A Comparison of the Effects of Betaine and S-Adenosylmethionine on Ethanol-Induced Changes in Methionine Metabolism and Steatosis in Rat Hepatocytes

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ABSTRACT Previous studies showed that chronic ethanol administration alters methionine metabolism in the liver, resulting in increased intracellular S-adenosylhomocysteine (SAH) levels and increased homocysteine release into the plasma. We showed further that these changes appear to be reversed by betaine administration. This study compared the effects of betaine and S-adenosylmethionine (SAM), another methylating agent, on ethanol-induced changes of methionine metabolism and hepatic steatosis. Wistar rats were fed ethanol or control Lieber-DeCarli liquid diet for 4 wk and metabolites of the methionine cycle were measured in isolated hepatocytes. Hepatocytes from ethanol-fed rats had a 50% lower intracellular SAM:SAH ratio and almost 2-fold greater homocysteine release into the media compared with controls. Supplementation of betaine or SAM in the incubation media increased this ratio in hepatocytes from both control and ethanol-fed rats and attenuated the ethanol-induced increased hepatic triglyceride levels by ∼20%. On the other hand, only betaine prevented the increase in generation of homocysteine in the incubation media under basal and methionine-loaded conditions. SAM can correct only the ratio and the methylation defects and may in fact be detrimental after prolonged use because of its propensity to increase homocysteine release. Both SAM and betaine are effective in increasing the SAM:SAH ratio in hepatocytes and in attenuating hepatic steatosis; however, only betaine can effectively methylate homocysteine and prevent increased homocysteine release by the liver. J. Nutr. 135: 519–524, 2005.

KEY WORDS: • hepatocytes • ethanol • S-adenosylhomocysteine • S-adenosylmethionine • betaine

Previous studies showed that chronic ethanol administration alters methionine metabolism in the liver (1–3). A major defect elicited by ethanol consumption appears to be the inhibition of methionine synthase (MS)3 activity, which results in impaired remethylation of homocysteine to form methionine (4–6). This impairment of MS activity ultimately results in detrimental consequences such as a decrease in the hepatic levels of the methylating agent, S-adenosylmethionine (SAM), and increased generation of the potentially toxic agent, homocysteine, which is released from the liver (1,7–10). Ethanol consumption also increases the intracellular levels of S-adenosylhomocysteine (SAH) (5,9,11), which is the metabolic precursor of homocysteine and is formed as a product of methyl transfer reactions involving SAM (2). The reaction that converts SAH to homocysteine and adenosine is reversible; it is catalyzed by SAH hydrolase and proceeds toward hydrolysis only if the products are removed (2). Because of inefficient removal of homocysteine due to the impairment of MS activity by ethanol, there is an increased accumulation of intracellular SAH that eventually leads to a lowering of the SAM:SAH ratio (5,9,11). The decrease in this ratio negatively affects the activity of many methyltransferases. The liver-specific SAM-dependent methyltransferase, phosphatidylethanolamine methyltransferase (PEMT), which generates phosphatidylcholine (PC) from phosphatidylethanolamine (PE) via methylation, is especially susceptible (12). It was reported recently that the generation of PC by the PEMT pathway is an obligatory event in the synthesis and secretion of VLDL (13). This process is essential for lipid export from the liver, and a defect in the PC generated via the PEMT pathway could, therefore, lead to abnormal lipid accumulation in the hepatocyte.

Betaine, by remethyllating homocysteine via betaine-homocysteine-methyltransferase (BHMT), was shown to mitigate the ethanol-induced changes in methionine metabolism by restoring hepatic SAM and glutathione levels (14–16). Additionally, betaine alleviates ethanol-induced changes in intracellular SAH levels and the SAM:SAH ratio (11) and prevents the increased release of homocysteine by the liver (7,10). Furthermore, betaine was also shown to diminish and reverse ethanol-induced hepatic steatosis in vivo (10,17). Therefore, betaine may diminish liver injury elicited by etha-
nol via its ability to correct alterations in methionine metabolic pathways and to restore crucial methylation reactions. SAM has also been used as a therapeutic agent in many toxin-induced liver injuries including those induced by alcohol (18). It is an essential metabolite in all cells and serves mainly as a methyl donor for many methylation reactions. Even though both betaine and SAM are advocated for use as potential therapeutic agents for alcoholic liver injury, the mechanism of their beneficial effects has not been clarified. Hence, in the present study, we investigated the efficacy of betaine compared with SAM in ameliorating ethanol-induced liver injury by concurrently examining the effects of these agents on hepatocellular SAM:SAH ratios, hepatic triglyceride levels, and homocysteine production.

**MATERIALS AND METHODS**

**Diet formulation.** Nutritionally adequate liquid diets were formulated according to the method of Lieber and De Carli (19) and purchased from Dyets. The ethanol-containing diet consisted of 18% of total energy as protein, 35% as fat, 11% as carbohydrate, and 36% as ethanol. All control rats were pair-fed the same diet as the ethanol-fed rats except that ethanol was replaced isocalorically with carbohydrate. Both ethanol-fed and control rats ingested identical amounts of all nutrients except carbohydrates.

**Ethanol feeding procedure.** Male Wistar rats (Charles River Laboratories) weighing 150–175 g were initially fed a rodent Purina 5001 diet (Ralston Purina) until they reached body weights of 200–220 g, and they were then divided into 2 groups (n = 8). The rats were housed individually and acclimated to the control diet for 3 d. These rats were then weight-matched and paired. One rat from each pair was fed the ethanol diet and the other received the isocaloric control diet. Ethanol-fed rats were acclimated to their diets over a 3-d period by feeding graded ethanol at 12% of total energy on d 1, 24% on d 2, and 36% of energy (full strength) on d 3 and thereafter. Pair-fed control rats received a volume of diet equal to the amount consumed by the ethanol-fed rat the previous day. The rats were pair-fed for 4 wk. During the 24 h before isolation of hepatocytes, the liquid diets were given in 3 portions: 25% at 0900 h, 50% at 1600 h, and the final 25% at 0700 h. This meal-feeding regimen was used to minimize variations in feeding patterns between the ethanol-fed rats and their pair-fed controls before isolation of hepatocytes. The care and the use of (as well as all procedures involving) rats were approved by the Institutional Animal Care and Use Committee at the Omaha Veterans Affairs Medical Center and in accordance with the guidelines of the Institutional Care and Use Committee of the National Institute on Drug Abuse, National Institutes of Health, and the Guide for the Care and Use of Laboratory Animals (20).

**Isolation of hepatocytes.** Hepatocytes were obtained from the livers of control and ethanol-fed rats by a modified collagenase-perfusion technique previously described by our laboratory (21). Animals were housed individually and acclimated to the control diet for 3 d. During the 24 h before isolation of hepatocytes, the liquid diets were given in 3 portions: 25% at 0900 h, 50% at 1600 h, and the final 25% at 0700 h. This meal-feeding regimen was used to minimize variations in feeding patterns between the ethanol-fed rats and their pair-fed controls before isolation of hepatocytes. The care and the use of (as well as all procedures involving) rats were approved by the Institutional Animal Care and Use Committee at the Omaha Veterans Affairs Medical Center and in accordance with the guidelines of the Institutional Care and Use Committee of the National Institute on Drug Abuse, National Institutes of Health, and the Guide for the Care and Use of Laboratory Animals (20).

**Incubation of hepatocytes.** Cell suspensions (1 × 10^7 cells/L) were incubated in Krebs-Henseleit buffer (KRH) containing 12.5 mmol/L HEPES and 2.5 mmol/L CaCl_2 under an atmosphere of 95% O_2/5% CO_2. Before any treatment, cells were equilibrated for 30 min at 37°C. The chosen concentrations were comparable to that used by us and many others in previous studies (7,11,22,23). In some experiments, additional incubations were done in the presence of methionine (0.5 mmol/L), methionine (0.5 mmol/L) plus betaine (1.0 mmol/L), or methionine (0.5 mmol/L) plus SAM (1.0 mmol/L). There was a slight decrease of <10% in cell viability over the 4 h of incubation time that was irrespective of the incubation condition. Betaine was obtained from Finnsugar Bioproducts. SAM (as L-toclenesulfonate salt), methionine, KRH, HEPES and all other chemicals were obtained from Sigma-Aldrich.

**Analysis of released homocysteine and cellular SAM and SAH.** At hourly intervals, hepatocyte suspensions were immediately centrifuged at 1000 × g for 10 min at 4°C to recover cell pellets as well as the supernatant. The supernatant was analyzed for homocysteine using HPLC (24). The cell pellets, in most cases, were treated with 0.5 mol/L HClO_4, and the filtered acid extracts were subjected directly to HPLC analysis for the determination of hepatocellular SAM and SAH levels (25). However, the cell pellets obtained after incubations with exogenous SAM were washed extensively with KRH, and the final pellets recovered after centrifugation were treated for HPLC analyses.

**Analysis of triglycerides.** At the end of the 4-h incubation period, the cells were centrifuged and the pellets collected and stored at −78°C. Lipids in cell pellets were extracted (26) and triglycerides in the extract were then saponified and quantified using diagnostic kit No. 320-UV (Sigma Chemical).

**Preparation of hepatocyte lysate and microsomal fraction.** Hepatocytes were sonicated in ice-cold 10 mmol/L Tris HCl (pH 7.4) containing 0.25 mol/L sucrose. The cell lysate was further processed to prepare the microsomal fraction. Briefly, the lysate was centrifuged at 16,000 × g for 20 min at 4°C and the supernatant was centrifuged again at 105,000 × g for 60 min at 4°C. The pellet from this last centrifugation (microsomal fraction) was resuspended in an appropriate volume of 0.25 mol/L sucrose. The protein content of the lysate and the microsomal fraction was determined by the bicinchoninic acid protein assay (Fierce Biotechnology) using bovine serum albumin as the standard.

**PEMT assay.** PEMT activity in the cell lysate and the microsomal fraction of hepatocytes isolated from control and ethanol-fed rats was determined by measuring the incorporation of the [3H]methyl group from S-adenosyl-L-(methyl-3H) methionine using endogenous microsomal PE as the substrate as previously described (11). Briefly, the assay mixture (in a total volume of 500 μL) contained 10 mmol/L HEPES, pH 7.3, 0.25 mmol/L dithiothreitol, 5 mmol/L MgCl_2, 10 μmol/L S-adenosyl-L-methionine, 74 kBq S-adenosyl-L-(methyl-3H) methionine (Amersham Biosciences), and 500 μg cellular or microsomal protein. The reaction was initiated by the addition of a mixture of unlabeled and labeled S-adenosyl-L-(methyl-3H) methionine, followed by a 10-min incubation at 37°C and was terminated by pipetting 100 μL of assay mixture, in duplicate, into 2 mL of chloroform:methanol:2 mol/L HCl (6:3:1) for lipid extraction. The aqueous phase was aspirated after centrifugation at 1800 × g for 5 min and the chloroform phase washed 3 times with 1 mL of 0.5 mol/L KCl in 50% methanol. After the final wash, the entire chloroform phase was pipetted into a counting vial, dried under a stream of nitrogen, dissolved into 8 mL of scintillation liquid, and counted. The PEMT activity was expressed as pmol methylated PE products formed/min (mg protein).

In addition to determining basal microsomal PEMT activity, experiments were also conducted in which S-adenosylhomocysteine was added in varying amounts to the microsomal assay reaction mixtures such that the ratio of SAM:SAH in the mixture was 2.5, 5, or 10.

**RESULTS**

SAM and SAH, respectively, are the substrate and the product of the cellular methyltransferase reactions and the ratio of these 2 metabolites (i.e., SAM:SAH) is frequently used as an indicator of cellular methylation capacity, whereby a decrease in this ratio predicts reduced cellular methylation potential (2,27). Therefore, this ratio was determined for all of the experimental conditions. After 4 wk of dietary treatment, the SAM:SAH ratio in freshly isolated hepatocytes from the ethanol-fed rats was lower than in the pair-fed controls, and
this difference was maintained during a 4-h incubation period of hepatocytes ($P < 0.0001$, Fig. 1A). Incubation of hepatocytes in the presence of betaine markedly increased this ratio in cells from both ethanol-fed and control rats within 1 h of supplementation, and was further improved with a more prolonged incubation time ($P < 0.03$). When SAM was added to the incubation medium, a more rapid increase in the ratio occurred within 1 h compared with the betaine-supplemented media ($P < 0.0005$, Fig. 1B); however, no further increase in the ratio occurred at the later incubation times. Overall, the effects of betaine or SAM supplementation on enhancing the hepatocellular SAM:SAH ratio were similar.

Hepatocytes isolated from control and ethanol-fed rats released homocysteine into the medium at a relatively constant rate over a 4-h incubation period (Fig. 2A). However, homocysteine release from hepatocytes of ethanol-fed rats occurred at a nearly 2-fold increased rate compared with the controls ($P < 0.002$, Fig. 2A). Under conditions of betaine supplementation, the accumulation of homocysteine in the incubation medium of hepatocytes from both control and ethanol-fed rats was attenuated ($P < 0.0001$, Fig. 2A). However, SAM exposure actually promoted homocysteine release from both cell types ($P < 0.0001$, Fig. 2B). This effect was in complete contrast to our observations with betaine supplementation.

Because methionine loading in fasting patients is used to stress homocysteine pathways in order to identify disturbances in methionine metabolism (28), the effect of methionine addition on the release of homocysteine by hepatocytes from control and ethanol-fed rats was also studied. Under conditions of exposing hepatocytes to excess methionine levels, homocysteine release was substantially increased in both cell types ($P < 0.0001$, Fig. 2C). Administration of betaine in to the incubation mix inhibited the increased generation of homocysteine with methionine loading in both cell types ($P < 0.0001$, Fig. 2D). In contrast to the effect of betaine, SAM was ineffective in limiting methionine-induced homocysteine release in both cell types ($P = 0.86$, Fig. 2E).

Because hepatocytes from ethanol-fed rats had a significantly lower SAM:SAH ratio, which in turn could affect methylation reactions, we examined whether ethanol feeding affects the methylation activity of PEMT. The activity of this enzyme was analyzed in cell homogenates as well as isolated microsomes. PEMT activity was lower in hepatocyte lysates from ethanol-fed rats ($P = 0.011$) compared with controls, although the magnitude of this difference was only ~20% (Fig. 3A). However, PEMT activity did not differ in microsomes isolated from hepatocytes of control and ethanol-fed rats (Fig. 3B). But, when these isolated microsomes were exposed to varying SAM:SAH ratios, there was a direct relation between the ratio and PEMT activity; i.e., a decrease in the ratio resulted in a corresponding decrease in PEMT activity ($P < 0.05$). Furthermore, the PEMT activity in isolated microsomes from hepatocytes of both control and ethanol-fed rats was affected similarly by the changes in the ratio.

We also investigated the effect of exposure to SAM or betaine on hepatocellular triglyceride levels. As was reported earlier (10,17), hepatocytes isolated from ethanol-fed rats had 2- to 3-fold greater levels of cellular triglycerides (Fig. 4; $P < 0.0001$). The difference was maintained over a 4-h incubation in vitro ($P < 0.0001$). However, when isolated hepatocytes from ethanol-fed rats were exposed to either betaine or SAM for a short period of the 4-h incubation, both were equally effective ($P < 0.05$) in reducing triglycerides by ~20%.

**DISCUSSION**

There have been numerous reports advocating the efficacy of both SAM (8,18,29–32) and betaine (7,10,11) on mitigating many aspects of ethanol-induced liver injury. To date, no side-by-side comparisons have been made. The present study compares, on the exact same isolated hepatocyte population incubated in vitro, the abilities of betaine and SAM to correct ethanol-induced defects in liver methionine metabolism and hypertriglyceridemia. The results of this study indicate that these 2 agents have similar beneficial effects in correcting most of these alterations; however, it appears that betaine has additional protective effects that do not occur with SAM.

Because the ratio of SAM:SAH in the cell is the prime regulator of the activities of most methyltransferases (27), a decrease in this ratio by ethanol consumption would be expected to result in reduced methylation reactions in the liver. In this study, when hepatocytes isolated from control and ethanol-fed rats were incubated in the presence of betaine or SAM, both agents were equally effective in increasing this
ratio. However, it appears that different mechanisms are responsible for this effect. In the case of betaine, the ratio is likely increased by lowering the intracellular levels of SAH, whereas in the case of SAM treatment, increased intracellular SAM levels contribute mainly to the elevation of this ratio.

An important methyltransferase in the liver whose activity is especially susceptible to regulation by the SAM:SAH ratio is SAM-dependent PEMT (12). This transferase catalyzes the methylation of PE to PC and was shown to play an important role in the synthesis and secretion of VLDL in the hepatocyte (13,33). In this study, PEMT activity was slightly lower in total hepatocyte lysates from ethanol-fed rats compared with controls, whereas activities in isolated microsomes obtained from both cell types did not differ. These results were not surprising because the enzyme assays were conducted in cell-free systems in the presence of excess SAM. However, when PEMT activity was assayed in the presence of varying SAM:SAH ratios, corresponding to the ratios in hepatocytes from control or ethanol-fed rats or these same hepatocytes incubated with betaine or SAM, PEMT activity declined with the decrease in the ratio. These results suggest that PEMT is unaffected by alcohol consumption per se, but the alcohol-induced decrease in the hepatocellular SAM:SAH ratio impairs PEMT activity, and that betaine and SAM, by virtue of their ability to increase the ratio, restore (increase) the activity of PEMT.

Because one of the earliest manifestations of ethanol-induced liver injury is the accumulation of triglycerides in the liver, recent reports that suggest an important role for the PC generated via the PEMT pathway in the formation of VLDL and transport of triglycerides from the liver (13,33) are relevant to the results obtained in this study. Reduced PC generation as a result of decreased PEMT activity caused by the decreased hepatocellular SAM:SAH ratios could ultimately produce a defect in the secretion of VLDL and contribute to hepatic steatosis in rats consuming alcohol. Furthermore, the

![FIGURE 2](https://example.com/figure2)

**FIGURE 2** Homocysteine release into the media by hepatocytes isolated from control and ethanol-fed rats and incubated with betaine (A), SAM (B), methionine (C), methionine + betaine (D), and methionine + SAM (E) for up to 4 h. Values are means ± SEM, n = 8. *Different from hepatocytes of control-fed rats incubated with and without betaine, SAM or methionine supplementation, P < 0.05; #different from hepatocytes of ethanol-fed rats incubated with and without betaine, SAM or methionine supplementation, P < 0.05.

![FIGURE 3](https://example.com/figure3)

**FIGURE 3** PEMT activity in rat liver cell homogenates (A) and isolated microsomes (B) from control and ethanol-fed rats and the effect of varying ratios of SAM:SAH on PEMT activity of isolated microsomes from both cell types (C). The concentration of SAM was maintained at 100 μmol/L. The concentration of SAH was varied to generate SAM:SAH ratios from 2.5 to 10, corresponding to the ratios in the hepatocytes of control or ethanol-fed rats under basal conditions and after betaine or SAM administration. Values are means ± SEM, n = 8. A: *different from control, P < 0.05. C: *#different from the SAM:SAH ratio of 5.0, control and ethanol, respectively, P < 0.05.
ability of betaine or SAM to increase this ratio and thus maintain optimal PEMT activity and restore VLDL secretion could explain the ability of these 2 agents to prevent and reverse the ethanol-induced fatty liver. Consistent with this conclusion are the data from this study showing that both betaine and SAM decreased the hepatocyte triglyceride level. Although the decrease in triglycerides by these 2 agents was small (~20%), it was significant; because isolated hepatocytes were used to test these agents, only a short incubation time of 4 h could be studied. Therefore, extension of the treatment period would likely be even more effective in reducing triglyceride levels as was indicated in previous in vivo studies showing the effectiveness of these agents in preventing and reducing hepatic steatosis over weeks of feeding (8,10,14,15,17,31).

Further consideration of the results of this study indicates that although the effects of both SAM and betaine were similar in correcting the ethanol-induced alterations in the SAM:SAH ratio and in attenuating hepatic steatosis, their effect on the release of the potentially toxic metabolite, homocysteine, by hepatocytes was different. Betaine effectively prevented the increased release of homocysteine from hepatocytes of ethanol-fed rats under basal conditions or after a methionine load, whereas SAM was completely ineffective in this regard. In fact, under basal conditions, SAM actually enhanced the release of homocysteine from the cells. A plausible explanation for this phenomenon is that by correcting the SAM:SAH ratio, both SAM and betaine promote the flux through the methylation pathway leading to the generation and accumulation of SAH, the by-product of transmethylation reactions, and its subsequent hydrolysis to homocysteine. Although betaine is able to remethylate the excess homocysteine to methionine via BHMT, thereby effectively preventing the ethanol-induced increased release of homocysteine under basal conditions or after methionine load, SAM was not capable of this reaction under any conditions.

Hyperhomocysteinemia is undesirable because of the toxicity of this amino acid (34). Although the detrimental effects of homocysteine were well studied in different cellular populations (35–42), very few studies were conducted concerning the toxicity of homocysteine on liver cell populations. Ji and Kaplowitz (10) reported that ethanol-induced hyperhomocysteinemia is associated with endoplasmic reticulum stress, leading to the activation of endoplasmic reticulum–dependent apoptosis and upregulation of lipid synthesis in hepatocytes. Another potentially serious consequence of increased homocysteine was reported by Torres et al. (43) who showed that exposure of cultured hepatic stellate cells to homocysteine resulted in increased collagen production and induction of tissue inhibitors of metalloproteinase. Both of these effects would favor the increased deposition of the extracellular matrix and progression of liver fibrosis.

In summary, the results of these studies indicate that betaine, by virtue of its ability to remethylate homocysteine, lowers SAH levels and in turn increases the reduced SAM:SAH ratio due to ethanol, stimulates cellular methylation, and attenuates the resultant sequela of events of steatosis and homocysteine-induced toxicities. SAM, on the other hand, can only correct the ratio and the methylation defects and may in fact be detrimental after prolonged use because of its propensity to increase homocysteine release. Thus, betaine could be very effective in treating liver diseases as well as other diseases associated with elevated homocysteine and defective methylation.

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LITERATURE CITED


