Methyltransferase SETD2 mediates hepatoprotection of berberine against steatosis

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Background: Berberine (BBR) can alleviate nonalcoholic fatty liver (NAFL), but the mechanisms remain uncertain. Mounting evidence has underscored the roles of epigenetic modulation in the development of NAFL. Increased expression of histone methyltransferase SET domain-containing protein 2 (SETD2) is found in the livers of diabetic animals with BBR administration. Whether SETD2 contributes to the protective effects of BBR against NAFL remains to be elucidated.

Methods: A C57BL/6 mouse model of NAFL induced by a high-fat high-sucrose diet (HFHS) and a palmitate-treated hepatocyte steatosis model were generated. The effects of BBR were evaluated by Oil Red O staining and the cell viability assay. Quantitative real-time polymerase chain reaction, Western blotting, and immunofluorescence staining were used to analyze the expression and activity of SETD2 and its downstream target trimethylation of lysine 36 on histone 3 (H3K36me3).

Results: BBR treatment induced the reduction of lipid droplets in both HFHS mouse livers and HepG2 cells, coincident with the elevation of the mRNA and protein expression of SETD2 and H3K36me3. Knockdown of SETD2 compromised the BBR-mediated inhibition of the accumulation of lipid droplets in the HepG2 cell model of steatosis. Moreover, upregulated SETD2 and H3K36me3 expression was also observed in intact HepG2 cells treated with BBR. The promoter assay indicated that BBR treatment increased the transcriptional activity of SETD2 and H3K36me3.

Conclusions: BBR confers hepatoprotection against steatosis through transcriptional regulation of SETD2 activity.

Keywords: Berberine (BBR); epigenetics; SET domain-containing protein 2 (SETD2); nonalcoholic fatty liver (NAFL); steatosis

Submitted Mar 16, 2022. Accepted for publication Apr 27, 2022.
doi: 10.21037/atm-22-1753
View this article at: https://dx.doi.org/10.21037/atm-22-1753

Introduction

Nonalcoholic fatty liver (NAFL) represents simple hepatic steatosis and is defined as triglyceride infiltration in more than 5% of hepatocytes (1). Hepatic steatosis has emerged as a predominant determinant for the progression of fibrosis in chronic hepatitis C patients (2). Patients with NAFL over 14.3 years progress 1 stage of fibrosis (3). Particularly, hepatic triglyceride accumulation contributes to systemic disorders, including insulin resistance in the liver, muscle, and adipose tissue (4). Although lifestyle countermeasures

such as weight loss, diet, and physical performance have been recommended as the first-line strategy for NAFL treatment (5), the noncompliance of patients urges the development of new therapies for NAFL.

Traditional Chinese medicine (TCM), which mainly comprises natural medicines and related extracts, has shown therapeutic advantage for NAFL. For instance, TCM improved hepatic steatosis and injury in both patients and animal models, as demonstrated by the reduction of alanine aminotransferase, aspartate aminotransferase (6), and serum triglycerides (7). Berberine (BBR), a kind of isoquinoline alkaloid (8) extracted from medicinal plants in the Berberidaceae family such as Rhizoma Coptidis and Phellodendron amurense Ruprin (9), has received increasing attention for attenuating NAFL. In patients with NAFL, BBR plus lifestyle intervention resulted in a significant reduction in the fat content of the liver and body weight, with better improvement in serum lipid profiles (10). In a rat model of NAFL, BBR treatment for 16 weeks decreased hepatic fat content by 14% (11). Current viewpoints regarding the mechanism of action of BBR-mediated protection against NAFL involve the inhibition of apoptosis, autophagy of hepatocytes, AMP-activated protein kinase (AMPK) activation, insulin receptor, low-density lipoprotein upregulation, downregulation of low-density lipoprotein receptor, and reduction of DNA hypermethylation and histone deacetylation (12,13).

The epigenetic regulation of NAFL has received particular attention (14). DNA methylation, histone acetylation, and long noncoding RNAs represent the most common epigenetic modifications. Among them, histone methylation could potentially contribute to the protection of BBR against NAFL. Knockout of histone methyltransferase SET domain-containing protein 2 (SETD2) increased the storage of hepatic triglycerides in mice under high-fat conditions (15). In the setting of diabetes, BBR administration ameliorated hepatic steatosis and induced an increase in the mRNA expression of SETD2 (16). These findings suggest the potential contribution of SETD2-dependent epigenetic modifications in hepatic steatosis and BBR-mediated hepatoprotection.

In the current study, we aimed to determine the role of SETD2 in the effects of BBR on hepatic lipid metabolism using a cell model of hepatic steatosis. Our results confirmed the downregulation of SETD2 in both mouse and cell models of NAFL, and indicated that the upregulation of SETD2 under BBR treatment was compensatory, in that its inactivation abolished the hepatoprotective effects of BBR. These findings not only for the first time illustrates a key role of histone methylation in the regulatory mechanism of BBR against hepatic steatosis, but also provides a new therapeutic target for NAFL. We present the following article in accordance with the ARRIVE reporting checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-22-1753/rc).

Methods

Reagents and antibodies

BBR (CAS: 633-65-8) was purchased from Sangon Biotech. Rabbit anti-SETD2 antibody (80290s), rabbit anti-H3 lysine 36 trimethylation antibody (8173S), and rabbit GAPDH (#AC006) were provided by Cell Signaling Technology (MA, USA).

Animal model of NAFL

Six-week-old male C57BL/6 mice (Shanghai SLAC Laboratory Animal Company) were divided into groups using a random number table. Mice (n=3) were fed by a high-fat, high-sucrose (HFHS) diet containing 40% carbohydrate, 40% fat, and 20% protein for 5 weeks, while BBR (5 mg/kg/day, n=3, or 10 mg/kg/day, n=3) or vehicle [phosphate buffered saline (PBS), n=3] was administered through intraperitoneal (i.p.) injection once per day. All animals were housed at a steady room temperature under a 12:12 hour light/dark cycle and were permitted free access to a certified chow diet and water.

The process for animal experiments was approved and conducted in the experimental animal center of Longhua Hospital, Shanghai University of Traditional Chinese Medicine (No. LHERAW-19004). All surgeries were rigidly performed in accordance with globally recognized and institutional guidelines for the care and use of animals.

Histological analysis

Livers from the mice were fixed in 4% paraformaldehyde and embedded in paraffin wax. Paraffin sections (5-μm thickness) were fixed on glass slides for immunohistochemistry. For evaluating hepatic steatosis, the liver was microdissected with scissors, put in a screw-cap tube, and frozen immediately in liquid nitrogen. The sections (8-μm thickness) cut in a cryostat at –20 ℃ were stained with Oil Red O following the manufacturer's

instructions.

Cell culture and treatment

Human HepG2 cells (American Type Cell Culture) were cultured in DMEM/L medium (Sigma, USA) containing 10% fetal bovine serum (FBS) (Sigma, USA) and 1% penicillin/streptomycin (Gibco, USA) at 37 ℃ with 5% CO_2. To establish the model of steatosis, serum-free DMEM was used to incubate the HepG2 cells for 12 hours, then the medium was exchanged to DMEM containing 100 μM palmitate, without or with BBR (10 or 20 μM), for an additional 24 hours. The influence of BBR on cell viability was determined with the Cell Counting Kit-8 (CCK8) assay.

Luciferase reporter assay

The reporter constructs consisted of the SETD2 promoter sequence. The segments of interest in the SETD2 promoter region were amplified utilizing polymerase chain reaction (PCR) and cloned in sense orientation into the Xho I and Hind III sites of the pGL4.17 vector (Promega). To detect the BBR induction of different constructs, HepG2 cells were incubated with 20 μM BBR or vehicle in DMEM medium without FBS 6 hours after transfection. Cells were then harvested 24 hours later, and the luciferase activity was detected as relative luminescence units (RLUs) using the Luciferase Assay System (Promega, Madison, WI, USA).

Flow cytometry

The Annexin V-FITC/PI Apoptosis Detection Kit (BioLegend, CA, USA) was used to stain cells according to the manufacturer's instructions, and a CytExpert flow cytometer (Beckman Coulter, USA) was used to count the percentage of apoptotic cells.

Cell transfection

HepG2 cells were seeded in 6-well plates and transfected either with scrambled siRNA or SETD2 siRNA (25 ng) for 36 hours using 6 L of Lipofectamine 3000 Reagent (Invitrogen, USA). The sequence of siRNAs against SETD2 was as follows: si-SETD2, 5'-CCGACCCCTGAGCAAAGATT-3'.

Oil Red O staining

Human HepG2 cells were fixed with 10% paraformaldehyde for 30 minutes, and then stained with Oil Red O dye solution (Sigma-Aldrich, USA) for another 30 minutes. After washing with 60% isopropanol for 10 minutes, a microplate reader was used to quantify the steatosis of HepG2 cells by measuring absorbance at 510 nm wavelength.

Triglycerides measurement

Triglycerides were extracted with chloroform/methanol (2:1 v/v) and dried in the chemical hood. Then, 1% Triton X-100-ethol was added to dissolve the triglycerides, and then the Triglyceride Reagent Kit (Nanjing Jiancheng Bioengineering Institute) was used to measure triglyceride concentration.

Quantitative real-time PCR

The TRIzol method was utilized to isolate total RNA from cells according to the manufacturer's specifications (Sigma, USA). Next, the Reverse Transcription Reagent Kit with genomic DNAeraser (Takala, Tokyo, Japan) transformed the total RNA into complementary DNA (cDNA). Quantitative real-time PCR was performed with an Applied Biosystems 7500 system (Applied Biosystems, USA) utilizing SYBR Green Premix Ex Taq (Takara, Tokyo, Japan).

Western blotting

Proteins were extracted from the cell pellet by RIPA buffer comprised of protease and phosphatase inhibitors. The concentration of the extracted proteins was measured using the BCA Protein Quantitative Analysis Kit (Thermo, MA, USA, China). A total of 20 μg lysates were loaded onto sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to nitrocellulose (NC) membranes (Sigma, USA). The membranes were blocked with 5% skim milk for 2 hours, and subsequently incubated with primary antibodies overnight. After incubation with horseradish peroxidase (HRP)-conjugated secondary antibodies for 3 hours, protein bands were detected with Immobilon Western Chemiluminescent HRP Substrate (Beyotime Biotechnology, China). Densitometric quantification was carried out using ImageJ software version 1.46r (ImageJ, USA).

Statistical analysis

Total data were expressed as mean ± standard error of the mean (SEM). Unpaired two-tailed *t*-test or one-way analysis of variance (ANOVA) was performed to analyze the significance between 2 groups or among numerous groups. $P<0.05$ was considered to be statistically significant.

Results

BBR treatment alleviates hepatic steatosis

To demonstrate the protective effect of BBR on hepatic steatosis, mice were fed with a HFHS diet (n=3) for 5 weeks, and in the meantime, low (5 mg/kg/day, n=3) and high doses (10 mg/kg/day, n=3) of BBR or vehicle (PBS, n=3) were administered through i.p. injection once a day (*Figure 1A-1F*). In mice given a HFHS diet, the content of lipid droplets was obviously lower in livers with low- and high-dose BBR treatment, as shown by the photomicrographs of Oil Red O staining (*Figure 1A*) and the measurement of triglycerides (*Figure 1B*). When high-dose BBR was used, circulating triglycerides significantly decreased (*Figure 1C*). These findings suggest a dose-dependent effect of BBR on hepatic steatosis and systemic lipid metabolism.

To further confirm whether BBR directly counteracts steatosis of hepatocytes by metabolic challenge, the human HepG2 hepatocyte cell line was exposed to palmitate (100 μM) with or without BBR. Firstly, we assessed the potential impact of BBR on hepatocyte viability. At the range of 1 to 20 μM, BBR did not significantly affect the viability of HepG2 cells, but BBR at 30 μM demonstrated a toxic effect (*Figure 2A*). This scenario was supported by the apoptosis assay (*Figure 2B*). Thus, we used 10 or 20 μM BBR to analyze its function against hepatic lipogenesis. Treatment of steatotic HepG2 cells with BBR at 10 or 20 μM obviously reduced the accumulation of lipid droplets induced by palmitate, as demonstrated by quantitative analysis of Oil Red O staining (*Figure 3A*) and intracellular triglyceride content (*Figure 3B*). Collectively, these results demonstrate that BBR confers resistance to hepatocyte steatosis.

BBR treatment transcriptionally activates SETD2 in steatotic hepatocytes

We further investigated whether BBR influences SETD2 activity and the potential underlying mechanism. In both HFHS-fed mouse livers (*Figure 1D-1F*) and HepG2 cells, the protein and mRNA levels of SETD2 and the downstream target trimethylation of lysine 36 on histone 3 (H3K36me3) were significantly downregulated compared to those in the corresponding controls (*Figure 4A*,*4B*). BBR treatment increased the expression of SETD2 and H3K36me3 at the transcriptional level under basal conditions (*Figure 4A*,*4C*). In HepG2 cells exposed to high palmitate, BBR administration also elevated the protein and mRNA expression of SETD2 and H3K36me3 (*Figure 4D*,*4E*). These results demonstrate the impact of the transcriptional regulation of BBR on SETD2 expression and activity.

SETD2 mediates the hepatoprotection of BBR against steatosis

Finally, we examined whether SETD2 mediates the hepatoprotection of BBR. We found that knockdown of SETD2 significantly increased the storage of lipid droplets in HepG2 cells exposed to palmitate, as detected by Oil Red O staining and triglyceride content (*Figure 5A*,*5B*). Moreover, in the presence of SETD2 knockdown, BBR treatment could not effectively reduce the generation of lipid droplets induced by palmitate challenge in HepG2 cells (*Figure 5A*,*5B*). Thus, SETD2 acts as a critical mediator for BBR to combat hepatic steatosis.

Discussion

The epigenetic mechanism of BBR in improving hepatic steatosis remains unknown. Our results revealed that BBR activated SETD2 and attenuated the generation of lipid droplets in hepatocytes. Importantly, SETD2 inactivation abolished the hepatoprotective effects of BBR, highlighting SETD2 as a crucial mediator. The modulation of BBR on SETD2 activity was demonstrated at the transcriptional level, underscoring the pleiotropic actions of BBR.

The anti-steatosis effect of BBR in hepatocytes has been documented in humans, mice, rats, hamsters, and fish (10,17-20), and in *in vitro* studies with normal human liver cells, primary mouse hepatocytes, and grass carp hepatocytes (21-23). In this study, we confirmed the hepatoprotective effects of BBR in a human liver cell line, namely HepG2 cells.

STED2 can inhibit the storage of hepatic triglyceride in NAFL. A previous study has suggested the potential importance of STED2 in the development of NAFL in a different model of metabolic disorders (16), despite the lack

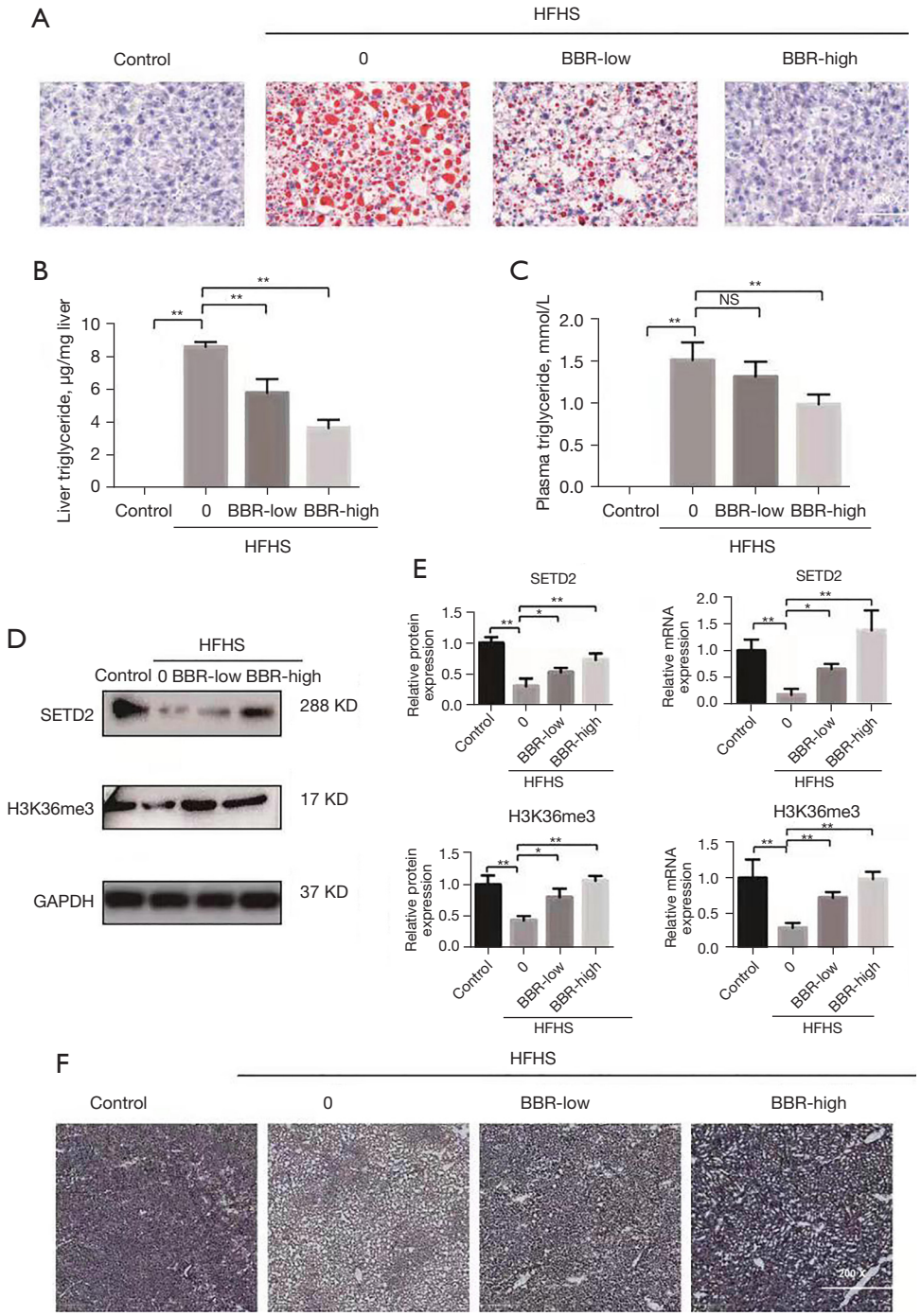

Figure 1 BBR attenuates the accumulation of triglycerides and increases the expression of SETD2 and H3K36me3 in HFHS diet-induced steatotic livers of mice. Male mice at 6 weeks old were fed by a HFHS diet for 12 weeks and then treated with high-dose BBR (10 mg/kg/day), low-dose BBR (5 mg/kg/day), or vehicle (PBS) by i.p. injection once daily for 5 weeks. (A) Representative photomicrographs of Oil Red O staining of the liver sections of mice (magnification, ×200). The levels of liver (B) and plasma (C) triglycerides were assessed in mice. Western blot analysis (D) and PCR analysis (E) of SETD2, H3K36me3, and GAPDH in mice. (F) Immunohistochemical staining of SETD2 in the livers of mice (magnification, ×200). The data are presented as mean ± SEM, n=3 each group. *, P<0.05; **, P<0.01. BBR, berberine; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; HFHS, high-fat, high-sucrose; H3K36me3, trimethylation of lysine 36 on histone 3; NS, not significant; PBS, phosphate buffered saline; SEM, standard error of the mean; SETD2, SET domain-containing protein 2.

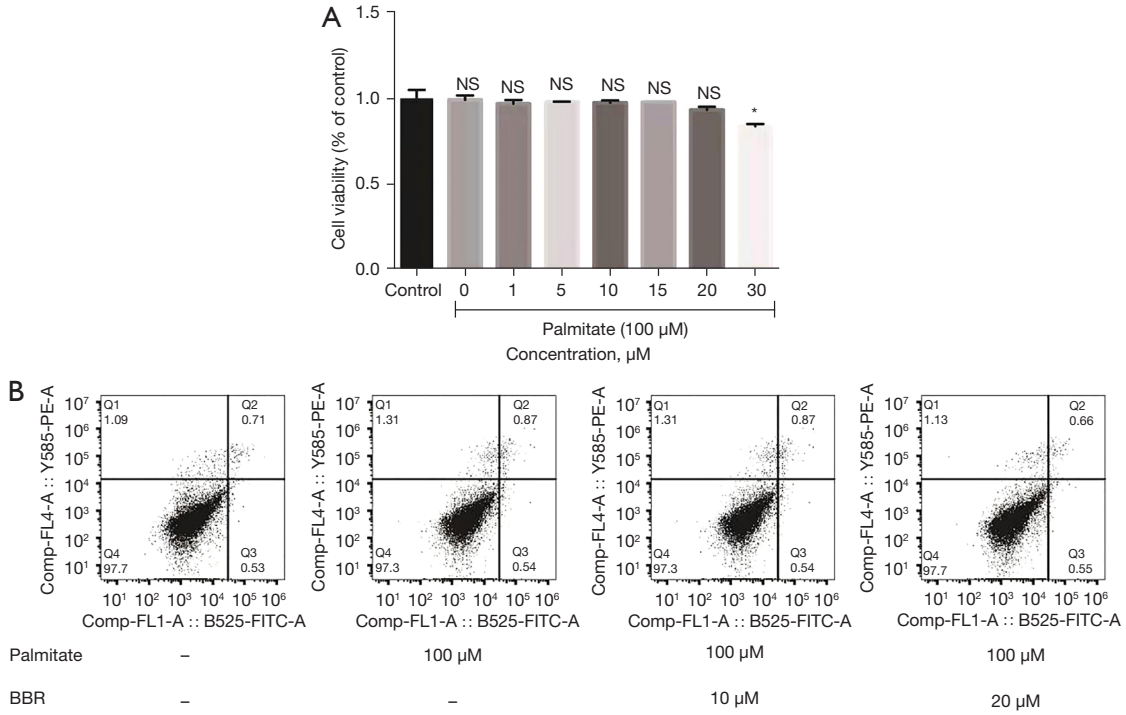

Figure 2 The toxic effect of BBR on HepG2 cells. Cell viability (A) and apoptosis (B) of HepG2 cells treated with varying concentrations of BBR (0, 1, 5, 10, 15, 20, and 30 μM) for 24 hours in serum-free media containing 100 μM palmitate. The data are presented as mean ± SEM, n=3 each group. *, P<0.05 versus control. BBR, berberine; NS, not significant; SEM, standard error of the mean.

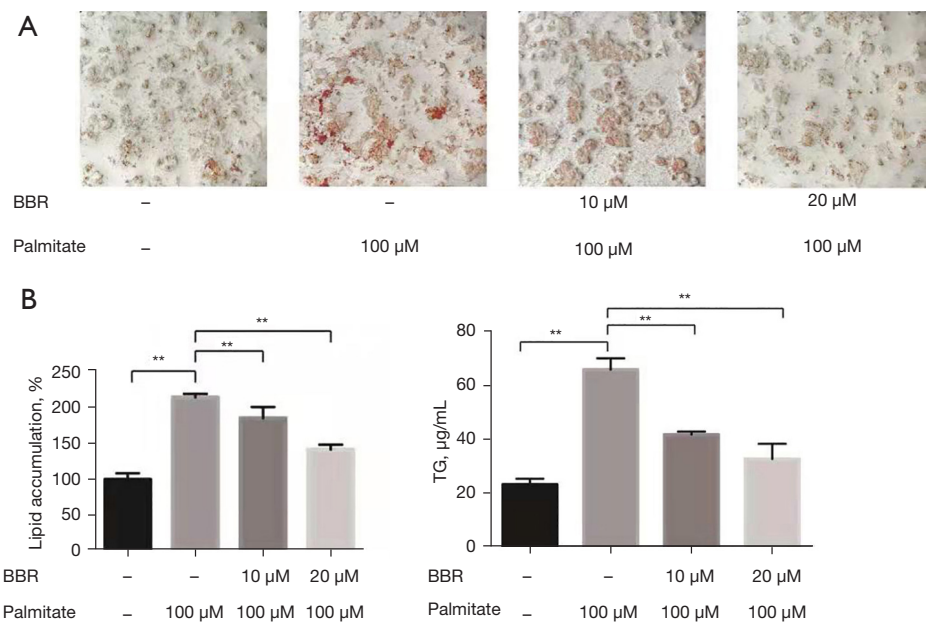

Figure 3 BBR alleviates high-lipid nutrition-induced lipid droplet accumulation of HepG2 cells. HepG2 cells were treated with or without BBR (10 or 20 μM) for 24 hours in serum-free media containing 100 μM palmitate. (A) Representative photomicrographs and quantitative analysis of Oil Red O staining (magnification, ×200). (B) Quantitative analysis of TG in HepG2 cells. The data are presented as mean ± SEM, n=3 each group. **, P<0.01. BBR, berberine; SEM, standard error of the mean; TG, triglyceride.

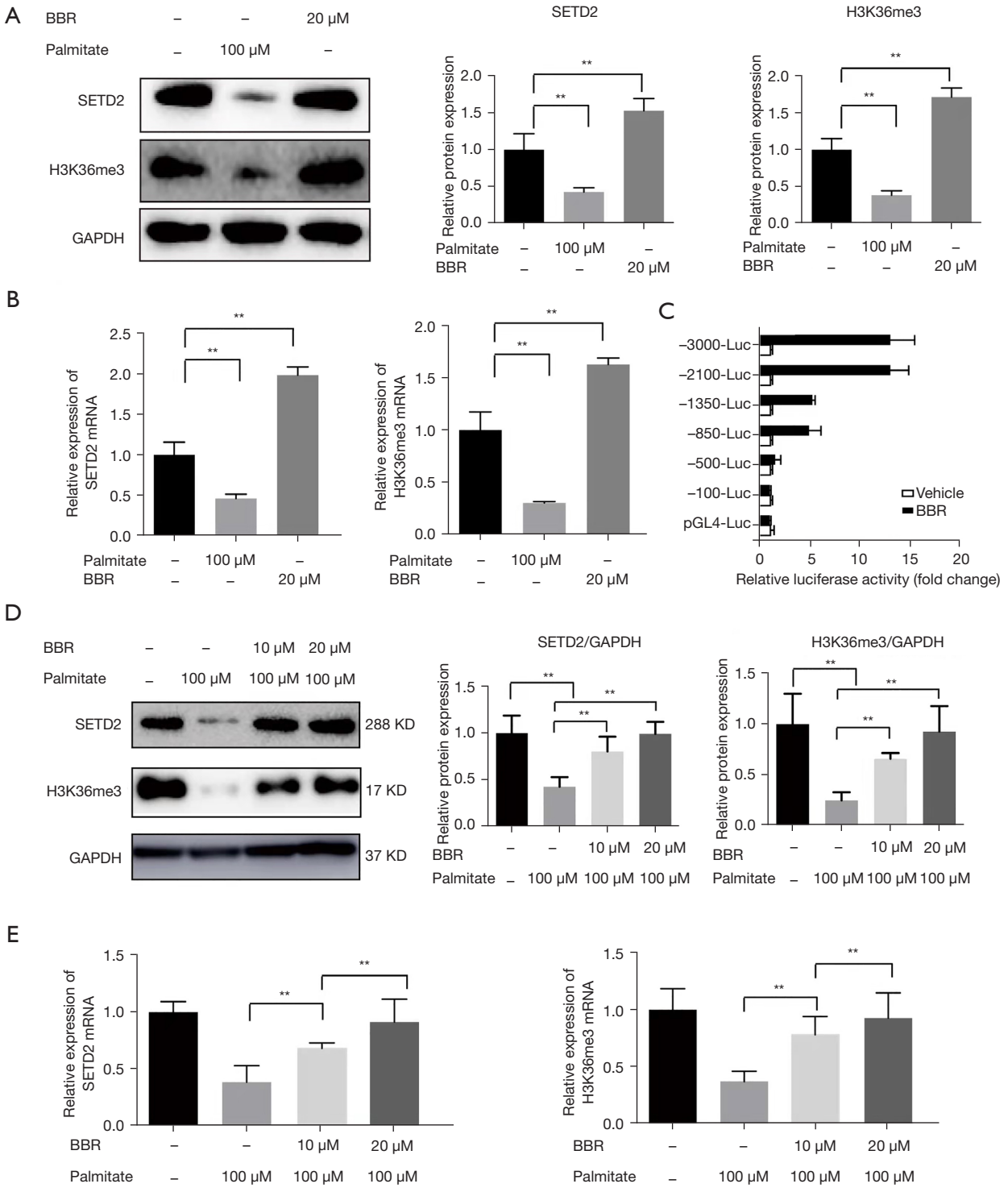

Figure 4 BBR increases the expression of SETD2 and H3K36me3 in HepG2 cells exposed to high-lipid nutrition. Western blot analysis (A,D) and PCR analysis (B,E) of SETD2 and H3K36me3 in HepG2 cells treated with or without BBR (10 or 20 μM) for 24 hours in serum-free media containing 100 μM palmitate. (C) Luciferase activity of SETD2 in HepG2 cells treated with BBR (20 μM). The data are presented as mean ± SEM, n=3 each group. **, P<0.01. BBR, berberine; SEM, standard error of the mean; SETD2, SET domain-containing protein 2.

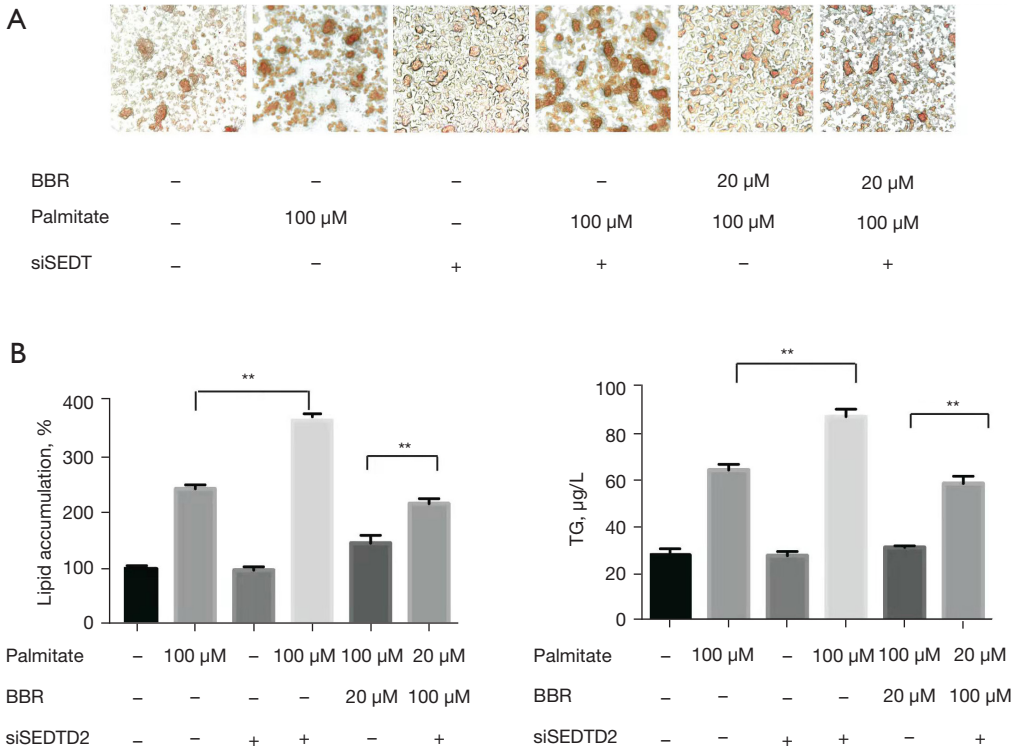

Figure 5 SETD2 mediates the effect of BBR on reducing high-lipid nutrition-induced the accumulation of lipid droplets in HepG2 cells. HepG2 cells were treated with 20 μM BBR and/or transfected with siRNA against SETD2 in serum-free media containing 100 μM palmitate. (A) Representative photomicrographs and quantitative analysis of Oil Red O staining (magnification, ×200). (B) Quantitative analysis of TG in HepG2 cells. The data are presented as mean ± SEM, n=3 each group. **, P<0.01. BBR, berberine; SEM, standard error of the mean; SETD2, SET domain-containing protein 2; TG, triglyceride.

of direct causality. In the current study, our data showed the downregulation of SETD2 in both mouse livers and HepG2 cells after metabolic challenge. Importantly, we identified that knockdown of SETD2 promotes the accumulation of lipid droplets in HepG2 cells. It is consistent with a recent report that a higher content of hepatic triglycerides and liver cancer were observed in liver-specific SETD2 knockout mice (15). The potential mechanism of above phenomenon are the decreased expression of cholesterol efflux genes and c-Jun/activator protein 1 activation (15).

Our evidence also suggests a potential mechanism underlying the regulation of BBR on SETD2 activity. First, the transcriptional upregulation of SETD2 by BBR was identified in intact hepatocytes. Second, promoter activity analysis confirmed this finding. Interestingly, the SETD2 downstream target H3K36me3 was also transcriptionally regulated by BBR, underscoring the dual control of BBR on SETD2 and its target.

In this study, we focused on the role of SETD2 in BBR-mediated hepatoprotection. In regards to the regulatory molecular network of SETD2 in the steatosis of hepatocytes, it seems plausible that lipogenesis-related genes are potential targets of the SETD2 signaling pathway, as previously reported (15). Moreover, to confirm the role of SETD2 in the anti-NAFL mechanism of BBR, using hepatocyte-specific SETD2 knockout mice is still required.

It is worthy to investigate in the future that whether histone demethylases, Jumonji domain containing (JMJD)-1C and plant homeodomain finger protein 2 (PHF2), participate in the anti-NAFL mechanism of BBR. Knockout of JMJD1C in mice lowered lipid accumulation in liver, whereas its overexpression contributed to hepatic lipogenesis (24). Increased expression of PHF2 also caused steatosis of liver in mice (25).

In conclusion, our findings suggest a critical role of SETD2 in the anti-NAFL effect of BBR. Pharmacological activation of SETD2 could be a novel anti-NAFL therapy.

Acknowledgments

Funding: This work was supported by the Traditional Chinese Medicine Discipline Program of Shanghai University of Traditional Chinese Medicine (grant No. Y1534).

Footnote

Reporting Checklist: The authors have completed the ARRIVE reporting checklist. Available at https://atm.amegroups.com/article/view/10.21037/atm-22-1753/rc

Data Sharing Statement: Available at https://atm.amegroups.com/article/view/10.21037/atm-22-1753/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://atm.amegroups.com/article/view/10.21037/atm-22-1753/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The process for animal experiments was approved and conducted in the experimental animal center of Longhua Hospital, Shanghai University of Traditional Chinese Medicine (No. LHERAW-19004). All surgeries were rigidly performed in accordance with globally recognized and institutional guidelines for the care and use of animals.

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(English Language Editor: C. Betlazar-Maseh)

Cite this article as: Dai L, Lu S, Shen T, Li Y, Chen J. Methyltransferase SETD2 mediates hepatoprotection of berberine against steatosis. Ann Transl Med 2022;10(10):552. doi: 10.21037/atm-22-1753

