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

ZHE PAN¹, JUE WANG², MIN Xu¹, SHIHONG CHEN¹, XIAOBO LI¹, AILI SUN¹, NENGIUN LOU¹ and YIHONG NI¹

¹Department of Endocrinology and ²Central Laboratory, The Second Hospital of Shandong University, Jinan, Shandong 250033, P.R. China

Received April 9, 2019; Accepted August 23, 2019

DOI: 10.3892/mmr.2019.10685

Abstract. Aberrant lipid metabolism contributes to the development of type 2 diabetes mellitus. The mechanisms by which hydrogen sulfide (H₂S), an endogenous gasotransmitter, regulates lipids metabolism remain unclear. The aim of the present study was to investigate if the protective effects of H₂S 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 H₂S were determined using adipogenesis colorimetric assay kits and H₂S 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 H₂S production in 3T3-L1 adipocytes. The H₂S 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 H₂S.

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 (H₂S) 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 H₂S decreases with disease progression (11,12). Moreover, exogenous H₂S decreases fatty liver development in obese rats (13). Clinical studies found that the levels of H₂S in patients with T2D and obesity are significantly decreased (14) and that high levels of H₂S may have protective effects against obesity and diabetes (15). However, the mechanisms underlying H₂S-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 H₂S on AMPK signaling have not been defined. In the present study, the role of H₂S 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 H₂S was examined.

Materials and methods

Cell culture. Mouse embryo 3T3-L1 preadipocytes 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% CO₂ and 37°C. Preadipocytes were induced to differentiate into
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 normal glucose (NG; 5.5 mM) or HG (25 mM) 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 (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 H₂S measurements. Endogenous H₂S 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 H₂S (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 manufacturer’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 a Tukey'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 H₂S 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 triglycerides. 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 H₂S 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 H₂S was responsible for...
HG-induced TG upregulation in adipocytes, NaHS was used as an exogenous donor to enhance H₂S production. NaHS pretreatment significantly reversed the HG-induced loss of H₂S in cultured adipocytes (Fig. 3A). More importantly, NaHS treatment significantly inhibited the HG-induced increase in TG in adipocytes (Fig. 3B).
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 H₂S in 3T3-L1 adipocytes. Secondly, the H₂S donor, NaHS, protected 3T3-L1 adipocytes against...
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 glycoen 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 H2S signaling, mediating the function of H2S in lipid metabolism of adipocytes. Further studies are required to prove whether H2S 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 H2S 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 H2S-induced lipid metabolic protection against HG injury. Furthermore, H2S-activated AMPK-dependent signaling protects against aberrant lipid metabolism. Thus, therapeutic H2S represents a promising therapeutic strategy for the treatment of diabetic lipid metabolic disturbances.

Acknowledgements

Not applicable.
Funding

This study was supported by the Shandong Provincial Medical and Health Science and Technology Development Plan (grant no. 2017WS626), the National Science Foundation of China (grant nos. 81670753 and 81070641), the Shandong Provincial Medical and Health Science and Technology Development Plan (grant no. 2014WS0156), the National Science Foundation for Young Scholars of China (grant no. 81500554) and the Scientific and Technological Projects of Shandong Province (grant no. 2014GSF118041).

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

MX, Sc, Xl, aS and nl helped to conduct research and wrote paper. ZP and JW conducted research and analyzed data. Authors' contributions available from the corresponding author on reasonable request.

Availability of data and materials

Projects of Shandong Province (grant no. 2014GSF118041).

Funding

1. Nadeem A, Mumtaz S, Naveed AK, Aslam M, Siddiqui A and Lodhi GM: Pattern of dyslipidaemia and impact of increasing age and duration of type 2 diabetes mellitus on dyslipidaemia, insulin levels and insulin resistance. J Pak Med Assoc 65: 928-932, 2015.

2. Tomkin GH and Owens D: Dyslipidaemia of diabetes and the intestine. World J Diabetes 6: 970-977, 2015.

3. Hermans MP and Valensi P: Elevated triglycerides and low insulin levels and insulin resistance. J Pak Med Assoc 65: 928-932, 2015.

4. Wong ND, Zhao Y, Patel R, Patao C, Malik S, Bertoni AG, Correa A, Folsom AR, Kachroo S, Mukherjee J, et al: Cardiovascular risk factor targets and cardiovascular disease event risk in diabetes: A pooling project of the atherosclerosis risk in communities study, multi-ethnic study of atherosclerosis, and Jackson heart study. Diabetes Care 39: 668-676, 2016.

5. Sylvestscy AV, Edelstein SL, Walford G, Boyko EJ, Horton ES, Ibebuogu UN, Knowler WC, Montez MG, Temprosa M, Hoskin M, et al: A high-carbohydrate, high-fiber, low-fat diet results in weight loss among adults at high risk of type 2 diabetes. J Nutr 147: 2060-2066, 2017.

6. van Goor H, van den Born JC, Hillebrands JL and Joles JA: Hydrogen sulfide in hypertension. Curr Opin Nephrol Hypertens 25: 107-113, 2016.

7. Xu Y, Dai X, Zhu D, Xu X, Gao C and Wu C: An exogenous hydrogen sulphide donor, NaHS, inhibits the apoptosis signaling pathway to exert cardioprotective effects in a rat hemorrhagic shock model. Int J Clin Exp Pathol 8: 6245-6254, 2015.

8. Nagpure BV and Bian JS: Brain, learning, and memory: Role of H2S in neurodegenerative diseases. Handb Exp Pharmacol 230: 193-215, 2015.

9. Gao SB, Duan ZJ, Wang QM, Zhou Q, Li Q and Sun XY: Endogenous carbon monoxide downregulates hepatic cystathionine-γ-lyase in rats with liver cirrhosis. Exp Ther Med 10: 2039-2046, 2015.

10. Aboubakr EM, Taye A, El-Moselhy MA and Hassan MK: Protective effect of hydrogen sulfide against cold stress-induced gastric mucosal injury in rats. Arch Pharm Res 36: 1507-1515, 2013.

11. Jin S, Pu SX, Hou CL, Ma FF, Li N, Li XH, Tan B, Tao BB, Wang MJ and Zhu YC: Cardiac H2S generation is reduced in aging diabetic mice. Oxid Med Cell Longev 2015: 758358, 2015.

12. Brancilione V, Rovierzo F, Vellocco V, De Gruttola L, Buccin M and Cirino G: Biosynthesis of H2S is impaired in non-obese diabetic (NOD) mice. Br J Pharmacol 155: 673-680, 2008.

13. Wu D, Zheng N, Qi K, Cheng H, Sun Z, Gao B, Zhang Y, Pang W, Huangfu C, Ji S, et al: Exogenous hydrogen sulfide mitigates the fatty liver in obese mice through improving lipid metabolism and antioxidant potential. Med Gas Res 5: 1, 2015.

14. Suzuki K, Sagara M, Aoki C, Tanaka S and Aso Y: Clinical implication of plasma hydrogen sulfide levels in Japanese patients with type 2 diabetes. Intern Med 56: 17-21, 2017.

15. Carter RN and Morton NM: Cysteine and hydrogen sulphide in the regulation of metabolism: Insights from genetics and pharmacology. J Pathol 238: 321-332, 2016.

16. Steinberg GR and Schertzer JD: AMPK promotes macrophage fatty acid oxidative metabolism to mitigate inflammation: Implications for diabetes and cardiovascular disease. Immunol Cell Biol 92: 340-345, 2014.

17. O’Neill HM, Holloway GP and Steinberg GR: AMPK regulation of fatty acid metabolism and mitochondrial biogenesis: Implications for obesity. Mol Cell Endocrinol 366: 135-151, 2013.

18. Coughlan KA, Valentine RJ, Ruderman NB and Saha AK: AMPK activation: A therapeutic target for type 2 diabetes? Diabetes Metab Syndr Obes 7: 241-253, 2014.

19. Lu S, Guan Q, Liu Y, Wang H, Xu W, Li X, Fu Y, Gao L, Zhao J and Wang X: Role of extrathyroidal TSHR expression in adipocyte differentiation and its association with obesity. Lipids Health Dis 11: 17, 2012.

20. Howell G III and Mangum L: Exposure to bioaccumulative organochlorine compounds alters adipogenesis, fatty acid uptake, and adipokine production in NIH3T3-L1 cells. Toxicol In Vitro 25: 394-402, 2011.

21. Li L, Bhatia M, Zhu YZ, Zhu YC, Ramnath RD, Wang ZJ, Ammar FB, Whiteman M, Salto-Tellez M and Moore PK: Hydrogen sulfide is a novel mediator of lipopolysaccharide-induced inflammation in the mouse. FASEB J 19: 1196-1198, 2005.

22. Pan Z, Wang H, Liu Y, Yu C, Zhang Y, Chen J, Wang X and Guan Q: Involvement of CSE/H2S in high glucose induced aberrant secretion of adipokines in 3T3-L1 adipocytes. Lipids Health Dis 13: 155, 2014.

23. Hardie DG: The AMP-activated protein kinase pathway—new players upstream and downstream. J Cell Sci 117: 549-547, 2004.

24. Saha AK and Ruderman NB: Malonyl-CoA and AMP-activated protein kinase: An expanding partnership. Mol Cell Biochem 253: 65-70, 2003.

25. Halse R, Fryer LG, McCormack JG, Carling D and Yeaman SJ: Regulation of glycogen synthase by glycogen and glucose: A possible role for AMP-activated protein kinase. Diabetes 52: 9-15, 2003.

26. Foret F, Ancellin N, Andreelli F, Saintpaul Y, Grondin P, Kalita A, Thorenz B, Vaulont S and Violette S: AMPK: A short-term overexpression of a constitutively active form of AMP-activated protein kinase in the liver leads to mild hypoglycemia and fatty liver. Diabetes 54: 1331-1339, 2005.

27. Sabater AG, Ribot J, Priego T, Vasquez J, Franck S, Palou A and Buchwald-Werner S: Consumption of a mango fruit powder protects mice from high-fat induced insulin resistance and hepatic fat accumulation. Cell Physiol Biochem 42: 564-578, 2017.

28. Andreelli F, Foret F, Knau C, Cani PD, Perrin C, Iglesias MA, Pilot B, Bado A, Tronche F, Mithieux G, et al: Liver adenosemonophosphate-activated kinase-alpha2 catalytic subunit is a key target for the control of hepatic glucose production by adiponectin and leptin but not insulin. Endocrinology 147: 2432-2441, 2006.

This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivatives 4.0 International (CC BY-NC-ND 4.0) License.