

UC Davis

UC Davis Previously Published Works

Title

Hepatic transmethylation reactions in micropigs with alcoholic liver disease

Permalink

<https://escholarship.org/uc/item/6r19w8r6>

Journal

Hepatology, 39(5)

ISSN

0270-9139

Authors

Villanueva, J A
Halsted, Charles H

Publication Date

2004-05-01

Peer reviewed

Halsted, Charles H.

Appendix 3

HEPATOLOGY IN PRESS 2004

HEPATIC TRANSMETHYLATION REACTIONS IN MICROPIGS WITH ALCOHOLIC LIVER
DISEASE

Jesus A.Villanueva and Charles H. Halsted

Department of Internal Medicine, University of California, Davis

Key words: S-Adenosylmethionine, homocysteine, S-adenosylhomocysteine, liver, alcohol

Corresponding author:

Charles H. Halsted, MD

TB 156, School of Medicine

University of California Davis

One Shields Avenue

Davis, CA 95616

Phone 530-752-6778; Fax: 530-752-3470; email: chhalsted@ucdavis.edu

Abbreviations: BHMT: betaine homocysteine methyl transferase; Cob: cobalamin; GNMT: glycine N-methyl transferase; GSH: glutathione; GSSG: oxidized glutathione; MAT: methionine adenosyl transferase; 5-MTHF: 5-methyltetrahydrofolate; MDA: malonodialdehyde; MS: methionine synthase; MTHFR: methylenetetrahydrofolate reductase; oxo(8)dG: 8-hydroxy-2'-deoxyguanosine; SAM: S-adenosylmethionine; SAH: S-adenosylhomocysteine; SAHH: S-adenosyl homocysteine hydrolase; THF: tetrahydrofolate

This work was supported by grants from the National Institutes of Health numbers AA014145 and DK35747. We are indebted to Jan Peerson for expert statistical consultation.

Abstract

Alcoholic liver disease is associated with abnormal hepatic methionine metabolism, including increased levels of homocysteine and S-adenosylhomocysteine (SAH) and reduced levels of S-adenosylmethionine (SAM) and glutathione (GSH). The concept that abnormal methionine metabolism is involved in the pathogenesis of alcoholic liver disease was strengthened by our previous findings in a micropig model that combining dietary folate deficiency with chronic ethanol feeding produced maximal changes in these metabolites together with early onset of microscopic steatohepatitis and an 8-fold increase in plasma aspartate transaminase (AST). The goal of the present study was to determine potential mechanisms for abnormal levels of these methionine metabolites by analyzing the transcripts and activities of transmethylation enzymes in the livers of the same micropigs. Ethanol feeding or folate deficiency, separately or in combination, decreased transcript levels of methylenetetrahydrofolate reductase (*MTHFR*), methionine adenosyltransferase (*MAT1A*), glycine-N-methyltransferase (*GNMT*) and S-adenosylhomocysteine hydrolase (*SAHH*). Ethanol feeding alone reduced the activities of MS and MATIII and increased the activity of GNMT. Each diet, separately or in combination, decreased the activities of MTHFR and SAHH. In conclusion, the observed abnormal levels of methionine metabolites in this animal model of accelerated alcoholic liver injury can be ascribed to specific effects of ethanol with or without folate deficiency on the expressions and activities of hepatic enzymes that regulate transmethylation reactions. These novel effects on transmethylation reactions may be implicated in the pathogenesis of alcoholic liver disease.

Abnormal hepatic methionine metabolism can result from chronic exposure to ethanol or from folate deficiency. Hyperhomocysteinemia has been described in chronic alcoholics (1, 2) and decreased liver SAM levels were found in experimental animals and cultured hepatocytes exposed to ethanol (3-6). Folate deficiency also results in abnormal methionine metabolism, since 5-methyltetrahydrofolate (5-MTHF) is substrate for the MS reaction that converts homocysteine to methionine, the substrate for SAM (Figure 1). Furthermore, folate deficiency is frequently associated with alcoholic liver disease, in part because chronic exposure to ethanol reduces the intestinal transport of folic acid and increases folate excretion in the urine (7-9).

Prior data support the concept that changes in methionine metabolism are involved in the development of alcoholic liver disease. Studies in rats and micropigs demonstrated that MS activity is reduced during chronic ethanol exposure (10-13). Rats that were fed ethanol by gastric tube demonstrated decreased MAT1/III activity with decreased SAM and SAM/SAH ratio in association with increased DNA strand breaks (5). A *MAT1A* knockout mouse model developed hepatic steatosis with reduced liver SAM and GSH and enhanced expression of several pro-inflammatory genes (14). The administration of SAM to chronic ethanol-fed baboons attenuated reductions in SAM and GSH as well as the histopathology of early alcoholic liver disease (3).

Recently, we explored the hypothesis that the combination of folate deficiency with chronic ethanol feeding would accelerate the development of alcoholic liver injury by enhancing abnormal methionine metabolism (15). This hypothesis is based on the integral role of folate in methionine metabolism (Figure 1) and prior evidence for the effects of ethanol on promoting folate deficiency and altering methionine metabolism (3-7, 16). We developed a micropig model for alcoholic liver disease based on 14 wk of feeding diets that either contained ethanol or were deficient in folate, or both factors in combination (15).

Compared to the control group fed a folate sufficient diet without ethanol, each folate deficient diet reduced liver folate by one half and the combined diet resulted in a 4-fold elevation in plasma homocysteine. Ethanol feeding alone increased liver homocysteine levels by one-half and reduced liver SAM by one-third and the GSH/GSSG ratio by one-half. The combined diet reduced mean liver methionine levels by two thirds and increased liver SAH by 2-fold, the urinary excretion of 8-hydroxy-2'-deoxyguanosine (oxo[8]dG), an index of DNA oxidation, by 2- fold, DNA strand breaks by one-third, and the mean level of plasma AST by 8-fold. Only the micropigs fed the combined diet developed the histopathology of alcoholic liver injury with steatosis, hepatocellular necrosis, and focal inflammation, whereas liver histology remained normal in the other three groups (15). This demonstration of abnormal histopathology after just 14 wk of the combined diet contrasts with a 1-year requirement to attain similar histopathology in micropigs fed ethanol with a folate sufficient diet (17), and strengthens the concept that abnormal levels of one or more methionine metabolites participate in the pathogenesis of alcoholic liver disease. The goal of the present study was to determine the bases for the observed changes in methionine metabolites by measuring the transcripts and activities of enzymes that regulate transmethylation reactions in liver tissues from the same experimental micropigs.

MATERIALS

Animals and diets. As previously described (15), 24 juvenile Yucatan micropigs were obtained from Sinclair Farms, Columbia, MO, USA, and pair-fed diets that provided 90 kcal/kg body weight with 15% of kcal as vitamin-free casein, 30 % of kcal as corn oil, and 55 % of kcal as carbohydrate (control), or diets in which carbohydrate was reduced to 15% and ethanol was substituted to provide 40% of kcal. Folic acid was absent or was added to diets at 14.5 µg/kg body weight, and each diet was supplemented with a vitamin and mineral mix that included levels that were in excess of pig requirements for choline at

60.3 mg and methionine at 675 mg/kg body weight (18) (Dyets, Inc. Bethlehem, Pennsylvania, USA). Thus, there were four feeding groups of six animals each: folate sufficient control (FS), folate deficient only (FD), folate sufficient with ethanol (FSE), and folate deficient with ethanol (FDE). The micropigs were housed in facilities approved by the National Institutes of Health and were cared for following standards and procedures outlined in the National Academy of Sciences "Guide for the Care and Use of Laboratory Animals." All procedures were reviewed and approved by the Animal Welfare Committee of University of California, Davis. After 14 weeks feeding, liver tissues were surgically removed from all animals under anesthesia, freeze-clamped in liquid nitrogen and frozen at -70° C for further analysis.

Chemicals. 5-[¹⁴C]-methyltetrahydrofolic acid and S-adenosyl-L-[methyl-³H]-methionine were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). [Methyl-³H]-L-methionine was purchased from Moraveck Biochemical, Inc. (Brea, CA). Cyto-Scin scintillation cocktail was purchased from Fisher Scientific (Houston TX). AG 50W-X4 resin and poly-prep chromatography columns were purchased from BioRad Laboratories Life Science Group (Richmond, CA). The TOPO TA-cloning kit was purchased from Invitrogen (Carlsbad, CA). QIAprep Spin Miniprep Kit was obtained from QIAGEN Inc. (Valencia, CA). All other chemicals and reagents were obtained from Sigma Chemical Co. (St. Louis, MO) and various commercial sources.

Isolation, purification and cloning of nucleic acids. Total RNA was isolated from liver tissue by acid guanidinium thiocyanate-phenol chloroform extraction (19) using Trizol reagent (Life Technologies). The quality of the RNA extracts was analyzed by gel electrophoresis in 1.0 % agarose gel. First strand cDNA was synthesized from 3 µg of total RNA by the SuperScript Preamplification System after DNase treatment and spectrophotometry reading at 260 nm according to the manufacturer (Life Technologies). Initially, we designed pig-specific sense and antisense primers to amplify 400-800 bp fragments within

the open reading frame of each gene. Primers for *MTHFR*, *MS*, *GNMT* and *18S* transcripts were obtained from pig-specific sequences, and primers for *MAT1A*, and *SAHH* were obtained from highly homologous regions of different species in Genbank and the multiple sequence alignment program Clustal version 1.8 (Table 1) (20). A fragment of the open reading frame of each gene was obtained by PCR using *Taq* polymerase, and each amplified cDNA fragment was ligated to TA-pCR-2.1 TOPO vector.

Transformation into chemically competent *E. coli* cells (Life Technologies) was followed by DNA isolation from transformants by the mini-preparation procedure described by QIAGEN Inc. Following sequence confirmation, each cDNA fragment was resolved in 1.0 % agarose gel, and specific bands were purified by the QIAEX II gel extraction procedure (QIAGEN Inc.).

Quantitation of transcripts by real time PCR. Pig-specific primers were designed to amplify 51 bp from the cDNA clone of each gene by Hot Start real-time PCR in the GeneAmp 5700 sequence detection system (Applied Biosystems) and are shown in Table 2. PCR products were detected by the increase in fluorescence caused by the binding of SYBR Green to double-stranded DNA. Transcripts of each gene were quantified using standard curves obtained with each purified cDNA fragment. The data are expressed as a ratio with 18S rRNA simultaneously amplified in separate PCR reactions (21).

Enzyme assays. The activities of transmethylation enzymes were measured in supernatant or cytosol fractions from each liver specimen. Each preparation contained the protease inhibitors aprotinin, leupeptin, benzamidine, and PMSF.

MTHFR Frozen liver tissue was homogenized in 10 volumes of 0.1 M potassium phosphate buffer pH 6.3. The liver homogenate was centrifuged at 105,000 g for 1 hr at 4° C and 500 µg protein of the cytosol fraction were incubated for 50 min, 37° C with 50 µl 1.0 M phosphate buffer pH 6.3, 40 µl 0.025 M menadione bisulfate, 4 µl 0.05M ascorbic acid, 10 µl 0.81 mM FAD, 4 µl 0.1 M EDTA pH 6.3 and 12 µl

5-[^{14}C]-methylTHF (1618 cpm/nmol) in a total volume of 280 μl . The reaction was stopped by the addition of 250 μl 0.6 M sodium acetate, 150 μl 0.4 M dimedone, and 100 μl 0.1 M formaldehyde. MTHFR activity was quantified by measuring the inverse enzyme reaction of radio-labeled product [^{14}C]-5-MTHF to [^{14}C]N⁵, N¹⁰-methyleneTHF using menadione as the electron acceptor (22). Oxidized [^{14}C]-N⁵, N¹⁰-methyleneTHF was dissociated into [^{14}C]-formaldehyde and THF, the [^{14}C] formaldehyde was coupled with dimedone, extracted into toluene, and the radioactivity was determined in a scintillation counter (23).

MS. As previously described (15), liver tissue was homogenized in 5 volumes of 0.028 M sodium phosphate buffer pH 7.0 followed by centrifugation at 40,000 g for 30 min. About 4.0 mg of supernatant protein were incubated with 25 μmol potassium phosphate buffer pH 7.5, 100 μmol sodium chloride, 100 μmol 2-mercaptoethanol, 1.25 μmol L-homocysteine, 50 nmol S-adenosylmethionine 37.5 nmol 5-methyl-THF, 7.5 μl 5-[^{14}C]-methylTHF (1233 cmp/nmol), 15 nmol cyanocobalamin and 50 nmol FADH₂ reduced with platinum oxide and hydrogen gas in a total volume of 0.5-ml. After 40 min incubation at 37° C, samples were boiled for 3 min, chilled in ice 5 min followed by addition of 500 μl of methionine-coated Norit-charcoal. After 10 min incubation at 25° C, samples were centrifuged at 16,000 g and 0.5 ml counted in a liquid scintillation counter (24).

MAT III. The synthesis of [^3H]-SAM by adenosine triphosphate activation of [methyl- ^3H]-L-methionine was measured according to described methods (25, 26). Liver tissue was homogenized in eight volumes of 10 mM Tris HCl pH 7.5 containing 0.3 M sucrose, 0.1% beta-mercaptoethanol and 0.1% of the protease inhibitors benzamidine and PMSF. The homogenate was centrifuged at 30,000 g for 60 min and 70 μl of supernatant normalized to about 800 μg of protein were incubated with 130 μl of a reaction mixture containing 80 mM TES pH 7.4, 50 mM KCl, 40 mM MgCl₂, 10 mM DTT, 0.5 mM

EDTA, 5 mM ATP, and 5 mM hot/cold [methyl-³H]-L-methionine. The reaction was incubated for 40 min at 37° C, stopped by adding 2 ml cold distilled water, and immediately applied to a column containing 1 ml of AG 50W-X4 cation exchange resin (BioRad) previously activated for 4 hr with ammonium hydroxide and equilibrated with distilled water to pH 7.0. After sample application, the column was washed with 20-ml distilled water and the [³H]-SAM generated was eluted with 4 ml of 3.0 N ammonium hydroxide. The eluate was neutralized with 1-ml acetic acid and counted in a liquid scintillation counter (Beckman model LS 3801).

GNMT. Activity was determined in liver homogenates by using a charcoal assay to adsorb unreacted S-adenosyl-L-[methyl-³H]-methionine(27) . Briefly, about 1200 µg whole homogenate protein were incubated with 10 µl 1.0 M Tris HCl buffer pH 7.5, 20 µl of 10 mM glycine, 20 µl of 1mM S-adenosyl methionine and 10 µl of S-adenosyl-L-[methyl-³H]-methionine(10226 dpm/nmol) in a total volume of 100 µl. After 15 min incubation at 25° C, the reaction was terminated by adding 50 µl of trichloroacetic acid followed by 350 µl of activated charcoal (38 mg/ml in 0.1 M acetic acid). Samples were incubated 20 min at 25° C and centrifuged 3 min at 14,000 g, and 200 µl of the supernatant fraction were counted in a liquid scintillation counter.

SAHH. Activity was measured in the forward hydrolysis direction (28). Briefly, 0.2 grams of liver tissue were homogenized in 4 ml of 50 mM potassium phosphate buffer pH 7.0. The homogenate was centrifuged at 30,000 g for 60 min at 4° C and 60 µl of supernatant normalized to about 800 µg protein were incubated with 860 µl of reaction mixture containing 50 mM potassium phosphate buffer pH 7.0, 1.2 mM MTT, 0.16% gelatin, 1.1 U adenosine deaminase, 0.45 U nucleoside phosphorylase and 0.1 U xanthine oxidase. Following 5-min incubation at 37° C, the SAHH reaction was started by the addition of 80 µl of 1.0 mM SAH to a total assay volume of 1-ml. SAHH-specific activity was related to the

production of formazan by chart-recording the change in absorbance at 728 nm at 37° C in a Gilford model 260 spectrophotometer.

Statistical analyses. All group values are expressed as mean $\pm$ SEM. Significant differences among the groups were determined by two-way ANOVA. If the interaction term for the effects of ethanol feeding and folate deficiency was significant, subgroup analyses were performed by one-way ANOVA with Tukey's test for post-hoc comparisons. Relationships among variables were determined by linear regression analysis using SPSS 10.0 for windows (SPSS Inc).

RESULTS

Table 3 illustrates changes in mean $\pm$ SEM levels of transmethylation enzyme transcripts, expressed as ratios with 18S. There was no effect of the feeding protocols on the expression of *MS* transcripts. Three of the transcript expressions in the combined group FDE were modified by interactions of the two feeding variables. Subsequent analyses showed that the reducing effects of ethanol feeding and folate deficiency on *MTHFR* and *SAHH* transcripts in the combined group FDE were similar but non-additive, and the effect of ethanol to reduce the expression of *MAT1A* was counteracted by the effect of folate deficiency. The transcript expressions of *GNMT* in group FDE were affected independently and additively by ethanol feeding and folate deficiency.

Table 4 shows changes in mean $\pm$ SEM values for transmethylation enzyme activities in each feeding group. Ethanol feeding acted independently to decrease *MS* and *MAT III* activities and to increase *GNMT* activity. *MTHFR* activity was decreased independently by ethanol feeding and folate deficiency in group FDE, while the two factors acted independently and additively to decrease *SAHH* activity.

Certain of the changes in transmethylation enzyme activities correlated with our prior findings on changes in metabolite levels (15), according to linear regressions using measurements from all 24 animals.

MTHFR activities were correlated negatively with homocysteine levels ($r = -0.655$, $p < 0.001$) and the SAM/SAH ratio ($r = 0.757$, $p < 0.0001$) and positively with GSH levels ($r = 0.703$, $p < 0.001$). MAT III activity correlated positively with the SAM/SAH ratio ($r = 0.532$, $p < 0.01$). GNMT activity was correlated negatively with SAM ($r = -0.515$, $p < 0.01$) and the SAM/SAH ratio ($r = -0.550$, $p < 0.01$). SAHH activity was correlated negatively with both SAH ($r = -0.467$, $p < 0.02$) and homocysteine ($r = -0.649$, $p < 0.001$), and positively with the SAM/SAH ratio ($r = 0.617$, $p < 0.001$).

DISCUSSION

In prior studies, we developed the micropig model of alcoholic liver disease and demonstrated that 1- year of ethanol feeding produced the histological features of inflammation, steatonecrosis, centrilobular fibrosis, cirrhosis and apoptosis in castrated males (13, 17, 29). Findings from our recent and much shorter 14-week study of uncastrated males strengthens the concept that abnormalities of certain methionine metabolites contribute to the pathogenesis of alcoholic liver disease. Summarizing, micropigs fed the combination of ethanol with a folate-deficient diet in group FDE demonstrated the greatest increase in SAH and decrease in the SAM/SAH ratio, the lowest GSH/GSSG ratio and the greatest increase in plasma AST, together with the histopathology of alcoholic liver injury (15). Finding a significant correlation between lowered levels of SAM and GSH in that study (15) provided a plausible link between abnormal methionine metabolism and antioxidant defense, since SAM is an essential activator of the transsulfuration pathway for GSH production (Figure 1). The findings that the DNA strand breaks and the urinary excretion of oxo(8)dG and were each maximally increased in pigs fed the

combined ethanol and folate-deficient FDE diet links abnormal methionine metabolism to DNA damage and increased susceptibility to apoptosis.

The present study shows that the single and combined effects of ethanol feeding and folate deficiency on methionine metabolites and alcoholic liver injury can be ascribed in part to specific effects on the expressions and activities of transmethylation enzymes. The prior finding of increased homocysteine in plasma and livers of the experimental animals (15) is consistent with present findings of decreased regulatory activities of MTHFR, MS, and reversible SAHH (Fig 1, Tables 3 and 4). Decreased SAM in the ethanol groups is consistent with reduced activity of MAT III and with increased activity of GNMT. Increased SAH in both folate-deficient and ethanol groups can be explained by the combination of increased GNMT activity and reduced activity of SAHH.

The observed suppression of transcript levels of four of the five transmethylation enzymes (Table 3) may be ascribed to the DNA oxidant effects of ethanol that were exacerbated by folate deficiency, as shown previously by enhanced oxo(8)dG excretion in the combined FDE group (15). Since SAM is the major methyl donor and SAH opposes its effects on DNA methylation (30), hypomethylation also may have played a part in altered transcription of the transmethylation genes in our study. In addition to its DNA effects, ethanol-induced oxidant stress can modify proteins in general by enhancing the irreversible covalent binding of acetaldehyde to lysine residues (31, 32).

The present finding of decreased MTHFR activity in response to both ethanol feeding and folate deficiency is novel and may constitute another explanation for low serum folate levels in chronic alcoholics (16). However, we cannot determine whether decreased MTHFR activity resulted in selectively decreased end product 5-MTHF in the experimental groups since folate analogs were not measured in their livers. In this regard, THF, but not 5-MTHF, is the major constituent of plasma folate in the pig (33),

whereas the distribution of pig liver folates is not known. The active site of MTHFR incorporates FAD, which interacts with NADPH to provide reducing equivalents for the synthesis of 5-MTHF (34, 35). The observed decreased GSH/GSSG ratio (15) is consistent with increased activity of glutathione reductase (36). Since glutathione reductase is also a flavoprotein (37), its presumed increased activity would predictably decrease the availability of FAD and NADPH for the MTHFR reaction. Furthermore, SAM is required to stabilize the binding of FAD to the active site of MTHFR (38). Therefore, decreased MTHFR activity could be ascribed to conformational changes in the presence of reduced SAM, FAD, and NADPH levels.

The observed decrease in MS activity could be explained in part by potential oxidant effects of ethanol feeding on the conformation of this protein in relation to its dependence on Cob and the interactive methionine synthase reductase (MSR) reaction (Fig 1). During transfer of the methyl group of 5-MTHF via Cob (III) to homocysteine, demethylated Cob (I) is oxidized to Cob (II), which is reduced back to the active Cob (III) state by methionine synthase reductase (MSR) with SAM as substrate (39). Therefore, decreased SAM that results from ethanol feeding could alter the activity of MSR. Also, others demonstrated irreversible inhibition of purified rat liver MS activity by acetaldehyde, suggesting another protein modification due to covalent binding to this metabolite (40)

Liver MAT is the product of *MAT1A* and encodes a dimer (MAT III) and a tetramer (MAT I) that catalyze the synthesis of SAM from methionine by the addition of an adenosyl group from ATP (41). The present finding of reduced MAT III activity in the ethanol-fed micropigs (Table 4) is consistent with findings of others in the ethanol-fed rat model (5). On the other hand, lack of correlation of product SAM levels with MAT III activity emphasizes that SAM is regulated both by its synthesis by MAT and its subsequent metabolism by GNMT (Figure 1). Inactivation of MAT III has been ascribed to covalent modification of

the cysteine 121 residue in the setting of oxidant stress and is specifically related to a decrease in the GSH/GSSG ratio (42, 43).

GNMT constitutes about 1% of all mammalian liver protein (44) and plays a pivotal role in regulation of the SAM to SAH ratio by catalyzing the conversion of SAM to SAH by donation of a methyl group to glycine for production of N-methylglycine (27). In the present experiment, the role of GNMT in regulation of the conversion of SAM to SAH was reflected by negative correlations of GNMT activity with both SAM and the SAM/SAH ratio. Although SAH is a potent inhibitor of most methyl transfer reactions (30), GNMT is relatively resistant to inhibition since its K_i for SAH is very high, 35 $\mu\text{mol/L}$, as compared to most other methyltransferases, for example the K_i of 1.4 $\mu\text{mol/L}$ SAH for DNA methyltransferase (45). Since GNMT transcripts were decreased independently and additively by folate deficiency and ethanol, the finding of increased GNMT activity in the presence of ethanol feeding must represent a posttranscriptional modification of the enzyme. A likely explanation for increased GNMT activity in the ethanol fed pigs is based on its known inhibition by avid binding to 5-MTHF (46). We can predict that hepatic 5-MTHF levels were decreased in the ethanol fed pigs, since they are regulated by the activity of MTHFR, which was decreased in the same animals (Table 4). The predictable low levels of 5-MTHF would account for release of inhibition of GNMT and its increased activity in the ethanol fed animals.

SAHH cleaves SAH to adenosine and homocysteine, but this reaction is readily reversible by the same enzyme (28) (Fig 1). The finding that both ethanol feeding and folate deficiency inhibited SAHH activity is novel and is consistent with the negative correlation of SAHH activity with both substrate SAH and product homocysteine. Under normal physiological conditions, the rapid metabolism of SAHH end products homocysteine and adenosine drives the SAHH reaction in the hydrolysis direction. On the other

hand, the accumulation of homocysteine and adenosine promotes the reversibility of SAHH towards the synthesis of SAH, since the affinity (K_m) and maximal velocity (V_{max}) of the reaction are more favorable in this reverse direction (28, 47). Under these circumstances, experimental alteration in the SAHH protein with reduction of its activity would be expected to promote the accumulation of SAH by a block in the hydrolysis direction and homocysteine by a block in the reverse direction.

In conclusion, the present observations on the effects of ethanol feeding with or without folate deficiency on the transcripts and activities of hepatic transmethylation enzymes are consistent with and explain our prior findings in the same experimental animals of abnormal levels of methionine metabolites that were associated with accelerated liver injury (15). The significance of the findings from both studies can be understood in the context of recent discoveries on the role of homocysteine in the induction of fatty liver and apoptosis through the endoplasmic reticulum (ER) stress pathway (48, 49). GRP78, a marker of ER stress, and SREBP, which enhances lipid synthesis, were up-regulated in a genetic mouse model of hyperhomocysteinemia that developed fatty liver (48). Others found that fatty liver in this model was associated with increased hepatocellular apoptosis by TUNEL assay and increased caspase 12 levels (49). There is no direct evidence that SAH is more or less effective than homocysteine as a trigger of the ER stress pathway. In this context, the present findings on ethanol induced changes in transmethylation reactions that resulted in increased levels of homocysteine and SAH assume importance for further understanding of the pathogenesis of alcoholic liver disease.

REFERENCES

1. Cravo M. Hyperhomocysteinemia in chronic alcoholism: correlation with folate, vitamin B-12, and vitamin B-6 status. *Am J Clin Nutr* 1996;63:220-224.
2. de la Vega MJ, Santolaria F, Gonzalez-Reimers E, Aleman MR, Milena A, Martinez-Riera A, Gonzalez-Garcia C. High prevalence of hyperhomocysteinemia in chronic alcoholism: the importance of the thermolabile form of the enzyme methylenetetrahydrofolate reductase (MTHFR). *Alcohol* 2001;25:59-67.
3. Lieber CS, Casini A, DeCarli LM, Kim CI, Lowe N, Sasaki R, Leo MA. S-adenosyl-L-methionine attenuates alcohol-induced liver injury in the baboon. *Hepatology* 1990;11:165-172.
4. Garcia-Ruiz C, Morales A, Colell A, Ballesta A, Rodes J, Kaplowitz N, Fernandez-Checa JC. Feeding S-adenosyl-L-methionine attenuates both ethanol-induced depletion of mitochondrial glutathione and mitochondrial dysfunction in periportal and perivenous rat hepatocytes. *Hepatology* 1995;21:207-214.
5. Lu SC, Huang ZZ, Yang H, Mato JM, Avila MA, Tsukamoto H. Changes in methionine adenosyltransferase and S-adenosylmethionine homeostasis in alcoholic rat liver. *Am J Physiol Gastrointest Liver Physiol* 2000;279:G178-185.
6. Colell A, Garcia-Ruiz C, Miranda M, Ardite E, Mari M, Morales A, Corrales F, et al. Selective glutathione depletion of mitochondria by ethanol sensitizes hepatocytes to tumor necrosis factor. *Gastroenterology* 1998;115:1541-1551.
7. Halsted CH, Robles EA, Mezey E. Decreased jejunal uptake of labeled folic acid (^3H -PGA) in alcoholic patients: roles of alcohol and nutrition. *N Engl J Med* 1971;285:701-706.
8. Villanueva JA, Devlin AM, Halsted CH. Reduced folate carrier: tissue distribution and effects of chronic ethanol intake in the micropig. *Alcohol Clin Exp Res* 2001;25:415-420.

9. McMartin KE, Collins TD, Eisenga BH, Fortney T, Bates WR, Bairnsfather L. Effects of chronic ethanol and diet treatment on urinary folate excretion and development of folate deficiency in the rat. *J Nutr* 1989;119:1490-1497.
10. Finkelstein JD, Cello JP, Kyle WE. Ethanol-induced changes in methionine metabolism in rat liver. *Biochem Biophys Res Commun* 1974;61:525-531.
11. Barak AJ, Beckenhauer HC, Tuma DJ, Badakhsh S. Effects of prolonged ethanol feeding on methionine metabolism in rat liver. *Biochem Cell Biol* 1987;65:230-233.
12. Trimble KC, Molloy AM, Scott JM, Weir DG. The effect of ethanol on one-carbon metabolism: increased methionine catabolism and lipotrope methyl-group wastage. *Hepatology* 1993;18:984-989.
13. Halsted CH, Villanueva J, Chandler CJ, Stabler SP, Allen RH, Muskhelishvili L, James SJ, et al. Ethanol feeding of micropigs alters methionine metabolism and increases hepatocellular apoptosis and proliferation. *Hepatology* 1996;23:497-505.
14. Lu SC, Alvarez L, Huang ZZ, Chen L, An W, Corrales FJ, Avila MA, et al. Methionine adenosyltransferase 1A knockout mice are predisposed to liver injury and exhibit increased expression of genes involved in proliferation. *Proc Natl Acad Sci U S A* 2001;98:5560-5565.
15. Halsted CH, Villanueva JA, Devlin AM, Niemela O, Parkkila S, Garrow TA, Wallock LM, et al. Folate deficiency disturbs hepatic methionine metabolism and promotes liver injury in the ethanol-fed micropig. *Proc Natl Acad Sci U S A* 2002;99:10072-10077.
16. Eichner ER, Hillman RS. Effect of alcohol on serum folate level. *J Clin Invest* 1973;52:584-591.
17. Halsted CH, Villanueva J, Chandler CJ, Ruebner B, Munn RJ, Parkkila S, Niemela O. Centrilobular distribution of acetaldehyde and collagen in the ethanol-fed micropig. *Hepatology* 1993;18:954-960.

18. Subcommittee on Swine Nutrition. Washington, DC: National Research Council/Board of Agriculture; 1998.
19. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987;162:156-159.
20. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 1994;22:4673-4680.
21. Heid CA, Stevens J, Livak KJ, Williams PM. Real time quantitative PCR. *Genome Res* 1996;6:986-994.
22. Weisberg IS, Jacques PF, Selhub J, Bostom AG, Chen Z, Curtis Ellison R, Eckfeldt JH, et al. The 1298A-C polymorphism in methylenetetrahydrofolate reductase (MTHFR): in vitro expression and association with homocysteine. *Atherosclerosis* 2001;156:409-415.
23. Sobti P, Rothenberg SP, Quadros EV. Radioenzymatic assay for reductive catalysis of N(5)N(10)-methylenetetrahydrofolate by methylenetetrahydrofolate reductase. *J Biochem Biophys Methods* 2000;46:11-20.
24. Kondo H, Osborne ML, Kolhouse JF, Binder MJ, Podell ER, Utley CS, Abrams RS, et al. Nitrous oxide has multiple deleterious effects on cobalamin metabolism and causes decreases in activities of both mammalian cobalamin-dependent enzymes in rats. *J Clin Invest* 1981;67:1270-1283.
25. Avila MA, Mingorance J, Martinez-Chantar ML, Casado M, Martin-Sanz P, Bosca L, Mato JM. Regulation of rat liver S-adenosylmethionine synthetase during septic shock: role of nitric oxide. *Hepatology* 1997;25:391-396.
26. Huang ZZ, Mao Z, Cai J, Lu SC. Changes in methionine adenosyltransferase during liver regeneration in the rat. *Am J Physiol* 1998;275:G14-21.

27. Cook RJ, Wagner C. Glycine N-methyltransferase is a folate binding protein of rat liver cytosol. *Proc Natl Acad Sci U S A* 1984;81:3631-3634.
28. Kloor D, Fuchs S, Petroktistis F, Delabar U, Muhlbauer B, Quast U, Osswald H. Effects of ions on adenosine binding and enzyme activity of purified S-adenosylhomocysteinehydrolase from bovine kidney. *Biochem Pharmacol* 1998;56:1493-1496.
29. Niemelä O, Parkkila S, Ylä-Herttuala S, Villanueva J, Ruebner B, Halsted CH. Sequential acetaldehyde production, lipid peroxidation, and fibrogenesis in micropig model of alcohol-induced liver disease. *Hepatology* 1995;22:1208-1214.
30. Yi P, Melnyk S, Pogribna M, Pogribny IP, Hine RJ, James SJ. Increase in plasma homocysteine associated with parallel increases in plasma S-adenosylhomocysteine and lymphocyte DNA hypomethylation. *J Biol Chem* 2000;275:29318-29323.
31. Tuma DJ, Thiele GM, Xu D, Klassen LW, Sorrell MF. Acetaldehyde and malondialdehyde react together to generate distinct protein adducts in the liver during long-term ethanol administration. *Hepatology* 1996;23:872-880.
32. Tuma DJ, Newman MR, Donohue TM, Jr., Sorrell MF. Covalent binding of acetaldehyde to proteins: participation of lysine residues. *Alcohol Clin Exp Res* 1987;11:579-584.
33. Natsuhori M, Shimoda M, Kokue E, Hayama T, Takahashi Y. Tetrahydrofolic acid as the principal congener of plasma folates in pigs. *Am J Physiol* 1991;261:R82-86.
34. Matthews RG, Vanoni MA, Hainfeld JF, Wall J. Methylene tetrahydrofolate reductase. Evidence for spatially distinct subunit domains obtained by scanning transmission electron microscopy and limited proteolysis. *J Biol Chem* 1984;259:11647-11650.
35. Jencks DA, Mathews RG. Allosteric inhibition of methylenetetrahydrofolate reductase by adenosylmethionine. Effects of adenosylmethionine and NADPH on the equilibrium between active and

inactive forms of the enzyme and on the kinetics of approach to equilibrium. *J Biol Chem* 1987;262:2485-2493.

36. Bailey SM, Patel VB, Young TA, Asayama K, Cunningham CC. Chronic ethanol consumption alters the glutathione/glutathione peroxidase-1 system and protein oxidation status in rat liver. *Alcohol Clin Exp Res* 2001;25:726-733.

37. Carlberg I, Mannervik B. Purification and characterization of the flavoenzyme glutathione reductase from rat liver. *J Biol Chem* 1975;250:5475-5480.

38. Yamada K, Chen Z, Rozen R, Matthews RG. Effects of common polymorphisms on the properties of recombinant human methylenetetrahydrofolate reductase. *Proc Natl Acad Sci U S A* 2001;98:14853-14858.

39. Matthews RG. Methylenetetrahydrofolate reductase: a common human polymorphism and its biochemical implications. *Chem Rec* 2002;2:4-12.

40. Kenyon SH, Nicolaou A, Gibbons WA. The effect of ethanol and its metabolites upon methionine synthase activity in vitro. *Alcohol* 1998;15:305-309.

41. Torres L, Avila MA, Carretero MV, Latasa MU, Caballeria J, Lopez-Rodas G, Boukaba A, et al. Liver-specific methionine adenosyltransferase MAT1A gene expression is associated with a specific pattern of promoter methylation and histone acetylation: implications for MAT1A silencing during transformation. *FASEB J* 2000;14:95-102.

42. Mingorance J, Alvarez L, Sanchez-Gongora E, Mato JM, Pajares MA. Site-directed mutagenesis of rat liver S-adenosylmethionine synthetase. Identification of a cysteine residue critical for the oligomeric state. *Biochem J* 1996;315:761-766.

43. Pajares MA, Duran C, Corrales F, Pliego MM, Mato JM. Modulation of rat liver S-adenosylmethionine synthetase activity by glutathione. *J Biol Chem* 1992;267:17598-17605.

44. Ogawa H, Fujioka M. Purification and properties of glycine N-methyltransferase from rat liver. *J Biol Chem* 1982;257:3447-3452.
45. Clarke S, Banfield K. S-Adenosylmethionine-dependent methyltransferases. In: R.Carmel and DW Jacobsen, eds. *Homocysteine in Health and Disease*. Cambridge: Cambridge University Press, 2001; pp63-78.
46. Wagner C, Briggs WT, Cook RJ. Inhibition of glycine N-methyltransferase activity by folate derivatives: implications for regulation of methyl group metabolism. *Biochem Biophys Res Commun* 1985;127:746-752.
47. Hoffman DR, Marion DW, Cornatzer WE, Duerre JA. S-Adenosylmethionine and S-adenosylhomocysteine metabolism in isolated rat liver. Effects of L-methionine, L-homocysteine, and adenosine. *J Biol Chem* 1980;255:10822-10827.
48. Werstuck GH, Lentz SR, Dayal S, Hossain GS, Sood SK, Shi YY, Zhou J, et al. Homocysteine-induced endoplasmic reticulum stress causes dysregulation of the cholesterol and triglyceride biosynthetic pathways. *J Clin Invest* 2001;107:1263-1273.
49. Ji C, Kaplowitz N. Betaine decreases hyperhomocysteinemia, endoplasmic reticulum stress, and liver injury in alcohol-fed mice. *Gastroenterology* 2003;124:1488-1499.

Table 1. Primers used for pig cDNA constructs

<u>Gene</u>	<u>Sense</u>	<u>Antisense</u>	<u>Fragment</u> (bp)
MTHFR [†]	CAGCACGGCCGTGAACTACTG	GTCTTTGATGGGCTCGATCACG	530
MS [†]	CTCTCCAGACAGGAATTAAGAGGT	GGTCTCATTTCGGCTGCACCCA	409
MAT1A*	CTCAAGCAAGACCCCAATGCCAA	CACAGAGATGACGATGGTGTGGA	585
GNMT [†]	CATGAGCCTGCTTTTCGACAAGTG	CGTCTTCCTGAGCACGTGGATG	573
SAHH*	CCAAGAGCAAGTTTGACAACCTCT	CTTGCCCAGGTGGGCTTCAGC	641
Ribosomal 18S [†]	CTCGATGCTCTTAGCTGAGTGTC	TAGTTAGCATGCCGAGAGTCTCG	648

* From homologous regions of different species. [†] From published sequences of pig

Table 2. Primers designed from authentic pig cDNA sequence for real time PCR quantitation

<u>Gene</u>	<u>Sense</u>	<u>Antisense</u>	<u>Fragment</u> (bp)
MTHFR	TGAACTACTGCGGCCTGGAG	TCTGATGGCAGCAGGTCATG	51
MS	TGTTGATAAAAGTGGGCGGAC	ACAAATGCCTCTCCGGTCTG	51
BHMT	AGAAGCAGTTCGCCAGCTTC	ACATTCGATCCGGCTCTGAG	51
MAT1A	ATCGCCCAGTGTGTCCATCT	ACCGGCACCAACATCTTCTT	51
GNMT	TGCTGTCATCTGCCAAGGAA	CCTCTGCAGTCTGGCAAGTG	51
SAHH	TGGATGAGGCCTGTCAGGAG	ATACAGCCCGTGGTGGTGA	51
Ribosomal 18S	CGGACACGGACAGGATTGAC	ACCCACGGAATCGAGAAAGA	51

Table 3. Transcripts of transmethylation enzymes

<u>Gene</u>	<u>Ratio multiple</u>	<u>Transcript level / 18S</u>			
		<u>FS</u>	<u>FD</u>	<u>FSE</u>	<u>FDE</u>
MTHFR	(10 ⁻⁵)	0.32±0.05	0.17±0.03*	0.16±0.04 [†]	0.18±0.03* ^{†‡}
MS	(10 ⁻⁴)	0.81±0.11	0.68±0.08	0.64±0.05	0.84±0.14
MAT1A	(10 ⁻³)	0.74±0.16	0.34±0.06*	0.27±0.06 [†]	0.48±0.06* [‡]
GNMT	(10 ⁻³)	0.72±0.16	0.35±0.07*	0.43±0.09 [†]	0.24±0.04* [†]
SAHH	(10 ⁻⁴)	0.38±0.05	0.24±0.02*	0.19±0.05 [†]	0.21±0.03* ^{†‡}

Values expressed as a ratio with the transcript level of 18S. * Effects of folate

deficiency; p<0.03, <0.05, <0.05, <0.02; † Effects of ethanol; p<0.03, <0.02, <0.02;

p<0.03 ‡ Interaction p<0.05, <0.005, <0.05).

Table 4. Activities of transmethylation enzymes

<u>Enzyme (SA)</u>	<u>Enzyme Activity</u>			
	FS	FD	FSE	FDE
MTHFR (pmol/mg/min)	22.6±0.99	16.8±1.34*	12.1±0.90 [†]	11.5±0.56* [†]
MS (pmol/mg/min)	8.2±0.44	8.9±0.60	5.3±0.53 [†]	6.1±0.31 [†]
MAT III (pmol/mg/min)	34.6±1.42	31.9±2.82	26.4±1.52 [†]	26.1±1.19 [†]
GNMT (pmol/mg/min)	2.4±0.66	3.5±0.53	7.9±0.65 [†]	6.4±1.00 [†]
SAHH (mU/mg/min)	18.7±0.92	15.3±1.33*	11.4±2.45 [†]	7.6±1.04* [†]

Values expressed as pmol/mg protein/min. * Effects of folate deficiency; p<0.005, <0.02;

[†] effects of ethanol; p<0.0001, <0.0001, <0.001, <0.0001; <0.0001

Figure 1. Transmethylation reactions in the liver. Arrows indicate the directions of change in methionine metabolites in response to the diets as previously described (15). MTHFR (methylenetetrahydrofolate reductase) converts 5,10-methylenetetrahydrofolate to 5-MTHF (5-methyltetrahydrofolate) in a FAD-dependent reaction that is normally down regulated by SAM (S-adenosylmethionine). 5-MTHF is substrate with co-factor Cob (Cobalamin) for MS (methionine synthase) that generates methionine from homocysteine. Demethylated Cob I is oxidized to Cob II, which is reduced to the methyl donor Cob III by FAD-dependent MSR (methionine synthase reductase) with SAM (S-adenosylmethionine) as substrate. In the alternate salvage pathway, betaine is substrate for BHMT (betaine homocysteine methyl transferase) in the conversion of homocysteine to methionine. MAT (methionine adenosyl transferase) adds ATP to methionine for generation of SAM, the methyl donor for multiple reactions. GNMT (glycine N-methyl transferase) converts SAM to SAH (S-adenosylhomocysteine) and is normally down regulated by 5-MTHF. SAH is substrate for SAHH (S-adenosyl homocysteine hydrolase) in the generation of homocysteine, but is reversible and may increase SAH during homocysteine accumulation.

