Hydrogen sulfide reduces serum triglyceride by activating liver autophagy via the AMPK-mTOR pathway

Li Sun,1* Song Zhang,1* Chengyuan Yu,1* Zhenwei Pan,3 Yang Liu,1 Jing Zhao,2 Xiaoyu Wang,1 Fengxiang Yun,1 Hongwei Zhao,1 Sen Yan,1 Yue Yuan,1 Dingyu Wang,1 Xue Ding,1 Guangzhong Liu,1 Wenpeng Li,1* Xuezhu Zhao,1 Zhaorui Liu,1 and Yue Li1,2

1Department of Cardiology, the First Affiliated Hospital, Harbin Medical University, Harbin, Heilongjiang Province, China; 2Key Laboratory of Cardiac Diseases and Heart Failure, Harbin Medical University, Harbin, Heilongjiang Province, China; and 3Department of Pharmacology, Harbin Medical University, Harbin, Heilongjiang Province, China

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Recent research suggests that autophagy participates in the regulation of liver lipid metabolism (5, 33, 34). Autophagy modulates hepatocyte lipid metabolism through lipophagy, which involves sequestration of lipid drops in double-membrane autophagosomes, followed by fusion with lysosomes to form autolysosomes, and subsequent degradation of TG by lipases within the autolysosomes. Reduction of liver cell autophagic activity causes decreased lipolysis and provokes free fatty acid (FFA) β-oxidation, resulting in hepatic steatosis and often progresses to NAFLD and HTG (34).

AMP-activated protein kinase (AMPK) is a pivotal regulator of cellular energy homeostasis, sensing cellular ATP starvation, and is an important regulator of autophagy. Its downstream targets include the negative regulation of the mammalian target of rapamycin (mTOR), whose inhibition promotes the formation of autophagosomes (17). Accordingly, AMPK-mediated inhibition of mTOR phosphorylation and activation induces autophagic responses in a variety of different cell types (3).

Conversely, inhibition of liver AMPK activation suppresses the autophagy ability of the liver and leads to the development of hepatic steatosis (21, 42). Hydrogen sulfide (H2S) is a novel signaling gasotransmitter and exerts many roles in both physiology and pathology. H2S induces autophagy in a variety of cell types including colonic epithelial cells through the AMPK/mTOR pathway, at least in vitro (38). A survey in the Beijing area showed that there was a significant decrease in plasma H2S level in children with dyslipidemia compared with that of healthy children, suggesting a generalized role of H2S in the regulating of plasma lipid level (23). Indeed, a recent study demonstrated that H2S played a hepatoprotective role in methionine-choline-deficient diet-induced nonalcoholic steatohepatitis (NASH) (31). However, the specific role of H2S on lipid metabolism, and especially the molecular mechanism by which this gasotransmitter influences hepatic autophagy, remains obscure at best.

The above-mentioned consideration prompted us to test the hypothesis that H2S can reduce the serum TG level and mitigate nonalcoholic fatty liver disease (NAFLD) through the activation of liver autophagy via the AMPK-mTOR pathway. The results show that H2S-mediated attenuation of hepatic mTOR activation is indeed a principal pathway by which this gasotransmitter affects hepatic lipid metabolism and autophagy.  

MATERIALS AND METHODS

Reagents. Sodium hydrosulfide (NaHS, H2S donor), chloroquine (CQ), and compound C were purchased from Sigma-Aldrich (Shang-
After being exposed to experimental agents for 6 wk, the mice were weighed weekly during the experiments. Mice were divided randomly into HFD control, NCD, or vehicle groups. The mice in the HFD group were raised for 8 wk (for diet composition and energy density, see Supplementary Table S1) and were then divided randomly into HFD control, NCD, or vehicle groups. The NCD group was assigned to the AIN-93G diet (18–20 g) and were obtained from Vital River (Beijing, China). C57BL/6 mice (18–20 g) were obtained from Vital River (Beijing, China). Male C57BL/6 mice (18–20 g) were obtained from Vital River (Beijing, China); AMPKα2−/− mice were kind gifts from Benoît Viollet (INSERM U567, Paris, France) and were generated as previously described (14, 35). Genotyping of mice by PCR was performed using the following three primers: 5'-GTTTACAGCTTA CCCTGGATGG-3', and 5'-GTTATCAGCACA ACTTAACTAC-3', 5'-GCATT GAACCAGTCCTTCTC-3'. All the mice were housed in a temperature-controlled setting under 12:12-h light-dark cycles. Thirty mice were divided into normal chow diet (NCD) and high-fat diet (HFD) groups. The NCD group was assigned to the AIN-93G diet during the entire experiment (n = 6). The mice in the HFD group were raised for 8 wk (for diet composition and energy density, see Supplementary Table S1) and were then divided randomly into HFD control, NaHS + CQ (50 mg·kg−1·day−1) and NaHS + CQ (50 mg·kg−1·day−1) groups (n = 6 mice in each group). NaHS and CQ were administered by intraperitoneal injection. The mice in the HFD control group received physiological saline. AMPKα2−/− mice were fed NCD or HFD for 8 wk and then challenged with NaHS (n = 3 mice in each group) for 6 wk. Mice were weighed weekly during the experiments. After being exposed to experimental agents for 6 wk, the mice were deeply anaesthetized with an intraperitoneal injection of pentobarbital sodium (60 mg/kg), and certainty was made that no response occurred after cornea stimulation. Blood samples were collected from abdominal aorta. Tissues were weighed immediately after sampling and then frozen at −80°C for subsequent analysis.

Histopathology. The livers were collected in 4% paraformaldehyde and then imbedded with paraffin. Tissues were cut into 5-μm sections and stained with haematoxylin and eosin (H&E). Histopathological changes were studied through light microscopy. For transmission electron microscope examination, the liver tissues were fixed in 2.5% glutaraldehyde and then sectioned and photographed with a transmission electron microscope according to routine procedures (JEOL, Tokyo, Japan).

Immunohistochemical analysis. Liver slides 3 mm thick were prepared and blocked with Sniper blocking buffer (Biocare, Concord, CA) for 20 min and incubated with rabbit p-AMPK (1:500) overnight at 4°C in a humidified chamber. The slides were rinsed with phosphate-buffered saline (PBS). After that, the slides were incubated with goat anti-rabbit polymer-horseradish peroxidase (HRP, Biocare) for 30 min at room temperature and rinsed again in PBS. Immunoreactivity was visualized with 3,3′-diaminobenzidine (Biocare). Sections were photographed using an Olympus AX70 microscope (Tokyo, Japan) (22).

Biochemical assay. Serum was collected by centrifugation at 4,000 g for 15 min at 4°C. Serum TG and liver tissue FFA were measured with ELISA kits (Blue Gene, Shanghai, China) according to the manufacturer’s instructions.

H2S measurements. Cystathionine γ-lyase (CSE) activity, which is equivalent to H2S production, was measured in liver tissue homogenates and human serum essentially as described previously (36). Briefly, the assay mixture contained tissue homogenate (1 ml), l-cysteine, and pyridoxal 5′-phosphate. Reactions were performed in 25-ml Erlenmeyer flasks. A small piece of filter paper was put into the central well of the flask, and 0.5 ml of zinc acetate was added in the central well for trapping evolved H2S in the mixture. After sealing and incubation (37°C, 90 min), the reactions were stopped by trichloroacetic acid. After 60 min at 37°C, the content of the central well was transferred to test tubes and mixed with 3 ml of distilled water, 0.5 ml N,N-dimethyl-p-phenylenediamine sulfate in 7.2 mol/l HCl and 50 μl of FeCl3. The absorbance was measured 10 min later at 670 nm with a spectrophotometer (Thermo Scientific Multiskan Spectrum). Serum (50 μl) was mixed with 100 μl of zinc acetate and then blended and deposited, 100 μl of sodium hydroxide (5M) was added into the mixture, and when the sedimentation was dissolved, it was centrifuged at 12,000 rpm for 5 min. The sediment was mixed with 100 μl of N,N-dimethyl-p-phenylenediamine sulfate and 100 μl of trichloroacetic acid, and centrifuged again. Then, 120 μl of supernatant was taken and 50 μl of FeCl3 was added. The absorbance was measured with a spectrophotometer 20 min later at 670 nm. The H2S concentration in the solution was calculated according to the calibration curve of the standard H2S solution.

Cell culture. The immortalized normal human hepatocyte-derived liver cell line (LO2) was purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Science, China, and human HCC cells (HePG2) were purchased from American Type Culture Collection. LO2 cells were cultured in 1640 medium (Hyclone, Beijing, China) with 10% fetal bovine serum (Gibco, Invitrogen) and 1% penicillin-amoxicillin in a humidified atmosphere of 5% CO2 in air at 37°C. HepG2 cells were cultured in DMEM (Hyclone, Beijing, China). The cells were treated with 20% fat emulsion at 1 μl/ml for 48 h to establish the nonalcoholic fatty liver cell model (44).

Immunofluorescence study. Immunofluorescence (IF) was performed according to routine protocols. To access autophagic flux, tandem green fluorescent protein/red fluorescent protein (GFP/RFP)-tagged LC3 plasmid was transfected into HepG2 and LO2 cells with Lipofectamine 2000 (Invitrogen) (8, 15, 19). Transfected cells were selected using G418 (400 nM; Roche, Basel, Switzerland) and then cultured with fat emulsion and/or NaHS or vehicle as appropriate. Autophagy was assessed by colocalization of the transgenes using ZEN software (Carl Zeiss, Jena, Germany) (19).

siRNA transfection. siRNA was transfected according to the manufacturer’s protocol (Santa Cruz Biotechnology). RNA (control) or pools of siRNAs for AMPKα1 or AMPKα2 were diluted into siRNA transfection medium to a final concentration of 2–20 nM. The diluted siRNA was incubated with 5 μl of Lipofectamine 2000 transfection reagent for 20 min at room temperature, and LO2 cells were washed with PBS twice and then incubated with siRNA in medium without streptomycin and penicillin for 8 h; then the medium was changed to growth medium for 48 h. After that, cells were treated with fat emulsion and NaHS as described previously (16).

Western blot analysis. Liver and LO2 protein were harvested using RIPA buffer containing 1% protease inhibitor (PMSF: Beyotime, Shanghai, China). Phosphatase inhibitor (10%) was added when phosphorylated proteins were to be detected. Protein samples were size-separated on 10% SDS-polyacrylamide and then transferred onto polyvinylidene difluoride membranes. Membranes were blocked with 10% nonfat milk for 1 h and then incubated with primary antibody against LC3B, AMPK, pAMPK, p62, pmTOR, and mTOR overnight at 4°C. After washing, the membrane was incubated with the secondary antibody for 1 h. Chemiluminescent signals were developed with
Table 1. Comparison of age, BMI, and biochemical parameters of control subjects and HTG patients

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>HTG</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>48.00 ± 10.00</td>
<td>47.00 ± 9.00</td>
<td>0.731</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>21.81 ± 1.57</td>
<td>25.51 ± 1.73**</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TG, mmol/l</td>
<td>1.25 ± 0.66</td>
<td>3.69 ± 1.16**</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>TC, mmol/l</td>
<td>4.80 ± 0.65</td>
<td>5.03 ± 0.70</td>
<td>0.194</td>
</tr>
<tr>
<td>VLDL, mmol/l</td>
<td>0.25 ± 0.10</td>
<td>0.72 ± 0.22**</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>GLC, mmol/l</td>
<td>5.30 ± 0.52</td>
<td>6.21 ± 1.30**</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>AST, U/l</td>
<td>22.76 ± 4.46</td>
<td>26.13 ± 6.99*</td>
<td>0.05</td>
</tr>
<tr>
<td>ALT, U/l</td>
<td>22.11 ± 8.85</td>
<td>31.46 ± 10.04*</td>
<td>0.05</td>
</tr>
<tr>
<td>BUN, mmol/l</td>
<td>4.94 ± 1.19</td>
<td>4.99 ± 0.81</td>
<td>0.426</td>
</tr>
<tr>
<td>Cr, mmol/l</td>
<td>74.72 ± 11.58</td>
<td>76.58 ± 8.14</td>
<td>0.267</td>
</tr>
</tbody>
</table>

Values are mean ± SD. HTG, hypertriglyceridemia; BMI, body mass index; TG, total triglyceride; TC, total cholesterol; VLDL, very low-density lipoprotein; GLC glucose. AST, aspartate aminotransferase; ALT, Alanine aminotransferase; BUN, urea nitrogen; Cr, creatinine. *P < 0.05, **P < 0.001.

RESULTS

Serum H₂S levels in HTG patients and mice. We tested the serum levels of TG and H₂S in three paired groups of human samples from 10 HTG patients and 10 healthy subjects (CTRL) in each group. As shown in Table 1, no statistical significance was detected with respect to age between HTG patients and control subjects. As expected, in HTG patients, BMI, serum TG levels, and VLDL levels were significantly higher than those of normal controls (P < 0.001). Serum levels of H₂S, however, in HTG patients were markedly lower than those of control subjects (P < 0.05; Fig. 1A). These data provide a first indication of the importance of H₂S as a regulator of lipid metabolism.

These data were supported by animal experimentation. We established an HTG model of C57BL/6 mice by feeding these animals a HFD. This provoked increased serum TG levels, but concomitantly H₂S content was lower in the HFD group than in the normal diet group (Fig. 1B). Accordingly, the level of H₂S in the liver was lower in the HFD group than that of normal diet group (P < 0.05; Fig. 1C). The most straightforward interpretation of these data is that H₂S is a regulator of hepatic metabolism in humans and experimental animals. Subsequently, experiments were initiated to substantiate this notion.

NaHS lowers serum TG levels and counteracts steatosis in HFD-fed mice. To investigate the relationship between H₂S and serum TG levels, HTG mice were challenged with NaHS, and subsequently its influence on serum TG level and liver histopathology was examined. We found that administration of NaHS significantly reduced serum TG level in HFD-fed mice (56 μM·kg⁻¹·day⁻¹, P < 0.05; Fig. 2A).

Chronic treatment with NaHS in the HFD group of mice significantly reduced the liver weight (Fig. 2B) and liver FFA (Fig. 2C). The ultrasound examination showed that the liver of HFD-treated mice had increased echogenicity with the liver appearing brighter than the cortex of the kidney. The increased hepatorenal echogenicity contrast of HFD-treated mice was obviously alleviated by NaSH (Fig. 2D). Consistently, histological observation of H&E-stained material revealed less lipid particles accumulating in hepatocytes of NaSH-treated mice (Fig. 2E).

Interestingly, administration of the autophagy inhibitor CQ significantly blocked all the beneficial effects of NaHS on liver pathology evoked by the HFD (Fig. 2, A–E). Thus, H₂S may exert its effects via the autophagic pathway.

NaHS induced autophagy in high-fat-treated mice and LO2 cells. We then examined the autophagic alterations in the mice hepatocytes and compared effects with LO2 cells treated with high fat. Electron microscopic (EM) imaging showed that H₂S treatment reduced the number of lipid droplets and autophagosomes in the hepatocytes of HFD mice, while conspicuously some lipid droplets appeared to be engulfed by the autophagosomes (Fig. 3A). In apparent agreement, we found that the ratio of LC3BII to LC3BI decreased in liver tissue of HTG mice and that treatment with NaHS increased the ratio of LC3BII to LC3BI (Fig. 3B). In addition, treatment with NaHS inhibited the expression of p62 in the liver of HFD-treated mice (Fig. 3C). Co-administration of CQ blocked the effects of NaHS on LC3 and p62 expression, confirming the efficacy of this compound with respect to autophagy.

Fig. 1. Serum H₂S levels in hypertriglyceridemia (HTG) patients and mice. A: serum H₂S levels of 30 male HTG patients and 30 controls from 3 tests, with 10 HTG patients and 10 controls in each test. Data are expressed as means ± SD. *P < 0.05, **P < 0.01 vs. control (CTRL) group. B: serum TG levels of male C57BL/6 mice treated with high-fat diet (HFD; HTG) (n = 4). C: H₂S synthesizing activity in liver of HTG mice (n = 5). Data are expressed as means ± SD. *P < 0.05, **P < 0.01 vs. normal chow diet (NCD) group.
Importantly, NaHS treatment rescued the reduction of LC3BII to LC3B observed in LO2 cultures subjected to high fat (Fig. 3D) and inhibited the expression of p62 (Fig. 3E), supporting that NaHS is capable of activating the autophagy of liver cells. Furthermore, we employed a GFP-tagged LC3 to detect autophagosomes and RFP to detect both autophagosomes and autolysosomes in HepG2 and LO2 cells. In merged images the red puncta overlays with the green ones, which appears as yellow dots indicating autophagosomes, while the other red puncta represent the late autolysosomes. Application of fatty emulsion reduced both autophagosomes (yellow dots) and autolysosomes (red dots) formation, and this effect was reversed by coadministration of H2S (Fig. 4, A and B). Collectively, these experiments show the power of H2S to provoke autophagy in a hepatic-relevant context.

NaHS activates AMPK and reduces mTOR to induce autophagy in liver cells. By molecular docking prediction, 23 potential binding sites in the AMPK protein were found potentially binding H2S (Fig. 5A), prompting investigation into the importance of AMPK in H2S effects. We observed that HFD treatment significantly decreased the p-AMPK level in the liver of HFD mice, and that this effect was counteracted by the administration of NaHS (Fig. 5, B and C). These effects did not involve a change in the expression of total AMPK (Fig. 5C) and can thus be attributed to differential enzymatic AMPK activity. mTOR is a key negative regulator of autophagy and is a canonical downstream mediator of AMPK, negatively regulated by this protein. As shown in Fig. 5D, HFD-fed mice had a higher level of p-mTOR than those fed NCD, and treatment with H2S decreased the phosphorylation and thus the inactivation of mTOR. Consistently, in cultured LO2 cells, we also observed decreased levels of p-AMPK and increased expression of p-mTOR following fatty emulsion treatment, which was reversed by the administration of NaHS (Fig. 6, A–D). Thus, the AMPK-mTOR pathway appears to be a major effector of H2S in hepatic physiology.

Activation of autophagy by NaHS is mediated through AMPKα2. To elucidate the potential role of AMPK in NaHS-induced activation of autophagy, we elected to use compound C to inhibit AMPK and examined the resulting autophagic condition of LO2 cells. We found that coapplication of compound C abolished the effect of NaHS on the ratio of LC3BII to LC3B treated with...
fatty emulsion (Fig. 3D). Successful inhibition of compound C on the expression of p-AMPK was confirmed by Western blot (Fig. 6A). Further confirmation of the role of AMPK in H$_2$S-induced autophagy came from experiments in which we fed the AMPKc2 knockout mice a HFD, administered NaHS, and checked the genotypes of the mice by PCR (Fig. 7E). As shown in Fig. 7A, knockout of AMPK abolished the TG lowering effect of NaSH. Moreover, no effects of NaSH were observed on the ratio of LC3BII to LC3B1 and p62 expression in the liver of AMPKc2 knockout mice fed HFD (Fig. 7, B and C). Finally, using the
siRNA technique to knockdown AMPK expression in vitro, we found that AMPK knockdown blocked the promoting effect of NaHS on the ratio of LC3BII to LC3BI in LO2 cells treated with fatty emulsion (Fig. 7D). In conjunction, our results provide compelling evidence that H2S counteracts steatosis through activation of AMPK, the inhibition of mTOR finally resulting in antisteatotic autophagy.

**DISCUSSION**

In this study, we observed that serum H2S level negatively correlated to TG levels using adult HTG patients and normal controls. Administration of NaSH promoted autophagy in liver cells, lowered serum TG, and mitigated NAFLD. These effects were sensitive to CQ, indicating that autophagy is indeed the
final effector here. In apparent agreement, knockdown of AMPK by siRNA in LO2 cells blocked the autophagy enhancement effects of NaSH, and such an effect was also observed in AMPKα2−/− mice. These data highlight the significant role of H2S in regulating TG metabolism through stimulation of AMPK and subsequent autophagic responses. HTG is the most common lipid metabolism disorder, and it is an independent risk factor for cardiovascular and cerebro-
vascular diseases. Previous study showed that the plasma H2S level in children with dyslipidemia was lower than that of healthy ones in the Beijing area (23). In the present study, we observed lower serum H2S levels in adult HTG patients (\geq 50 yr old). Moreover, in HTG mice serum TG levels are higher than those of control mice whereas correspondingly liver H2S synthesizing activity is decreased in mice fed HFD. The synthesis of H2S is regulated by two pyridoxal-5-phosphate-dependent enzymes, cystathionine β-synthase (CBS) and cystathionine γ-lyase (CSE), the former expressed mainly in the brain and the latter in the vasculature and liver. (12, 18) The microflora in the gastrointestinal system might synthesize H2S and release it to the portal circulation, which means that conceivably under physiological conditions the liver is exposed to a high level of H2S. Reduction of H2S is involved in the pathogenesis of lipid disorders. Mani et al. (27) found that CSE gene knockout mice fed an atherogenic diet developed elevated plasma levels of cholesterol and low-density lipoprotein cholesterol. In this study, we have found that administration of NaHS dramatically reduces serum TG levels of mice fed HFD and promotes TG lipolysis in the liver.

The liver plays a critical role in lipid metabolism, and increased production and/or decreased clearance of TG in the liver can result in HTG (13, 30). Conversely, TG accumulating in the liver leads to NAFLD. H2S has been shown to be protective against NAFLD (25, 32). NaHS prevents nonalcoholic steatohepatitis induced by a methionine-choline-deficient diet in rats by suppressing oxidative stress and inflammation (25). Very recently, Wu et al. (37) found that NaHS mitigated the fatty liver in obese mice through antioxidant potential. Consistently, in this study we found that NaSH alleviated the pathological changes of mice subjected to HFD, manifested by reduced liver weight-to-body weight ration and lipid accumulation in liver tissue.

Although recent studies show the protective effect of H2S on NAFLD, the underlying mechanism remains unclear. In this study we observed that CQ (an inhibitor of autophagic and lysosomal function), when coadministered with NaHS, counteracts the beneficial effect of NaHS on liver pathology as induced by a HFD. Autophagy is a lysosomal degradative pathway responsible for the removal and breakdown of cellular components. Presently, three types of autophagy are well known: macroautophagy, chaperone-mediated autophagy, and microautophagy (28). Among them, macroautophagy plays its role by engulfing a portion of cytosol by means of a double-membrane structure called an autophagosome. When the autophagosome fuses with the lysosome, lysosomal enzymes degrade the cellular components engulfed in the autophagosome.
HFD impairs autophagic function in liver cells (20, 34). Reducing autophagy is accompanied by aging, which may explain, at least in part, the increased occurrence of NAFLD with increasing age (4, 9). Singh et al. (34) showed that inhibiting macroautophagy by knocking down the autophagy gene ATG5 or its pharmacological inhibition by 3-methyladenine, increases TG content of hepatocytes cultured with fatty acid, causing decreased FFA β-oxidation. A further in vivo study demonstrates that starvation-induced stimulation of the liver macroautophagy causes LC3II association to lipid droplets, and concomitantly, autophagic vacuoles containing such lipid droplets increase. These observations demonstrate that macroautophagy can mediate the breakdown of TG-containing lipid droplets in the liver and, thus, that activation of liver autophagy may be a therapeutic target for HTG and NAFLD.

In line with these previous studies, we found that high fat diet impairs the liver autophagy. In the liver of HFD-fed mice, the level of LC3BII, a key protein required for autophagosome formation, is considerably decreased, and p62, one of the specific autophagy substrates, is increased. NaHS administration promotes liver autophagy flux in mice fed HFD as it does in human LO2 and HepG2 cells. These data imply that activation of the autophagy-lysosome pathway may contribute to the serum TG lowering effect of exogenous H2S.

Autophagy is regulated by multiple signaling pathways. Activation of AMPK blocks the mTOR pathway and thus inhibits activation of autophagy (25, 41). Previous studies have shown that neuronal tuberous sclerosis complex (TSC1/2) is

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Fig. 7. Role of AMPK in NaSH-associated autophagy. A: serum TG concentrations in AMPKα2−/− mice subjected to HFD and/or NaSH. Data are given as means ± SD (n = 3). *P < 0.05 vs. NCD group. B: LC3BII/LC3BII ratio in AMPKα2−/− mice treated with HFD + NaSH. Data are given as means ± SD (n = 3). *P < 0.05 vs. NCD group. C: p62 expression in AMPKα2−/− mice subjected to HFD + NaSH. Data are given as means ± SD (n = 3). *P < 0.05 vs. NCD group. D: effects of AMPKα1 and AMPKα2 siRNA knockdown by on LC3BII/LC3BII ratio in cultured LO2 cells. Data are given as means ± SD (n = 3). *P < 0.05, **P < 0.01, ***P < 0.001 vs. HFD+NaHS group. E: PCR analysis of genomic DNA extracted from mouse tails and Western blot analysis of AMPKα2 expression in mouse liver. F: proposed model summarizing the effects of NaSH in autophagy and TG metabolism. H2S activates AMPK, in turn negatively regulating mTOR and thus provoking autophagosome formation. The autophagosomes engulf and clear TG-enriched lipid droplets, which will be cleared following fusion with lysosomes. CQ pharmacologically blocks autophagosome fusion with the lysosomes, thus abolishing H2S-mediated TG degradation. Breakdown of TG yields FFA, which can undergo β-oxidation in the mitochondria.
required for the regulation of autophagy by AMPK. TSC2 could negatively regulate mTOR activity when complexed with TSC1 and thus inhibits activation of mTOR and the downstream pathway members (6, 24).

AMPK is composed of α, β, and γ subunits, and each subunit exists as two or three kinds of isomerides (α1, α2, β1, β2, γ1, γ2). The liver has a high expression of AMPKα2. In a previous study, Wu et al. (38) found that when H2S was administered to colon epithelial cells, the expression of AMPK was increased, indicating that AMPK is a critical signaling molecule in mediating the autophagy effects of H2S; hence, we explored whether NaHS activates liver autophagy through the AMPK–mTOR pathway. Accumulating reports suggest that mTOR can regulate the activity of the serine/threonine kinase ULK1 (7, 11). In its active state, mTOR inhibits the ULK1 kinase activity, which regulates the membrane protein required for autophagosome formation. AMPK may also promote phosphorylation of ULK1 and induce autophagy directly (39). By molecular docking prediction, we found that H2S may bind to 23 sites of AMPK protein, and in agreement, we demonstrated that H2S increased the phosphorylation of AMPK at Thr172 (α-subunit). Treating the LO2 cells with a specific AMPK inhibitor (compound C) markedly reduces NaHS-induced LC3BII increase. In addition, AMPKα2 knockout in mice abrogated the H2S-induced activation of autophagy in liver and serum TG reduction. AMPKα2 RNA interference further confirmed the importance of AMPK in the regulation of liver autophagy by H2S. Furthermore, we found that H2S may promote AMPK phosphorylation by activating TSC2, and ULK1 may also play a role in H2S-induced autophagy (Figs. S5 and Figure S6 in the supplement data).

In conclusion, our studies demonstrate that H2S plays a protective role against HTG and NAFLD by activating liver autophagy flux and that the AMPK–mTOR pathway is essential for the H2S-induced autophagy (Fig. 7F). Our findings highlight the potential of H2S-releasing drugs in the treatment of HTG and NAFLD. Currently, several H2S-releasing drugs have been developed. Further studies are needed to explore their serum TG lowering and NAFLD ameliorating effects in detail.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: L.S. drafted manuscript; S.Z., C.Y., Y. Liu, X.W., F.Y., H.Z., S.Y., Y.Y., D.W., X.D., W.L., G.L., X.Z., and Z.L. performed experiments; Z.P. revised and approved final version of manuscript; J.Z. and Y. Li conception and design of research.

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