Hydrogen sulfide protects against high glucose-induced lipid metabolic disturbances in 3T3-L1 adipocytes via the AMPK signaling pathway

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Abstract. Aberrant lipid metabolism contributes to the development of type 2 diabetes mellitus. The mechanisms by which hydrogen sulfide (H_2S), an endogenous gasotransmitter, regulates lipid metabolism remain unclear. The aim of the present study was to investigate if the protective effects of H_2S during high glucose (HG)-induced lipid accumulation in 3T3-L1 adipocytes may be mediated by AMP-activated protein kinase (AMPK). Triglyceride (TG) content and the production of H_2S were determined using adipogenesis colorimetric assay kits and H_2S synthesis methods. The levels of monocyte chemoattractant protein-1 and adiponectin were evaluated by ELISA. Total AMPK and phosphorylated AMPK levels were assessed by western blot analysis. HG increased the cellular level of TG and decreased H_2S production in 3T3-L1 adipocytes. The H_2S donor, sodium hydrosulfide (NaHS) protected against the HG-induced accumulation of TG in 3T3-L1 adipocytes. Furthermore, NaHS suppressed HG-induced TG accumulation by activating AMPK. Collectively, the findings of the present study suggested that HG induced lipid accumulation in 3T3-L1 adipocytes, and AMPK activation may underlie the lipid-lowering effects of H_2S.

Introduction

Diabetes mellitus (DM) is a serious threat to human health and is complicated by cardiovascular and cerebrovascular diseases. Disturbances in lipid metabolism are a primary risk factor for DM and dyslipidemia is a risk factor for type 2 DM (T2DM) complicated with coronary heart disease (1,2). Dyslipidemia is characterized by hypertriglyceridemia and increased lipoprotein levels (3). Long-term hyperglycemia and hyperlipidemia may cause atherosclerosis and fatty liver (4). A low-fat and high-fiber diet can decrease weight gain and the risk of diabetes (5). Targeted interventions to correct diabetes-associated dyslipidemia can lower lipid toxicity and delay the progression of diabetes and its associated complications.

Hydrogen sulfide (H_2S) is a gaseous signaling molecule that improves the pathophysiology of hypertension, chronic obstructive pulmonary disease, sepsis, hemorrhagic shock, Alzheimer's disease, gastric mucosal injury and liver cirrhosis (6-10). In mouse models of diabetes, the biosynthesis of H_2S decreases with disease progression (11,12). Moreover, exogenous H_2S decreases fatty liver development in obese rats (13). Clinical studies found that the levels of H_2S in patients with T2DM and obesity are significantly decreased (14) and that high levels of H_2S may have protective effects against obesity and diabetes (15). However, the mechanisms underlying H_2S-regulated lipid metabolism in T2DM remain poorly understood.

AMP-activated protein kinase (AMPK) is an evolutionarily conserved serine/threonine protein kinase that promotes short-term energy metabolism, glucose uptake and glycolysis. AMPK improved insulin resistance by increasing fatty acid oxidation and decreasing triglyceride (TG) and cholesterol synthesis (16,17). AMPK was studied as a potential target for the treatment of T2DM (18); however, the effects of H_2S on AMPK signaling have not been defined. In the present study, the role of H_2S in the regulation of lipid and TG metabolism in 3T3-L1 adipocytes under high glucose (HG) conditions was investigated, and the role of AMPK signaling in mediating the effects of H_2S was examined.

Materials and methods

Cell culture. Mouse embryo 3T3-L1 preadipoctyes were obtained from the American Type Culture Collection and were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Lonsa Science Srl), 0.1 mg/ml streptomycin and 100 U/ml penicillin at 5% CO_2 and 37˚C. Preadipocytes were induced to differentiate into

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mature adipocytes as described by Lu *et al* (19). Confluent preadipocytes were treated with DMEM containing 10% FBS, 10 μM insulin, 0.5 mM isobutylmethylxanthine and 0.25 μM dexamethasone for 2 days, followed by 2 days of treatment with DMEM containing 10% FBS and 10 μM insulin alone. Cells were replenished with fresh media every other day until day 12 and ≥90% of cells differentiated into mature adipocytes.

Oil red O staining. Cells were cultured in 6-well plates and Oil Red O staining was performed as previously described (20). Briefly, cells were fixed in 4% formalin for 30 min, permeabilized in 60% isopropanol for 20 min, and stained with Oil Red O for 20 min at room temperature. Cells were washed 3 times with distilled water, air dried and counterstained with hematoxylin for 3 min at room temperature. Slides were imaged on a Nikon 80i microscope (magnification, x10). Counts and lipid droplet areas were analyzed using MetaMorph software (version 6.2; Molecular Devices, LLC). Quantitative analysis of the lipid droplet in adipocytes was measured by spectrophotometry. In brief, Oil Red O staining was dissolved with isopropyl alcohol and the optical density was measured at 510 nm by spectrophotometry.

Glucose and sodium hydrosulfide (NaHS) treatment. Mature adipocytes were grown in 60 mm cell culture dishes and were incubated overnight in M199 medium (Gibco; Thermo Fisher Scientific, Inc.) containing 2% FBS. Cells were then treated with normal glucose (NG; 5.5 mM) or HG (25 mM) for the indicated time periods at 37˚C. For NaHS experiments, cells were pretreated with NaHS (50 μM) for 30 min at 37˚C. Mannitol (24.5 mM) was added to medium containing NG to maintain osmotic pressure (5.5 mM). For experiments with Compound C (cat. no. 11452, MedChemExpress), the cells were pre-treated with Compound C (10 μmol/l) at 37˚C for 1 h before stimulation with NG or HG according to the experimental design.

Endogenous H_2S measurements. Endogenous H_2S was measured as previously described (21) with some modifications. Briefly, cells were homogenized in 50 mM potassium phosphate buffer at 4˚C, pH 6.8. Tissue homogenates (0.1 ml) were mixed with 2.5 ml of distilled water, 0.5 ml of 1% zinc acetate, 0.4 ml of 1.2 M hydrochloric acid containing 30 mM iron trichloride and 0.5 ml of 7.2 M hydrochloric acid containing 20 mM N, N-dimethyl-p-phenylenediamine sulfate salt for 20 min at room temperature. Trichloroacetic acid (1 ml of 10% stock) was added to a total reaction volume of 5 ml. Mixtures were centrifuged at room temperature at 4,000 x g for 5 min and absorbance values were measured at 670 nm. The concentrations of H_2S (nM) were calculated against a NaHS calibration curve.

Endogenous TG extraction and measurements. TG extractions and measurements were performed using adipogenesis colorimetric/fluorometric assay kits (cat. no. K610-100; BioVision, Inc.) according to the manufacture's protocols. Briefly, cells cultured in 96-well plates were washed in PBS and 100 μl Lipid Extraction (BioVision, Inc.) solution was added to each well. Plates were heated at 90-100˚C for 30 min until the solution in the wells became cloudy. Plates were cooled at room temperature and mixed by shaking for 1 min. For the colorimetric assays, 1 mM TG was used to generate the TG standard curves. Lipase was added to each well to convert TG to glycerol and fatty acids. TG reagent (50 μl) was added to each well and plates were incubated at 37˚C for 30 min in the dark. Absorbances were read at 570 nm and TG concentrations were calculated from standard curves.

ELISA for monocyte chemoattractant protein-1 (MCP-1) and adiponectin. Cell culture supernatants were collected and MCP-1 and adiponectin were measured by ELISA assay (cat. nos. CSB-E07430m and CSB-E07272m; CUSABIO Technology LLC) according to the manufacturer's protocol.

Western blot analysis. Cells were homogenized for proteome extraction in TNE lysis buffer (10 mM Tris at pH 7.4, 150 mM NaCl, 1 mM EDTA and 1% Nonidet P-40) containing protease and phosphatase inhibitors. Protein concentrations were determined using BCA assays, 20 mg per protein sample was used for western blot analysis. Equal amounts of proteins were subject to 10% SDS-PAGE, transferred to polyvinylidene fluoride membranes, and were blocked in 5% fat-free milk for 1 h at room temperature. Membranes were probed with anti-AMPKα (1:1,000; Cell Signaling Technology, Inc.); anti-phosphorylated (p)-AMPKα (Thr172; 1:1,000; Cell Signaling Technology, Inc.), anti-β-actin (1:2,000; Santa Cruz Biotechnology, Inc.), and anti-MCP1 antibodies (cat. no. ab25124; Abcam) overnight at 4˚C. Following three washing with TBST, membranes were labeled with HRP-conjugated secondary antibodies (1:5,000, SA00001-1, HRP-conjugated Affinipure Goat Anti-Mouse IgG (H+L); 1:5,000, SA00001-2, HRP-conjugated Affinipure Goat Anti-Rabbit IgG (H+L), ProteinTech Group, Inc.) for 1 h at room temperature. Proteins were detected using the enhanced chemiluminescence system and immunoreactive bands were quantified using ImageJ software (version 1.42; National Institutes of Health).

Statistical analysis. Data were analyzed using a Student's t-test or one-way ANOVA followed by aTukey's post hoc test using the SPSS software (version 16.0; SPSS, Inc.). All values are presented as the mean ± SEM. $P<0.05$ was considered to indicate a statistically significant difference.

Results

HG increases TG content by downregulating H_2S production. The effects of HG on TG levels in mature adipocytes were investigated. 3T3-L1 cells were differentiated into mature adipocytes, which demonstrated typical characteristic morphology, round cells containing a high accumulation of Oil Red stained lipid droplets (Fig. 1A) (19). TG levels were significantly higher in induced mature adipocytes compared with preadipocytes (Fig. 1B and C).

Subsequently, adipocytes were treated with HG for 6, 12, 24 or 48 h. HG significantly increased the content of TG in cultured adipocytes in a time-dependent manner (Fig. 2A). Interestingly, HG also significantly decreased the production of H_2S in mature adipocytes (Fig. 2B).

NaHS inhibits HG-induced TG accumulation and the aberrant secretion of adipokines in mature adipocytes. In order to investigate whether the loss in H_2S was responsible for

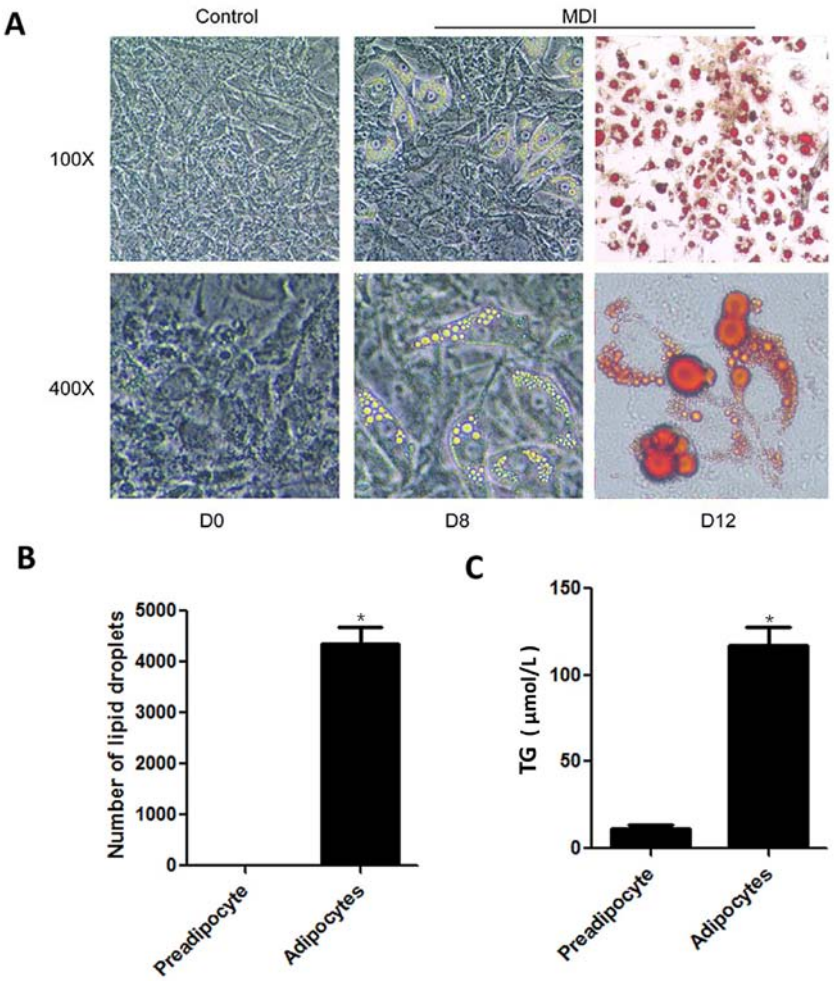

Figure 1. Adipose differentiation of 3T3-L1 preadipocytes. (A) Adipose differentiation of 3T3-L1 preadipocytes was induced by MDI for 12 days. Cell differentiation was evaluated by Oil Red O staining. (B) Quantification of the number of lipid droplets in preadipocytes and adipocytes. (C) Quantification of the level of TG in preadipocytes and adipocytes. *P<0.05 vs. preadipocyte group. NG, normal glucose; HG, high glucose; TG, triglyceride; MDI, differentiation medium; p-, phosphorylated.

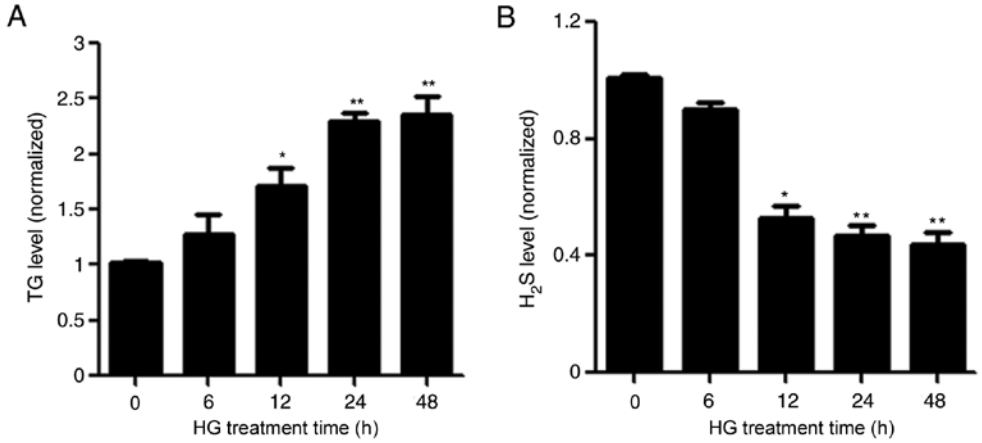

Figure 2. HG increases the content of TG in adipocytes by decreasing the level of H_2S. (A) HG increases the level of TG in adipocytes in a time-dependent manner. (B) HG decreases the production of H_2S in adipocytes. Data from three independent experiments are presented as the mean ± SEM. *P<0.05 and **P<0.01 vs. 0 h (normal glucose group). HG, high glucose; TG, triglyceride.

HG-induced TG upregulation in adipocytes, NaHS was used as an exogenous donor to enhance H_2S production. NaHS pretreatment significantly reversed the HG-induced loss of H_2S in cultured adipocytes (Fig. 3A). More importantly, NaHS treatment significantly inhibited the HG-induced increase in TG in adipocytes (Fig. 3B).

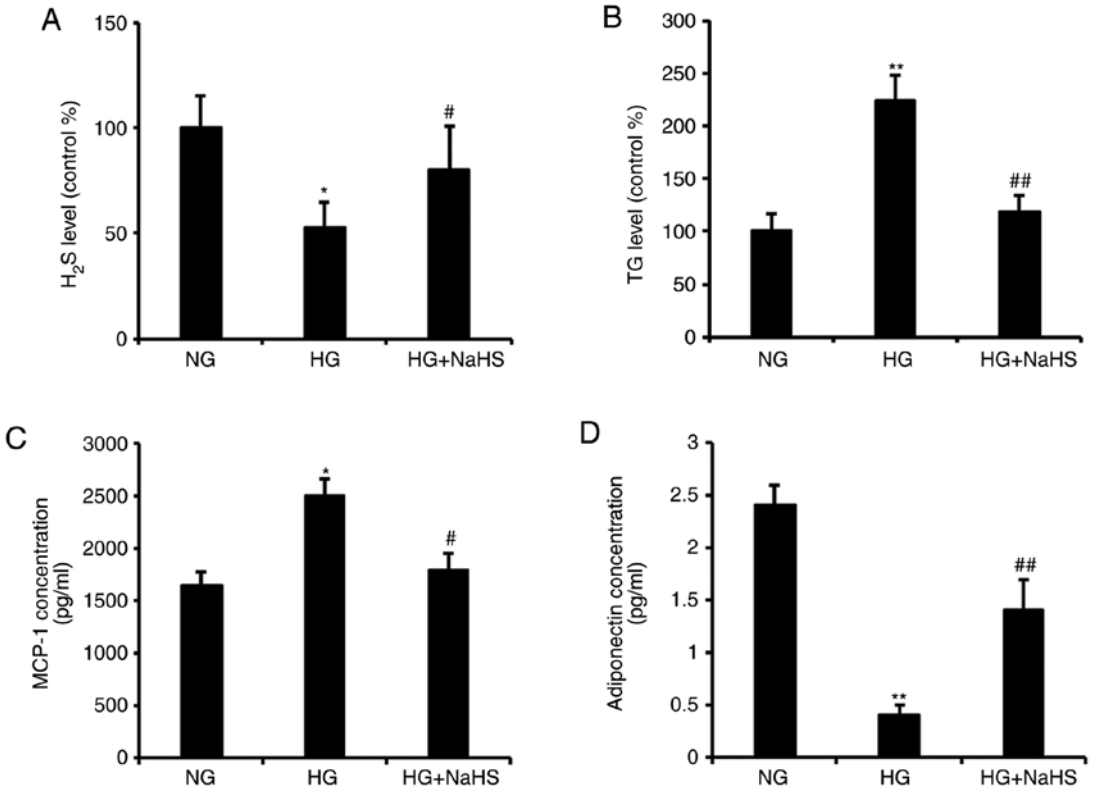

Figure 3. NaHS inhibits the HG-induced accumulation of TG and aberrant secretion of adipokines in adipocytes. (A) Production of H_2S in adipocytes. (B) Content of TG in cultured adipocytes. (C) Concentration of MCP-1 in the medium of cultured adipocytes. (D) Concentration of adiponectin in the medium of cultured adipocytes. All data are from three independent experiments and are presented as the mean ± SEM. n=3. *P<0.05 and **P<0.01 vs. NG group. $^{\#}$P<0.05 and $^{\#\#}$P<0.01 vs. HG group. MCP-1, monocyte chemoattractant protein-1; NG, normal glucose; HG, high glucose; TG, triglyceride.

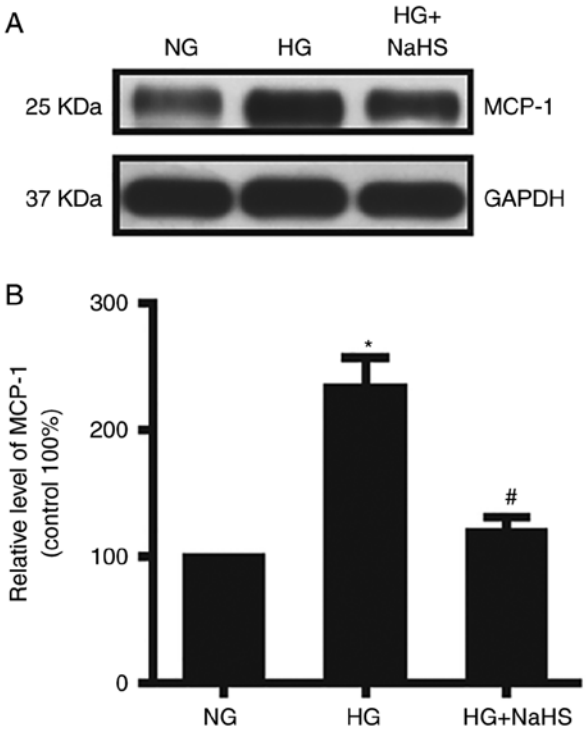

Figure 4. NaHS inhibits the HG-induced aberrant secretion of MCP-1 in adipocytes. (A) Effects on MCP-1 following NaHS treatment in adipocytes detected by western blot analysis. Data are representative of three independent experiments. (B) Relative MCP-1 protein levels as determined by western blot analysis are presented as the mean ± SEM. n=3. *P<0.05 vs. NG group. $^{\#}$P<0.05 vs. HG group. MCP-1, monocyte chemoattractant protein-1; NG, normal glucose; HG, high glucose.

The protective effect of NaHS against HG-induced adipokine secretion was then investigated. HG significantly increased the secretion of MCP-1 and decreased the secretion of adiponectin, which was reversed by the addition of NaHS (Fig. 3C and D).

MCP-1 was highly expressed in HG conditions as assessed by western blot analysis. NaHS treatment decreased MCP-1 levels in mature adipocytes (Fig. 4).

NaHS suppresses the HG-induced increase in TG through AMPK activation. AMPK activation was previously shown to decrease lipid synthesis and enhance fatty acid oxidation (18). Therefore, the role of AMPK in the suppression of HG-induced increase in TG by NaHS was investigated in the present study. Western blot analysis showed that HG significantly decreased the phosphorylation on Thr172 of AMPKα in mature adipocytes, which was counteracted by NaHS (Fig. 5). Moreover, the effects of HG and NaHS on AMPKα phosphorylation were reversed by treatment with compound C (10 μmol/l), an AMPK inhibitor (Fig. 5A and B). Therefore, the inhibitory effects of NaHS on the HG-induced increase of TG may be AMPK-dependent.

Discussion

A comprehensive understanding of the mechanisms underlying the pathogenesis of diabetic disturbances in lipid metabolism is required. The present study provided three new insights into lipid metabolism during diabetes. Firstly, HG treatment increased TG levels and decreased H_2S in 3T3-L1 adipocytes. Secondly, the H_2S donor, NaHS, protected 3T3-L1 adipocytes against

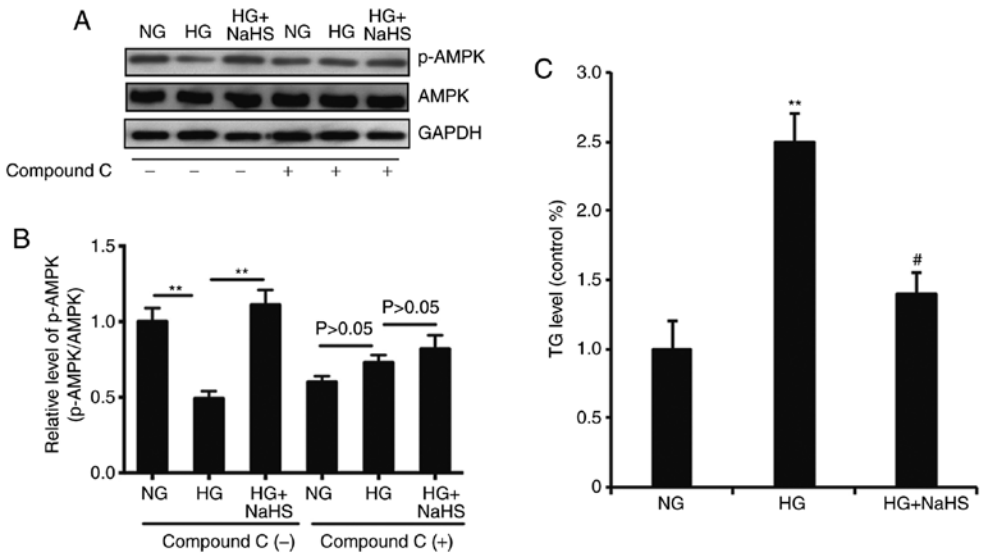

Figure 5. NaHS inhibits HG-induced increases in TG in adipocytes by activating AMPK signaling. (A) Adipocytes cultured with HG or NG were treated with or without NaHS for 48 h. Cell lysates were analyzed for the phosphorylation of AMPK. (B) Immunoblotting indicated that HG significantly decreased p-AMPK, which was reversed by NaHS. (C) Quantification of TG in cultured adipocytes. Data are from three independent experiments and are presented as the mean ± SEM. **P<0.01 vs. NG group. n=3. $^{\#}$P<0.05 vs. HG group. NG, normal glucose; HG, high glucose; AMPK, AMP-activated protein kinase; TG, triglyceride; p-, phosphorylated.

HG-induced accumulation of TG. Finally, NaHS suppressed the HG-induced increase in TG by activating AMPK.

A previous study showed the association of lipid metabolic disturbance in DM with the decreased expression of H_2S (15). Previous studies found that H_2S protected against HG-induced aberrant secretion of adipokines in cultured 3T3-L1 adipocytes (22). In the present study, the protective effects of H_2S against HG-induced aberrant lipid metabolism were investigated. The findings of the present study indicated that HG increased the TG level in cultured adipocytes and promoted the aberrant secretion of adipokines. Importantly, the production of H_2S also decreased following HG treatment and exogenous H_2S protected against HG-induced lipid metabolic disturbances in 3T3-L1 adipocytes. Previous studies revealed that H_2S can improve the health of obese individuals with diabetes (15-17) by promoting, at least in part, the degradation of TG.

A recent study showed that adipose tissue is not only an energy-storing organ, but also an important endocrine organ that secretes adipocytokines including adiponectin, leptin, interleukin (IL)-1, IL-6 and MCP-1 (4). In the present study, HG treatment increased the levels of MCP-1 and decreased the secretion of adiponectin in adipocytes. Moreover, NaHS pretreatment blocked the effects of HG on adipocytokine secretion, which suggested that NaHS exerted protective effects.

AMPK plays an important role in regulating energy metabolism. When the ratio of AMP/ATP increases, AMPK is phosphorylated and activates an array of downstream targets, increasing the cell catabolism by inhibiting the synthesis of glycogen and fat, and promoting fatty acid oxidation. In contrast, when the AMP/ATP ratio decreases, AMPK activity is inhibited and cell anabolism increases (23). AMPK is a heterologous trimer consisting of three subunits: α, β and γ (24). The α subunit plays a catalytic role, whereas the β and γ subunits play a regulatory role. AMPKα can be activated by phosphorylation at Thr172. Activated AMPK subsequently promotes phosphorylation of the downstream substrate acetyl coenzyme A carboxylase (ACC), which inhibits ACC activity, thus inhibiting the synthesis of fatty acids and cholesterol, and increasing fatty acid oxidation (24). In skeletal muscle cells *in vitro*, AMPK promotes cellular uptake of sugar, inhibits glycogen synthesis and promotes glycolysis (25). In hepatocytes, AMPK inhibits hepatocyte gluconeogenesis and glycolysis (26). Notably, in the liver, AMPK activation resulted in decreased fat accumulation by upregulating the expression of lipid oxidation genes (27). Liver-specific AMPKα deletion in mice led to increased plasma TG content and hepatic lipogenesis (28). Thus, AMPK is also an important regulator of lipid metabolism. In the present study, HG led to decreased phosphorylation of AMPKα and increased the accumulation of TG in adipocytes, which could be reversed by NaHS pretreatment, without affecting AMPKα expression. Therefore, AMPKα may be downstream of H_2S signaling, mediating the function of H_2S in lipid metabolism of adipocytes. Further studies are required to prove whether H_2S promoted lipid metabolism through the activation of AMPKα and to determine the underlying mechanism of this activation. Hormone-sensitive lipase and adipose triglyceride lipase were associated with the mobilization of stored triglycerides from adipose tissue. The lipolysis-associated genes and proteins include peroxisome proliferator-activated receptor γ, visfatin and Insig-2. The protective mechanisms of H_2S require further investigation in the future.

In conclusion, the *in vitro* experiments of the present study have shown that AMPK signaling regulates lipid metabolism and is essential for H_2S-induced lipid metabolic protection against HG injury. Furthermore, H_2S-activated AMPK-dependent signaling protects against aberrant lipid metabolism. Thus, therapeutic H_2S represents a promising therapeutic strategy for the treatment of diabetic lipid metabolic disturbances.

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Availability of data and materials

All data sets used and/or generated during the current study are available from the corresponding author on reasonable request.

Authors' contributions

YN made substantial contributions to the design of the study and wrote paper. ZP and JW conducted research and analyzed data. MX, SC, XL, AS and NL helped to conduct research and analyzed data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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