

Berberine reduces methylation of the *MTTP* promoter and alleviates fatty liver induced by a high-fat diet in rats^S

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Abstract High-calorie food leads to nonalcoholic fatty liver disease (NAFLD) through dysregulation of genes involved in lipid metabolism, but the precise mechanism remains unclear. DNA methylation represents one of the mechanisms that contributes to dysregulation of gene expression via interaction with environmental factors. Berberine can alleviate fatty liver in *db/db* and *ob/ob* mice. Here, we investigated whether DNA methylation is involved in the pathogenesis of NAFLD induced by a high-fat diet (HFD) and whether berberine improves NAFLD through influencing the methylation status of promoters of key genes. HFD markedly decreased the mRNA levels encoding *CPT1α*, *MTTP*, and *LDLR* in the liver. In parallel, DNA methylation levels in the *MTTP* promoter of rats with NAFLD were elevated in the liver. Interestingly, berberine reversed the downregulated expression of these genes and selectively inhibited HFD-induced increase in the methylation of *MTTP*. Consistently, berberine increased hepatic triglyceride (TG) export and ameliorated HFD-induced fatty liver. Furthermore, a close negative correlation was observed between the *MTTP* expression and its DNA methylation (at sites -113 and -20). These data indicate that DNA methylation of the *MTTP* promoter likely contributes to its downregulation during HFD-induced NAFLD and, further, that berberine can partially counteract the HFD-elicited dysregulation of *MTTP* by reversing the methylation state of its promoter, leading to reduced hepatic fat content.—Chang, X., H. Yan, J. Fei, M. Jiang, H. Zhu, D. Lu, and X. Gao. Berberine reduces methylation of the *MTTP* promoter and alleviates fatty liver induced by a high-fat diet in rats. *J. Lipid Res.* 2010. 51: 2504–2515.

Supplementary key words DNA methylation • microsomal triglyceride transfer protein • nonalcoholic fatty liver disease

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Nonalcoholic fatty liver disease (NAFLD), characterized by excessive accumulation of triglyceride (TG) in the hepatocytes, affects from 10% to ~39% of people worldwide (1, 2). It is closely associated with obesity (3), insulin resistance (4), and type 2 diabetes (5). We previously reported that in NAFLD patients without type 2 diabetes, up to 31.4% of individuals meet the criteria of metabolic syndrome, and 43.2% meet the criteria of impaired glucose regulation, of which 14.4% are newly diagnosed with diabetes (6). In a hamster model, preventing intrahepatic lipid accumulation abrogates the development of hepatic insulin resistance; a dose-dependent relationship exists (7). Shulman et al. reported that moderate weight loss normalizes fasting hyperglycemia and improves hepatic insulin sensitivity in patients with poorly controlled type 2 diabetes by reducing hepatic triglyceride content (8). Several prospective studies (4–6, 9) have also shown that NAFLD can predict type 2 diabetes and metabolic syndrome. Thus, reducing hepatic fat accumulation can be an effective strategy to prevent type 2 diabetes. As the pathogenesis of NAFLD remains unclear, no drug is generally accepted, and the only effective treatment is lifestyle intervention, including low-calorie diet, weight loss, and exercise (10).

Abbreviations: ACC, acetyl CoA carboxylase; ALT, alanine transaminase; Apo, apolipoprotein; AST, aspartate aminotransferase; BBR, berberine; BRL, buffalo rat liver; CpG, cytosine phosphodiester bond guanine; CPT1α, carnitine palmitoyltransferase-1α; DGAT, diacylglycerol O-acyltransferase; DNMT, DNA methyltransferase; FPLC, fast protein liquid chromatography; GPAT, glycerol-3-phosphate acyltransferase; HFD, high-fat diet; LDLR, low-density lipoprotein receptor; MTTP, microsomal triglyceride transfer protein; NAFLD, nonalcoholic fatty liver disease; ND, normal diet; PPAR, peroxisome proliferator-activated receptor; SAH, S-adenosylhomocysteine; SAM, S-adenosylmethionine; SCD-1, stearoyl-CoA desaturase-1; 5-Aza-CdR, 5-aza-2'-deoxycytidine; SD, Sprague-Dawley; TC, total cholesterol; TG, triglyceride; UCP, uncoupling protein.

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Therefore, it is necessary to understand the pathogenesis of NAFLD to seek a safe and effective drug for reducing hepatic fat accumulation.

Berberine (BBR) is an alkaloid originally isolated from Huanglian (*Coptis chinensis*). Recent studies have shown that BBR can reduce body weight and improve dyslipidemia and insulin sensitivity in *db/db* mice (11), hamsters fed a high-fat diet, and patients with type 2 diabetes and dyslipidemia (12, 13). Moreover, BBR reduces serum cholesterol and LDL-cholesterol by elevating hepatic low-density lipoprotein receptor (*LDLR*) expression through a posttranscriptional mechanism that stabilizes its mRNA (12). Intraperitoneal injection of BBR for three weeks has been shown to alleviate hyperlipidemia and fatty liver in obese *db/db* and *ob/ob* mice; BBR is associated with changes in the mRNA levels of genes involved in hepatic and muscular lipid metabolism that enhance fatty acid oxidation and reduce lipogenesis (14). However, because the *ob/ob* and *db/db* mice are animal models that contain an inactivating mutation in the *leptin* or *leptin receptor* genes, respectively, the pathogenesis of fatty liver in these mice is greatly different from that of human nonalcoholic fatty liver disease. In addition, it has yet to be established whether BBR can improve fatty liver in the wild-type animal model of NAFLD induced by a high-fat diet (HFD).

A set of genes involved in hepatic β -oxidation and lipid export is decreased in the liver of patients with NAFLD (1). Recent studies have shown that modification by DNA methylation of the genes that regulate oxidative phosphorylation is associated with their decreased expression in human skeletal muscle (15, 16) and islets (17) in patients with type 2 diabetes, which increases their susceptibility to insulin resistance. The liver is a central organ in lipid and glucose metabolism, and it remains unclear whether DNA methylation plays a role in dysregulation of these genes in HFD-induced NAFLD and whether BBR can reverse fatty liver by influencing their methylated state.

In the present study, we attempted to investigate 1) whether abnormal expression of key genes involved in lipid metabolism in the liver of Sprague-Dawley (SD) rats with HFD-induced NAFLD is associated with DNA methylation modifications in their promoters; 2) whether BBR-mediated improvement of fatty liver is related to the demethylation within the promoter regions of these genes; and 3) how BBR affects the DNA methylation levels of certain genes.

EXPERIMENTAL PROCEDURES

Animal studies

Healthy male SD rats (5–6 weeks old) weighing 190–210 g were obtained from the Animal Development Center, Chinese Academy of Sciences, Shanghai, and acclimated for 1 week before initiation of the experiment. Rats were given free access to food and water and were maintained on a 12/12-h light/dark cycle. Rats received either a regular rodent chow (normal diet: 62.3% carbohydrate, 12.5% fat, 24.3% protein calories) or a high-fat diet (32.6% carbohydrate, 51.0% fat, 16.4% protein calories) for 24 weeks. Lard was the major constituent of the high-fat

diet. After 8 weeks of feeding, rats on the HFD were randomized to receive either BBR (Sigma-Aldrich, Steinheim, UK) at a dose of $200 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{day}^{-1}$ (BBR+HFD group) or an equal volume of vehicle (0.5% methylcellulose, HFD group) by gavage for 16 weeks. Rats fed the normal diet received the equal volume of vehicle (0.5% methylcellulose, ND group) as a control group. Body weight and food intake were monitored weekly. Fasting serum insulin (Rat insulin RIA kit, Linco Research, St Charles, MO) and glucose were measured every 4 weeks. At 16 weeks, an intraperitoneal glucose tolerance test (IPGTT) was performed for evaluating insulin sensitivity. After a fasting for 14 h, all rats were euthanized, and their livers were removed and stored in liquid nitrogen for quantitative real-time PCR (qPCR) analysis, hepatic fat content measurement, and DNA methylation analysis. Visceral fat mass, including mesenteric fat pad, epididymal fat pad, and perirenal fat tissue, was weighed. Total blood samples were also collected for measurement of fasting serum total cholesterol (TC), low density lipoprotein cholesterol (LDL-c), and TG levels. Serum TG, TC, and LDL-c were measured using commercially available kits. All experimental procedures involving the use of animals were conducted in conformity with PHS policy and were approved by the Animal Use and Care Committee of Fudan University.

Histological analysis

After the rats were sacrificed, the livers were removed and subsequently fixed in phosphate-buffered 10% formalin. The right lateral lobule of the liver was then divided into 2 sections at the long middle line, one of which was embedded in paraffin blocks and the other in O.C.T. compound. A section from each paraffin block was stained with hematoxylin and eosin (HE) to examine the pathologic structures of the liver and serial cryosections were stained with Sudan III to evaluate lipid droplets.

Liver lipid content

Hepatic lipids were extracted according to the method of Folch et al. (18). The TG content was determined as described previously (19). Briefly, lipid was extracted from frozen liver tissues (30 mg) by homogenization in 1 ml of 2:1 chloroform:methanol, followed by shaking at room temperature for overnight and centrifugation at 3000 rpm for 10 min. Aliquots (400 μl) of the organic-extract lipid suspension were used for the measurement of triglyceride concentrations (TG kit, Sysmex, Japan). Hepatic lipid content was defined as mg of triglyceride per gram of the liver.

Real-time quantitative RT-PCR analysis

Key enzymes of lipid metabolism, which were analyzed by qPCR, were selected as candidate genes for assessment of their mRNA expression levels in the liver of these rats. Those genes that were significantly downregulated in the HFD group and upregulated by BBR treatment were subsequently chosen as targets for further DNA methylation analysis. Total RNA was isolated from liver tissues using Trizol reagent (Invitrogen, Carlsbad, CA). cDNA was synthesized by reverse transcription using ReverTra Ace (Toyobo, Osaka, Japan). The SYBR Green PCR Master Mix (Toyobo, Osaka, Japan) was used for qPCR with a sequence detection system (ABI PRISM7900, Applied Biosystems, Foster City, CA). The 8 μl reaction mixture contained 1 μl of cDNA and 125 nmol/l of primers. The specific primers used for qPCR are shown in supplementary Table I. The same reaction was performed in triplicate with rat β -actin as an internal control. Fluorescent signals were normalized to an internal reference (ΔR_n), and the threshold cycle (C_t) was set within the exponential phase of PCR. The relative gene expression was calculated using the $2^{-\Delta\Delta C_t}$ as described previously (20).

Western immunoblot analysis

Protein from rat liver samples was extracted with RIPA buffer [50 mmol/l Tris-HCl (pH 7.4), 1% NP-40, 0.5% sodium deoxycholate, 150 mmol/l NaCl, 0.1% SDS, EDTA, etc.] containing protease and phosphatase inhibitors. Protein concentrations were measured using a BCA-100 Protein Quantitative Analysis Kit. After denatured, protein samples were subjected to SDS-PAGE and blotted onto polyvinylidene difluoride (Millipore) membranes. Nonspecific binding sites were blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween 20 for 1 h and then incubated with primary antibodies against MTTP (Santa Cruz Biotechnology, Santa Cruz, CA), CPT-1 α (Santa Cruz), SCD-1 (Santa Cruz), LDLR (Abcam) overnight at 4°C. After three washes in Tris-buffered saline containing 0.1% Tween 20, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (anti-mouse or anti-rabbit IgG) for 1 h and visualized by ECL detection (Pierce Biotechnology, Rockford, IL). Quantitation was performed by Fujifilm Las-3000 Luminescent Image Analyzer.

Analysis of DNA methylation within the promoters of target genes in the liver

All reagents were purchased from Sigma (St Louis, MO). Genomic DNA was isolated from the livers of the three groups of rats using the SDS and proteinase K methods, and DNA bisulfite modification was performed as previously described (20). Bisulfite-modified DNA was amplified with primers (see supplementary Table I) designed using the MethPrimer program (<http://www.urogene.org/methprimer/index.html>). Direct sequencing and classic cloning/sequencing methods were used to detect DNA methylation. Briefly, promoters of target genes were amplified with Taq polymerase (Tiangen Biotech, Beijing, China). Then 2 μ l of the PCR products were purified by shrimp alkaline phosphatase-exonuclease I (SAP-ExonI) and subjected to sequencing using a DNA sequencer (ABI PRISM 3730, Applied Biosystems) with Bigdye terminator v3.1 cycle sequencing kit (Applied Biosystems). If there was a single "C" at a cytosine phosphodiester bond guanine (CpG) site, the site was defined as complete methylation; if there was a single "T" at the CpG site, it was considered as no methylation. Overlapping of "C" and "T" was ranked as partial methylation. Classic cloning/sequencing was also used to accurately measure the levels of DNA methylation. The PCR products ($n = 4$) were cloned into plasmid vectors (pGM-T Cloning kit; Tiangen Biotech, Beijing, China), which were subsequently transformed into *Escherichia coli*. Plasmid DNA of ten clones derived from each individual hepatic sample was extracted (AxyPrep Plasmid Miniprep Kit, Axygen Bioscience) and sequenced, and the number of methylated sites was determined. The mean methylation level for each rat was calculated as the total number of methylation sites divided by the total number of possible methylation sites in all clones sequenced, and then multiplied by 100. The proportion of methylation for each CpG site was calculated as the number of methylated cytosine divided by total number of samples in each rat.

Serum lipoprotein-associated TG analysis

Equal volumes of serum samples were pooled from rats for three groups in the fasting states at end of 16 weeks of BBR administration. Lipoproteins were separated using fast protein liquid chromatography (FPLC) (21) on a Superose 6 10/300 GL column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Samples were chromatographed at a flow rate of 0.5 ml/min, and fractions of 500 μ l each were collected and assayed for TG. The contents of apolipoprotein (apo)B100, apoB48, and apoE in the fractions were analyzed by Western immunoblotting. Proteins in

the fractions were separated by SDS-PAGE using 4–5% or 5–12% gradient gels and transferred to polyvinylidene difluoride (Millipore). The membranes were incubated with primary antibodies against apoB (RayBiotech, Norcross, GA) and apoE (Abcam), followed by donkey anti-rabbit IgG horseradish peroxidase and visualized by ECL detection (Pierce Biotechnology).

In vivo VLDL triglyceride production and serum lipoprotein fractionation

Male SD rats (5–6 weeks old, 190–210 g) fed a HFD (20% carbohydrate, 59% fat, 21% protein calories) were randomized to receive either BBR (Sigma-Aldrich, Steinheim, UK) at a dose of 200 mg \cdot kg⁻¹ \cdot day⁻¹ (BBR+HFD group) or an equal volume of vehicle (0.5% methylcellulose, HFD group) by gavage for 8 weeks, and rats fed regular rodent chow received the vehicle as control (ND group). After BBR treatment, rats were injected with tyloxapol (Sigma-Aldrich) at 600 mg \cdot kg⁻¹ via tail vein after a fasting period of 4 h. Orbital venous samples were taken at 0, 1, 2, and 3 h after tyloxapol injection. After centrifugation, serum was kept at -80°C until use. Serum TG was determined with a commercial kit (Sigma kit TR0100). VLDL triglyceride production was assessed from the triglyceride concentrations. At 24 h after tyloxapol injection, serum was collected and then analyzed for lipoproteins by FPLC.

Cell culture experiments

Buffalo rat liver (BRL) cells were cultured in 6-well plates at 2×10^6 cells per well in DMEM containing 10% FBS in an atmosphere of 10% CO₂ at 37°C. After 24 h, the monolayer was washed with PBS and incubated with 1% FBS-DMEM containing 20 μ M BBR or 5 μ M 5-aza-2'-deoxycytidine (5-Aza-CdR, Sigma). Palmitate (Sigma) was complexed with essentially fatty acid-free BSA (BSA) to generate a stock solution of 11% (w/v) BSA and 10 mM palmitate in serum-free medium, which was diluted into 1% FBS-DMEM for preparation of 0.1 mM palmitate. BRL cells were incubated with 1% FBS-DMEM containing 100 μ M palmitate and 20 μ M BBR or 5 μ M 5-Aza-CdR for 72 h. Cells were washed with PBS and used for RNA extraction and qPCR analysis.

Statistical analysis

All data were presented as mean \pm SEM. Significance was assessed among three groups by one-way ANOVA followed by Tukey's multiple comparison test and LSD. To compare food intake, serum glucose, and insulin levels between BBR- and vehicle-treated groups, independent-samples *t*-test was used, depending on a preliminary F-test for homogeneity of variance. To determine correlations between gene expression levels and DNA methylation degrees, we used Pearson correlation coefficients for normally distributed values or Spearman correlation coefficients when normality was rejected. All *P* values were two-tailed; *P* values less than 0.05 were considered statistically significant.

RESULTS

Effects of BBR on body weight, visceral fat, and serum lipid profiles of HFD-fed rats

Rats fed a HFD tended to develop obesity (Fig. 1A). After 11 weeks of BBR treatment, body weights of HFD-fed rats were significantly reduced, reaching similar levels of ND-fed control animals ($P < 0.05$, Fig. 1B). BBR did not result in dramatic changes in food intake, although slight differences were observed at weeks 10 and 13 (Fig. 1C). Notably, BBR treatment for 16 weeks significantly lowered visceral fat

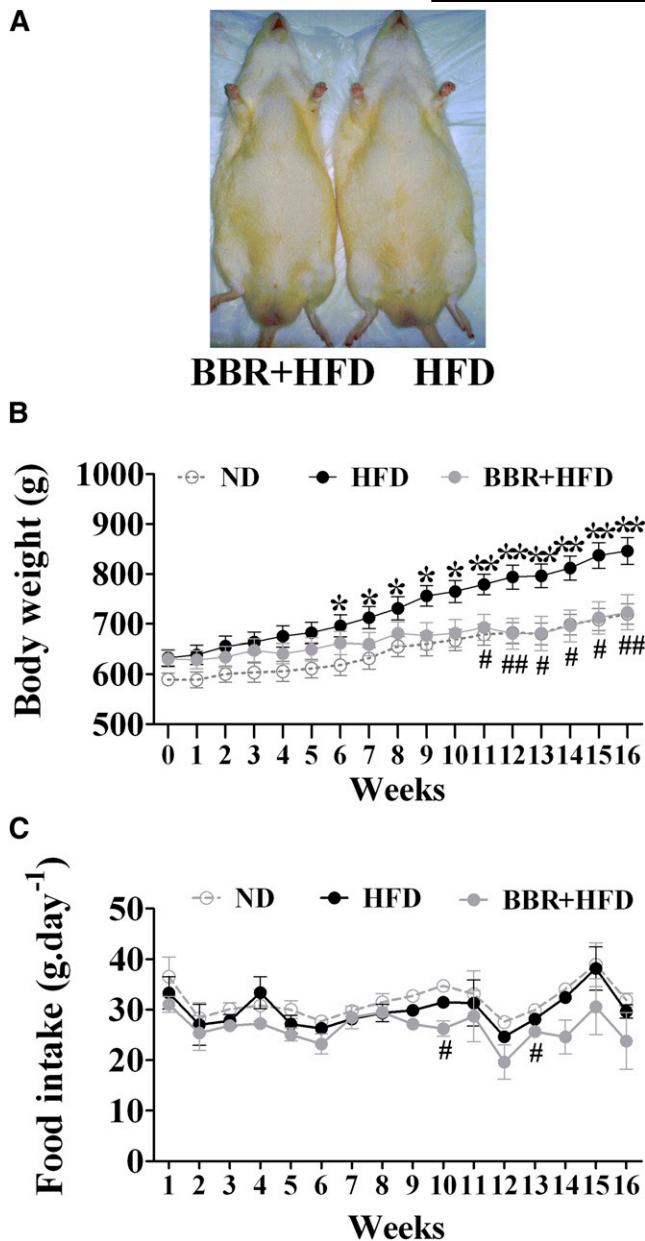


Fig. 1. Effects of BBR on body weight of rats fed a high-fat diet. Male SD rats at 6 weeks of age received either a regular rodent chow (ND) or a HFD. After 8 weeks of feeding, rats on the HFD were treated for 16 weeks with BBR or vehicle ($n = 8$ per group). **A:** Gross appearance of HFD-fed rats at the end of treatment with BBR (BBR+HFD) or vehicle (HFD). **B:** Changes of body weight were monitored during BBR treatment. **C:** Effects of BBR on food intake. Values are mean \pm SEM. * $P < 0.05$, ** $P < 0.01$ versus ND-fed control group (ND); # $P < 0.05$, ## $P < 0.01$ versus HFD-fed vehicle group (HFD). BBR, berberine; HFD, high-fat diet; ND, normal diet; SD, Sprague-Dawley.

mass by up to 42.8% ($P < 0.05$) and reduced the serum levels of TC and LDL-c (Table 1), whereas BBR did not significantly affect serum TG levels (HFD group, 1.02 ± 0.10 mmol/l, $n = 8$; BBR+HFD group, 0.95 ± 0.12 mmol/l, $n = 7$; $P = 0.32$) or HDL-c levels (supplementary Table II). Further analysis of the lipid profiles at 4, 8, and 16 weeks after BBR treatment showed that BBR did not exhibit significant effects on serum TC and LDL-c until at 16 weeks (supple-

TABLE 1. General parameters of BBR treatment for 16 weeks and control groups

Parameter	ND	HFD	BBR+HFD
Visceral fat (g)	45.1 \pm 6.0	74.7 \pm 8.2 ^a	42.7 \pm 5.8 ^b
Serum TC (mmol/l)	2.17 \pm 0.13	2.81 \pm 0.28 ^a	2.02 \pm 0.21 ^b
Serum LDL-c (mmol/l)	0.54 \pm 0.04	2.10 \pm 0.31 ^a	1.22 \pm 0.19 ^b
Serum TG (mmol/l)	2.01 \pm 0.51	1.02 \pm 0.10 ^a	0.95 \pm 0.12 ^a
Serum ALT (IU/l)	68.0 \pm 4.3	106.7 \pm 15.6 ^a	69.0 \pm 6.4 ^b
Serum AST (IU/l)	194.7 \pm 17.3	239.9 \pm 43.9	167.3 \pm 13.8

Data are presented as mean \pm SEM ($n = 8$). BBR, berberine; HFD, high-fat diet; ND, normal diet; TG, triglyceride; ALT, alanine transaminase; AST, aspartate aminotransferase.

^a $P < 0.05$ versus ND (normal diet + vehicle group).

^b $P < 0.05$ versus HFD (high-fat diet + vehicle group).

mentary Table II). These data suggest that BBR exerts antagonizing effects on HFD-induced obesity, and long-term BBR treatment can improve serum TC and LDL-c.

BBR alleviates liver steatosis in HFD-fed SD rats

Histological analysis by HE or Sudan III staining of the liver sections showed derangement of liver cells and excessive lipid droplets in the hepatocytes of SD rats fed the HFD (Fig. 2A), which were alleviated by BBR treatment. Consistently, treatment of HFD-fed rats with BBR for 16 weeks significantly lowered liver weight by 21% ($P < 0.05$, Fig. 2B) and decreased hepatic triglyceride contents (by 14%, $P < 0.01$, Fig. 2C). Serum alanine transaminase (ALT) and aspartate aminotransferase (AST) levels were also reduced by BBR treatment (Table 2). Thus, BBR alleviates HFD-induced liver steatosis.

BBR improves insulin sensitivity in SD rats with HFD-induced NAFLD

Whereas fasting serum glucose levels were slightly lower in BBR-treated, HFD-fed rats than those of the vehicle-treated group (Fig. 3A), treatment with BBR for 4–16 weeks resulted in sustained reduction in fasting serum insulin levels (Fig. 3B). Analysis by IPGTT showed that the area under curve (AUC) of serum glucose level ($P < 0.05$, Fig. 3C) was significantly decreased in BBR-treated rats, and notably, the AUC of serum insulin levels in the vehicle-treated, HFD-fed group was significantly higher than that of BBR-treated group ($P < 0.05$, Fig. 3D). These results demonstrate that BBR improves insulin resistance associated with HFD-induced obesity and fatty liver.

BBR reverses HFD-induced abnormal expression of some key genes associated with lipid metabolism in the liver

Next we tested by qPCR analysis whether BBR affects HFD-induced dysregulation of key genes known to be involved in lipid homeostasis. The relative mRNA levels of *CPT-1 α* ($P < 0.05$, Fig. 4A), *MTTP*, and *LDLR* ($P < 0.05$, Fig. 4B) were significantly downregulated in the livers of HFD-fed rats relative to the ND control group. BBR treatment significantly reversed the downregulating effects of HFD on the expression of *CPT-1 α* and *MTTP* ($P < 0.05$). Meanwhile, BBR upregulated *LDLR* gene expression, although there is no statistical significant difference between BBR-treated group and HFD control group ($P > 0.05$). On the

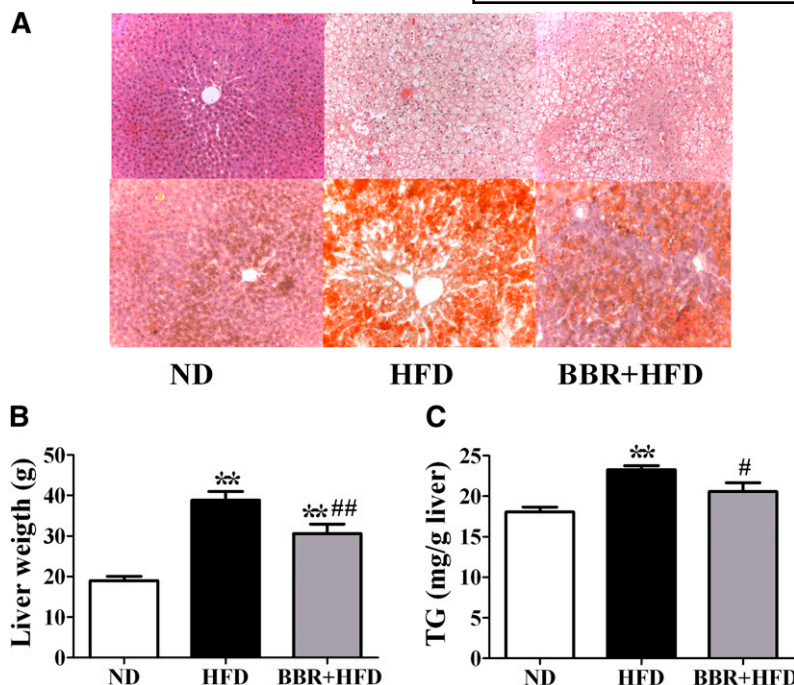


Fig. 2. BBR alleviates HFD-induced fatty liver in SD rats. After BBR treatment for 16 weeks, rats were euthanized after a 14 h fast. A: Histological analysis of livers of ND-fed, vehicle-treated rats (ND group) and HFD-fed rats treated with vehicle (HFD group) or BBR (BBR+HFD group). Liver sections were stained with hematoxylin and eosin (upper panel) or with Sudan III (lower panel). Photographs are at 200 \times magnification. B: Effects of BBR on liver weight. C: Effects of BBR on hepatic TG contents. Significance was assessed by one-way ANOVA followed by Tukey's multiple comparison test. Data are mean \pm SEM. * P < 0.05, ** P < 0.01 versus ND; # P < 0.05, ## P < 0.01 versus HFD. BBR, berberine; HFD, high-fat diet; ND, normal diet; SD, Sprague-Dawley; TG, triglyceride.

other hand, the level of *SCD-1* mRNA was increased in rats with NAFLD, which was markedly lowered by BBR treatment (P < 0.05, Fig. 4D). We also found that the *PPAR γ* and *GPAT* mRNAs were significantly altered by HFD feeding (P < 0.05, Fig. 4C), but these were not affected by BBR treatment. Despite these changes, BBR did not influence the expression of other lipogenic and fatty acid oxidation genes, such as *ACC*, *DGAT1*, *DGAT2*, *PPAR α* , and *UCP-2*. Consistent with alterations in their mRNA expression levels, Western immunoblot analysis of liver protein extracts also revealed corresponding changes in the protein levels of *MTTP*, *CPT-1 α* , *LDLR*, and *SCD-1* (Fig. 5A), with *CPT-1 α* , *LDLR*, and *MTTP* significantly increased and *SCD-1* prominently decreased in BBR-treated, HFD-fed rats (Fig. 5B). Thus, these data suggested that BBR might selectively alter the expression of certain target genes, which prompted us to examine whether the expression of these genes in the liver are influenced by DNA methylation.

Analysis of methylation in the promoter regions of select genes in lipid metabolism

Next we chose *MTTP*, *CPT-1 α* , and *LDLR* as target genes for further DNA methylation analysis. As indicated in Fig. 6A, the sequence analyzed for the *MTTP* promoter (+1 indi-

cates the start site of transcription) contains a CpG island with five potential methylation sites. Genomic sequencing chromatograms for each of the three treatment groups showed that all nonCpG cytosines were replaced by thymine, and that the ratio of C:(C+T) (i.e., methylation) was markedly altered at each CpG site among the three treatment groups of rats (Fig. 6B). Sequences located at -260 to +165 (Fig. 6C) in the *CPT-1 α* promoter region and at -389 to +76 (Fig. 6E) in the *LDLR* promoter, which contain 37 and 23 potential methylation sites, respectively, were also analyzed for DNA methylation using specific bisulfate-sequencing primers (supplementary Table I). In contrast to the results for the *MTTP* promoter, direct sequencing indicated that nearly all C's were completely replaced by T in all of the CpG sites within the *CPT-1 α* (Fig. 6D) and *LDLR* (Fig. 6F) promoter sequences analyzed, suggesting that DNA methylation modifications of the *CPT-1 α* and *LDLR* promoters were not efficiently upregulated in the livers of the HFD-fed rats.

BBR treatment reduces HFD-elicited increases in the level of DNA methylation in the *MTTP* promoter region

To examine further whether BBR exerts an inhibitory effect on HFD-dependent methylation changes in the *MTTP* promoter, we used the classic cloning/sequencing method to quantitatively analyze the methylation levels within the *MTTP* promoter region in the livers of rats. Sequencing of ten clones of the PCR products from each rat revealed that the mean methylation level of the *MTTP* promoter was higher in HFD-fed rats than that in the ND-fed control group ($37.6 \pm 6.3\%$ versus $11.8 \pm 0.6\%$, P < 0.01) (Fig. 7A). When methylation at each CpG site was analyzed individually among the three groups, the degrees of DNA methylation at -174, -113, and -20 CpG sites were significantly increased by HFD feeding (site -174: ND, $9.4 \pm 5.9\%$ versus HFD, $48.3 \pm$

TABLE 2. General parameters of rats treated with BBR or vehicle for 8 weeks

Parameter	ND	HFD	BBR+HFD
Body weight(g)	487.4 \pm 13.4	525.5 \pm 10.1	472.8 \pm 12.4 ^b
Visceral fat (g)	11.2 \pm 0.9	27.8 \pm 3.8 ^a	13.5 \pm 3.8 ^c
Liver weight (g)	17.7 \pm 0.8	19.3 \pm 0.5	18.0 \pm 1.0
Fast serum TG (mmol/l)	4.0 \pm 0.2	7.0 \pm 0.7 ^a	4.2 \pm 0.4 ^c

Data are presented as mean \pm SEM (n = 6). BBR, berberine; HFD, high-fat diet; ND, normal diet; TG, triglyceride.

^a P < 0.01 versus ND (normal diet + vehicle group).

^b P < 0.05.

^c P < 0.05 versus HFD (high-fat diet + vehicle group).

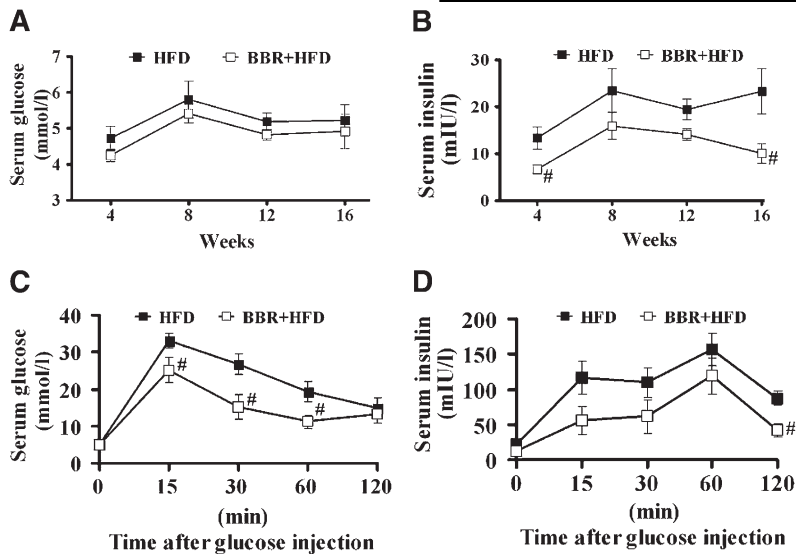


Fig. 3. BBR lowers serum insulin levels and improves insulin resistance in NAFLD rats. Fasting serum glucose (A) and insulin (B) were measured every 4 weeks during 16-week BBR treatment of HFD-fed rats. Serum glucose (C) and insulin (D) levels were determined during intraperitoneal injection of glucose ($2\text{g} \cdot \text{kg}^{-1}$ body weight) in HFD-fed rats at 16 weeks of BBR treatment. Values are mean \pm SEM. * $P < 0.05$, ** $P < 0.01$ versus HFD group. BBR, berberine; HFD, high-fat diet; NAFLD, nonalcoholic fatty liver disease.

10.7%, $P < 0.05$; site -113: $9.4 \pm 3.1\%$ versus $33.3 \pm 2.4\%$, $P < 0.01$; site -20: $3.1 \pm 3.1\%$ versus $35.6 \pm 4.4\%$, $P < 0.01$; respectively; Fig. 7B). Importantly, BBR treatment resulted in lower mean methylation levels (HFD, $37.6 \pm 6.3\%$ versus BBR+HFD, $16.5 \pm 3.4\%$, $P < 0.05$) (Fig. 7A) and lower levels of DNA methylation at -174, -113, and -20 CpG sites (site -174: HFD, $48.3 \pm 10.7\%$ versus BBR+HFD, $15.0 \pm 5.0\%$, $P < 0.05$; site -113: $33.3 \pm 2.4\%$ versus $7.5 \pm 4.9\%$, $P < 0.01$; and site -20: $35.6 \pm 4.4\%$ versus $15.0 \pm 2.9\%$, $P < 0.01$, respectively; Fig. 7B) within the *MTTP* promoter in the liver of HFD-fed rats with NAFLD. Of particular note, an inverse correlation was found between the expression level of *MTTP* mRNA and the degrees of DNA methylation at -113 and -20 CpG sites (site -113, $r = -0.636$, $P = 0.026$, Fig. 7C; site -20, $r = -0.726$, $P = 0.008$, Fig. 7D). On the other hand, no significant correlation was observed between the *MTTP* mRNA level and the DNA methylation at -174 CpG site ($r = -0.481$, $P = 0.1135$) or the mean DNA methylation degree ($r = -0.434$, $P = 0.158$). These data

suggest that BBR may influence the expression of genes involved in lipid metabolism by affecting the methylation modifications of their promoters, which contributes to its alleviating actions in reducing HFD-induced hepatic lipid overload.

Direct effects of BBR on the expression of *MTTP* in cultured rat liver cells

To confirm further that BBR affects *MTTP* transcription through DNA methylation inhibition, we investigated whether BBR could directly upregulate the expression of *MTTP* compared with 5-aza-2'-deoxycytidine (5-aza-CdR, a potent demethylating agent) in a rat liver cell line, BRL cells. Treatment of BRL cells with 20 μM BBR or 5 μM 5-aza-CdR caused similarly time-dependent increases in the mRNA expression of *MTTP* as determined by qPCR (Fig. 8A). In addition, incubation of BRL cells with palmitate (100 μM) resulted in reduced *MTTP* mRNA abundance, and treatment by 20 μM BBR or 5 μM 5-aza-2'-deoxycytidine effectively reversed the sup-

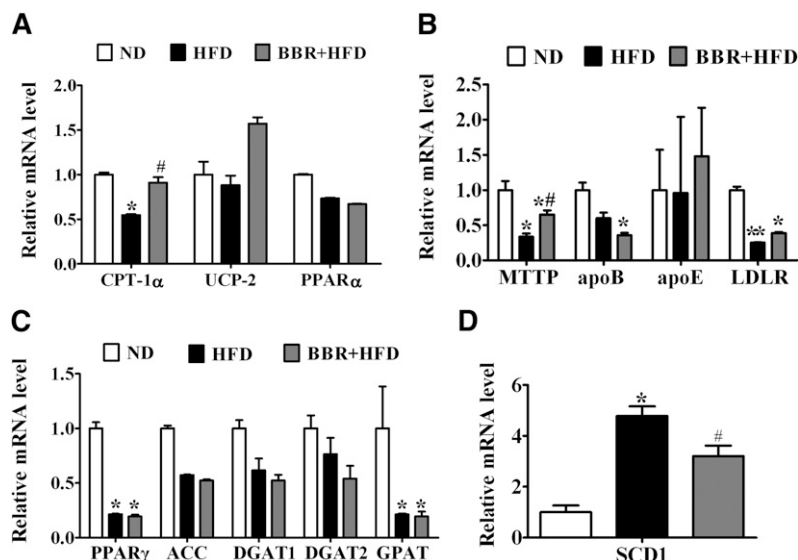


Fig. 4. BBR reverses HFD-induced dysregulation of key genes in lipid metabolism in the liver of SD rats. Real-time quantitative PCR analysis of fatty acid oxidation (A), VLDL assembly and secretion (B), and lipogenesis (C and D) in the livers of SD rats of ND, HFD, and BBR+HFD groups. Relative mRNA amounts of each gene were normalized to that of β -actin. Values are mean \pm SEM. * $P < 0.05$, ** $P < 0.01$ versus ND; # $P < 0.05$, ## $P < 0.01$ versus HFD. ACC, acetyl CoA carboxylase; Apo, apolipoprotein; BBR, berberine; CPT-1 α , carnitine palmitoyltransferase-1 α ; DGAT, diacylglycerol O-acyltransferase; HFD, high-fat diet; ND, normal diet; LDLR, low density lipoprotein receptor; SCD-1, stearoyl-CoA desaturase-1; SD, Sprague-Dawley; UCP, uncoupling protein.

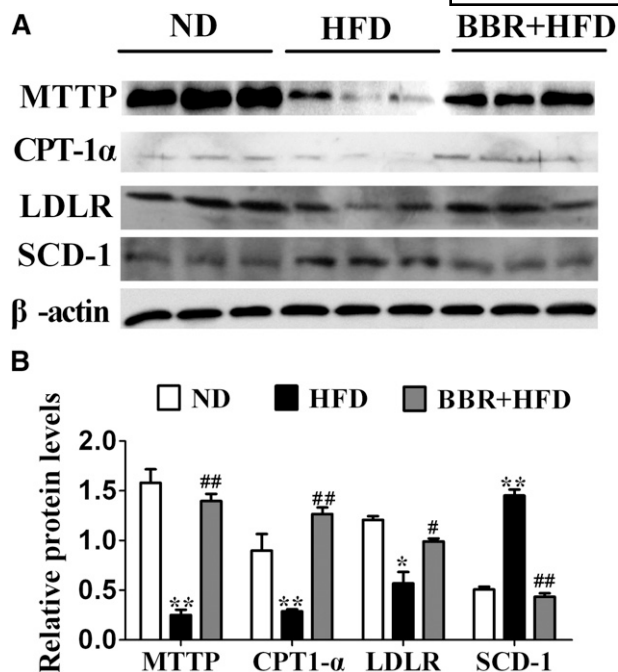


Fig. 5. BBR normalizes the liver expression levels of MTTP, CPT-1α, LDLR, and SCD-1 proteins in HFD-fed rats. A: Western immunoblot analysis of the liver protein extracts from ND, HFD, and BBR+HFD group (n = 3 per group). B: Quantitation of the protein levels from the immunoblots after normalization to β-actin as the loading control. BBR, berberine; CPT-1α, carnitine palmitoyltransferase-1α; HFD, high-fat diet; LDLR, low density lipoprotein receptor; MTTP, microsomal triglyceride transfer protein; ND, normal diet; SCD-1, stearoyl-CoA desaturase-1.

pressive effect of palmitate on *MTTP* mRNA expression (Fig. 8B). These data indicate that BBR is able to up-regulate *MTTP* expression in a cell-autonomous manner, likely through affecting its methylation status.

BBR does not affect hepatic DNA methyl transferases but reduces serum homocysteine levels

Next we tested whether BBR affected *MTTP* expression through regulating DNA methyl transferases. No significant differences were detected in the mRNA and protein levels of DNA methyltransferase (DNMT)1 and DNMT3b from the liver of HFD-fed rats upon BBR treatment (supplementary Fig. 1). Moreover, BBR treatment did not alter the total hepatic DNMT enzyme activity ($P > 0.05$, supplementary Table III). On the other hand, in rats with HFD-induced fatty liver, the serum level of homocysteine was strikingly higher than that in ND group ($P < 0.01$, supplementary Table III). Interestingly, BBR treatment significantly reduced serum homocysteine to the level of the ND group, whereas no significant differences were found in the hepatic contents of S-adenosylmethionine (SAM), S-adenosylhomocysteine (SAH), or the SAM/SAH ratio between BBR-treated, HFD-fed rats and control rats fed the HFD or ND ($P > 0.05$, supplementary Table III). These data imply that BBR might suppress DNA methylation through its effect on the pool of methyl donors that remain to be further defined.

BBR treatment for 16 weeks increased serum VLDL-associated TG levels

MTTP is required for the secretion of VLDL from the liver, and the amount of triglycerides in VLDL fractions is strikingly decreased in the liver of tissue-specific MTTP knockout mice (22). Given our observation that BBR treatment resulted in upregulated hepatic *MTTP* expression and increased MTTP protein abundance in HFD-fed rats, we examined whether BBR could increase the TG content in VLDL fractions. Indeed, measurement of serum lipoprotein-associated TG (Fig. 9A) by FPLC showed that BBR treatment for 16 weeks significantly increased VLDL-TG levels, by ~26% according to the area under curve (AUC) (Fig. 9B) compared with vehicle-treated animals fed a HFD. On the other hand, HFD feeding in the absence of BBR treatment led to decreased serum VLDL-TG levels by ~41% compared with ND control group (Fig. 9A, B). Moreover, Western immunoblot analysis showed higher levels of apoB100 and apoB48 in VLDL fractions from the BBR-treated group than in those from the control group (Fig. 9C), whereas no apparent alterations were observed for apoE protein from these VLDL fractions. These data indicate that BBR treatment caused an increase in the triglyceride-rich VLDL particles, which was decreased upon long-term HFD feeding, consistent with the observed changes of hepatic MTTP expression levels.

Effect of BBR treatment for 8 weeks on hepatic VLDL production

To directly test the effect of BBR treatment on VLDL production from the liver, we measured serum TG and fractionated VLDL-TG from BBR-treated SD rats following intravenous injection of tyloxapol, which can prevent the metabolism and removal of plasma lipoprotein by inhibiting lipoprotein lipase activity (23). Rats that were fed HFD and simultaneously treated with BBR for 8 weeks exhibited decreased body weight and serum TG by 18% and 41%, respectively (Table 2). Notably, more increasingly elevated serum TG levels were observed in BBR-treated, HFD-fed rats (by ~55%, ~195%, and ~257% at 1, 2, and 3 h) relative to the vehicle-treated control group (by ~40%, ~132%, and ~160% at 1, 2, and 3 h) after tyloxapol administration (Fig. 10A), suggesting the tendency of increased hepatic TG production upon BBR treatment. While we observed considerably higher amounts of TG in the VLDL fractions in HFD-fed rats than in those of ND-fed control animals (Fig. 10B, C), markedly increased TG levels (~2-fold) in VLDL particles were observed in BBR-treated, HFD-fed rats compared with the vehicle-treated group (Fig. 10B, C). These results further indicated that BBR treatment led to increased secretion of VLDL-TG from the liver.

DISCUSSION

In this study we have shown that BBR could alleviate HFD-induced fatty liver and reversed the abnormal expression of *MTTP*, *CPT-1α*, *SCD-1*, and *LDLR* in SD rats with HFD-induced NAFLD. BBR treatment increased TG-rich VLDL particles from the liver of HFD-fed rats. Impor-



The precise mechanisms by which HFD causes the development of NAFLD or BBR improves fatty liver remain largely unclear. Defects in lipid metabolism pathways, including de novo lipogenesis, fatty acid oxidation, hepatic fatty acid influx, and/or fat export in the form of VLDL, are closely linked to hepatic steatosis (1). Our results revealed dramatically reduced expression level of *MTTP* in the liver of HFD-fed rats. In 1984, Wetterau et al. found that an intracellular protein accelerates the transfer of

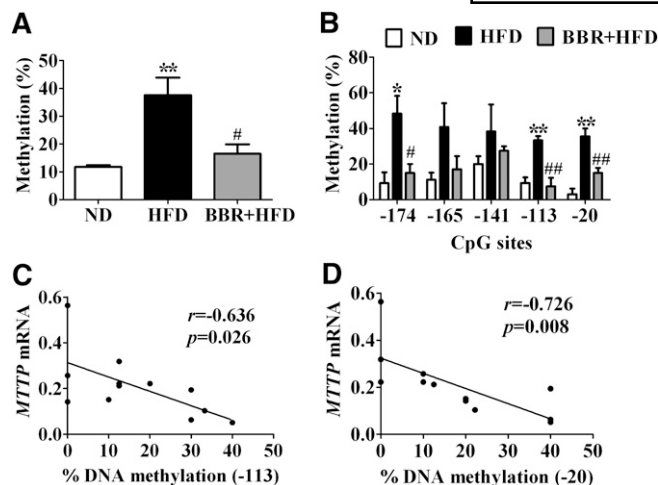


Fig. 7. BBR inhibits HFD-induced upregulation of DNA methylation in the *MTTP* promoter. A: Percentage of DNA methylation was determined from subcloned PCR products in the pGM-T vector (10 clones from each rat, $n = 4$ per group). Shown is the mean methylation level. B: Methylation degree (%) of each indicated cytosine phosphodiester bond guanine (CpG) site was determined for rats of the three indicated groups, calculated as the ratio of the number of methylated cytosine to the total number of samples. C–D: Negative correlations between the *MTTP* mRNA expression level and the degree of DNA methylation at –113 (C) and –20 (D) CpG sites in the liver. Values are mean \pm SEM. * $P < 0.05$, ** $P < 0.01$ versus ND; # $P < 0.05$, ## $P < 0.01$ versus HFD. BBR, berberine; HFD, high-fat diet; *MTTP*, microsomal triglyceride transfer protein; ND, normal diet.

triglyceride and cholesteryl ester, which was subsequently named microsomal triglyceride transfer protein (*MTTP*). Later studies have demonstrated that *MTTP* is necessary for the assembly and secretion of apoB-containing lipoproteins (e.g., VLDL and LDL), whereby lipids are normally exported from the liver (24). A common polymorphism exists within the *MTTP* promoter, a G to T transition at 493 nucleotides upstream of the initiation start site in different populations (25–28). The G allele, which is responsible for a decrease in the *MTTP* gene transcription, has been shown to be prone to developing increased intrahepatic triglycerides content (25, 28). In *ob/ob* mice, downregulation of *MTTP* gene expression facilitates intracellular fat accumulation in hepatocytes, increasing the susceptibility to hepatic steatosis (29). Therefore, a decrease in the *MTTP* mRNA may play a critical role in the pathogenesis of obesity-associated NAFLD. Interestingly, we observed that BBR treatment led to upregulated expression of *MTTP* mRNA. Further, the amount of triglyceride and apoB in the VLDL fractions were increased in SD rats upon BBR treatment for 16 weeks, indicating that BBR-upregulated hepatic *MTTP* expression led to increased *MTTP* function. Consistent with this, we found that BBR increased the amount of TG in VLDL particles, particularly in HFD-fed rats treated with tyloxapol (21), which can decrease TG hydrolysis by inhibiting lipoprotein lipase (LPL). Moreover, we found that BBR could also increase *LDLR* protein levels in the liver, which is in line with previously reported studies (12, 30). Hepatic *LDLR* is known to regulate human plasma LDL-c homeostasis and

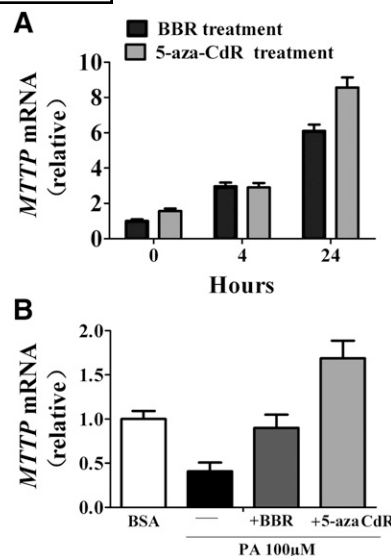


Fig. 8. Direct effect of BBR and 5-aza-CdR on *MTTP* mRNA expression. A: The expression levels of *MTTP* mRNA were determined by qPCR from BRL rat liver cells treated with BBR (20 μ M) or 5-aza-CdR (5 μ M) for 4 or 24 h. B: Analysis of the relative *MTTP* mRNA levels in BRL cells treated with 5 μ M 5-aza-CdR or 20 μ M BBR for 72 h in the presence 100 μ M PA. BBR, berberine; BRL, buffalo rat liver; *MTTP*, microsomal triglyceride transfer protein; PA, palmitate.

a high level of hepatic *LDLR* mRNA is associated with improved clearance of plasma LDL-c. Thus, BBR might result in more clearance of serum LDL-c through its action on *LDLR*. These results suggest that *MTTP* is an important target of BBR in reducing hepatic triglyceride content, while more physiological targets are most likely to exist in mediating the effects of BBR on lipid homeostasis.

In addition, the present study revealed decreased expression of *CPT-1 α* , a key rate-limiting enzyme in mitochondrial β -oxidation, in the liver of rats with HFD-induced NAFLD, which is consistent with the results reported in patients with NAFLD (1). HFD-induced elevation in the expression of *SCD-1*, the main enzyme responsible for the conversion of saturated fatty acyl-CoAs, stearoyl-CoA (18:0) and palmitoyl-CoA (16:0), to their respective monounsaturated fatty acyl-CoAs, oleyl-CoA (18:1) and palmitoleyl-CoA (16:1) (31), was also observed in the liver of SD rats with NAFLD. Lack of *SCD-1* expression in mice has been shown to result in reduced tissue lipid content and protect against both diet-induced and genetically induced obesity (32). Therefore, HFD-elicited upregulation of *SCD-1* as well as repressed expression of *CPT-1 α* (33) could suppress fatty acid β -oxidation in the mitochondria and thus facilitate the development of NAFLD. More importantly, we observed that BBR could reverse the HFD-induced dysregulation of these genes and improve fatty liver. A previous study (34) demonstrated that overexpression of *CPT-1 α* was accompanied by a 69% reduction in hepatic TG accumulation in obese SD rats. Thus, the regulatory effects of BBR on the expression of *MTTP*, *CPT-1 α* , and *SCD-1* in the liver could contribute to its reducing effects on hepatic TG in rats with NAFLD.

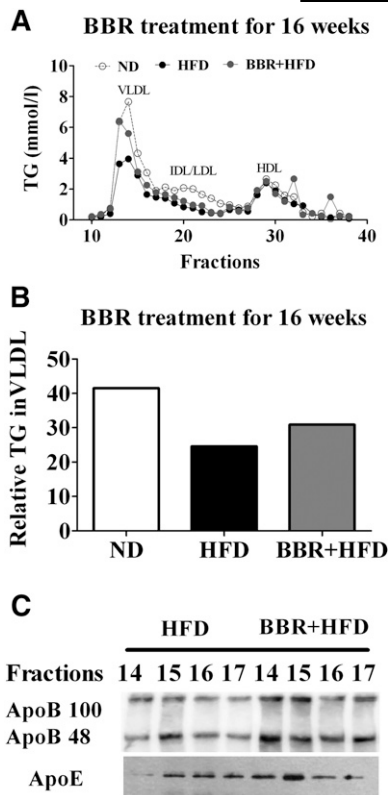


Fig. 9. Effects of BBR on the plasma apoB-containing lipoproteins. A: Analysis of lipoprotein profiles from ND-fed control group or HFD-fed rats treated by BBR or vehicle for 16 weeks. Pooled serum samples ($n = 6$ per group) were fractionated by FPLC, and TG from each fraction was measured. Fractions corresponding to VLDL, IDL/LDL, and HDL are indicated. B: Relative TG contents in VLDL fractions were calculated using AUCs of the FPLC fractions in (A). C: Western immunoblot analysis of apoB100/48 and apoE protein levels in the indicated VLDL fractions (14–17). Apo, apolipoprotein; AUC, area under curve; BBR, berberine; FPLC, fast protein liquid chromatography; HFD, high-fat diet; MTTP, microsomal triglyceride transfer protein; ND, normal diet; TG, triglyceride.

How does high-fat diet feeding cause abnormal expression of these genes and how can BBR reverse their dysregulated expression? DNA methylation is usually involved in the interaction between genes and environment factors. Cytosine residues occurring in CG dinucleotides are targets for DNA methylation, and gene expression is usually downregulated when DNA methylation modification occurs at its promoter. Ling et al. reported that DNA methylation of *NDUFB6* (15), *COX7A1* (16), and *PPARGC1A* (17) is involved in the pathogenesis of insulin resistance and type 2 diabetes, suggesting that DNA methylation may play a role in regulating the expression of these genes in the diseases influenced by environmental and genetic factors. Promoters of *MTTP*, *LDLR*, and *CPT-1 α* genes are rich in CpG sites and susceptible to DNA methylation modifications. This prompted us to hypothesize that DNA methylation may be involved in altering hepatic *MTTP*, *CPT-1 α* , and *LDLR* expression in SD rats with HFD-induced NAFLD and that BBR may counteract HFD-induced dysregulation of these genes through promoting demethylation of

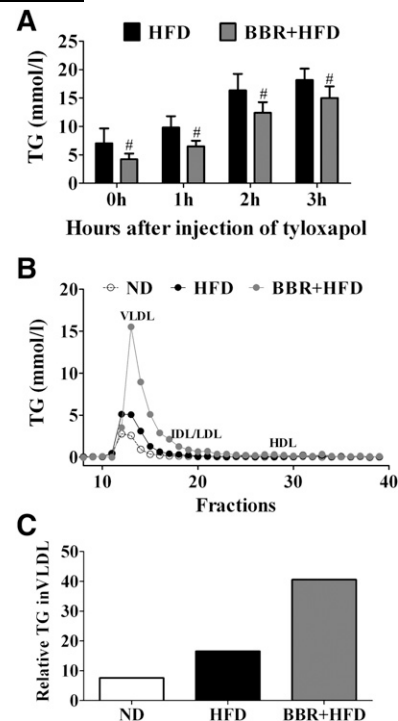


Fig. 10. Effects of BBR on the hepatic TG production in HFD-fed rats. Male SD rats were fed ND or a HFD, with the HFD-fed animals simultaneously treated with vehicle or BBR for 8 weeks ($n = 6$ per group). Rats were then treated via tail vein injection with 600 mg/kg tyloxapol after a 4 h fast. A: Serum TG was measured at 0, 1, 2, and 3 h after tyloxapol injection for the indicated groups. Data are shown as mean \pm SEM. $\#P < 0.05$ versus HFD. B: Analysis of lipoprotein profiles from the indicated groups. Pooled serum samples ($n = 6$ per group) were fractionated by FPLC, and TG in each fraction was measured. Fractions corresponding to VLDL, IDL/LDL, and HDL are indicated. C: Relative TG contents in the VLDL fractions were calculated using AUCs from the FPLC fractions in (B). AUC, area under curve; BBR, berberine; FPLC, fast protein liquid chromatography; HFD, high-fat diet; ND, normal diet; SD, Sprague-Dawley; TG, triglyceride.

their promoters. Indeed, our results showed that the mean methylation levels of *MTTP* promoter and three out of five CpG sites (–174, –113, and –20) were increased in the liver of HFD-fed rats. Even more interestingly, BBR was able to decrease the mean methylation level as well as methylation at the three CpG sites (the greatest by up to 30%) in the *MTTP* promoter region. Furthermore, the levels of DNA methylation of two CpG sites (–113 and –20) were inversely correlated with the expression level of *MTTP* mRNA in the liver of rats of the three treatment groups. These results suggest that HFD resulted in DNA hypermethylation of the *MTTP* promoter region, causing a decrease in its mRNA expression level. The relatively lower abundance of *MTTP* protein might block the assembly and secretion of VLDL, resulting in defective fat export from the liver. BBR treatment led to decreased level of DNA methylation in the *MTTP* promoter region and thus upregulated the expression of *MTTP*, which contributed to triglyceride export from the liver and improved fatty liver. Thus, the methylation modifications within the sequence located at –113 to –20 of *MTTP* promoter region

may play a prominent role in regulation of its transcription, which includes several positive elements, such as HNF1, LRH-1, HNF4, and DR1 (35). Whether DNA methylation of these CpG sites directly blocks the binding of transcription factors to these response elements within the *MTTP* promoter or changes the molecular conformation of the *MTTP* promoter has yet to be further investigated. On the other hand, no methylation modifications were found in the potential CpG sites within the *CPT-1α* and *LDLR* promoter sequences analyzed. Epigenetic regulatory mechanisms include histone modifications and microRNAs besides DNA methylation, which are also emerging as important players in polygenic human diseases and are influenced by a complex interaction of environmental, dietary, and exercise factors (36). Therefore, it remains to be understood whether the expression of *CPT-1α* and *LDLR* might be regulated by other mechanisms, such as histone modification or microRNAs.

In contrast to our results from the present study, elevated *MTTP* mRNA levels have been reported in the liver from OLETF rats (37) and Syrian Golden hamsters (38) when fed HFD for 28 days. Quesada et al. (39) also reported increased *MTTP* expression (by ~1.5-fold) in the liver of Wistar rats fed HFD for 13 weeks. While we found HFD feeding for 24 weeks led to severe hepatic steatosis in SD rats, the amount of VLDL-TG was increased in the early stage of HFD feeding (at 8 weeks) as opposed to a decline of VLDL-TG in the late stage of NAFLD (at 24 weeks). Although it is likely that the regulation of hepatic *MTTP* expression may respond differently to short-term versus long-term HFD intake, this inconsistency might also result from the different genetic background of animals or differences in the high-fat diets used in these different experimental settings.

In keeping with a direct effect of BBR on the expression of *MTTP* by influencing DNA methylation, we observed that treatment of rat liver BRL cells with both BBR and 5-aza-CdR could lead to time-dependent increases in the *MTTP* mRNA expression. Moreover, both could reverse the suppressive effect of palmitate on *MTTP* expression. Given the fact that 5-aza-CdR can strongly inhibit DNA methyltransferase activity and induce the expression of many genes through reversing gene-specific hypermethylation (40, 41), BBR might regulate *MTTP* gene expression through a similar mechanism, i.e., DNA demethylation. In our attempt to explore the mechanism(s) by which BBR alters the DNA methylation level of *MTTP* promoter, we found that the serum homocysteine level was markedly elevated in SD rats with HFD-induced NAFLD, which was accompanied by a significant hypermethylation of *MTTP* promoter in the liver. Moreover, treatment with BBR lowered the serum homocysteine content as well as the methylation level of *MTTP* promoter. On the other hand, we observed that BBR neither significantly altered hepatic SAM or SAH concentrations nor affected the total DNMT enzyme activities. Although it remains unclear how BBR treatment led to lower serum homocysteine levels or how this might relate to hypomethylation of certain gene promoters, these results suggest that BBR may exert impor-

tant effects on the pool of these species involved in the transfer of methyl group, thereby altering the methylation status of select genes that include those associated with lipid metabolism. While the exact mechanisms underlying BBR's actions remain to be elucidated, it is possible that BBR may also exert its demethylation effects on the promoters of other genes involved in modulation of DNA methylation (e.g., DNA demethylases).

In summary, the present study has demonstrated that BBR reduces hepatic fat content in rats with HFD-induced NAFLD by increasing TG export from hepatocytes, at least partially through decreasing the methylation of the *MTTP* promoter. Our findings not only illustrate that BBR represents a promising agent for the treatment of NAFLD but also provide novel insights into the importance of epigenetic factors such as DNA methylation in the pathogenesis of NAFLD induced by excessive intake of a high-calorie diet. **BB**

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