary antibody (cat. no. E031620) was purchased from EarthOx Life Sciences (Millbrae, CA, USA). Dulbecco’s modified Eagle’s medium (DMEM) was purchased from HyClone (Logan, UT, USA). Fetal bovine serum (FBS) was purchased from Hangzhou Siqing Biocompany (Hangzhou, China). The Hoechst Staining kit was purchased from Beyotime Institute of Biotechnology (Jiangsu, China). 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate-labeled LDL (DiI-LDL) was purchased from Beyotime Institute of Biotechnology, Co., Ltd. (Guangzhou, China). siRNAs were synthesized by Ruibo Biotechnology Co., Ltd. (Guangzhou, China).

Cells and cell culture. The HepG2 human liver cancer cell line was obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and maintained in tissue culture bottles with DMEM supplemented with 10% heat-inactivated FBS at 37°C under an atmosphere of 5% CO₂ and 95% air. The cell culture medium was replaced with fresh medium 3 times a week.

Oil Red O staining protocol. Oil Red O staining was used to quantify intracytoplasmic lipid droplets. The HepG2 cells were seeded in a 6-well plate covered with coverslips. After the cells had become adherent, they were incubated for 12 h at 37°C in serum-free medium, treated with various concentrations (0, 50, 100 and 200 µmol/l) of NaHS (a H₂S donor) for 24 h, the cells were rinsed 3 times with PBS, fixed with 4% paraformaldehyde for 30 min, permeabilized with 0.5% Triton X-100 in PBS for 30 min, and incubated with anti-LDLR antibody (1:1,000) overnight at 4°C. The cells were rinsed 3 times for 5 min with PBS, followed by incubation with the appropriate fluorophore-conjugated secondary antibody (SA012; 1:1,000; Aijia Biological Technology Co., Ltd.) for 2 h. The nuclei were counterstained with Hoechst 33258 (250 nM; Beyotime Institute of Biotechnology). Immunofluorescence was visualized using a Nikon TE2000-U fluorescence microscope (Nikon).

DiI-LDL uptake assay. The HepG2 cells were seeded in 24-well plates. Following treatment with various concentrations of NaHS (0, 50, 100 and 200 µmol/l) for 24 h, the cells were rinsed twice with phosphate-buffered saline (PBS). Subsequently, the cells were incubated with 25 µg/ml DiI-LDL for 4 h at 37°C, followed by fixation in 3% paraformaldehyde for 20 min. The nuclei were counterstained with Hoechst 33258 (250 nM; Beyotime Institute of Biotechnology) for 5 min at room temperature. Immunofluorescence was visualized using a Nikon TE2000-U fluorescence microscope (Nikon, Tokyo, Japan).

Western blot analysis. The cells were washed twice with PBS and incubated on ice with cell lysis buffer and phenylmethanesulfonyl fluoride for 30 min, followed by cell lysis collection and centrifugation at 10,000 × g for 10 min at 4°C. The BCA protein assay kit was used for the quantification of total protein in cell lysates (Beyotime Institute of Biotechnology) according to the manufacturer's instructions. Proteins were separated by 12% SDS-PAGE and electrotransferred onto a polyvinylidene fluoride membrane. The membrane was incubated in a blocking buffer (0.1% Tween-20, 150 mM NaCl, 20 mM Tris base, pH 7.6 and 5% non-fat milk) for 2 h at 37°C and subsequently with the appropriate primary antibodies (PCS9, 1:400; LDLR, 1:1,000; SREBP-2, 1:5,000; SREBP-1c, 1:2,000; Akt, 1:400; phosphorylated Akt, 1:2,000; and β-actin, 1:1,000) in blocking buffer at 4°C overnight. The membranes were then incubated with anti-rabbit or anti-mouse horseradish peroxidase-conjugated secondary antibodies (SA009 and SA001; 1:1,000; Aijia Biological Technology Co., Ltd.) for 2 h. Proteins were visualized by chemiluminescence using AURA ECL (Aijia Biological Technology Co., Ltd.) following the manufacturer’s instructions. Image Pro-Plus 6.0 software (Media Cybernetics) was used to analyze relative protein band density.

siRNA transfection. The PCS9 mRNA sequence was retrieved from GeneBank (no. NM 199253). The target sequences of PCS9 were as follows: siPCS9 sense strand, 5'-GGC AGAGACUGAUCCACUdTdT-3' and siPCS9 antisense strand, 5'-AAGUGGAAUCAGUCUCUGCCTdTdT-3'. The sequences of the negative control siRNA were as follows: Sense strand, 5'-UAUAGCUGUGCGAGCAGGdTdT-3' and antisense strand, 5'-GUAGUGAGCGAGCUAATdTdT-3'. The HepG2 cells were seeded in 6-well plates. siRNA transfection was performed using riboFFECT™ CP (Ruibo Biotechnology Co., Ltd.) following the manufacturer's instructions. Cells were harvested for the subsequent experiments at 48 h following transfection.

Statistical analysis. Quantitative data are presented as the means ± standard error of the mean, with the significance determined by a Student's t-test or one-way analysis of variance with Tukey's multiple comparisons post hoc test. The results were plotted using GraphPad Prism 5.0 software. Differences between datasets were considered statistically significant at P<0.05.
Results

$H_2S$ inhibits PCSK9 expression in HepG2 cells. To elucidate the role of $H_2S$ in PCSK9 expression, we first incubated the HepG2 cells with various concentrations of NaHS (0, 50, 100 and 200 µmol/l) for 24 h. PCSK9 protein expression was detected by western blot analysis. As shown in Fig. 1, $H_2S$ inhibited PCSK9 expression in the HepG2 cells in a concentration-dependent manner. Subsequently, we incubated the HepG2 cells with 200 µmol/l NaHS for the indicated periods of time (0, 6, 12 and 24 h). As shown in Fig. 2, $H_2S$ inhibited PCSK9 expression in the HepG2 cells in a time-dependent manner.

$H_2S$ inhibits the expression of PCSK9, upregulating the expression of LDLR and increase lipid uptake in HepG2 cells. The above-mentioned results indicated that $H_2S$ inhibited PCSK9 expression. Thus, the LDLR levels may be increased in the HepG2 cells. To determine whether $H_2S$ inhibits the expression of PCSK9 to influence the expression of LDLR and lipid uptake, we first examined the effects of $H_2S$ on LDLR expression in the HepG2 cells. The HepG2 cells were incubated with various concentrations of NaHS (0, 50, 100 and 200 µmol/l) for 24 h. LDLR expression was detected by western blot analysis and immunofluorescence assay. As shown in Fig. 3, $H_2S$ upregulated LDLR expression in the HepG2 cells in a concentration-dependent manner. In addition, as shown in Fig. 4, red fluorescence intensity increased with the increasing NaHS concentrations. This finding indicates that $H_2S$ upregulates the LDLR protein levels.

It is well known that PCSK9 can promote LDLR degradation (9). Thus, to determine whether $H_2S$ upregulates LDLR expression by inhibiting PCSK9 expression, we first used RNA interference to suppress PCSK9 expression, and LDLR expression was detected. The results of western blot analysis revealed that PCSK9 inhibition was maintained following transfection for 48 h (Fig. 5A and B). Subsequently, DiI-LDL was incubated with the HepG2 cells for 4 h, and DiI-LDL uptake by the HepG2 cells was detected. The uptake of DiI-LDL at 37°C represented the cell surface-bound DiI-LDL. As shown in Figs. 5 and 6, compared with the cells treated with 200 µmol/l NaHS, those that were transfected with PCSK9 siRNA exhibited an increased LDLR expression and increased lipid uptake. This result indicated that $H_2S$ reduced the effects of PCSK9 on LDLR degradation by inhibiting PCSK9 expression, thereby increasing lipid uptake by HepG2 cells.

$H_2S$ regulates the expression of PCSK9 by affecting the P38/Akt-SREBP-2 signaling pathway in HepG2 cells. It has been reported that SREBP-2 may upregulate PCSK9...
expression (10). Thus, to explore the potential mechanisms underlying the H$_2$S regulation of PCSK9 expression, we investigated whether H$_2$S regulates PCSK9 expression by regulating SREBP-2 expression. Following treatment with various concentrations of NaHS (0, 50, 100 and 200 µmol/l) for 24 h, SREBP-1c and SREBP-2 expression was detected by western blot analysis. As shown in Fig. 7, SREBP-1c expression increased and that of SREBP-2 decreased with the increasing NaHS concentration.

PI3K/Akt is known to regulate the expression of SREBPs (11). Thus, in this study, we treated the HepG2 cells with various concentrations of NaHS (0, 50, 100 and 200 µmol/l) for 24 h, and phosphorylated Akt protein expression was detected by western blot analysis. As shown in Fig. 8, phosphorylated Akt protein expression increased with the increasing NaHS concentration. Following treatment with 200 µmol/l NaHS, the level of Akt phosphorylation increased, and SREBP-2 and PCSK9 expression decreased accordingly (Fig. 9).

H$_2$S reduces lipid accumulation in HepG2 cells. The above-mentioned results indicated that H$_2$S increased lipid uptake. Therefore, we hypothesized that lipid accumulation may be increased in the HepG2 cells. To explore whether H$_2$S influences lipid accumulation in the HepG2 cells, the HepG2 cells were incubated with culture medium supplemented with 30% FBS for 24 h, and intracellular lipids were detected by Oil Red O staining. Surprisingly, as shown in Fig. 10, lipid accumulation in the HepG2 cells exhibited no increase, but exhibited a decrease with the increasing H$_2$S concentration. Subsequently, Dil-LDL was incubated with the HepG2 cells for 24 h, and intracellular red fluorescence was also reduced, suggesting the reduced lipid accumulation in HepG2 cells (Fig. 11). The above-mentioned results indicate that H$_2$S can inhibit lipid accumulation in HepG2 cells induced by high serum concentrations.

Taken together, these results indicated that H$_2$S downregulated the expression of PCSK9 in a time- and concentration-dependent manner, thereby regulating HepG2 lipid metabolism (Fig. 12).

**Discussion**

H$_2$S inhibits PCSK9 expression to regulate lipid metabolism in HepG2 cells. H$_2$S has emerged as an important cardiovascular signaling molecule similar to nitric oxide and carbon monoxide (12). H$_2$S carries out a wide range of physiological and pathological functions in the cardiovascular system, including maintaining endothelial homeostasis (13), inhibiting the proliferation of vascular smooth muscle cells (14), and regulating lipid metabolism (15). However, the precise mechanisms through which H$_2$S carries out its functions remain unknown. In this study, we found that H$_2$S inhibited the expression of PCSK9 and increased lipid uptake by HepG2 cells.

The PCSK9 gene was discovered in 2003 (16); it plays an important role in lipid metabolism (17). PCSK9 accelerates the degradation of hepatic LDLR and prevents its recycling to the cell surface, which reduces lipid removal from the liver and increases serum lipid levels, thus promoting atherogenesis (18).
Figure 5. PCSK9 siRNA reduces PCSK9 expression, which in turn increases the LDLR levels. (A-F) The protein levels of PCSK9 and LDLR were detected by western blot analysis (*P<0.05 and **P<0.01 vs. the control group; ***P<0.001 vs. the control siRNA group; #P<0.05, PCSK9 siRNA+200 µmol/l NaHS group vs. control siRNA + 200 µmol/l NaHS group) (n=3). NaHS, sodium hydrosulfide; LDLR, low-density lipoprotein receptor; PCSK9, proprotein convertase subtilisin/kexin type 9.

Figure 6. PCSK9 siRNA increases the uptake of DiI-LDL by HepG2 cells (magnification, x400). DiI-LDL was incubated with the HepG2 cells for 4 h, and the DiI-LDL uptake by HepG2 cells was detected. PCSK9, proprotein convertase subtilisin/kexin type 9; DiI-LDL, 1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate-labeled LDL.
PCSK9 mutations cause autosomal dominant hypercholesterolemia (19). Numerous factors, such as statins (20) and berberine (21), regulate the expression of PCSK9. This study demonstrated that H$_2$S downregulated the expression of PCSK9 in HepG2 cells in a concentration- and time-dependent manner. To the very best of our knowledge, this study is the first to report that H$_2$S can regulate the expression of PCSK9. Previous studies have confirmed that PCSK9 regulates cellular lipid metabolism by degrading LDLR on the cell surface (22,23). The overexpression of PCSK9 has been shown to significantly increase LDLR degradation in HepG2 cells, thus reducing LDLR expression (24). In this study, H$_2$S treatment upregulated the expression of LDLR by decreasing the expression of PCSK9 in HepG2 cells. The expression of LDLR further increased after the expression of PCSK9 was interfered with by siRNA. Consistent with this result, following incubation with Dil-LDL for 4 h, Dil-LDL uptake by HepG2 cells increased in the 200 µmol/l NaHS and PCSK9 siRNA groups compared with that in the 200 µmol/l NaHS group. This result indicates that H$_2$S reduces the expression of PCSK9, thereby inhibiting the degradation of LDLR by PCSK9, and increasing the expression of LDLR on the cell surface to enhance lipid uptake. In addition, the highest concentration of NaHS (200 µmol/l) used in the treatment of HepG2 cells was confirmed by previous experiments, and this concentration does not cause toxic effects on cells (25).

**PI3K/Akt-SREBP-2 is involved in the regulation of PCSK9 and LDLR expression by H$_2$S.** SREBPs play an important role in lipid metabolism by regulating the expression of lipid metabolism-related genes (26). SREBP-1c primarily enhances the synthesis of fatty acids and the transcription of metabolic enzymes, while SREBP-2 primarily regulates the transcription of cholesterol-metabolizing enzymes (27). SREBP-1c and SREBP-2
are the predominant isomers in the liver (27). The promoter region of PCSK9 contains a sterol-regulatory element, which implies that PCSK9 transcription depends on sterols (28). Dubuc et al demonstrated that PCSK9 is a target gene of SREBP-2 (29), whereas SREBP-2 may participate in lipid metabolism by regulating PCSK9 expression. In this study, following treatment with various concentrations of H2S, the expression of SREBP-1c and SREBP-2 was detected in HepG2 cells. The results revealed that the expression of SREBP-1c increased, while that of SREBP-2 decreased with the increasing H2S concentration. This opposite result may be due to the fact that SREBP-1c primarily enhances the synthesis of fatty acids and triglycerides, while SREBP-2 preferentially synthesizes cholesterol. Chae et al demonstrated that LDLR was increased by downregulating the expression of hepatic PCSK9 via SREBP-2 (30). In this study, H2S inhibited the expression of SREBP-2 to inhibit the expression of PCSK9 and reduced the degradation of LDLR by PCSK9 to increase the LDLR level on the cell surface, consistent with the findings in the study by Chae et al (30).

PI3K/Akt can upregulate SREBP-2 expression and participate in lipid metabolism regulation (31). In this study, following treatment with various concentrations of NaHS, the levels of phosphorylated Akt were detected in the HepG2 cells. The results revealed that H2S increased the content of phosphorylated Akt protein to activate the PI3K/Akt signaling pathway, consistent with the findings of the study by Zheng et al (32). The increased Akt phosphorylation resulted in the decreased expression of SREBP-2. As a positive transcriptional regulator of PCSK9, the inhibition of SREBP-2 by phosphorylated Akt also downregulated the expression of PCSK9. The above-mentioned results indicate that PI3K/Akt-SREBP-2 is involved in the regulation of PCSK9 and LDLR expression by H2S.

Notably, this study demonstrated that H2S inhibited PCSK9 expression to reduce LDLR degradation at the HepG2 cell membrane to increase lipid uptake in the HepG2 cells. However, following incubation with 30% FBS or DiI-LDL, lipid accumulation in the HepG2 cells exhibited no increase, but instead decreased. This result suggests that H2S may also promote lipid efflux in addition to promoting lipid uptake through the SREBP-2 PCSK9/LDLR pathways in HepG2 cells. Gong et al (33) reported that H2S upregulated ATP-binding cassette transporter A1 (ABCA1) expression, whereas ABCA1 played an important role in promoting intracellular cholesterol efflux, which may explain why H2S increases lipid uptake, but causes no increase in lipid accumulation in HepG2 cells.
Notably, H$_2$S can affect pH and Na$^+$, but whether pH or Na$^+$ affects lipid metabolism in HepG2 cells remains unclear. Therefore, further studies are required to determine the effects of pH or Na$^+$ on lipid metabolism in HepG2 cells and whether other liver cancer cells have the same effect as HepG2.

In conclusion, the findings of this study demonstrate that H$_2$S downregulates the expression of PCSK9 in a time- and concentration-dependent manner, thereby regulating HepG2 lipid metabolism (Fig. 12). To the very best of our knowledge, this study is the first to report that H$_2$S can regulate the expression of PCSK9. Our findings provide new insight into the understanding of H$_2$S-regulated lipid metabolism and anti-atherosclerosis.

More importantly, the findings of this study suggest that H$_2$S may be a novel candidate as PCSK9 inhibitor in addition to PCSK9 monoclonal antibodies and may provide a novel method with which to discover novel PCSK9 inhibitors (34).

Acknowledgements

Not applicable.

Funding

The present study was supported by grants from the National Natural Science Foundation of China (no. 81370376), the Natural Science Foundation of Hunan province (nos. 2018JJ2343 and 2015JJ4097), the Key Project of the Educational Department of Hu-nan Province (no. 15A137), the Visiting Scholar Foundation of Key Laboratory of Bioreheological Science and Technology (Chongqing University), Ministry of Education (nos. CQKLBST-2014-002 and CQKLBST-2015-004), and the first-class discipline in Hunan Province, and Undergraduate Innovative Experiment Project (no. 2018XJXZ369).

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

LSL and MMW contributed to the conception and design of the experiment. JX, XQB, LL, MZ, JP and QX performed the experiments and collected the data. ZR, ZSJ, ZHT, HYW and MMW analyzed the data and helped interpret the results. JX,
The authors declare that they have no competing interests.

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