Involvement of AMP-activated protein kinase in beneficial effects of betaine on high-sucrose diet-induced hepatic steatosis

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Submitted 22 March 2007; accepted in final form 13 August 2007

Am J Physiol Gastrointest Liver Physiol 293: G894–G902, 2007. First published August 16, 2007; doi:10.1152/ajpgi.00133.2007.—Although simple steatosis was originally thought to be a pathologically inert histological change, fat accumulation in the liver may play a critical role not only in disease initiation, but also in the progression to nonalcoholic steatohepatitis and cirrhosis. Therefore, prevention of fat accumulation in the liver may be an effective therapy for multiple stages of nonalcoholic fatty liver disease (NAFLD). Promising beneficial effects of betaine supplementation on human NAFLD have been reported in some pilot clinical studies; however, data related to betaine therapy in NAFLD are limited. In this study, we examined the effects of betaine on fat accumulation in the liver induced by high-sucrose diet and evaluated mechanisms by which betaine could attenuate or prevent hepatic steatosis in this model. Male C57BL/6 mice weighing 20 ± 0.5 g (means ± SE) were divided into four groups (8 mice per group) and started on one of four treatments: standard diet (SD), SD+betaine, high-sucrose diet (HS), and HS + betaine. Betaine was supplemented in the drinking water at a concentration of 1% (wt/vol) (anhydrous). Long-term feeding of high-sucrose diet to mice caused significant hepatic steatosis accompanied by markedly increased lipogenic activity. Betaine significantly attenuated hepatic steatosis in this animal model, and this change was associated with increased activation of hepatic AMP-activated protein kinase (AMPK) and attenuated lipogenic capability (enzyme activities and gene expression) in the liver. Our findings are the first to suggest that betaine might serve as a therapeutic tool to attenuate hepatic steatosis by targeting the hepatic AMPK system.

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accumulation in the liver induced by high-sucrose diet and to identify mechanisms by which betaine could attenuate or prevent hepatic steatosis in this model.

**MATERIALS AND METHODS**

**Animal model and experimental protocol.** Male C57BL/6 mice weighing 20 ± 0.5 g (means ± SE) were obtained from the Jackson Laboratory (Bar Harbor, ME). The mice were housed in the animal quarters at the University of Louisville Research Resources Center, and the studies were approved by the Institutional Animal Care and Use Committee, which is certified by the American Association of Accreditation of Laboratory Animal Care. Initially, all mice were housed in conventional conditions and fed standard diet and water ad libitum at the animal facility (Research Resource Facility) for 1 wk before experiments began. Thereafter, the mice were divided into four groups (n = 8 per group) and started on one of four treatments: standard diet (SD), SD supplemented with betaine (SD + BT), high-sucrose diet (HS), and HS supplemented with betaine (HS + BT). Grain-based standard diet (2018, Harlan Teklad, Madison, WI) contained (by weight) 18.9% crude protein, 5% fat, and 55.7% carbohydrate (65% as sucrose and 2.6% as corn starch), identical to that used by Feldstein et al. (10) (TD 02366; Harlan Diagnostic Instruments, Sterling Heights, MI).

The dosing of betaine utilized in this experiment was based on previous report by Ji et al. (16) showing that betaine at this dose had protective effects on alcohol-induced fatty liver and liver injury. Mice were maintained on the treatments for 16 wk before being killed.

**Cells and culture conditions.** HepG2 cells, a human hepatoma cell line, were obtained from the American Type Culture Collection (Manassas, VA) and were cultured in DMEM containing 10% (vol/vol) fetal bovine serum, 2 mM/l glutamine, 5 U/ml penicillin, and 50 μg/ml streptomycin at 37°C in a humidified O2-CO2 (19:1) atmosphere.

**Histological examination.** At the time of killing, the liver was harvested and small pieces were fixed immediately in 10% buffered formalin. After paraffin embedding, 5-μm sections were deparaffinized in xylene and were rehydrated through a series of decreasing concentrations of ethanol. Sections were stained with hematoxylin and eosin. Alternately, portions of fresh liver were flash frozen and cryostat sections were cut and prepared for staining with Oil Red O. Alternatively, portions of fresh liver were flash frozen and homogenized in 5 ml 1 × hypotonic buffer supplemented with 1 mM DTT and 1% detergent, and put on ice for 30 min. After centrifugation at 10,000 rpm at 4°C for 10 min, supernatants (cytoplasmic fraction) were removed and 500 μl of complete lysis buffer was added to the nuclei and incubated for 30 min at 4°C with rocking. The samples were vortexed and centrifuged at 14,000 rpm for 10 min at 4°C. Supernatants containing nuclear proteins were harvested and transferred to prechilled tubes.

**Western blot analysis.** Tissues or cells were lysed in Western lysis buffer consisting of the following: 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10% glycerol, 2% Nonidet P-40, 1 mM EDTA, pH 8.0, 20 mM sodium fluoride, 30 mM sodium pyrophosphate, 0.2% sodium dodecyl sulfate, 0.5% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 1 mM sodium vanadate, 50 μM leupeptin, and 5 μM aprotinin. Samples were incubated on ice with frequent vortexing for 15 min and centrifuged for 20 min at 18,000 g. The protein content of each supernatant was quantified via a protein assay reagent from Bio-Rad Laboratories (Hercules, CA) in accordance with the manufacturer’s instructions.

Sixty micrograms of each supernatant sample of proteins were separated by electrophoresis through a 12% polyacrylamide gel [8% gel for acetyl-CoA carboxylase (ACC)] and transferred to 0.45 μM Immobilon-P polyvinylidene difluoride membrane (PerkinElmer Life Science). After transfer, membranes were blocked in 5% (wt/vol) nonfat dry milk in PBS-0.1% Tween 20 and probed with the antibodies specified. Horseradish peroxidase-conjugated secondary antibodies (Sigma) and enhanced chemiluminescence substrate kit (PerkinElmer Life Science) were used in the detection of specific proteins.

**HPLC assay.** Deproteinized tissue homogenates (4% metaphosphoric acid) were prepared and hepatic S-adenosylhomocysteine (SAH), S-adenosylhomocysteine (SAM), reduced form of glutathione (GSH), and homocysteine levels were determined by a HPLC method using a 5 μm Hypersil C-18 column (250 × 4.6 mm). The mobile phase consisted of 40 mM/l ammonium phosphate, 8 mM/l heptane sulfonic acid (ion-pairing reagent, pH 5.0), and 6% acetonitrile and was delivered at a low rate of 1.0 ml/min. SAM and SAH were detected using a Waters 740 UV detector (Milford, MA) at 254 nm. An internal standard, S-adenosylmethionine, was added to all samples and standard solutions to a concentration of 100 nmol/ml. For GSH and homocysteine assays, 20 μl samples were injected onto a reversed-phase C18 column (Val-U-Pak HP, fully endcapped octadecyl
Betaine activates hepatic AMPK

Silane, 5 μm, 250 × 4.6 mm; ChromTech, Apple Valley, IN). The mobile phase, which consisted of a solution of 0.1 M monochloroacetic acid and 2 mmol/l heptane sulfonic acid at pH 2.8 (98%) and acetonitrile (2%), was delivered at a low rate of 1 ml/min. The compounds were detected in the eluant with a Bioanalytical Systems (West Lafayette, IN) dual LC4B amperometric detector using two Au-Hg electrodes in series with potentials of 1.2 and 0.15 V for the upstream and downstream electrodes, respectively. Standard curves for the analytes were plotted as peak area vs. concentration of the analyte. Protein concentrations were measured by a protein assay kit from Bio-Rad Laboratories in accordance with the manufacturer’s instructions.

Intracellular triglyceride measurement. Liver tissues were homogenized and hepatic total lipids were extracted according to Bligh and Dyer (4) and redissolved in 2% Triton X-100 in water. Hepatic triglyceride content was determined by enzymatic colorimetric methods using commercially available kits (Sigma). For the in vitro study, HepG2 cells were plated into a six-well plate for overnight. Twenty hours after treatments, cells were washed twice with 1 ml of cold PBS. Then 0.3 ml of 0.25 M NaOH was added to each well. Samples were collected by scraping cells into a 2-ml tube and total lipids were extracted according to Bligh and Dyer (4). The triglyceride content was determined by enzymatic colorimetric methods using commercially available kits (Sigma).

Statistical analysis. All data were expressed as means ± SD. Statistical analysis was performed using a one-way ANOVA and was analyzed further by Newman-Keuls test for statistical difference. Differences between treatments were considered to be statistically significant at P < 0.05.

RESULTS

Body weight. Absolute body weight of each mouse from each group was measured at the end of the feeding period, and body weight gain was calculated and shown in Fig. 1. No difference in either absolute body weight or body weight gain between the SD and SD+BT groups was observed at the end of feeding period. Compared with the SD group, mice in the HS group had significantly larger body weight and greater body weight gain. Sixteen-week high-sucrose diet feeding was accompanied by 35% (11.36 vs. 15.39 g) more body weight gain than those in the SD group. Supplementation of betaine in the HS group (HS+BT) caused a numerically less body weight gain when compared with the HS group; however, this decrease was not statistically significant.

Fat accumulation in the liver. We next examined changes of hepatic triglyceride content in different groups. As we expected, mice in the HS group displayed significantly increased hepatic triglyceride content when compared with those in the SD group (Fig. 2A). The histological examination confirmed this and showed that the liver from the SD group was normal whereas obvious hepatic fat infiltration was observed in the HS group, but no inflammation or obvious cell death were detected (Fig. 2B and C). Betaine significantly reduced hepatic triglyceride accumulation induced by high-sucrose feeding, which was confirmed by both hematoxylin and eosin and Oil Red O staining. Since betaine supplementation had no effects on either body weight or hepatic triglyceride content of mice fed standard diet, we excluded this group in all following measurements to simplify our description.

Blood biochemistry. The data in Table 1 showed the effects of high-sucrose diet feeding with and without betaine supplementation on some routine plasma parameters. After 16-wk feeding, mice in the HS group had increased levels of glucose, cholesterol, and insulin in plasma. Unexpectedly, free fatty acids levels in plasma were significantly lowered in the HS group compared with the SD group. No significant changes were observed in alanine aminotransferase, triglyceride, TNF-α, and adiponectin levels between the SD group and the HS group. Betaine supplementation alleviated elevations of plasma glucose, cholesterol, and insulin levels resulting from high-sucrose feeding, with no effects on free fatty acid and triglyceride changes. Plasma adiponectin levels in HS+BT group were slightly increased compared with those in both SD and HS groups.

Hepatic methionine metabolism. Since betaine is an essential biochemical component of the hepatic methionine-homocysteine cycle and acts as a donor of methyl groups, the effects of high-sucrose feedings with and without betaine supplementation on hepatic methionine metabolism were examined by measuring concentrations of several metabolites in the hepatic methionine metabolic pathway, particularly SAM, SAH, homocysteine, and GSH levels. The status of transmethylation reactions in the liver was gauged by the SUM-to-SAH ratio. As
depicted in Fig. 3, prolonged high-sucrose feeding did not produce significant effects on hepatic SAM (Fig. 3A), SAH, and GSH levels (data not shown); however, it induced a slight increase in hepatic homocysteine levels (Fig. 3B). Betaine markedly increased hepatic SAM levels (Fig. 3A) with no effects on SAH levels (data not shown), leading to a significant elevation in hepatic SAM-to-SAH ratio (data not shown). Furthermore, betaine abolished high-sucrose diet-induced ele-
vation of hepatic homocysteine levels (Fig. 3B). No effects on GSH levels were found following betaine supplementation (data not shown).

**Hepatic AMPK and ACC activation.** AMP-activated protein kinase (AMPK) has been proposed to act as a fuel gauge in mammalian cells and plays a critical role in the repression of glycolytic and lipogenic pathways and the promotion of fatty acid oxidation in the liver. To determine whether the antisteatotic functions of betaine in our animal model could be ascribed to its effects on AMPK activation, we examined AMPK phosphorylation (activation) in each group by detecting the amount of phosphorylated AMPK proteins by Western blot analysis. As shown in Fig. 4, whereas long-term high-sucrose diet feeding had no obvious effects on the activity of AMPK compared with SD feeding, betaine supplementation to the high-sucrose diet resulted in enhanced activation of AMPK (Fig. 4, A and B), as demonstrated by substantially increased protein phosphorylation. ACC is a rate-limiting enzyme in hepatic de novo fatty acid synthesis and a well-identified

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Values are means ± SD, n = 8. SD, standard diet; HS, high-sucrose diet; BT, betaine supplementation; ALT, alanine aminotransferase; TG, triglyceride; NEFA, nonesterified fatty acid. Means in a row with superscripts without a common letter differ, P ≤ 0.05.

![Fig. 3. Changes of hepatic methionine metabolism. Male C57BL/6 mice were fed with high-sucrose diet in the presence or absence of betaine supplementation (1%) in the drinking water for 16 wk. Liver samples were homogenized with 4% metaphosphoric acid and hepatic S-adenosylmethionine (SAM) and homocysteine contents were assayed by high-performance liquid chromatography. A: changes of hepatic SAM levels. Data are means ± SD (n = 8). Bars with different letters differ significantly (P < 0.05). B: hepatic homocysteine levels. Data are means ± SD (n = 8). Bars with different letters differ significantly (P < 0.05).](image-url)

![Fig. 4. Betaine supplementation resulted in increased phosphorylation (p-) of hepatic AMP-activated protein kinase (AMPK) and inhibited acetyl-CoA carboxylase (ACC) activation. Male C57BL/6 mice were fed with high-sucrose diet in the presence or absence of betaine supplementation (1%) in the drinking water for 16 wk. Total protein extracts from liver tissues were prepared thereafter. Forty micrograms of protein were subjected to Western blot analysis for AMPK or ACC phosphorylation using antibodies specific for AMPK phosphorylated at Thr172 and ACC phosphorylated at Ser79. A: Western blot assay for AMPK. Betaine supplementation increased phosphorylation of liver AMPK (activation). B: quantification of p-AMPK protein levels in different groups. Levels were normalized to total AMPK. Data are means ± SD (n = 6–7). Bars with different letters differ significantly (P < 0.05). C: Western blot assay for ACC. Betaine supplementation attenuated the increase of ACC protein content induced by high-sucrose feeding and increased phosphorylation of ACC (inhibition) in the liver. D: quantification of p-ACC and total ACC protein levels in different groups. Levels were normalized to actin. Data are means ± SD (n = 6–7). Bars with different letters differ significantly (P < 0.05).](image-url)
protein among the large number of AMPK targets. Therefore, we examined protein levels of ACC in the liver and its phosphorylation (deactivation) status in different groups. As shown in Fig. 4, C and D, high-sucrose feeding resulted in increased ACC protein levels in the liver, which was attenuated by betaine supplementation. Concomitant with its effect on AMPK activity, betaine supplementation markedly alleviated hepatic ACC activation, shown by Western blot as significantly increased protein phosphorylation.

**SREBP and ChREBP activation in the liver.** Sterol regulatory element-binding protein-1c (SREBP-1c) and carbohydrate response element-binding protein (ChREBP) are two critical transcriptional factors for gene expression [e.g., ACC, fatty acid synthase (FAS)] in the hepatic lipogenic pathway. The mature SREBP-1 and ChREBP proteins in nuclear extracts of mouse liver from the three different groups were examined by Western blot. As shown in Fig. 5, long-term high-sucrose diet feeding led to an obvious increase of mature SREBP-1 and ChREBP protein in the nucleus compared with the standard diet feeding. This increase was inhibited by betaine supplementation.

**Gene expression of ACC, FAS, and SREBP-1d in the liver.** The effects of high-sucrose diet feeding with or without betaine supplementation on gene expression of ACC, FAS, and SREBP-1d in liver tissue were examined by real-time PCR analysis. As shown in Fig. 6, high-sucrose feeding caused significant elevation in the expression of all three genes, which was significantly attenuated by betaine supplementation.

**Betaine activated AMPK and reduced lipid accumulation in HepG2 cells.** To determine the effect of betaine on the activation of AMPK in vitro, the betaine was added to the culture...
medium of HepG2 cells. Both time-course and dose-response analyses were performed by Western blot assay using anti-phospho-AMPK antibody. As shown in Fig. 7A, betaine at 2 mM and 5 mM activated AMPK (shown by increased phosphorylation) at the 1-h time point. At 2 h, all levels (0.5, 1, 2, and 5 mM) of betaine activated AMPK, and this activation displayed a dose-dependent pattern. The effects of betaine on AMPK activation subsided after 4 h.

To determine whether betaine could attenuate lipid accumulation induced by extracellular glucose, HepG2 cells were incubated with either normal (5.5 mM) or high (25 mM) glucose concentrations in the absence or presence of betaine (2 mM) for 24 h. At the normal concentration of glucose, the intracellular contents of triglyceride (Fig. 7B) and cholesterol (Fig. 7C) were lowered ~30% by betaine. A high concentration of glucose in the medium elevated intracellular triglyceride and cholesterol levels, which was also reduced by betaine supplementation.

DISCUSSION

In mammals, the liver is the principal organ responsible for the conversion of excess dietary carbohydrate into triglycerides. Both glucose and fructose are metabolized in the liver to provide the precursor, acetyl CoA, for fatty acid synthesis. Fatty acids are then incorporated into triglycerides that function as a long-term energy reservoir. Using an experimental model with high-sucrose feeding, we demonstrated that long-term (16 wk) feeding of high-sucrose diet to mice induced remarkable hepatic steatosis. Betaine, when supplemented in the drinking water, significantly attenuated hepatic steatosis in this animal model, and this change was associated with increased activation of hepatic AMPK in the liver. Furthermore, betaine supplementation attenuated ACC activation (increased phosphorylation) and increased gene expressions of SREBP-1c, ACC, and FAS in the liver induced by prolonged high-sucrose feeding. Therefore, our results are the first to suggest that betaine might serve as a therapeutic tool to attenuate hepatic steatosis by targeting the hepatic AMPK system.

AMPK is a phylogenetically conserved serine/threonine protein kinase that has been proposed to act as a “metabolic master switch” mediating the cellular adaptation to environmental or nutritional stress factors (6). AMPK has been implicated in the control of hepatic glucose and lipid homeostasis via multiple effects on genes and on short-term regulation of specific enzymes. Activation of AMPK (phosphorylation of its α-subunit at site Thr172) leads to an inhibition of fatty acid synthesis by phosphorylating critical enzyme(s) involved in the process of lipogenesis. Among these is ACC, which is the rate-limiting enzyme for the synthesis of long-chain fatty acids (28, 29). Malonyl-CoA is not only a critical precursor for the biosynthesis of fatty acids, but also a potent inhibitor of mitochondrial fatty acid oxidation through its inhibitory action on carnitine palmitoyltransferase-1 (18). Moreover, the long-term activation of AMPK plays an important role in repressing expression of genes encoding enzymes involved in glycolytic and lipogenic processes in the liver. Activation of AMPK inhibits transcriptional stimulation of ACC and FAS induced by insulin or glucose in primary hepatocytes (11). Sustained activation of hepatic AMPK decreases transcriptional activation of SREBP-1c and ChREBP (12). Furthermore, activation of AMPK leads to decreased DNA binding activity of ChREBP and subsequent transcriptional inhibition of lipogenesis in the liver (17). Recent studies suggest that activation of AMPK accounts for some of the anti-diabetic and antisteatotic actions of a number of drugs, such as metformin and thiazolidinediones, and is required for the lipid-lowering effects of metformin in cultured hepatocytes (22, 39, 40). Taken together, the overall effects of AMPK activation in the liver are to inhibit lipogenesis and stimulate fatty acid oxidation. Therefore, attempts to find or generate novel, safe, and
In addition, betaine supplementation efficiently improved hepatic methionine metabolism status, as reflected by a substantially increased SAM-to-SAH ratio. Abnormal lipid metabolism deriving from increased hepatic homocysteine levels has been reported in various studies. Although hepatic homocysteine levels in all these animal models were much higher than observed in our study, a recent study showed that incubation of HepG2 cells with homocysteine at 50–100 μM could induce cAMP response element-binding protein (CREB) phosphorylation and subsequently increased CREB/DNA binding activity (35), implying that modest elevation of hepatic homocysteine levels could lead to prominent changes in intracellular signal transductions involved in lipid metabolism. Therefore, the contribution of abnormal hepatic methionine metabolism resulting from high-sucrose diet feeding in the development of fatty liver should also be taken into account, and further investigations are still required.

Taken together, our data demonstrate that long-term feeding of a high-sucrose diet to mice induced obesity, hepatic steatosis, and insulin resistance. Betaine supplementation in the drinking water resulted in remarkable improvement of hepatic steatosis, which was associated mainly with significant enhancement of hepatic AMPK activation. Moreover, betaine supplementation also rescued abnormalities in hepatic methionine metabolism and alleviated insulin resistance. Unlike other AMPK activators [such as metformin and 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR)] with potential side effects, oral anhydrous betaine solution is safe in doses ranging from 3 to 30 g/day, as has been shown by both experimental and clinical studies. Thus the present study provides strong evidence for further evaluation of the potential therapeutic role of betaine in NAFLD.

GRANTS

This research was supported by grants from the National Institutes of Health (Z. Song, C. J. McClain, I. Deaciuc, D. Hill, and Z. Zhou) and the Department of Veterans Affairs (C. J. McClain).

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