Betaine improved adipose tissue function in mice fed a high-fat diet: a mechanism for hepatoprotective effect of betaine in nonalcoholic fatty liver disease

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diseases, including alcoholic liver disease and bile acid-induced liver injury (4, 11, 15, 18), with different mechanisms involved. The therapeutic effects of betaine on NAFLD have also been investigated and reported in both clinical and experimental studies (1, 17, 21, 24, 28). Although the overall results are promising, data relating betaine to NAFLD are limited, and cellular and molecular mechanisms remain elusive because of the lack of detailed experimental investigations. Thus detailed studies are warranted before any recommendations regarding the use of betaine in NAFLD can be made.

The beneficial effects of betaine on an animal model of NAFLD were previously shown by our laboratory using a long-term high-carbohydrate (sucrose) diet-feeding mouse model (38). We found that betaine supplementation alleviated hepatic fat accumulation via direct activation of the hepatic AMP-activated protein kinase (AMPK) system and subsequent inhibition of the de novo lipogenesis pathway in the liver. At the same time, we also observed increased circulating adiponectin levels and improved plasma insulin and glucose levels by betaine supplementation, implying that betaine had the potential to improve adipose tissue function and insulin sensitivity. In this study, the experimental model of long-term (12-wk) high-fat diet feeding to mice was used. Our aims were: 1) to investigate the hepatoprotective effects of betaine in a well-established animal model of NAFLD; 2) to evaluate beneficial effects of betaine on adipose tissue function, i.e., insulin sensitivity and adipokine production; and 3) to identify the underlying mechanisms involved.

MATERIALS AND METHODS

Animal model and experimental protocol. Male C57BL/6 mice (8 wk) weighing 25 ± 0.5 (SD) g 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/group) and started on one of four treatments: control diet (Con), control diet supplemented with betaine (BT), high-fat diet (HF), and high-fat diet supplemented with betaine (HB). Both control (D12450B) and high-fat diet (D12451) were obtained from Research Diets (New Brunswick, NJ). The detailed diet composition was shown in the formula table (Table 1). Betaine was supplemented in the drinking water at a concentration of 1% (wt/vol) (anhydrous; Sigma, St. Louis, MO) and started simultaneously with high-fat diet feeding. All animals had access to diet and water ad libitum. Mice were maintained on the treatments for 12 wk before being killed. At the end of the experiment, the mice were anesthetized with Avertin (300 mg/kg body wt) after 4 h fasting, and plasma, liver, and epididymal fat pad samples were harvested for assays.

Isolation and culture of primary adipocytes. High-fat diet-fed mice were used to obtain primary adipose cells at the end of the experimental period. Briefly, the mice were anesthetized and killed via cervical dislocation. Epididymal fat pads were harvested, washed in PBS (pH 7.4) buffer at room temperature, and minced thoroughly (2–3 mm in diameter) in the collagenase solution (0.2 mg/ml collagenase A; 4 mg/l of adipose tissue). This mixture was incubated at 37°C with shaking at 120 rpm for 30 min. After digestion, the mixture was filtered through a 250-μm gauze mesh in a 50-ml conical polypropylene tube and allowed to stand for 2–3 min. The floating layer of adipocytes was washed three times and incubated at 37°C in Dulbecco’s modified Eagle’s medium containing 5% BSA. The cells and conditioned medium were taken at various times as indicated in the legends for Figs. 1–6.

Measurements of liver injury and hepatic fat content. Liver injury was determined by measuring plasma alanine aminotransferase (ALT) activities using a commercially available kit (Infinity; Thermo Electron, Melbourne, Australia). Hepatic fat accumulation was determined by measuring total hepatic triglyceride content and by histopathological evaluation. For intrahepatic triglyceride measurement, liver tissues were homogenized, and hepatic total lipids were extracted according to Bligh and Dyer (5) and redissolved in 2% Triton X-100 in water. Hepatic triglyceride content was determined by enzymatic colorimetric methods using a commercially available kit (Infinity; Thermo Electron). For histopathological evaluation, fresh liver were flash-frozen, and cryostat sections were cut and prepared for staining with oil red O. Photographs were taken on an Nikon Eclipse E600 microscope (Fryer, Cincinnati, OH) equipped with a digital camera (SPOT; Diagnostic Instruments, Sterling Heights, MI).

Plasma biochemical assays. The plasma biochemical assays were performed with commercially available kits: glucose, triglyceride, cholesterol (Infinity; Thermo Electron), free fatty acids (Waco Chemicals, Richmond, VA), adiponectin, leptin, resistin, and insulin (Linco Research, St. Charles, MO).

The homeostasis model assessment of basal insulin resistance (HOMA-IR) was used to calculate an index from the product of the fasting concentrations of plasma glucose (mmol/l) and plasma insulin (μU/ml) divided by 22.5 (23). Lower HOMA-IR values indicated greater insulin sensitivity, whereas higher HOMA-IR values indicated lower insulin sensitivity (insulin resistance).

Glucose tolerance test. The glucose tolerance tests were conducted 1 wk before the end of feeding. After a 6-h fast, the mice were anesthetized, and, after the collection of an unchallenged sample (time 0), a solution of 50% glucose (2.0 g/kg body wt) was administered by oral gavage. During the test, blood was collected from the tail vein at 30, 60, and 120 min after glucose administration. All blood glucose measurements were performed using a handheld glucometer.

Western blotting detections. Epididymal fat pads 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% SDS, 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

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<th>Table 1. Diet formula</th>
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<td>Casein (80 mesh)</td>
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<td>l-Cystine</td>
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<td>Corn starch</td>
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<td>Maltdextrin 10</td>
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<td>Sucrose</td>
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<td>Cellulose, BW200</td>
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<td>Soybean oil</td>
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<td>Lard</td>
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<td>Mineral mix S10026</td>
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<td>Dicalcium phosphate</td>
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<td>Calcium carbonate</td>
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<td>Potassium citrate, H2O</td>
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the manufacturer’s instructions. Proteins were separated by SDS-PAGE and transferred to a 0.45-μm Immobilin-P polyvinylidene difluoride membrane (PerkinElmer Life Sciences). 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.

**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 changes and food consumptions.** 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 Con and BT groups was observed at the end of the feeding period. Compared with the Con group, mice in the HF group had significantly larger body weight and greater body weight gain. Twelve-week high-fat diet feeding was accompanied by 53% (8.92 vs. 5.81 g) more body weight gain than those in the Con group. Supplementation of betaine in the HF group (HB) had no significant effects on either body weight or average weight gain when compared with the HF group. Food consumptions were monitored every week, and average daily food consumptions during the entire experimental period were calculated. As shown in Fig. 1C, there was no significant difference in daily food consumptions among groups.

**Betaine supplementation alleviated high-fat diet-induced fatty liver and liver injury.** The pathological alterations of livers from different groups were evaluated by measuring hepatic triglyceride contents and circulating liver enzyme levels. Consistent with previous studies (20, 33), long-term high-fat diet feeding induced liver injury (Fig. 2A) and fatty liver (Fig. 2B) in mice, which were manifested by increased plasma ALT levels and hepatic triglyceride contents, respectively. Moreover, massive fat accumulation in the liver of a mouse fed the high-fat diet was also observed via oil red O staining. When betaine was supplemented to the high-fat diet in the drinking water, both hepatic triglyceride accumulation and liver injury were significantly reduced. Because betaine supplementation to the control diet (BT group) had no effects on body weight changes, hepatic triglyceride content, and liver injury when compared with the Con group, we excluded this group in all of the following measurements to simplify our description.

**Betaine supplementation alleviated insulin resistance.** Insulin resistance plays a central role in the pathogenesis of NAFLD. To test whether the hepatoprotective effects of betaine supplementation are associated with the improved insulin resistance in the high-fat-fed mouse model, we evaluated insulin sensitivity by measuring three critical parameters: HOMA-IR, glucose tolerance test, and plasma NEFA levels. As shown in Fig. 3, high-fat diet feeding led to significantly higher HOMA-IR (Fig. 3A), severe hyperglycemia upon glucose administration, and impaired glucose tolerance (Fig. 3B), as well as significantly increased circulating NEFA levels (Fig. 3C), compared with control animals, which were consistent with previous studies. However, all of these alterations were alleviated through betaine supplementation.

**Betaine improved adipokine production.** To examine the effects of betaine supplementation on adipokine production,
circulating levels of three adipokines, adiponectin, leptin, and resistin, were measured. As shown in Fig. 4, compared with the control group, high-fat diet feeding caused a significant decrease of plasma adiponectin levels (Fig. 4A), whereas leptin and resistin levels were increased significantly (Fig. 4, B and C). With betaine supplementation in the drinking water, all of these abnormalities were attenuated substantially.

Fig. 2. Betaine supplementation attenuated liver injury and hepatic fat accumulation in mice fed a high-fat diet. A: plasma alanine aminotransferase (ALT) levels. B: hepatic triglyceride content. C57BL/6 mice were fed a high-fat diet with/without betaine supplementation (1%) in the drinking water for 12 wk. Data are means ± SD (n = 8). Bars with different letters differ significantly (P < 0.05). C: fresh-frozen liver sections were stained with oil red O. Control group animals with/without betaine supplementation had no obvious fat accumulation in the liver. High-fat diet feeding resulted in profound hepatic steatosis, featured by a remarkable amount of fat droplet in the liver. Betaine supplementation significantly decreased hepatic fat accumulation induced by high-fat diet feeding.
Betaine supplementation improved insulin sensitivity in adipose tissue. We next examined the effects of betaine supplementation on insulin-signaling pathways in adipose tissue by Western blotting. As shown in Fig. 5, high-fat diet feeding impaired insulin signaling in adipose tissue, and this was illustrated by suppressed activations (phosphorylations) of both protein kinase B (Akt) (Fig. 5A) and extracellular signal-regulated kinases (ERK; Fig. 5B), two major downstream kinases in the insulin signaling pathway, in epididymal fat pad. With betaine supplementation, the suppressive effects of high-fat diet on both Akt and ERK activations were rescued. To further investigate the effect of betaine on insulin signaling in adipocytes, we then examined the effect of betaine on insulin-induced Akt phosphorylation status in primary adipocytes isolated from epididymal fat pads of mice fed the high-fat diet. Betaine pretreatment significantly improved insulin-stimulated Akt phosphorylation (Fig. 5C). Furthermore, compared with untreated adipocytes, betaine pretreatment increased adiponectin and decreased resistin productions from primary adipocytes, both in a dose-dependent manner (Fig. 5, D and E).

Betaine supplementation attenuated endoplasmic reticulum stress in adipose tissue. Endoplasmic reticulum (ER) stress plays a critical role in adipose tissue dysfunction and insulin resistance in a variety of experimental models, including high-fat diet-fed mice (13, 42). To test if the observed beneficial

**Fig. 3.** Betaine supplementation alleviated insulin resistance in mice fed a high-fat diet. A: homeostasis model assessment of basal insulin resistance (HOMA-IR). B: glucose tolerance test. *P < 0.05 and **P < 0.01 compared with the control group or betaine supplementation group. C: circulating nonesterified free fatty acid (NEFA) levels. C57BL/6 mice were fed a high-fat diet with/without betaine supplementation (1%) in the drinking water for 12 wk. Data are means ± SD (n = 8). Bars with different letters differ significantly (P < 0.05).

**Fig. 4.** Betaine supplementation improved adipokine production in mice fed a high-fat diet. A: circulating adiponectin levels. B: circulating leptin levels. C: circulating resistin levels. C57BL/6 mice were fed a high-fat diet with/without betaine supplementation (1%) in drinking water for 12 wk. Data are means ± SD (n = 8). Bars with different letters differ significantly (P < 0.05).

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effects of betaine supplementation in our study are related to its alleviative effects on ER stress response in adipose tissue, we measured three representative proteins induced/activated during ER stress, glucose-regulated protein 78 (GRP78), C/EBP homologous protein (CHOP), and c-Jun NH2-terminal kinase (JNK), in epididymal fat pad. As shown in Fig. 6, long-term high-fat diet feeding induced both GRP78 and CHOP protein productions and JNK activation, indicative of the ER stress response. With betaine supplementation, the abnormal changes in all three proteins were rescued, suggesting that alleviating ER stress may play an important role in betaine’s beneficial effects on adipose tissue function.

DISCUSSION

We reported previously that betaine supplementation alleviated hepatic steatosis in mice fed a high-sucrose diet. Mechanistic studies revealed that activation of the hepatic AMPK
system and subsequent inhibition of de novo lipogenesis contributed, at least partially, to betaine’s hepatoprotective effects (37). In addition to AMPK activation in the liver, we also found, in that study, that betaine supplementation increased circulating adiponectin levels, whereas insulin and glucose levels were decreased, implying that betaine had the potential to improve adipose tissue function. Our results showed that betaine, when supplemented in the drinking water, significantly attenuated hepatic steatosis and injury, and these changes were associated with improved insulin sensitivity and adipokine production. Mechanistic investigations revealed that betaine supplementation alleviated the ER stress response in adipose tissue of mice fed a high-fat diet. Taken together, our results suggest that multiple mechanisms are involved in the hepatoprotective effects of betaine in NAFLD. Apart from acting on hepatocytes to activate the AMPK system, betaine also improved adipose tissue function.

Insulin resistance has been well-recognized to play a central role in the development of steatosis and the transitional process to steatohepatitis in NAFLD (16, 34, 40). Because of the strong relationship between insulin resistance and NAFLD, pharmacological treatment using insulin sensitizers, such as TDZs and metformin, has been shown to be beneficial in the prevention or improvement in NAFLD (22, 26–28, 33), although the varying degrees of side effects, specifically increases in body weight and body fat content (29, 35), have also been reported. There is currently no FDA-approved treatment for NAFLD or nonalcoholic steatohepatitis; therefore, searching for the safe compound with high preventive and/or therapeutic efficacies is urgently needed. In the present study, we demonstrated that long-term high-fat diet feeding resulted in significantly increased fat accumulation in the liver and plasma ALT levels, an indicator of liver injury. These changes were associated with increased body weight gain and systemic insulin resistance, which was manifested by significantly elevated HOMA-IR and circulating NEFA levels, as well as impaired blood glucose clearance compared with those in mice fed control diet. Betaine supplementation alleviated hepatic steatosis and lowered plasma ALT levels compared with the high-fat group, which were concomitant with improved HOMA-IR, circulating NEFA levels, and glucose tolerance test without affecting body weight changes and daily food consumptions, suggesting that betaine may act as an insulin sensitizer.

Although there had been some debates with regard to the primary site of insulin resistance in NAFLD (peripheral insulin resistance vs. hepatic insulin resistance), recent data indicate the periphery, primarily adipose tissue insulin resistance, to be the initial site, followed by hepatic steatosis, which in turn exacerbates the degree of insulin resistance (39). In normal circumstances, insulin inhibits NEFA release (lipolysis) from adipose tissues via suppressing hormone-sensitive lipase. As insulin resistance develops, insulin-mediated suppression of lipolysis is overwhelmed, and increased release of NEFA in the portal circulation ensues. Enhanced exposure and uptake of NEFA by the liver lead to not only excessive fat accumulation in the hepatocyte but also hepatic insulin resistance (6). Although beneficial effects of betaine in NAFLD were reported in both clinical and experimental studies (1, 17, 21, 24, 37), the underlying mechanisms remain to be clearly defined. Clinically, betaine supplementation improved aminotransferases and hepatic steatosis in nonalcoholic steatosis patients (1, 24). Experimentally, a couple of very recent studies showed that betaine alleviated fatty liver and liver injury by correcting abnormal hepatic methionine metabolism and improving hepatic insulin sensitivity in mice fed a high-fat diet (17, 21). Considering the critical role of adipose tissue in the initiation and progression of NAFLD, in present study, we examined the effects of betaine supplementation on insulin sensitivity in epididymal fat by detecting activations of Akt kinase and ERK, two major downstream kinases activated by insulin binding to its receptors. High-fat diet feeding decreased phosphorylated (p)-Akt and p-ERK42/44 protein abundance in epididymal fat pad, indicative of dampened insulin signal transduction in adipose tissue. Importantly, our study showed that suppression of both kinases was rescued by betaine supplementation in the drinking water, suggesting that betaine may act directly on adipose tissue to prevent insulin resistance induced by high-fat diet feeding.

Mechanism(s) involved in the induction of insulin resistance in adipocytes are multifactorial, with ER stress, specifically JNK activation, being well-established (13, 38, 41). ER stress is initiated by protein overload or misfolding in the ER. Cells cope with ER stress by an adaptive protective response termed unfolded protein response (UPR), which includes enhancing protein folding and degradation in the ER and downregulating overall protein synthesis. When the UPR adaption to ER stress is insufficient, the ER stress response unleashes pathological consequences. The link between ER stress and adipose tissue insulin resistance has been shown in a variety of experimental models, including high-fat diet-fed mice and ob/ob mice (12, 30, 30). Furthermore, treatment with orally active chemical chaperones reversed ER stress and insulin resistance in high-fat diet-fed mice (30), suggesting that the ER stress response plays a mechanistic role in adipose tissue insulin resistance. In the present study, we characterized the ER stress response in adipose tissue via Western blot detection of three representative proteins induced/activated by the ER stress response, GRP78, CHOP, and JNK. Among these proteins, JNK activation represents a critical link between the ER stress response and insulin resistance in that JNK can directly phosphorylate insulin receptor substrate (IRS)-1 at several sites, including serine-307 (2), and this modification leads to reduced insulin-stimulated IRS-1 tyrosine phosphorylation. In obese animals, deletion of the JNK gene reversed the obesity-induced increase in JNK activity and IRS-1 serine phosphorylation and protected against insulin resistance (13). In the present study, we showed that high-fat diet feeding increased protein productions of both GRP78 and CHOP and activation of JNK, which were in line with previous studies that high-fat diet feeding induced the ER stress response in adipose tissue (30). With betaine supplementation, the high-fat diet-induced ER stress response in adipose tissue was significantly alleviated. Our data clearly showed that betaine supplementation not only reduced GRP78 and CHOP abundance but also prevented JNK activation in the epididymal fat pad. Therefore, alleviation of the ER stress response in adipose tissue represents an important mechanism underlying the beneficial effects of betaine on adipose tissue function.
Apart from insulin resistance, another critical mechanism implicated in the pathogenesis of NAFLD is dysregulated adipokine production. Adipokines are implicated in the pathogenesis of NAFLD, through their metabolic and pro-/anti-inflammatory activity (9, 25). Accumulated evidence supports that decreased adiponectin levels are related to hepatic steatosis and inflammation in NAFLD (25). Leptin promotes insulin resistance and hepatocyte injury/fibrogenesis in cell cultures and animal models through activation of the transforming growth factor-β axis and stellate cells (10). In animal models, circulating resistin levels link adiposity and insulin resistance (31). In the present study, we measured circulating levels of three adipokines in high-fat diet-feeding mice with/without betaine supplementation. We found that long-term high-fat diet feeding significantly decreased circulating adiponectin levels, whereas leptin and resistin levels were significantly elevated compared with control animals. Importantly, we found that all changes were recovered by betaine supplementation, implying that betaine can prevent not only the early stage steatosis but also its transition to nonalcoholic steatohepatitis and fibrosis.

Betaine is a metabolite in the choline metabolism pathway and an important participant in methionine metabolism. Via a reaction catalyzed by betaine homocysteine methyltransferases, betaine can convert homocysteine to methionine, thereby preventing homocysteinemia accumulation. Although the beneficial effects of betaine supplementation on fatty liver diseases have been reported, exact mechanisms remain elusive. Correction of abnormal methionine metabolism seems to represent a major mechanism proposed by these studies. For instance, using a mouse model of alcoholic liver disease, Ji et al. (15) demonstrated that betaine prevented fatty liver and liver injury induced by chronic alcohol consumption through attenuating hyperhomocysteinemia and subsequent ER stress in the liver. Moreover, a very recent study demonstrated that betaine alleviated high-fat diet-induced NAFLD in rats by correcting abnormal hepatic methionine metabolism (21). Although we also showed that improved liver abnormalities by betaine supplementation were associated with improved hepatic methionine/homocysteine metabolism in a high-sucrose diet feeding model of NAFLD (37), our results revealed that betaine could directly activate the AMPK system in hepatocytes, implying that the mechanisms underlying the beneficial effects of betaine on NAFLD may be multifactorial.

Although the detailed mechanism(s) involved in the association between betaine supplementation and improvement of adipose tissue dysfunction remain to be fully understood, our data clearly demonstrate that long-term feeding of a high-fat diet to mice induced fatty liver and liver injury, which were associated with obesity, insulin resistance, and dysregulated adipokine production, and betaine supplementation in the drinking water alleviated hepatic steatosis and liver injury, which were associated with improved adipose tissue function, including insulin resistance and abnormal adipokine production. Our further investigations revealed that alleviation of the ER stress response represented a critical mechanism underlying the beneficial effects of betaine on adipose tissue function. Betaine is a nature component of many foods and safe in doses ranging from 3 to 30 g/day; therefore, unlike other insulin sensitizers with potential side effects, betaine may represent an ideal therapeutic agent for NAFLD. Our present study provides strong evidence for further evaluation of the potential therapeutical role of betaine.

GRANTS
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DISCLOSURES
No conflicts of interest are declared by the authors.

REFERENCES


