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Effect of Transgenic Extrahepatic Expression of Betaine-Homocysteine Methyltransferase on Alcohol or Homocysteine-Induced Fatty Liver

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Abstract

Background—Chronic alcohol feeding induces hyperhomocysteinemia (HHcy). Previously, we reported a protective role of betaine-homocysteine methyltransferase (BHMT) in homocysteine-induced injury in cultured hepatocytes. In this study, we investigated the direct role of BHMT in alcohol or homocysteine-induced liver injury.

Methods—Betaine-homocysteine methyltransferase transgenic (Tg) mice were generated. Comparisons were made between the Tg and wild type (WT) mice in their response to intragastric alcohol infusion or to oral feeding of a high methionine low folate diet (HMLF).

Results—Expression of the Tg BHMT was increased in organs peripheral to the liver. The alcohol infusion for 4 weeks increased: plasma ALT by 5-fold in WT mice and 2.7-fold in Tg mice; plasma homocysteine by 7-fold in WT mice and 2-fold in Tg mice; liver triglycerides by 4-fold in WT mice and 2.5-fold in Tg mice. The alcohol-induced fatty liver was more severe in WT than in Tg mice based on H&E staining. The HMLF feeding for 4 weeks increased plasma ALT by 2-fold in WT mice and 1-fold in Tg mice; plasma homocysteine by 21-fold in WT mice and 3.3-fold in Tg mice; liver triglycerides by 2.5-fold in WT mice and 1.5-fold in Tg mice. HMLF induced accumulation of macro fat droplets in WT but not Tg mice. Betaine supplementation decreased partially the alcohol or HMLF-induced increase of ALT, homocysteine and liver lipids in WT mice. However, Tg mice were normal when fed both HMLF and betaine. In WT mice, both alcohol and HMLF induced moderate increase of sterol regulatory element binding protein 1 (SREBP1) protein which was partially reduced by betaine supplementation. In Tg mice, alcohol but not HMLF increased SREBP1. Carbohydrate responsive element-binding protein was increased by alcohol in either WT or Tg mice which was not affected by betaine supplementation. Ratio of *S*-adenosylmethionine (SAM) to *S*-adenosylhomocysteine (SAH) was reduced by 50% in WT and by 20% in Tg mice fed alcohol. Ratio of phosphatidylcholine (PC) to phosphatidylethanolamine (PE) was reduced in WT but not Tg mice fed alcohol. Changes in PE methyltransferase activities were not detected in response to alcohol or HMLF feeding but were increased by betaine.

Conclusions—The BHMT Tg mice are resistant to alcohol or HMLF-induced HHcy and liver steatosis indicating that peripheral metabolism of homocysteine protected the liver without a direct effect of BHMT in the liver. Multiple mechanisms are involved in protection by betaine including increased SAM/SAH and PC/PE ratios.

Keywords

Alcohol; Hyperhomocysteinemia; BHMT; Betaine; SAM; PC/PE Ratio

IN THE LIVER, the essential amino acid methionine is converted to homocysteine after removal of its methyl group by the conversion of *S*-adenosylmethionine (SAM) to *S*-adenosylhomocysteine (SAH) (Finkelstein, 2006; Stead et al., 2006). SAM is a methyl donor for numerous methylation reactions that play major roles in biosynthesis, regulation, and detoxification. Homeostasis of SAM is maintained by glycine methyltransferase (GNMT), which utilizes glycine as the methyl receptor to consume excess SAM and forms a nontoxic product. SAH is reversibly converted to homocysteine catalyzed by SAH hydrolase. Two other methylation reactions also contribute significantly to homocysteine production in the liver: the methylation of phosphatidylethanolamine (PE) forming phosphatidylcholine (PC) which is catalyzed by phosphatidylethanolamine methyltransferase (PEMT) and the methylation of guanidinoacetic acid forming creatine which is catalyzed by guanidinoacetic acid methyltransferase. Homocysteine can be metabolized through either transsulfuration or remethylation. Transsulfuration converts homocysteine to cysteine for glutathione (GSH) production which is initiated by cystathionine β -synthase. Remethylation of homocysteine back to methionine is catalyzed by cobalamin-dependent methionine synthase using 5-methylfolate as co-substrate supplied by 5, 10-methylenetetrahydrofolate reductase and by betaine-homocysteine methyltransferase (BHMT) using betaine (trimethylglycine) as a methyl donor. These reactions maintain homeostasis of methionine, homocysteine, SAM, and GSH.

Alcohol induced liver injury starts with fatty liver which is followed by steatohepatitis, fibrosis, and cirrhosis. Factors including oxidative stress, acetaldehyde toxicity, endotoxins, cytokines, impaired immune response, and nutritional deficiencies have been suggested to contribute to the injury. Recent evidence indicates that disturbance of methionine and homocysteine homeostasis may contribute to the development of alcoholic liver disease (ALD) (Lu et al., 2002; Halsted et al., 2002; Ji and Kaplowitz, 2004). For example, chronic ethanol exposure has been shown to decrease hepatic concentrations of SAM and folate but increase concentrations of plasma homocysteine and hepatic SAH in animal and human studies (Lieber et al., 1990; Barak et al., 1994; Cravo et al., 1996; Halsted et al., 1996; de la Vega et al., 2001; Ji and Kaplowitz, 2003; Barak et al., 2003). The changes are associated with different degrees of liver injury. Exogenous administration of SAM or betaine has been shown to prevent alcoholic liver injury in animal studies (Feo et al., 1986; Lieber et al., 1990; Ji and Kaplowitz, 2003; Song et al., 2003). The mechanisms by which SAM exerts its protection include attenuation of oxidative stress by restoring GSH concentrations, inhibition of inflammation by down-regulating tumor necrosis factor α and up-regulating interleukin-10 synthesis, prevention of apoptosis by reducing mitochondrial cytochrome *c* release and caspase-3 activation in hepatocytes, and induction of apoptosis of liver tumor cells by increasing DNA oxidation and strand breaks (Feo et al., 1986; Fernandez-Checa et al., 2002; Ishii et al., 2003; Song et al., 2003; Yang et al., 2004; Barve et al., 2006). How betaine ameliorates ALD is not fully understood. We have previously studied the direct role of BHMT/betaine in cell protection by over-expressing BHMT in HepG2 cells and found that BHMT expression inhibited homocysteine-induced endoplasmic reticulum (ER) stress response, lipid accumulation, and cell death (Ji et al., 2007). Suppression of BHMT expression in primary mouse hepatocytes potentiated homocysteine-induced, but not tunicamycin-induced, ER stress response and cell injury. In addition, in the presence of betaine, expression of BHMT correlated with increased apolipoprotein B (ApoB) expression and increased SAM to SAH ratio. The evidence suggests that BHMT/betaine has multiple beneficial effects in cultured hepatocytes. To know whether the protective effects also occur in vivo, we produced transgenic (Tg) mice expressing human BHMT and compared the Tg and wild type (WT) mice in their response to

intra-gastric alcohol infusion or to oral feeding of a high methionine low folate diet (HMLF). These mice had a significant expression of the transgene only in extrahepatic tissues allowing us to assess its effect independent of a direct effect of transgene expression in the liver. We found that in conjunction with lowering homocysteine and increasing ratios of SAM/SAH and PC/PE, the BHMT Tg mice were resistant to alcohol or diet-induced hyperhomocysteinemia (HHcy) and liver steatosis.

METHODS

Generation of BHMT Transgenic Mice

An open-reading frame (ORF) of the human BHMT gene was blunt-ended and subcloned into an expression vector-pcDNA3.1 (Invitrogen, Carlsbad, CA) as described previously (Ji et al., 2007). The vector contains the cytomegalovirus (CMV) promoter for constitutive expression in mammalian cells. The DNA of the expression vector-pcDNA3.1 containing human BHMT ORF was digested with the restriction enzyme, *DrdI*, which yielded 2 DNA fragments. One larger DNA fragment (~3 kb) covers the CMV promoter, the BHMT ORF, and a portion of the C-terminal peptide encoding the V5 epitope. The larger DNA fragment was visualized on agarose gel by the crystal violet from Invitrogen and purified subsequently using QIAquick gel extraction kit (Qiagen, Valencia, CA). The purified DNA fragment was then injected into the pronucleus of fertilized ova from B6D2F mice. The injected eggs that survived to 2 cell stage were implanted into pseudopregnant females. Twenty-three pups were initially obtained. The human BHMT Tg positive mice were then identified with polymerase chain reaction (PCR) genotyping of mouse tail DNA samples using primers: forward, CGCAAATGGGCGGTAGGCGTG and reverse, GCTCGCCCTTCTGTGATTTGA. Two of the 23 were Tg BHMT positive and were used as founder strains. The Tg positive mice were backcrossed with WT C57BL/6 for at least five generations before they were used. All animal protocols were in full compliance with the Guide for Care and Use of Laboratory Animals (NIH, Bethesda, MD; Publication 86 to 23, 1985) and were approved by the Animal Care Committee.

Intra-gastric Ethanol Infusion

The intra-gastric ethanol infusion model was described previously (Ji and Kaplowitz, 2003; Ji et al., 2004). Total caloric intake derived from the diet and ethanol was set at 533 cal/kg and the caloric percentages of ethanol, dietary carbohydrate (dextrose), protein (lactalbumin hydrolysate), and fat (corn oil) were 24.3, 15.7, 25, and 35%, respectively. The highest ethanol dose at the end of 4 weeks accounted for 35% of calories. Adequate vitamin and salt mix were included at the recommended amounts by the Committee on Animal Nutrition of the National Research Council (AIN-76A, 4,421 and 15.4 g/l, respectively, Dyets Inc., Bethlehem, PA). In some experiments, animals were simultaneously fed with 0.5% (w/v) betaine.

Feeding of a High Methionine and Low Folate Diet

Mice were fed orally a HMLF (TD 98272) or equal amount of control diet (TD 05552) from Harlan (Madison, WI). The high methionine diet contained 2% (w/w) of L-methionine, 1.5 ppm of folic acid, and 1% (w/w) of succinylsulfathiazole, which was used to interfere further with folate metabolism and inhibit the production of folate by gut bacteria. In some experiments, betaine was added to the drinking water (5%, w/v). Serum and tissue samples were taken for analysis after 4 week feeding.

Histological and Serum Analysis

Procedures of liver histological staining and measurements of plasma alanine aminotransferase (ALT) and homocysteine were described previously (Ji and Kaplowitz, 2003).

DNA and RNA Isolation and Analysis

Genomic DNA was extracted from the tails of Tg mice using the Qiagen DNeasy Tissue Kit. The Tag PCR Master Mix Kit from Qiagen was used for PCR. Total hepatic RNA was isolated from 300 mg of fresh liver tissues using TRIzol reagent by Invitrogen and purified by the RNeasy Mini Kit from Qiagen following the manufacture's instructions and with an addition of 500 U of an RNase inhibitor (RNAguard; Amersham Pharmacia Biotech, Piscataway, NJ) to the starting materials. RNA was stored at -80°C until use. The Qiagen OneStep RT-PCR Kit was used for the RT-PCR. The primer sequences used are: apolipoprotein A1 (ApoA1), GTATGGCAGCAAGATGAACCC and TCGCCAAGTGTCTTCAGGTG; ApoB, TCAAGGACGCAAAGGCAGAA and GCACTAACTTGTATGAAGGCACC; ApoE, AAAGCAACCAACCCTGGGAG and CAGGCGTATTTGCTGGGTCT; GNMT, AAGAGGGCTTCAGCGTGATG and TTTACTCCGTTTGCCCCGACC; microsomal triglyceride transfer protein (MTP), TCTCCTCCTACTCTGCTTCCGT and TTTGAAGTACGCCCAGCAC; sterol regulatory element-binding protein 1 (SREBP1), ACACTCAGCAGCCACCATCTA and TCTCCACCACTTCGGGGTTTCA; carbohydrate responsive element-binding protein (ChREBP), AGCCCAGTGTGTGGTTTCGT and GGCGTGTATTCCCTGAGTGA. The PCR optimal cycle number for each gene was predetermined to obtain detectable but nonsaturating PCR product. The relative expression was normalized to the expression of β -actin or 18S in that same sample and compared with control.

Protein and Enzyme Analysis

Proteins were extracted according to the method previously reported (Ji et al., 2007). Proteins were routinely analyzed by immunoblotting using horseradish peroxidase-labeled (Santa Cruz Biotechnology Inc., Santa Cruz, CA) or alkaline phosphatase-labeled (Cell Signaling Technology, Danvers, MA) second antibodies. Proteins were visualized using LumiGLO Reagent (Cell Signaling Technology) on CL-Xposure films (Pierce, Rockford, IL) at an optimized time point. Antibodies against ApoB, ApoE, SREBP1, and ChREBP were purchased from Santa Cruz Biotechnology Inc. The intensity of protein bands on the Western blots were quantified by ImageQuant Version 5.2 (Molecular Dynamics, Sunnyvale, CA). Generation and purification of the BHMT antibodies and assay of BHMT activities were described previously (Ji et al., 2004, 2007). Anti-GNMT antibodies were provided by Dr. C. Wagner from Vanderbilt University, TN. PEMT activities in liver homogenates were measured according to the methods by Ridgway and Vance (1992; Li et al., 2005). Briefly, phosphatidylmonomethylethanolamine was used as an exogenous substrate. Hepatocyte membranes or liver homogenates (25 μ g) were assayed for activity in 125 mM Tris-HCl, pH 9.5, 5 mM dithiothreitol, 1 mM Triton X-100, and 0.4 mM phosphatidyl-*N*-monomethylethanolamine for 20 minutes at 37°C. The assay was initiated by the addition of [methyl-³H] AdoMet (21 μ Ci/ μ mol) to a final concentration of 200 μ M. The final volume of all assays was 150 μ l. When the assay was completed, labeled phospholipids were extracted and specific activity expressed as nmol of methyl groups transferred/min/mg of protein.

Analysis of Liver Lipid, SAM, SAH, and Betaine

For lipid extraction, livers were homogenized with a Polytron homogenizer in 5 volumes of buffer (10 mM Tris-HCl, pH 7.2, containing 150 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1:100 protease inhibitor mixture (P8340; Sigma) followed by sonication for 20 s. The homogenates were centrifuged for 5 minutes at 600 $\times$ g, and supernatants were collected. Total lipids were extracted from liver homogenates by the method of Bligh and Dyer (1959). Phospholipids were separated by high performance liquid chromatography (HPLC) and quantified with a light scattering detector (McCluer et al., 1986). Phosphatidylidimethylethanolamine was used as an internal standard for quantification.

Hepatic SAM, SAH, and betaine were determined with HPLC (Ji et al., 2004, 2007). Briefly, liver tissues (100 to 300 mg) were homogenized in 500 μ l of 0.5 M HClO₄ (perchloric acid). The homogenates were centrifuged at 14,000 rpm (21,920 $\times$ g) for 5 minutes. To 200 μ l of the supernatant 10 μ l of the 10 N KOH was added, mixed, and recentrifuged. One hundred microliters of the resultant supernatant was injected into the HPLC. One hundred micrometers of 50 μ M SAM, SAH, or betaine standards were injected into the HPLC for reference.

Statistical Analysis

Experiments were performed with 4 to 6 mice per group with values presented as mean plus or minus SD. Statistical analysis was performed using three-way ANOVA and the Tukey-Kramer Multiple Comparisons Test post hoc. A value of $p < 0.05$ was considered significant.

RESULTS

Peripheral Expression of Human BHMT in Mice

Comparing the BHMT Tg mice and WT littermates, we observed increased expression of the Tg BHMT at protein level. The size of cloned human BHMT is larger than that of mouse BHMT which is represented by the upper protein band on the Western blots (Fig. 1A). There was no significant difference in liver BHMT activities between WT and Tg mice. BHMT enzyme activities in protein extracts from brain, kidney, lung, and heart were increased significantly in Tg mice (Fig. 1B). Tg mice appeared normal based on liver to body ratio ($4.5\% \pm 0.5$, w/w) and ALT value (19.5 ± 4.3 U/l). In addition, the peripheral BHMT expression did not cause significant alterations in the levels of liver betaine (2.9 ± 0.8 nmol/g tissue) and plasma homocysteine (3.2 ± 0.5 μ M) in Tg mice when compared with those in WT littermates (betaine, 3.3 ± 0.7 nmol/g tissue and homocysteine, 3.7 ± 1.5 μ M).

Resistance of Tg Mice to Alcoholic or Nonalcoholic Hyperhomocysteinemia and Liver Steatosis

To test whether Tg mice were resistant to diet or alcohol induced HHcy and liver steatosis, we treated the animals by intragastric infusion of alcohol or by oral feeding of a HMLF. After 4 weeks, the alcohol treatment increased serum ALT by more than 5-fold, plasma homocysteine by nearly 6-fold, liver triglycerides by more than 4-fold, and cholesterol by 2-fold in WT littermates when compared with pair-fed controls (Table 1). A partial reduction of these parameters was observed when the mice were fed alcohol and betaine simultaneously, which is consistent with our previous findings (Ji and Kaplowitz, 2003; Ji et al., 2004). In Tg mice fed alcohol alone, the increase in serum ALT, liver triglycerides, and cholesterol were of lower magnitude compared with the WT mice fed alcohol. When they were fed alcohol and betaine simultaneously, Tg mice were nearly normal in terms of ALT, homocysteine and liver lipids when compared with pair-fed controls (Table 1). The HMLF treatment increased plasma ALT by 2-fold, plasma homocysteine by 21-fold, and liver triglycerides by nearly 2-fold in WT littermates. The HMLF-induced increase of plasma homocysteine was decreased by 70% in Tg mice. Hepatic triglycerides were also significantly decreased in Tg mice fed HMLF when compared with those of the WT littermates fed HMLF (Table 1). Interestingly, in contrast to the partial response to betaine with alcohol feeding, in both WT and Tg mice all the parameters were normal when they were fed HMLF and betaine simultaneously. In liver histology, alcohol infusion induced fatty liver was more severe in WT littermates than that in Tg mice (Fig. 2A and B). HMLF feeding induced an accumulation of macro fat droplets in the liver of WT littermates but it only caused an accumulation of micro fat droplets in the liver of the BHMT Tg mice (Fig. 2C and D).

Expression of Genes Involved in Lipid Synthesis and Secretion

To investigate possible mechanisms responsible for alcohol and/or homocysteine induced liver steatosis, we examined mRNA and protein expression of SREBP1 and ChREBP that regulate lipogenic pathways in the liver. Both alcohol and HMLF induced a moderate increase of SREBP1 protein in WT mice (Fig. 3). In Tg mice, alcohol but not HMLF induced increase of SREBP1. Alcohol-induced SREBP1 was partially reduced in the WT and nearly fully reduced in Tg mice when the animals were supplemented with betaine (Fig. 3). ChREBP was induced by alcohol in both Tg and WT mice but betaine supplementation did not have any significant effect on the increased ChREBP (Fig. 3). In contrast to alcohol, HMLF did not increase ChREBP in either WT or Tg mice.

Previously, we and others reported that expression of genes that regulate lipid transport or secretion was altered by BHMT expression in cultured hepatocytes (Sowden et al., 1999; Ji et al., 2007). To know if this occurred in vivo, we examined expression of MTP, ApoA1, ApoE, and ApoB. Figure 4 demonstrates that alcohol but not HMLF slightly reduced the expression of MTP in both genotypes whereas HMLF but not alcohol reduced the expression of ApoB in WT mice. No other changes were detected between alcohol and HMLF treatments or between the 2 genotypes (not shown).

Ratios of SAM/SAH and PC/PE and Expression of GNMT and PEMT

S-adenosylmethionine/SAH ratio was reduced by about 50% in WT littermates in response to alcohol feeding (Table 2) whereas it was reduced by only 20% in Tg mice fed alcohol. Betaine supplementation through intragastric alcohol infusion increased SAM/SAH in both WT and Tg mice. Betaine exerted its effects on the SAM/SAH ratio by increasing SAM levels in alcohol-fed WT mice. Interestingly, SAM/SAH ratio was increased by more than 200% in the WT fed both alcohol and betaine which was exceptionally higher than that of pair-fed controls or that of Tg mice fed both alcohol and betaine. As GNMT maintains homeostasis of SAM levels (Finkelstein, 2006; Stead et al., 2006), dramatic increase of SAM/SAH would not occur under normal conditions. The exceptional high value of SAM/SAH in animals fed both alcohol and betaine prompted us to examine the expression of GNMT. Figure 5 shows that there was a trend for reduction of GNMT expression in response to alcohol in both genotypes in the presence or absence of betaine supplementation. With respect to the HMLF feeding (Table 2), a significant increase of SAM/SAH was observed in WT. This was because both SAM and SAH were increased but the increase of SAM was greater than that of SAH. Betaine supplementation through oral feeding increased SAM/SAH in both genotypes. Betaine exerted its effects on the SAM/SAH ratio by decreasing both SAM and SAH levels in WT mice whereas by decreasing only SAH level in Tg mice. The HMLF feeding exerted no effects on GNMT expression in both genotypes (Fig. 5).

Phosphatidylcholine and PE are major components of mammalian membrane. The ratio of PC/PE reflects membrane integrity. PC/PE was increased in both genotypes fed betaine alone (Table 2). In WT the PC/PE was significantly reduced in response to alcohol feeding and betaine supplementation restored the ratio (Table 2). In Tg mice fed alcohol, no reduction of the PC/PE was observed but betaine supplementation significantly increased PC/PE when compared with pair-fed controls. PEMT activity which catalyzes the conversion of PE to PC was also examined. Alcohol or HMLF did not have any significant effects on the activity of PEMT in either WT or Tg mice. However, betaine increased PEMT activity in WT and increased PEMT activity more in the Tg mice regardless of whether the animals were fed alcohol or HMLF.

DISCUSSION

Dietary betaine can be absorbed from the intestine and transported to the liver where, under the function of BHMT, it can transfer its 1 methyl group to homocysteine to form methionine. The transmethylation results in decreased concentrations of homocysteine and SAH and increased concentrations of methionine and SAM in the liver. This protective role of the BHMT/betaine system has often been observed in animal and human studies (Ji and Kaplowitz, 2006; Purohit et al., 2007; Ji, 2008). However, most of previous studies focused on betaine supplementation and the direct effects of BHMT have not been studied. Previously, we initiated this kind of study by expressing BHMT in cultured hepatocytes that have impaired methionine metabolism (Ji et al., 2007). In this study, we continued investigating the direct role of BHMT in alcoholic and nonalcoholic liver injury by generating Tg mice that expressed human BHMT gene in peripheral organs but had minimal expression in the liver. The alcohol- or HMLF-induced pathological changes including HHcy, increased ALT values, ER stress response (increased SREBP1 expression), altered SAM/SAH and PC/PE, and increased fat accumulation were ameliorated in the Tg mice. In the presence of betaine, the Tg mice were completely resistant to the alcohol infusion induced or dietary HHcy and were partially protected against alcoholic liver steatosis and fully against the high methionine diet-induced fatty liver.

The main significance of this study is 3-fold. First, consistent with our in vitro study, multiple mechanisms are involved in the protection by BHMT. By catalyzing the remethylation of homocysteine using betaine as a substrate, BHMT directly lowers homocysteine levels. The remethylation yields methionine which could contribute to increased SAM levels. BHMT may indirectly affect many biological processes as SAM and/or SAM/SAH ratio influence numerous methylation reactions of DNA, lipids, and proteins along with PC/PE ratio, PEMT activity (Kharbanda et al., 2007). Second, although either betaine or BHMT alone exerts certain protective effects in the liver, maximal protection required both betaine supplementation and Tg expression of BHMT. Thus, betaine supplementation was more effective in Tg mice reflecting greater overall capacity to lower homocysteine. Third, peripheral expression of BHMT could ameliorate alcohol- or homocysteine-induced injury in the liver without supplementation with betaine. This selective increased expression of BHMT in extrahepatic tissues without supplemental betaine was able to increase the SAM/SAH ratio in the liver (1.2 to 1.96) and prevent the hepatic alterations in PC/PE, and lessen the hepatic lipid accumulation, serum ALT and HHcy. This provides strong evidence that HHcy makes significant contribution to alcohol liver injury as removal of plasma homocysteine through extrahepatic metabolism of BHMT in Tg mice protects the liver.

The molecular mechanisms leading to the development of alcoholic and nonalcoholic hepatic steatosis is still not fully understood. We and others have suggested that the transcription factor SREBP contributes to the lipid accumulation based on observations that SREBP mRNA and proteins are moderately up-regulated and are associated with genes encoding lipogenic enzymes in livers of alcoholic and nonalcoholic mice (Crabb and Liangpunsakul, 2006; Ji and Kaplowitz, 2006; Esfandiari et al., 2007). Our results from the present study support the role of SREBP1 in liver steatosis. Because of emerging evidence for the role of the transcription factor ChREBP in the control of lipogenic gene expression in liver (Postic et al., 2007; Denechaud et al., 2007), we examined the expression of ChREBP in mice fed alcohol or the high methionine diet. Both ChREBP mRNA and protein were moderately increased in the alcohol infused mice but not in the mice fed the HMLF diet. The Tg expression of BHMT and/or betaine supplementation did not have any effect on the expression of ChREBP suggesting that factors other than alcoholic or nonalcoholic HHcy may cause the induction of ChREBP. ChREBP is usually phosphorylated and kept inactive in the cytosol and activation of ChREBP requires dephosphorylation (Denton et al., 2006; Denechaud et al., 2008). As one of AMP-

activated protein kinase (AMPK) substrates is ChREBP (Kawaguchi et al., 2002) and AMPK is inhibited by chronic alcohol feeding (Crabb and Liangpunsakul, 2006), chronic alcohol might up-regulate ChREBP via inhibition of AMPK leading to up-regulation of lipogenic gene transcription. Alternatively, chronic alcohol might cause insulin resistance (Onishi et al., 2003; Wan et al., 2005) and increased hepatic glucose activates ChREBP as it has been shown that high concentration of glucose activates protein phosphatase 2A which dephosphorylates ChREBP (Denechaud et al., 2007). Whether it is true or not needs further studies. The important point with respect to present study is that attenuation of liver steatosis by betaine supplementation was not due to suppression of ChREBP.

A few interesting observations in this study deserve comment. HMLF feeding induced more striking HHcy with less lipid accumulation and ALT than alcohol. This suggests that either alcohol exerts other effects independent of HHcy partially leading to fat and injury or somehow modifies the pathological response of the homocysteine pathway to fat and injury. One obvious difference between alcohol and HMLF is the effect on SAM/SAH, reduced by alcohol and raised by HMLF (presumably because of methionine over-load). The increase in SAM/SAH may exert protective effects which dampen the pathological effects of HHcy. Curiously, the Tg mice fed HMLF without supplemental betaine had a decrease in SAM/SAH to normal perhaps because removal of homocysteine outside the liver decreased the recycling of homocysteine to SAM in the liver. Betaine supplementation of WT mice fed HMLF decreased SAM and SAH levels but did not lower the SAM/SAH ratio. We do not understand the mechanism for this paradoxical effect on SAM levels at present. Of note, in this study, a greater than 6-fold increase of SAM/SAH ratio was observed in WT mice fed alcohol and betaine. As GNMT maintains homeostasis of SAM, it is possible that the alcohol infusion induced alterations in GNMT expression shown in Fig. 5 might contribute to the abnormal accumulation of SAM. Thus decreased GNMT expression might be a protective mechanism to sustain SAM and SAM/SAH and decrease homocysteine production. Alternatively, there may be an optimal ratio of SAM/SAH in the liver as a SAM/SAH ratio higher than 5 is rarely seen in published reports. GNMT null mice have been shown to have very high SAM levels and develop steatosis (Luka et al., 2006; Liu et al., 2007). Another finding of interest was that the PC/PE ratio was reduced in response to alcohol feeding. PEMT activity was not significantly reduced. In fact, the PEMT pathway only accounts for 20% to 30% of the hepatic PC synthesis but is the only pathway for conversion of PE to PC (Vance et al., 1997). Considering that PC is made primarily via the Kennedy pathway (CDP-choline pathway) in all mammalian tissues, the altered PC/PE ratio might be caused by alcoholic effects on the Kennedy pathway enzymes such as CTP:phosphocholine cytidylyltransferase (CT). It will be interesting to know whether CT or other enzymes of Kennedy pathway are affected by the chronic alcohol feeding. We observed increased PEMT activity in response to betaine which may be another protective effect for betaine. The mechanism for this effect will require further research. Finally, ApoB expression in the liver was not affected by the peripheral expression of BHMT which differs from findings with BHMT expression in cultured cells (Ji et al., 2007). This difference likely relates to lack of expression of the transgene in liver and suggest a direct role of BHMT in ApoB expression.

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A. Western blot **B. BHMT activity** **Fig. 1.**

Expression patterns of the transgenic betaine-homocysteine methyltransferase (BHMT) in mice. **(A)** Western blot of BHMT proteins and **(B)** enzyme activity of BHMT. * $p < 0.05$ compared with wild type littermates ($n = 4$).

A. WT_EtOH

B. Tg_EtOH

C. WT_HMLF

D. Tg_HMLF

Fig. 2.

Resistance of the BHMT transgenic mice to alcohol or a diet-induced fat accumulation. H&E staining (100×); WT_EtOH, wild type fed alcohol; Tg_EtOH, transgenic mice fed alcohol; WT_HMLF, wild type fed a high methionine low folate diet; Tg_HMLF, transgenic mice fed a high methionine low folate diet.

Fig. 3.

Expression of SREBP1 and ChREBP in mice fed alcohol or a high methionine low folate diet. (A) RT-PCR; (B) Western blots; (C) relative levels of proteins; Tg, betaine-homocysteine methyltransferase (BHMT) transgenic mice; WT, wild type littermates; SREBP1, sterol regulatory element-binding protein 1; ChREBP, carbohydrate responsive element-binding protein; Ce, pair-fed control for intragastric infusion; E, intragastric alcohol infusion; EB, intragastric alcohol infusion plus betaine supplementation (0.5%, w/v); Ch, pair-fed control for oral feeding; H, fed orally a high methionine low folate (HMLF) diet; HB, HMLF plus betaine supplementation (0.5%, w/v); $^{\alpha}p < 0.05$ compared with pair-fed control and $^{\beta}p < 0.05$ compared with Tg ($n = 4$).

Fig. 4. Expression of the genes related to lipid secretion in mice fed alcohol or a high methionine low folate diet. **(A)** RT-PCR; **(B)** Relative expression of ApoB analyzed by Western blotting; Tg, betaine-homocysteine methyltransferase (BHMT) transgenic mice; WT, wild type littermates; MTP, microsomal triglyceride transfer protein; ApoA1, apolipoprotein A1; ApoB, apolipoprotein B; ApoE, apolipoprotein E; C, pair-fed control; E, intragastric alcohol infusion; H, fed orally a high methionine low folate (HMLF) diet. * $p < 0.05$ compared with pair-fed control ($n = 4$).

Fig. 5.

Expression of glycine methyltransferase (GNMT) in mice fed alcohol or a high methionine low folate diet. **(A)** RT-PCR; **(B)** Western blots; transgenic, betaine-homocysteine methyltransferase (BHMT) transgenic mice; Ce, pair-fed control for intragastric infusion; E, intragastric alcohol infusion; EB, intragastric alcohol infusion plus betaine supplementation (0.5%, w/v); Ch, pair-fed control for oral feeding; H, fed orally a high methionine low folate (HMLF) diet; HB, HMLF + betaine supplementation (0.5%, w/v). * $p < 0.05$ compared with pair-fed control ($n = 5$).

Table 1
Levels of Plasma ALT and Homocysteine and Liver Lipids in Mice Fed Alcohol or a HMLF Diet

	Control_1	EtOH	EtOH + Betaine	Control_2	HMLF	HMLF + Betaine
Wild type						
ALT (U/l)	20.5 ± 3.7	115.8 ± 23.5 ^{aaa}	53.0 ± 16 ^a	23.8 ± 7.1	44.3 ± 11.0 ^a	24.3 ± 14.5
Hcy (μM)	3.7 ± 1.5	23.4 ± 5.1 ^{aaa}	5.2 ± 0.9	4.1 ± 1.2	87.2 ± 19.2 ^{aaa}	7.7 ± 5.4
TG (mg/g liver)	23.3 ± 3.3	91.5 ± 7.2 ^{aaa}	41.3 ± 6.2 ^a	22.6 ± 2.1	56.9 ± 5.6 ^a	28.4 ± 2.7
Chol (mg/g liver)	2.9 ± 0.3	5.3 ± 0.5 ^{aaa}	3.5 ± 0.4	3.0 ± 0.3	4.4 ± 0.7 ^a	4.0 ± 0.8
Transgenic (Tg)						
ALT (U/l)	18.0 ± 3.3	50.1 ± 25 ^{ab}	30.2 ± 3.7 ^{abβ}	19.2 ± 3.3	38.7 ± 22.8	20.1 ± 12.3
Hcy (μM)	3.2 ± 0.5	4.6 ± 0.8 ^{ab}	2.8 ± 0.3 ^β	2.5 ± 0.4	9.7 ± 1.2 ^a	3.0 ± 0.9
TG (mg/g liver)	24.4 ± 1.3	68.6 ± 6.9 ^{abβ}	35.9 ± 2.8 ^{abβ}	24.4 ± 1.2	40.1 ± 3.3 ^{abβ}	22.2 ± 1.8 ^β
Chol (mg/g liver)	2.5 ± 0.5	4.1 ± 0.2 ^{abβ}	3.5 ± 0.2 ^{abβ}	2.5 ± 0.3	4.0 ± 0.6 ^a	2.6 ± 0.4 ^β

Tg, betaine-homocysteine methyltransferase transgenic; HMLF, high methionine low folate.

^a
p < 0.05

^{aaa}
p < 0.01 compared with pair-fed control

^β
p < 0.05 compared with pair-fed wild type (*n* = 6).

Table 2
Ratios of SAM to SAH and PC to PE and PEMT Enzyme Activities in BHMT Tg Mice When Compared With Wild Type

	C1	C1 + Betaine	EtOH	EtOH + Betaine	C2	C2 + Betaine	HMLF	HMLF + Betaine
Wild type								
SAM (nmol/g liver)	56.3 ± 11.2	96.2 ± 26.6 ^α	49.5 ± 12.5	230.8 ± 141.3 ^{αα}	61.2 ± 17.4	84.8 ± 24.1	340.3 ± 110.8 ^{αα}	135.2 ± 59.8 ^α
SAH (nmol/g liver)	26.2 ± 6.3	29.2 ± 11.3	41.3 ± 11.7 ^α	35.1 ± 14.8	28.7 ± 9.7	25.5 ± 11.1	89.0 ± 29.8 ^α	39.7 ± 16.2
SAM/SAH	2.15 ± 0.10	3.29 ± 0.31 ^α	1.2 ± 0.15 ^α	6.58 ± 1.16 ^{αα}	2.13 ± 0.11	3.33 ± 0.24 ^α	3.82 ± 0.17 ^α	3.4 ± 0.36 ^α
PC (nmol/mg protein)	125.4 ± 36.2	121.8 ± 28.5	92.3 ± 44.7	131.1 ± 19.5	117.6 ± 28.2	149.8 ± 35.4	92.3 ± 55.3	110.9 ± 39.1
PE (nmol/mg protein)	88.3 ± 33.5	79.6 ± 41.7	85.9 ± 37.2	93.6 ± 25.8	81.7 ± 23.1	97.3 ± 40.5	68.9 ± 42.6	78.7 ± 31.9
PC/PE	1.42 ± 0.19	1.53 ± 0.13	1.08 ± 0.11 ^α	1.49 ± 0.12	1.44 ± 0.30	1.54 ± 0.16	1.34 ± 0.21	1.41 ± 0.17
PEMT	1.33 ± 0.14	1.62 ± 0.09 ^α	1.31 ± 0.22	1.67 ± 0.17 ^α	1.33 ± 0.17	1.66 ± 0.15 ^α	1.41 ± 0.09	1.63 ± 0.11 ^α
Transgenic								
SAM (nmol/g liver)	59.6 ± 13.4	101.8 ± 21.0 ^α	76.2 ± 16.2	112.3 ± 35.7 ^α	57.2 ± 12.2	99.7 ± 28.8 ^α	171.4 ± 22.6 ^{αα}	165.5 ± 38.9 ^α
SAH (nmol/g liver)	24.7 ± 6.5	30.0 ± 12.3	38.9 ± 13.1 ^α	31.2 ± 5.1	24.9 ± 5.2	30.2 ± 6.3	81.2 ± 24.8 ^α	35.2 ± 14.7
SAM/SAH	2.41 ± 0.17	3.43 ± 0.18 ^α	1.96 ± 0.07 ^{αβ}	3.6 ± 0.19 ^{αβ}	2.30 ± 0.32	3.30 ± 0.31 ^α	2.11 ± 0.41	4.7 ± 0.31 ^{αβ}
PC (nmol/mg protein)	126.3 ± 31.4	158.7 ± 26.9	115.4 ± 36.8	165.1 ± 24.2	137.6 ± 28.5	177.2 ± 39.9	124.2 ± 26.7	160.7 ± 19.1
PE (nmol/mg protein)	92.2 ± 27.5	98.6 ± 25.6	87.4 ± 37.1	90.2 ± 28.5	101.2 ± 23.8	95.3 ± 30.4	98.6 ± 28.2	91.3 ± 36.1
PC/PE	1.37 ± 0.14	1.61 ± 0.09 ^α	1.32 ± 0.18	1.83 ± 0.12 ^{αβ}	1.36 ± 0.19	1.86 ± 0.22 ^α	1.26 ± 0.23	1.76 ± 0.17 ^{αβ}
PEMT	1.31 ± 0.28	1.78 ± 0.11 ^α	1.33 ± 0.25	2.18 ± 0.21 ^{αβ}	1.39 ± 0.23	2.01 ± 0.28 ^α	1.31 ± 0.25	1.86 ± 0.14 ^{αβ}

SAM, S-adenosylmethionine; SAH, S-adenosylhomocysteine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; BHMT, betaine-homocysteine methyltransferase; PEMT, PE methyltransferase; HMLF, high methionine low folate diet; C1, pair-fed control for alcohol; C2, pair-fed control for HMLF.

^α
 $p < 0.05$

^{αα}
 $p < 0.01$ compared with pair-fed control

^β
 $p < 0.05$ compared with pair-fed wild type ($n = 4$).