ABSTRACT
Emerging evidence indicates that ethanol-induced alterations in hepatic methionine metabolism play a central role in the pathogenesis of alcoholic liver disease (ALD). Because malnutrition is a universal clinical finding in this disease and hepatic methionine metabolism is dependent upon dietary folate and vitamins B-6 and B-12, ALD can be considered an induced nutritional disorder that is conditioned by alcohol abuse. The present review describes the etiologies of these 3 vitamin deficiencies in ALD and how they interact with chronic ethanol exposure to alter hepatic methionine metabolism. Subsequent sections focus on molecular mechanisms for the interactions of aberrant methionine metabolism with ethanol in the pathogenesis of ALD, in particular the role of S-adenosylmethionine (SAM) in regulating the epigenetic expressions of genes relevant to pathways of liver injury. The review will conclude with descriptions of studies on the efficacy of SAM in the treatment of ALD and with discussion of potentially fruitful future avenues of research.

Introduction
Chronic alcoholism affects up to 10% of the U.S. population, of whom up to 8% develop the complication of ALD, which is among the top 10 causes of death in the United States (1,2). Conventional understanding of the pathogenesis of ALD involves the ethanol-induced translocation of LPS enterotoxin from the intestine to the liver, where it stimulates the production and release of cytokines, in particular TNFα, from specialized in-residence macrophages called Kupffer cells, which then facilitate ethanol-induced injury pathways in the hepatocyte (3). These pathways include CYP2E1 (cytochrome P450 2E1) that both metabolizes ethanol and generates ROS that lead to inflammation of the liver and necrosis of hepatocytes (4). Oxidative responses to ethanol metabolism are counterbalanced by the actions of the principal antioxidant, GSH, which is generated through the metabolism of methionine. In addition, the metabolism of alcohol by alcohol dehydrogenase generates acetaldehyde and an excess of reducing equivalents, mainly reduced NADH, and the altered NADH/nicotinamide adenine dinucleotide redox potential promotes steatosis, i.e. the accumulation of fat in the liver (5). Hepatic steatosis represents a balance among lipogenesis, fatty acid oxidation, and lipid export. Hepatic lipogenesis is activated through the ER stress pathway that is stimulated by many factors, including ROS and homocysteine to activate stearoyl response element binding protein (SREBP 1-c) and its downstream genes (6) and by the effect of ethanol-induced TNFα on reducing the production of adiponectin from peripheral adipocytes and its activation of hepatic AMPK (adenosine monophosphate kinase) dependent protein kinase (5). Fatty acid oxidation occurs within mitochondria and is impaired by the effects of ethanol metabolism on reducing levels of PPARα (7). SIRT1 (Sirtuin1) is a deacetylase that is decreased by ethanol exposure and regulates fatty acid oxidation through effects on PPARα (8). The transport of fatty acids into the mitochondria is regulated by CPT1 (carnitine palmitoyl transferase), which is inhibited by the effects of ethanol on AMPK and its downstream pathways (7,9). Lipid export involves the synthesis of VLDL from phosphatidylcholine, which is synthesized within the methionine cycle by PEMT (phosphatidylethanolamine methyltransferase) (10,11). Apoptosis, or hepatocyte death, is mediated through several ER stress pathways that are activated by homocysteine and ROS (6). Cirrhosis, the end stage of progressive fibrosis, involves the transformation of vitamin A storing transitional cells to...
collagen secreting stellate cells, and may be regulated by the oxidation stress pathway gene NOX (nicotinamide adenine dinucleotide phosphate oxidase) (12). All of these processes potentially involve intricate and epigenetically regulated balances of gene activation and suppression that may be modulated epigenetically by the availability of methyl groups for methyltransferase reactions within the liver.

Whereas ALD was considered for decades strictly a disease of alcohol abuse, there is now abundant evidence for multiple nutritional deficiencies in this clinical disorder, including protein calorie malnutrition and many vitamin deficiencies such as those of folate, vitamin B-6, and vitamin B-12 (13). Mechanistically, it is known that vitamin B-6 deficiency is caused by the effect of the ethanol metabolite acetaldehyde on displacing pyridoxal phosphate from its intrahepatic protein binding sites (14), whereas folate deficiency in chronic alcoholism results from combinations of poor diet (15), intestinal malabsorption (16), reduced liver uptake (17), and increased renal excretion of this vitamin (18,19). Vitamin B-12 levels are decreased in livers of ALD patients, although serum levels are normal or elevated due to decreased uptake and retention by damaged hepatocytes (20).

Aberrant hepatic methionine metabolism has now emerged as both a result of alcohol abuse and as a major factor in the pathogenesis of ALD (21). More specifically, ethanol-induced lowering of SAM levels in the liver appears to play a major role in weakening defense mechanisms against oxidative liver injury and in epigenetic modification of the expressions of genes involved in the progression of ALD. This review will summarize established evidence for the causative effects of alcohol abuse with deficiencies of folate, vitamin B-6, and vitamin B-12 on altered hepatic methionine metabolism. In addition, we will discuss the effects of aberrant methionine metabolism with reduced levels of SAM and the SAM:SAH methylation ratio on the different mechanisms in pathogenesis of ALD, and on the potential role of SAM in the clinical treatment of ALD.

**Current status of knowledge**

**Hepatic methionine metabolism**

Methionine metabolism occurs predominantly in the liver and consists of two components, the transmethylation cycle for the production of SAM and its metabolism to homocysteine, and the transsulfuration pathway for the reduction of homocysteine in the production of GSH (Fig. 1). Whereas 5-methyltetrahydrofolate is substrate for the initial transmethylation reaction of methionine synthase (MS), it is also the principal source of dietary methyl groups in the production of SAM. In the transmethylation pathway, homocysteine with cosubstrate 5-methyltetrahydrofolate is converted to methionine by MS (methionine synthase) with vitamin B-12 as its cofactor. The endogenous synthesis of methionine and reduction of its precursor homocysteine is also facilitated by the rescue pathway of BHMT (betaine homocysteine methyl transferase) that uses the alternate substrate and methyl donor betaine, which is both a dietary component and a product of choline. Methionine is metabolized by MAT, a product of the MAT1A gene, to yield SAM, which is generated and consumed in the liver at a rate of 6–8 g/d (22,23). SAM is the universal methyl donor for all methylation reactions, including those for DNA, histones, glycine n-methyltransferase, and PEMT, all of which produce SAH, which, conversely, is the principal inhibitor of all methylation reactions (24). Because the Km of many methyltransferases reactions is similar to their inhibitory Ki (24), the ratio of SAM to SAH may be considered an index of methylation capacity. Although SAH is the precursor of homocysteine, the SAHH (S-adenosylmethionine hydrolase) reaction is reversible so that SAH levels may increase in the presence of elevated homocysteine. In the transsulfuration pathway, SAM also regulates the synthesis of GSH by its facilitation
of the cystathionine $\beta$S (cystathionine $\beta$ synthase) reaction (25,26), so that reduced SAM leads to reduced production of GSH. Vitamin B-6 is cofactor for 2 reactions, $\beta$S and cystathionase, to produce cysteine and GSH from their precursor homocysteine.

**Effects of ethanol exposure on methionine metabolism**

Studies in both clinical and animal models have demonstrated diverse effects of chronic ethanol exposure on the regulatory enzymes in methionine metabolism that result in elevations in homocysteine and SAH and reductions in SAM and antioxidant GSH levels. As shown in Figure 1, elevated homocysteine can result from reduced activities of both MS and $\beta$S, whereas reduced SAM can result from reduced activities of MS and MAT. Elevated levels of SAH with reduction in the SAM:SAH ratio could result both from decreased SAHH forward activity or elevation in homocysteine with increased SAHH reverse activity. Studies of liver biopsies from ALD patients demonstrated reduced transcripts of MS, MAT, and $\beta$S (27), and similar findings of reduced synthesis and activities of MS and MAT as well as SAHH with increased glycine N-methyltransferase were found in chronic ethanol-fed micropigs with the histopathology of ALD (28). Others found reductions in MS activity with compensatory increase in BHMT activity in ethanol fed rats (29). As reviewed (23), MAT is generated by 2 separate genes, MAT1A, which is expressed in adult liver, and MAT2A that is expressed in fetal liver and extrahepatic tissues. MAT1A is susceptible to inactivation by ROS that are generated by ethanol induction of CYP2E1 through nitrosylation or oxidation of one or more amino acid residues (30,31). Because SAM also facilitates the generation of the main antioxidant GSH by activation of $\beta$S (25,26), ethanol-induced ROS that inactivate MAT1A expression and decrease the production of SAM predictably decrease the production of GSH. Studies in ethanol-fed baboons and micropigs found strong correlations between reduced liver levels of SAM and antioxidant GSH (32,33). Lowered SAM levels in hepatocytes from ethanol-fed rats were linked to increased CYP2E1 and ROS production, whereas SAM levels were preserved through inhibition of CYP2E1 (34).

**Effects of ethanol-induced aberrant methionine metabolism on the pathogenesis of ALD**

Separate and interactive pathways for alcoholic injury that are known to be influenced by ethanol-induced aberrant methionine metabolism include those for steatosis, apoptosis, and oxidative liver injury. Both lipogenesis and apoptosis are regulated in part by the ER stress pathway that is activated by many factors, including ROS and homocysteine (6). A study of C57BL/6 mice fed intragastric control or ethanol diets for 4 wk associated with typical ALD histopathology and hyperhomocysteinemia with activation of the ER stress pathway, including upregulation of the lipogenesis transcription factor SREBP-1c and the growth arrest and DNA damage (GADD) 153 pathway for apoptosis, all of which were prevented by concomitant provision of betaine, which lowered homocysteine through the BHMT pathway (Fig. 1) (35). Others associated ethanol-induced ALD in the ethanol-fed rat with elevated liver homocysteine and apoptosis, whereas steatosis in the same model was related to reduced lipid export according to decreased PEMT activity and VLDL secretion (36–38). Additional studies in ethanol-fed mice associated elevated liver SAH with enhanced sensitization of hepatocytes to the effects of TNF-α on apoptosis pathways (39). SAM is also transported across the mitochondrial membrane by a carrier-mediated pathway that is inhibited by SAH (40). Studies in ethanol-fed mice showed that elevated cytoplasmic SAH inhibited the mitochondrial transport of SAM, resulting in reduced mitochondrial SAM and increased mitochondrial oxidative reactivity (41). These findings on the significance of mitochondrial SAM to oxidative stress are relevant to prior discoveries of a mitochondrial transporter for the antioxidant GSH that is inactivated by ethanol but is sustained by the coadministration of SAM (42).

Further evidence for a central role of aberrant methionine metabolism in the pathogenesis of ALD consists of observations from several laboratories on the preventive effects of the coadministration of SAM in diets of ethanol-fed experimental animals. These include the original finding that coadministration of SAM to ethanol-fed baboons sustained liver GSH levels with partial maintenance of normal histology (33). The development of acute liver injury in mice by 3 large gavage doses of ethanol was prevented by predadministration of SAM, which also sustained liver SAM and mitochondrial levels of antioxidant GSH (43). Others showed that SAM prevented decreased mitochondrial respiration and ribosomal dissociation in ethanol-fed rats (44) as well as increased mitochondrial superoxide and iNOS (inducible NO synthase) (45) and alterations in the mitochondrial proteome (46).

**Potential roles for altered gene methylation in pathogenesis of ALD**

Our laboratory explored the effects of chronic ethanol exposure on the relationships of aberrant methionine metabolism to the expressions of genes relevant to steatosis, apoptosis, and oxidative liver injury in ethanol-fed micropigs and genetically altered mice. Initially, we developed the experimental model of ALD in micropigs that were fed ethanol at 40% of energy for 12 mo, including characteristic histological features of steatosis, inflammation, and centrilobular fibrosis that progressed to cirrhosis over 24 mo (47). A subsequent 12-mo study showed that ethanol feeding induced progressive elevation in serum homocysteine levels together with lowered activity of liver MS and a reduction in the liver SAM:SAH ratio and increased apoptotic bodies in terminal hepatocytes (48). To establish a central role for ethanol-induced aberrant methionine metabolism in the pathogenesis of ALD, the next study tested the effects of the combination of a low-folate diet and ethanol feeding in the micropig (32). This model is relevant to clinical
ALD in view of the established association of chronic alcoholism with clinical deficiency of folate (15,49), which is the initial methyl donor in the methionine cycle (Fig. 1). In contrast to our prior finding of the histopathology of ALD after 12 mo of feeding (47), these pathological features were established in just 3 mo of the combined ethanol- and folate-deficient diet and were associated with a greater increase in plasma homocysteine and greater reductions in liver folate, the SAM:SAH ratio, and GSH levels, along with increased apoptosis and DNA strand breaks (32). Using liver samples from the same animals, we found enhanced transcript and protein expressions of CYP2E1 and ER stress genes, including the chaperone glucose regulated protein (GRP) 78, lipogenesis transcription factor SREBP 1-c and its target fatty acid synthase, and the apoptosis mediator caspase 12 in both ethanol groups with greatest expressions in the combined diet group, each of which negatively correlated with the SAM:SAH ratio of methylation capacity (50). This observation was strengthened by 2 subsequent studies that tested the potential preventive effect of SAM on the development of ALD in the ethanol-fed micropig. Evaluating lipid synthesis, we found that ethanol feeding enhanced and supplemental SAM completely prevented the clinical and histological development of ALD in the micropig as well as ethanol-induced reduction in the SAM:SAH ratio, elevations in liver TG and expressions of SREBP 1-c and its downstream genes, and also prevented changes in the adiponectin pathway for lipogenesis, including ethanol-induced lowering of serum adiponectin and liver phosphorylated AMPK (51). Using the same liver samples to evaluate the protective effect of SAM on pathways of oxidative liver injury, we found that ethanol feeding enhanced and SAM supplementation prevented reduction in GSH and increases in the transcript and protein expressions of liver CYP2E1 and activities of NOX1 and iNOS (52). Summarizing, studies in the micropig model established a central role for ethanol-induced aberrant methionine metabolism in the pathogenesis of ALD by showing acceleration of liver injury when ethanol feeding was combined with a folate-deficient diet, by demonstrating relationships of relevant gene activations to changes in the SAM:SAH ratio, and by preventing ALD and its gene activations by coadministration of SAM in the ethanol-containing diet.

Role of aberrant methionine metabolism in epigenetic regulation of ASH

The epigenetic regulation of gene expression involves remodeling of chromatin by either the addition of methyl groups to DNA and/or the posttranslational modification of histone amino acid residues. As described in a recent review, the effects of chronic ethanol exposure on the activation or suppression of selected ALD-related genes has been ascribed to acetylation at H3K9 and by methylation at H3K4 and H3K9 residues (53). Whereas acetylation at histone H3K9 and methylation at H3K4 result in gene activation, methylation at H3K9 downregulates gene expression. Conversely, reduced methylation at H3K4 associates with gene suppression, whereas reduced methylation at H3K9 associates with gene activation. Histone methylation is closely linked to hepatic methionine metabolism, because the level of substrate SAM is critical as substrate for histone methyltransferases, whereas the product SAH is a potent inhibitor of the same reactions (24) (Fig. 1).

We explored these mechanisms in CbS wild-type and heterozygous C57Bl6 mice that were fed control or ethanol diets by intragastric infusion for 4 wk followed by assessment of the activations of relevant genes and their regulation by histone methylation. Because others had shown that CbS-deficient mice demonstrate marked hyperhomocysteinemia (54), we predicted that the addition of ethanol exposure to the model would enhance homocysteine elevation even further through its known inhibitory effect on MS expression and activity (27–29), whereas elevated homocysteine would have the secondary effect of elevating SAH through the reverse SAHH pathway, thereby reducing the SAM:SAH ratio (Fig. 1). Summarizing the results, intragastric feeding of ethanol to mice of either wild-type or heterozygous CbS genotype over 4 wk resulted in all features of alcoholic liver injury (55). Both ethanol and genotype interacted to decrease the SAM:SAH ratio as well as enhancing ER stress marker GRP78, SREBP-1c for lipogenesis, and GADD153 for apoptosis. Epigenetic effects were assessed by immunohistochemical staining of liver slices for histone modifications, finding no changes in H3K4 but decreased intensity of H3K9 in the centrilobular areas of heterozygous mice that were most damaged by ethanol feeding. Subsequent analysis by chromatin immunoprecipitation with antibody to H3K9 showed its decreased presence in the promoter regions of the ER stress genes GRP78, SREBP-1c, and GADD 153. Because H3K9 suppresses gene activities, its reduced presence in the promoter regions of these genes was consistent with epigenetic regulation to increase their expressions. The study also demonstrated reduction in expressions of the histone methyltransferase gene G9a for H3K9. Correlations of these findings with SAM:SAH ratios confirmed the association of these epigenetic observations with altered hepatic methionine metabolism (55).

Clinical studies on the relationship of aberrant methionine metabolism to the pathogenesis and treatment of ALD

Several groups studied the significance of abnormal methionine metabolism in the pathogenesis and treatment of clinical ALD. Low levels of SAM and activities of MAT and PEMT were found in 2 studies of liver biopsies from cirrhotic patients of mixed etiology, including ALD (56,57), whereas reduced expressions of liver MS, MAT1A, and CbS were found in another study of ALD patients (27). A study of cirrhotic patients of diverse etiology found significant elevations in serum homocysteine and cystathionine, suggestive of a block in the transsulfuration pathway (58). The importance of vitamin deficiencies relevant to methionine metabolism in ALD was demonstrated in baseline data from our recent clinical study (59). Serum levels of folate,
vitamin B-6, vitamin B-12, and methionine metabolites were compared in 40 ALD patients, 26 active drinkers without ALD, and 28 healthy participants. Serum folate levels were within normal range but were relatively decreased in both alcoholic groups compared to healthy participants, whereas serum vitamin B-6 levels were lower and vitamin B-12 levels were higher in ALD patients. Although lower serum folate levels are a well-known finding in chronic alcoholics with or without ALD (15), this is the first description to our knowledge of relatively low folate levels in chronic alcoholics after mandatory fortification of folate in the U.S. diet, which underscores the importance of nondietary causes of folate deficiency in this disease. Low vitamin B-6 levels have been well established in alcoholic patients (60,61) and elevated serum vitamin B-12 levels in ALD are known to result from decreased retention of this vitamin in the liver (20). Serum homocysteine levels were elevated in all alcoholics with or without liver disease, as previously described in correlation with low folate and vitamin B-6 levels (62), and were predictable as a consequence of impaired MS expression and activity (27–29) and a potential block in the vitamin B-6–dependent transsulfuration pathway (58). Serum SAM levels were unexpectedly increased in ALD patients and serum SAH was elevated in both alcoholic groups, consistent with elevated homocysteine levels (Fig. 1). Serum cystathionine was markedly increased by >2-fold in ALD patients, whereas ABU, a by-product of the conversion of cystathionine to cysteine by γ-cystathionase (Fig. 1), and its ratio with cystathionine were markedly reduced in ALD patients, consistent with reduced activity of the vitamin B-6–dependent enzyme cystathionase. Notably, serum levels of vitamin B-6 negatively correlated with cystathionine and positively with the ABU:cystathionine ratio in all participants. In addition, we found a positive correlation between cystathionine levels and the severity of fibrosis, whereas the ABU:cystathionine ratio was a positive predictor of the presence of ALD among alcohol drinkers (59). These findings confirm the presence of relative folate and vitamin B-6 deficiencies in ALD and point to the importance of alteration in the vitamin B-6–dependent homocysteine transsulfuration pathway in its pathogenesis.

There have been a number of clinical trials of SAM in treatment of ALD. An early study of 20 ALD patients showed that RBC GSH levels increased and cysteine levels decreased after 15 d of i.v. SAM at 2 g/d (63). In another placebo controlled study, a 6-mo treatment with SAM at 1.2 g/d increased hepatic GSH levels in 9 patients with ALD and 7 patients with other causes of chronic liver disease (64). A multicenter, 2-y European trial in 123 ALD patients found that SAM at 1.2 g/d reduced mortality or liver transplant incidence from 30% in the placebo group to 16% in the SAM group but was significant only when patients with more advanced clinical disease were excluded from the analysis (65). However, none of these studies included an analysis of the effect of SAM on liver histopathology, and a subsequent meta-analysis of 9 studies could not confirm whether SAM is effective in the treatment of ALD (66). The data from the Veterans Affairs Cooperative Study is also of interest (67), because it tested the efficacy of polyenylphosphatidylcholine, which had been shown to restore liver SAM levels in ethanol-fed rats (68). However, this double-blind, 24 mo placebo-controlled trial found no effect of polyenylphosphatidylcholine on improving liver histopathology in 412 ALD patients (67).

Recently, we completed a double-blind trial of SAM treatment in 37 ALD patients who received 1.2 g/d of SAM or placebo for 6 mo (69). The participants were required to abstain from drinking alcohol and 11 were discharged mainly due to drinking relapse, leaving 26 evenly distributed patients who completed the study. Among 14 patients who underwent liver biopsies, 8 presented with advanced stages of fibrosis at baseline. Serum methionine metabolites, including SAM, SAH, and homocysteine, were measured before and at monthly intervals during the study. Ultimately, SAM was no more effective than placebo in the treatment of ALD according to unchanged histological scores and no differences in the improvements in biochemical parameters of liver disease over the course of the study. Whereas serum SAM levels were progressively increased after oral SAM, indicative of a systemic response, the levels of SAH, homocysteine, and other methionine metabolites were unchanged by either treatment. Despite the limitation of the small number of patients, our study was the first to our knowledge to provide liver histology data and extensive analysis of serum methionine metabolites levels in response to SAM treatment (69).

To summarize, clinical trials of SAM in the treatment of ALD have provided mixed results. Several positive studies were compromised by inclusion of patients with additional causes of liver disease and lack of histological data. Our study is limited by a relatively short exposure to SAM and relatively few participants with an excessive number of dropouts. However, the study documents the lack of effect of SAM on liver histopathology and lack of difference from placebo on changes in liver function tests and is the first to our knowledge to study the effect of SAM on serum methionine metabolites. There are several possible explanations for the lack of efficacy of SAM in our study that could apply to other future clinical trials. First, unlike trials in animal models showing the efficacy of SAM in the prevention of ALD (33,51,52), the use of SAM in treatment of established ALD may be compromised by a lack of retention of SAM by injured hepatocytes, as was shown by elevated serum SAM levels in our study (59,69) and by decreased numbers of hepatocytes due to the presence of fibrosis. Our data from both baseline and treatment studies suggest a potential requirement for vitamin B-6 supplementation in addition to SAM in ALD patients that present with subnormal vitamin B-6 levels at baseline (59,69).

Conclusions

Abundant evidence now links ethanol-induced aberrant methionine metabolism with deficiencies of folate and vitamins B-6 and B-12 as a key factor in the pathogenesis of
ALD. In particular, reduced liver SAM can lead to 2 avenues for the progression of liver injury that include metabolic insufficiency of antioxidant defense mechanisms and abnormal epigenetic regulation of genes relevant to alcoholic liver injury. The doors have now been opened for potentially productive research into the relationship of epigenetic changes in SAM-regulated gene methylation to all pathways of liver injury in ALD. Furthermore, the inconclusive results of trials in SAM treatment of ALD suggest that provision of other nutritional factors involved in SAM metabolism, such as vitamin B-6, should be included with SAM in larger and more prolonged clinical trials.

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**Literature Cited**