Inter-Strain Differences in Liver Injury and One-Carbon Metabolism in Alcohol-Fed Mice

Masato Tsuchiya[1,4], **Cheng Ji**[2], **Oksana Kosyk**[1], **Svitlana Shymonyak**[1], **Stepan Melnyk**[3], **Hiroshi Kono**[4], **Volodymyr Tryndyak**[5], **Levan Muskhelishvili**[5], **Igor P. Pogribny**[5], **Neil Kaplowitz**[2], and **Ivan Rusyn**[1]

[1]Department of Environmental Sciences and Engineering, University of North Carolina, Chapel Hill, NC

[2]Department of Medicine, Keck School of Medicine, University of Southern California, Los Angeles, CA

[3]University of Arkansas for Medical Sciences, Little Rock, AR

[4]First Department of Surgery, University of Yamanashi, Chuo, Yamanashi, Japan

[5]National Center for Toxicological Research, U.S. FDA, Jefferson, AR

Abstract

Alcoholic liver injury is a major public health issue worldwide. Even though the major mechanisms of this disease have been established over the past decades, little is known about genetic susceptibility factors that may predispose individuals who abuse alcoholic beverages to liver damage and subsequent pathological conditions. We hypothesized that a panel of genetically diverse mouse strains may be used to examine the role of ER stress and one-carbon metabolism in the mechanism of inter-individual variability in alcoholic liver injury. We administered alcohol (up to 27 mg/kg/d) in high fat diet using intragastric intubation model for 28 days to male mice from 14 inbred strains (129S1/SvImJ, AKR/J, BALB/cJ, BALB/cByJ, BTBR T+tf/J, C3H/HeJ, C57BL/10J, DBA/2J, FVB/NJ, KK/HIJ, MOLF/EiJ, NZW/LacJ, PWD/PhJ, and WSB/EiJ). Profound inter-strain differences (more than 3-fold) in alcohol-induced steatohepatitis were observed among the strains in spite of consistently high levels of urine alcohol that was monitored throughout the study. We found that endoplasmic reticulum stress genes were induced only in strains with the highest liver injury. Liver glutathione and methyl donor levels were affected in all strains, albeit to a different degree. Most pronounced effects that were closely associated with the degree of liver injury were hyperhomocysteinemia and strain-dependent differences in expression patterns of one-carbon metabolism-related genes.

Conclusion—Our data demonstrate that strain differences in alcohol-induced liver injury and steatosis are striking and independent of alcohol exposure and the most severely affected strains exhibit major differences in the expression of ER stress markers and genes of one-carbon metabolism.

Keywords

alcohol-induced liver injury; homocysteine; methyl donors; endoplasmic reticulum stress

Alcohol abuse continues to be a major social and clinical problem as excessive drinking is regarded to be a major risk factor for morbidity, disability and death globally[1]. Liver is a

Corresponding author: Ivan Rusyn, University of North Carolina, Chapel Hill, NC 27599; Phone/Fax (919) 843-2596; iir@unc.edu.

major, but not the only, target organ for alcohol-induced injury and a statistically significant relationship between per capita consumption of alcohol and mortality from liver cirrhosis, one of the major alcohol-related disease diagnoses, exists in all countries with published data[2]. Alcoholic liver disease represents a spectrum of clinical illnesses that range from fatty liver to hepatitis, fibrosis, cirrhosis and cancer[3].

Not all alcohol abusers develop alcoholic liver disease, especially pathology more severe than steatosis[4], and the contribution of genetic and other risk factors for disease development and the mechanisms by which it occurs remain unclear[1]. The major pathways of alcohol's adverse effect on the liver is through de-regulation of the metabolism, immune system response and oxidative stress[5,6]. Both "candidate gene" and "genome-wide association" approaches have been used to study gene-environment interactions that may exacerbate the risk of liver damage and promote clinically-evident disease[1]. Many of the candidate gene-based epidemiology studies suggested that polymorphisms in genes for alcohol (e.g., *ADH* and *ALDH*, etc.) and folate metabolism (e.g., *MTHFR*), as well as oxidative stress (e.g., *MNSOD*) and immune response (e.g., *CD14*, tumor necrosis factor α), are likely to be genetic modifiers of alcohol-related diseases[7]. The strongest evidence, confirmed in large meta-analyses of the data, exists for a role of polymorphisms in *ADH1B* and *ALDH2* in alcohol-related cancer risk[8]. Recent advances in genotyping technologies and their embrace by clinicians are likely to bring additional information through the genome-wide association studies on large human cohorts. For example, a polymorphism in patatin-like phospholipase domain containing 3 gene, the product of which is involved in energy homeostasis, has been identified as strongly associated with the severity of both non-alcoholic fatty liver disease[9] and alcohol-related cirrhosis[10].

This study evaluated key molecular events postulated to play a role in alcoholic liver injury: ER stress, lipid and one-carbon metabolism. Specifically, we tested a hypothesis that a panel of genetically diverse mouse strains may be used to examine the role of one carbon metabolism in the mechanism of inter-individual variability in alcoholic liver injury. The rationale for the focus of this study is the key role that one-carbon metabolism plays in susceptibility to liver steatosis, alcoholic liver injury and carcinogenesis[11-13].

Experimental Procedures

Animals, Diets and Treatment

Male mice (8-14 wks old at the start of the study, n=3-9 per strain/treatment group, Jackson Laboratory, Bar Harbor, ME) from 14 inbred strains (priority strains for the Mouse Phenome Project that are densely genotyped[14]: 129S1/SvImJ, AKR/J, BALB/cJ, BALB/cByJ, BTBR T+tf/J, C3H/HeJ, C57BL/10J, DBA/2J, FVB/NJ, KK/HIJ, MOLF/EiJ, NZW/LacJ, PWD/PhJ, and WSB/EiJ) underwent surgical intra-gastric intubation[15]. Following surgery, mice were housed in individual metabolic cages and allowed a week to recover with *ad libitum* access to food and water. Next, mice were administered via gastric cannula a high-fat liquid diet prepared as detailed elsewhere[16]. Animals had free access to water and non-nutritious cellulose pellets throughout the study. Control groups received high-fat diet supplemented with isocaloric maltose-dextrin and lipotropes[15], while alcohol groups received high-fat diet containing ethyl alcohol. Alcohol was delivered initially at 17.3 g/kg/day and was gradually increased 1.3 g/kg every 2 days until day 8. The dose was then raised by 1.2 g/kg every 4 days until the dose reached 27 g/kg/day. Mice were monitored at least 4 times daily and sacrificed after 28 days of treatment. All animals were given humane care in compliance with NIH guidelines and severe alcohol intoxication was assessed carefully to evaluate the development of tolerance using a 0-3 behavioral scoring system[17]. This work was approved by the institutional animal care and use committee at the University of North Carolina.

Sample collection, histological evaluation and biochemical measurements

Urine was collected daily using metabolism cages and stored at −80°C. Blood was collected at sacrifice into heparin tubes, and serum was isolated. A section of the median and left lateral liver lobes was fixed in formalin and embedded in paraffin, and the remaining liver was frozen and stored at −80°C.

Formalin-fixed/paraffin-embedded liver sections were stained with hematoxylin/eosin. Liver pathology was evaluated in a blind manner by a certified veterinary pathologist and scored[18] as follows: steatosis (% of hepatocytes containing fat): <25%=1+, <50%=2+, <75%=3+, >75%=4+; inflammation and necrosis: 1 focus per low-power field=1+, 2 or more foci=2+.

Alcohol concentrations in serum and urine were determined as described elsewhere[19]. Serum transaminase levels were determined spectrophotometrically with the Thermo Scientific Infinity ALT Liquid stable reagent (Thermo Electron, Melbourne, Australia). The content of one-carbon metabolites in liver tissue extracts and plasma was determined using high-performance liquid chromatography with coulometric electrochemical detection as previously described[20].

Molecular Assays

Tissues collected from 3 representative (based on liver pathology phenotypes) mice per group were used in these experiments.

Western blot—Proteins were extracted from the liver and analyzed by immunoblotting as detailed previously[21]. Primary antibodies against actin, Grp78, Chop, Bhmt and nSrebp1 were from Santa Cruz Biotechnology (Santa Cruz, CA). IRDye680- and IRDye800-conjugated secondary antibodies were from LiCor (Lincoln, NE). Blots were scanned using the Odyssey system (LiCor) and intensity of the bands was quantified with ImageJ. The intensity of protein bands on the blots was normalized to actin and to corresponding strain's HFD samples.

RNA Isolation and Gene Expression Analysis—Total RNA was extracted from liver using the RNeasy Mini kit (Qiagen, Valencia, CA) and used for quantitative real-time Polymerase-Chain Reaction as detailed in Supplemental Methods. Genes assayed and their primer information is included in Supplemental Methods.

Statistical analyses

Results are presented as mean ± S.D. Comparisons between groups within strain was done using Student's t-test. *P*-values <0.05 were considered significant. Correlation analysis was performed using SAS (Cary, NC) 9.2 software.

Results

Inter-strain Variability in the Severity of Alcohol-Induced Liver Injury in the Mouse

The intra-gastric sub-chronic infusion model[15] was used to study the population-wide effects of alcohol on the liver since it standardizes the animal's environment, allows control of the dose, and assures adequate nutritional status. All phenotypic, biochemical and molecular data collected in this study is available for individual animals as Supplemental Table 1. Alcohol (up to 27 g/kg/day) treatment for 28 days resulted in the development of pronounced steatohepatitis, consisting of steatosis, inflammation and necrosis, in animals of the majority of strains used, as compared to the strain-matched animals on a high fat corn oil-based diet (Figures 1A and B, see Supplemental Figure 1 for serum alanine aminotransferase and individual components of the pathology score). Notably, NZW/LacJ

was one of the most sensitive to the alcohol-induced liver injury and WSB/EiJ was one of the most resistant strains.

Micro- and macro-vesicular fat accumulation in the liver was exacerbated by alcohol feeding in all strains, except for WSB/EiJ, MOLF/EiJ and DBA/2J (Figure 2A), data which is supported by measurements of liver triglyceride content in select strains (Figure 2B). Accordingly, we examined several pathways for hepatic fat metabolism. Mttp is responsible for very low density lipoprotein secretion and impairment of Mttp-dependent lipoprotein secretion in the liver increases liver injury caused by lipopolysaccharide[22] and its expression was induced in strains that were resistant to alcohol-induced fatty liver (Figure 2C). Alcohol is also known to decrease peroxisomal lipid metabolism[23] and we found decreased expression of *Acox1* in strains with severe fatty liver (Figure 2D). Finally, the fat-derived hormone adiponectin alleviates alcoholic fatty liver disease in mice[24] and liver *Adipor2* expression was decreased by alcohol treatment in mice[25], an effect that was not observed in alcoholic liver injury-resistant strains (Figure 2E).

Mice of different strains received the same dose of alcohol under identical experimental conditions, and the daily urine concentrations of alcohol were measured. In all mice, a characteristic cyclic fluctuation in urine alcohol concentration[26] was observed (Figure 1C). Importantly, peak urine alcohol concentration (in treated animals) was not significantly correlated with the severity of steatohepatitis or other markers of liver injury (see Supplemental Table 2 for the correlation analysis matrix).

Endoplasmic Reticulum (ER) Stress Response and Dysregulation of Lipid Metabolism

Chronic alcohol-induced liver injury has been associated with ER stress and alterations in lipid synthesis pathways[27]. In addition, it has been shown that unresolved ER stress may also lead to steatosis through inhibition of lipid oxidation, instead of *de novo* lipogenesis as down-regulation of Srebf1 and Cebpa, key transcription factors involved in fatty acid metabolism were observed[28]. In some strains that exhibited the greatest degree of alcohol-induced liver injury, a concordant induction of ER stress factors Grp78 (Figure 3A) and Chop (Figures 3B and C), and dysregulation of *Cebpa* (Figure 3D) and *Srebf1* (Figure 3E), as well as a decrease activated cleaved Srebp1 (Figure 3F), was observed.

Changes in Hepatic GSH and GSSG

Oxidative stress and lipid peroxidation are well-established hallmarks of alcohol-induced liver injury[29]. Hepatic GSH depletion after chronic alcohol consumption was shown both in experimental animals and in humans[30]. We evaluated content of GSH and GSSG in livers of alcohol- and high fat diet-fed mice (Figure 4). GSH depletion was observed in most of the strains (Figure 4A), and the level of GSH was significantly inversely correlated with the severity of liver injury only when both control and alcohol-fed groups were considered. While in most strains a modest increase in GSSG was observed (Figure 4B), the effect was not significant and no correlation with liver injury was observed. Reduction in GSH/GSSG ratio (Figure 4C) across the panel of strains followed closely the changes observed with GSH.

Changes in Methionine Cycle Metabolites

Alterations of methionine metabolism have been suggested to play an important role in the pathogenesis of alcoholic liver disease[11]. To determine what changes in liver methionine cycle metabolites are elicited by alcohol in different individuals under the conditions of control exposure, concentrations of methionine, SAM, SAH, and homocysteine were measured in the liver (Figure 5). Overall, little inter-strain difference in hepatic levels of methionine and SAM, or the effect of alcohol feeding (with the exception of a non-

significant, yet consistent decrease in SAM) was observed (Figures 5A and B). Liver SAH (Figure 5C) and homocysteine (Figure 5E) levels were elevated as a consequence of alcohol feeding in most strains with several strains showing a significant effect. Liver SAM/SAH ratios were decreased (Figure 5D). Liver injury scores were significantly correlated with SAM/SAH ratio (inverse correlation) and liver homocysteine content only when both control and alcohol-fed groups were considered.

Plasma hyperhomocysteinemia has been observed in mice but not rats treated intragastrically with alcohol-containing diet[21]. In addition, hyperhomocysteinemia has been associated with the degree of liver injury[27]. We observed that plasma levels of homocysteine are elevated in alcohol-fed mice (Figure 6A) and that the degree of hyperhomocysteinemia is correlated significantly with both overall liver injury (Figure 6B) and steatosis (Figure 6C). These correlations remained significant when only alcohol-fed animals were considered (Supplemental Table 2).

Inter-strain Differences in Alcohol-induced Alterations in One-carbon Metabolism Pathway

Homocysteine metabolism is dependent on the concordant action by a number of enzymes in one-carbon metabolism pathway. To evaluate the mechanisms of inter-strain differences in hyperhomocysteinemia, we evaluated expression of genes or protein levels of major enzymes responsible for the maintenance of the methyl donor pool in the liver (Figure 7). It has been previously shown that expression of *Bhmt* is not affected in alcohol-fed C57BL6 mice[21]. However, in our study we did observe changes in Bhmt protein in the liver of alcohol-fed mice of some strains (Figure 7A). There was a significant decreasing non-linear relationship between alcohol-induced change in liver Bhmt and plasma homocysteine (Supplemental Table 2).

Changes in other regulators of one-carbon metabolism were assessed using gene expression as mRNA, protein and activity levels of these enzymes correlate closely[31]. Genes encoding Mtr, an enzyme that catalyzes the final step in methionine biosynthesis, and Mthfr, an enzyme that is involved in homocysteine-methionine transition, were generally down-regulated in alcohol-fed mice, especially in strains that exhibited higher liver injury (Figures 7B-C). Mat1a, an enzyme that converts methionine into SAM, was markedly induced in strains with low liver injury (Figure 7D). Gnmt, an enzyme that converts SAM to SAH, was also induced in strains that had little liver injury and down-regulated in strains that had most severe injury (Figure 8A). Similar trends were observed in the expression of Ahcy (Figure 8B), Cbs (Figure 8C) and Cth (Figure 8D), enzymes responsible for SAH-homocysteine conversion and downstream homocysteine catabolism, respectively.

Discussion

Alcoholic liver disease is a complex pathological condition which depends on both parenchymal and nonparenchymal cells, and involves multiple pathways. Much is known about the roles of alcohol metabolism[32], oxidative stress and inflammation[33], ER stress[34], apoptosis[35], as well as disruptions in lipid[36], glutathione[37] and methionine metabolism[11]. Even though our understanding of the molecular underpinnings of this devastating human disease is considerable, the ability to translate these discoveries into successful therapies for progressive liver damage and prevention of fibrosis, cirrhosis and hepatocellular carcinoma is less obvious.

Alcoholic liver disease requires sustained alcohol consumption; however, only a fraction of individuals who abuse alcoholic beverages develop clinically prominent disease[38]. It has been proposed that factors other than alcohol itself can be involved in the progression of the disease, yet little is known about how the paucity of the genetic variation that exists in

human population influences the response of each individual. Several genetic susceptibility factors have been identified (reviewed in[39]); however, none of those findings are conclusive. It is likely that alcoholic liver disease is a complex trait whereby multiple genetic factors may be involved.

Population-based mouse models have been used in studies of the genetic factors that may confer susceptibility to human disease[40]. Genetic variation across the inbred mouse strains is at least as large, if not greater, as the variation observed in the human population[41], which provides opportunities for assessing the role of genetics in disease. In this study, we used the intragastric enteral alcohol feeding model in the mouse[15] because it (i) closely mirrors pathophysiology of human alcoholic steatohepatitis, (ii) is amenable to multi-strain studies of liver injury independent of alcohol preference, and (iii) allows to control the dose and animal's nutrition.

Most notable observation from this study is that in spite of a very high dose of alcohol being delivered to all inbred strains, minimal liver injury developed in some strains. Considerable inter-strain variability in sensitivity to alcoholic liver disease demonstrates that with a relatively limited number of individuals (*i.e.*, inbred strains), it is possible to experimentally model the effect of genetic differences on a disease outcome. We posit that the observed differences in the effects on the liver were not a factor of alcohol dose, because all animals had high daily, average (over the 28-day period) and peak urine alcohol concentrations. Furthermore, the inter-strain variability in the disease phenotype affords a unique opportunity to establish whether ER stress, fatty acid synthesis, and one-carbon metabolism play a role in the susceptibility to alcoholic liver injury.

Alcoholic liver disease has been associated with the accumulation of unfolded proteins in the ER of hepatocytes[34]. Several strains that developed the most pronounced liver injury, C57BL/10J and NZW/LacJ, also exhibited increased levels of Grp78 and Chop and an increase in *Chop* transcript. Notably, these ER stress markers were not induced consistently in other strains with high liver injury which suggests that ER stress may not be a requisite event in alcoholic liver disease. Alternatively, it is also likely that selective persistence of ER chaperone and CHOP expression is an evidence of failure to adapt to chronic unfolded protein response[42], thus serving as a pro-death factor that exacerbates liver injury caused by alcohol.

ER stress has also been implicated as one of the regulatory mechanisms in hepatocyte lipid metabolism[28]. A key interconnectedness between hepatic steatosis and ER stress, including the physiological role of the ER stress protein sensors in lipid homeostasis, has been demonstrated in several recent publications[43]. In this study, we observed an un-expected down-regulation of *Srebf1* and lack of induction of *Cebpa* in strains with high liver injury and liver steatosis. In prior work, up-regulation of SREBP1 and lipogenesis has been observed, albeit in a mouse strain not studied here. The difference may be related to the severity of ER stress or other unknown factors. A down-regulation of transcription factors involved in lipid synthesis has also been suggested as a sign of failure to adapt to chronic ER stress. For example, steatosis develops in the liver of tunicamycin-treated mice and is associated with unresolved ER stress, prolonged up-regulation of Chop, and inhibition of metabolic master regulators[28]. In addition, silencing of SREBP1 *in vitro* has led to dramatic loss of cell viability via induction of apoptosis[44]. Most recent studies demonstrated that the decreased SAM/SAH ratio as a consequence of hyperhomocysteinemia appears to have a key role as it can affect the ratio of phosphatidylcholine to phosphatidylethanolamine in ER membrane which could either lead to increased processing of SREBP1[45] or ER stress response[46]. In *C.elegans*, decreased SAM/SAH leads to decreased phosphatidylcholine/phosphatidylethanolamine ratio in ER resulting in transcription-independent activation of

SREBP1 and induction of lipogenesis and one-carbon metabolism[45]. However, the latter compensatory attempt to correct SAM/SAH may be impaired by the effects of alcohol.

While the precise mechanism of alcohol-induced effects on the one carbon metabolism remain to be determined and additional studies are needed to further investigate the differences in the role of ER stress in apoptosis and steatohepatitis among susceptible and resistant strains, our data clearly points to the genetic factors that may control adaptation to ER stress as one of the key events in the predisposition to alcoholic liver disease.

Significant reduction in the amount of liver GSH was observed in most of the strains upon treatment with alcohol, consistent with chronic oxidative stress elicited by the intragastric feeding[15]. A drop in the GSH/GSSG ratio was also detected in all but one strain. The consistency in reduction in the liver GSH and GSH/GSSG ratio among strains, and the negative correlation of these biomarkers with the liver pathology, only when both HFD and alcohol-fed groups were considered, are indicative of the fact that oxidative stress is a common feature across the individuals exposed to alcohol, but is not associated strongly with the degree of liver injury.

Similar to the observations with GSH, liver concentrations of SAM, SAH and homocysteine exhibited similar trends across all strains. Specifically, liver SAM/SAH ratio was lower and liver homocysteine was increased by ~5-30% in alcohol-treated mice. However, plasma homocysteine was highly significantly correlated with both total liver pathology and steatosis scores, in concert with previous reports on the key role of hyperhomocysteinemia in experimental alcoholic liver disease[21,27,34]. These results are strongly suggestive that hyperhomocysteinemia is a key molecular event and a potential biomarker of the severity of liver disease. The observation of hyperhomocysteinemia in rodent models of alcoholic liver injury is highly relevant to human disease. Hyperhomocysteinemia is a common clinical observation in alcoholics and is a risk factor for neurological complications[47]. Importantly, a large human study found that hyperhomocysteinemia was not only common in chronic alcoholics, but was also associated with the severity of liver disease[48].

Impairment in remethylation secondary to folate deficiency was suggested as the mechanism for hyperhomocysteinemia in chronic alcoholics[48]. Indeed, inter-strain differences in susceptibility to alcohol-induced liver injury were associated with different expression patterns of one-carbon metabolism-related genes. Specifically, strains resistant to alcoholic liver injury, such as WSB/EiJ, PWD/PhJ, 129S1Sv/ImJ, and AKR/J, were characterized by a significant up-regulation of *Mat1a, Ahcy*, and *Cth*. Increased expression of these genes indicates up-regulation of the transmethylation and transsulfuration pathways leading consequently to enhanced liver protection and/or attenuation of liver injury. In contrast, in sensitive strains, including FVB/NJ, KK/HIJ, C57BL/10J, and NZW/LacJ, alcohol exposure did not have an effect on expression of *Mat1a, Ahcy*, and *Cth*, while expression of *Cbs* was significantly down-regulated. The *Cbs* gene encodes one of the two pyridoxal phosphate-dependent enzymes; another one is cystathionine γ-lyase, which plays a key role in the proper function of the transulfuration pathway. Therefore, a decreased expression of *Cbs* gene may consequently lead to a lower protein level and activity of Cbs, substantially altering the biosynthesis of glutathione via transulfuration pathway and compromising antioxidant defenses. In addition to down-regulation of *Cbs*, a marked down-regulation of *Mtr* and *Mthfr* was also observed in strains sensitive to alcoholic liver injury indicating that removal of homocysteine via Mtr-dependent pathway is also compromised. Thus, dysregulation of one-carbon metabolism genes may lead not only to a more severe alcoholic liver injury, but also to hyperhomocysteinemia in sensitive strains.

Several aspects of our study deserve further comment. First, we have only examined one time point and the changes we observed may not be reflective of what occurred earlier in response to alcohol, nor what might occur later, as the complex adaptive, metabolic, and injury pathways adjust or maladjust. Second, although some of our findings demonstrate striking differences between alcohol and control but no correlation with disease severity, this should be not be taken as evidence that these changes are unimportant; the results simply suggest that these are not determinants of strain differences. Nevertheless, they could reflect important universal effects of alcohol which are prerequisites for the additional genetic responses to influence disease severity. For example, hepatic levels of GSH, SAM/SAH and homocysteine show marked differences across most of the alcohol versus pair-fed strains. Third, we have not measured protein expression and enzyme activity of most of the various apparently dysregulated gene transcripts so our findings do not take into consideration translational or posttranslational effects on these systems of lipid and one carbon metabolism and such effects could also be genetically determined. Nevertheless, notwithstanding the limitations, the findings of our initial approach indicate that genetic strain differences in liver injury and steatosis are striking and independent of alcohol exposure and the most severely affected strains exhibit major differences in the expression of ER stress markers and genes of one carbon metabolism. The significant correlation across species in plasma homocysteine and alcohol induced steatohepatitis stands out as a marker of dysregulated one carbon metabolism and confirms earlier studies in one mouse strain. These findings support the hypothesis that alcohol-induced hyperhomocysteinemia is not simply a marker of disturbed one carbon metabolism but reflects an integral aspect of the pathogenesis of steatohepatitis. The contribution of homocysteine induced homocysteinylation, redox effects, or mass effect on SAH to lower SAM/SAH in mediating effects on ER stress or other epigenetic effects requires additional investigation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Abbreviations

ADH	alcohol dehydrogenase
ALDH	aldehyde dehydrogenase
HFD	high fat diet
GSH	reduced glutathione
GSSG	oxidized glutathione
SAM	S-adenosylmethionine
SAH	S-adenosylhomocysteine
ER	endoplasmatic reticulum
Srebf1	sterol regulatory element binding transcription factor 1
Cebpa	CCAAT/enhancer-binding protein alpha
Grp78	glucose-regulated protein 78

Chop	C/EBP-homologous protein
Bhmt	betaine-homocysteine methyltransferase
Mtr	5-methyltetrahydrofolate-homocysteine methyltransferase
Mthfr	methylenetetrahydrofolate reductase
Mat1a	methionine adenosyltransferase 1 alpha
Gnmt	glycine N-methyltransferase
Ahcy	adenosylhomocysteinase
Cbs	cystathionine-beta-synthase
Cth	cystathionase
Gusb	beta glucuronidase
Adipor2	adiponectin receptor 2
Mttp	microsomal triglyceride transfer protein
Acox1	acyl-Coenzyme A oxidase 1, palmitoyl

References

1. Li TK. Quantifying the risk for alcohol-use and alcohol-attributable health disorders: present findings and future research needs. J Gastroenterol Hepatol. 2008; 23(Suppl 1):S2–S8. [PubMed: 18336658]

2. Norstrom T, Ramstedt M. Mortality and population drinking: a review of the literature. Drug Alcohol Rev. 2005; 24:537–547. [PubMed: 16361210]

3. McKillop IH, Schrum LW. Alcohol and liver cancer. Alcohol. 2005; 35:195–203. [PubMed: 16054981]

4. Yip WW, Burt AD. Alcoholic liver disease. Semin Diagn Pathol. 2006; 23:149–160. [PubMed: 17355088]

5. Breitkopf K, Nagy LE, Beier JI, Mueller S, Weng H, Dooley S. Current experimental perspectives on the clinical progression of alcoholic liver disease. Alcohol Clin Exp Res. 2009; 33:1647–1655. [PubMed: 19645734]

6. Albano E, Vidali M. Immune mechanisms in alcoholic liver disease. Genes Nutr. 2010; 5:141–147.

7. Wilfred de Alwis NM, Day CP. Genetics of alcoholic liver disease and nonalcoholic fatty liver disease. Semin Liver Dis. 2007; 27:44–54. [PubMed: 17295176]

8. Druesne-Pecollo N, Tehard B, Mallet Y, Gerber M, Norat T, Hercberg S, et al. Alcohol and genetic polymorphisms: effect on risk of alcohol-related cancer. Lancet Oncol. 2009; 10:173–180. [PubMed: 19185835]

9. Romeo S, Kozlitina J, Xing C, Pertsemlidis A, Cox D, Pennacchio LA, et al. Genetic variation in PNPLA3 confers susceptibility to nonalcoholic fatty liver disease. Nat Genet. 2008; 40:1461–1465. [PubMed: 18820647]

10. Tian C, Stokowski RP, Kershenobich D, Ballinger DG, Hinds DA. Variant in PNPLA3 is associated with alcoholic liver disease. Nat Genet. 2010; 42:21–23. [PubMed: 19946271]

11. Lu SC, Tsukamoto H, Mato JM. Role of abnormal methionine metabolism in alcoholic liver injury. Alcohol. 2002; 27:155–162. [PubMed: 12163143]

12. Mato JM, Martinez-Chantar ML, Lu SC. Methionine metabolism and liver disease. Annu Rev Nutr. 2008; 28:273–293. [PubMed: 18331185]

13. Obeid R, Herrmann W. Homocysteine and lipids: S-adenosyl methionine as a key intermediate. FEBS Lett. 2009; 583:1215–1225. [PubMed: 19324042]

14. Frazer KA, Eskin E, Kang HM, Bogue MA, Hinds DA, Beilharz EJ, et al. A sequence-based variation map of 8.27 million SNPs in inbred mouse strains. Nature. 2007; 448:1050–1053. [PubMed: 17660834]

15. Kono H, Bradford BU, Rusyn I, Fujii H, Matsumoto Y, Yin M, et al. Development of an intragastric enteral model in the mouse: studies of alcohol-induced liver disease using knockout technology. J Hepatobiliary Pancreat Surg. 2000; 7:395–400. [PubMed: 11180860]

16. Thompson JA, Reitz RC. Effects of ethanol ingestion and dietary fat levels on mitochondrial lipids in male and female rats. Lipids. 1978; 13:540–550. [PubMed: 703531]

17. Kono H, Enomoto N, Connor HD, Wheeler MD, Bradford BU, Rivera CA, et al. Medium-chain triglycerides inhibit free radical formation and TNF-alpha production in rats given enteral ethanol. Am J Physiol Gastrointest Liver Physiol. 2000; 278:G467–G476. [PubMed: 10712267]

18. Nanji AA, Mendenhall CL, French SW. Beef fat prevents alcoholic liver disease in the rat. Alcohol Clin Exp Res. 1989; 13:15–19. [PubMed: 2646971]

19. Bergmeyer, HU. Methods of enzymatic analysis. Academic Press; New York: 1988.

20. Trasler J, Deng L, Melnyk S, Pogribny I, Hiou-Tim F, Sibani S, et al. Impact of Dnmt1 deficiency, with and without low folate diets, on tumor numbers and DNA methylation in Min mice. Carcinogenesis. 2003; 24:39–45. [PubMed: 12538347]

21. Shinohara M, Ji C, Kaplowitz N. Differences in betaine-homocysteine methyltransferase expression, endoplasmic reticulum stress response, and liver injury between alcohol-fed mice and rats. Hepatology. 2010; 51:796–805. [PubMed: 20069651]

22. Bjorkegren J, Beigneux A, Bergo MO, Maher JJ, Young SG. Blocking the secretion of hepatic very low density lipoproteins renders the liver more susceptible to toxin-induced injury. J Biol Chem. 2002; 277:5476–5483. [PubMed: 11739387]

23. Fischer M, You M, Matsumoto M, Crabb DW. Peroxisome proliferator-activated receptor alpha (PPARalpha) agonist treatment reverses PPARalpha dysfunction and abnormalities in hepatic lipid metabolism in ethanol-fed mice. J Biol Chem. 2003; 278:27997–28004. [PubMed: 12791698]

24. Xu A, Wang Y, Keshaw H, Xu LY, Lam KS, Cooper GS. The fat-derived hormone adiponectin alleviates alcoholic and nonalcoholic fatty liver diseases in mice. J Clin Invest. 2003; 112:91–100. [PubMed: 12840063]

25. Qin Y, Tian YP. Exploring the molecular mechanisms underlying the potentiation of exogenous growth hormone on alcohol-induced fatty liver diseases in mice. J Transl Med. 2010; 8:120. [PubMed: 21087523]

26. Li J, Nguyen V, French BA, Parlow AF, Su GL, Fu P, et al. Mechanism of the alcohol cyclic pattern: role of the hypothalamic-pituitary-thyroid axis. Am J Physiol Gastrointest Liver Physiol. 2000; 279:G118–G125. [PubMed: 10898753]

27. Ji C, Kaplowitz N. Betaine decreases hyperhomocysteinemia, endoplasmic reticulum stress, and liver injury in alcohol-fed mice. Gastroenterology. 2003; 124:1488–1499. [PubMed: 12730887]

28. Rutkowski DT, Wu J, Back SH, Callaghan MU, Ferris SP, Iqbal J, et al. UPR pathways combine to prevent hepatic steatosis caused by ER stress-mediated suppression of transcriptional master regulators. Dev Cell. 2008; 15:829–840. [PubMed: 19081072]

29. Shaw S, Jayatilleke E, Ross WA, Gordon ER, Lieber CS. Ethanol-induced lipid peroxidation: potentiation by long-term alcohol feeding and attenuation by methionine. J Lab Clin Med. 1981; 98:417–424. [PubMed: 7264437]

30. Lauterburg BH, Velez ME. Glutathione deficiency in alcoholics: risk factor for paracetamol hepatotoxicity. Gut. 1988; 29:1153–1157. [PubMed: 3197987]

31. Christensen KE, Wu Q, Wang X, Deng L, Caudill MA, Rosen R. Steatosis in mice is associated with gender, folate intake, and expression of genes of one-carbon metabolism. J Nutr. 2010; 140:1736–1741. [PubMed: 20724492]

32. Bradford BU, Rusyn I. Swift increase in alcohol metabolism (SIAM): understanding the phenomenon of hypermetabolism in liver. Alcohol. 2005; 35:13–17. [PubMed: 15922133]

33. Cohen JI, Chen X, Nagy LE. Redox signaling and the innate immune system in alcoholic liver disease. Antiox Redox Signal. 2011; 15:523–534.

34. Ji C, Kaplowitz N. ER stress: can the liver cope? J Hepatol. 2006; 45:321–333. [PubMed: 16797772]

35. Ji C. Dissection of endoplasmic reticulum stress signaling in alcoholic and non-alcoholic liver injury. J Gastroenterol Hepatol. 2008; 23:S16–24. [PubMed: 18336657]

36. Lieber CS. Alcoholic fatty liver: its pathogenesis and mechanism of progression to inflammation and fibrosis. Alcohol. 2004; 34:9–19. [PubMed: 15670660]

37. Han D, Hanawa N, Saberi B, Kaplowitz N. Mechanisms of liver injury. III. Role of glutathione redox status in liver injury. Am J Physiol Gastrointest Liver Physiol. 2006; 291:G1–7. [PubMed: 16500922]

38. Gramenzi A, Caputo F, Biselli M, Kuria F, Loggi E, Andreone P, et al. Review article: alcoholic liver disease--pathophysiological aspects and risk factors. Aliment Pharmacol Ther. 2006; 24:1151–1161. [PubMed: 17014574]

39. Stickel F, Osterreicher CH. The role of genetic polymorphisms in alcoholic liver disease. Alcohol Alcohol. 2006; 41:209–224. [PubMed: 16492723]

40. Harrill AH, Watkins PB, Su S, Ross PK, Harbourt DE, Stylianou IM, et al. Mouse population-guided resequencing reveals that variants in CD44 contribute to acetaminophen-induced liver injury in humans. Genome Res. 2009; 19:1507–1515. [PubMed: 19416960]

41. Roberts A, Pardo-Manuel d V, Wang W, McMillan L, Threadgill DW. The polymorphism architecture of mouse genetic resources elucidated using genome-wide resequencing data: implications for QTL discovery and systems genetics. Mamm Genome. 2007; 18:473–481. [PubMed: 17674098]

42. Rutkowski DT, Arnold SM, Miller CN, Wu J, Li J, Gunnison KM, et al. PLoS Biol. 2006; 4:e374. [PubMed: 17090218]

43. Malhi H, Kaufman RJ. Endoplasmic reticulum stress in liver disease. J Hepatol. 2011; 54:795–809. [PubMed: 21145844]

44. Calvisi DF, Wang C, Ho C, Ladu S, Lee SA, Mattu S, et al. Increased lipogenesis, induced by AKT-mTORC1-RPS6 signaling, promotes development of human hepatocellular carcinoma. Gastroenterology. 2011; 140:1071–1083. [PubMed: 21147110]

45. Walker AK, Jacobs RL, Watts JL, Rottiers V, Jiang K, Finnegan DM, et al. A Conserved SREBP-1/Phosphatidylcholine Feedback Circuit Regulates Lipogenesis in Metazoans. Cell. 2011; 147:840–852. [PubMed: 22035958]

46. Fu S, Yang L, Li P, Hofmann O, Dicker L, Hide W, et al. Aberrant lipid metabolism disrupts calcium homeostasis causing liver endoplasmic reticulum stress in obesity. Nature. 2011; 473:528–531. [PubMed: 21532591]

47. Hultberg B, Berglund M, Andersson A, Frank A. Elevated plasma homocysteine in alcoholics. Alcohol Clin Exp Res. 1993; 17:687–689. [PubMed: 8392819]

48. Blasco C, Caballeria J, Deulofeu R, Lligona A, Pares A, Lluis JM, et al. Prevalence and mechanisms of hyperhomocysteinemia in chronic alcoholics. Alcohol Clin Exp Res. 2005; 29:1044–1048. [PubMed: 15976531]
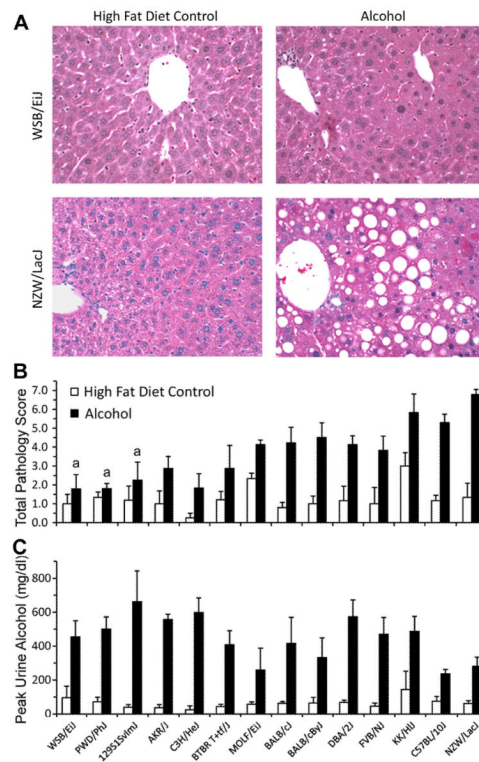
Figure 1. Inter-strain differences in alcoholic liver injury
(A) H&E staining (200x) of liver tissues from mice of 2 representative strains. Total
pathology (B) and peak urine alcohol (C) in mice fed intragastrically with control (white
bars) or alcohol-containing (black bars) diets. [a], No significant differences between control
and alcohol groups (mean±SD, n=3-9 per group). All other differences are significant
(P<0.05). See Supplemental Table 1 for raw data and Supplemental Figure 1 for graphs of
serum ALT and scores for liver inflammation and necrosis.
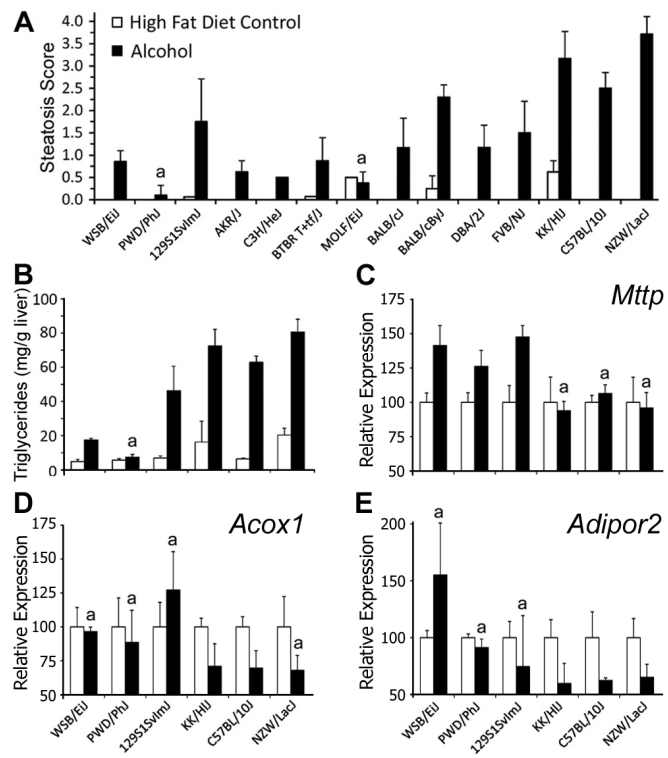
Figure 2. Inter-strain differences in steatosis and liver fat metabolism
Steatosis scores (A), liver triglycerides (B) and relative mRNA abundance of *Mttp* (C), *Acox1* (D) and *Adipor2* (E) in mice fed intragastrically with control (white bars) or alcohol-containing (black bars) diets. [a], No significant differences between control and alcohol groups (mean±SD, n=3). All other differences are significant (*P*<0.05).
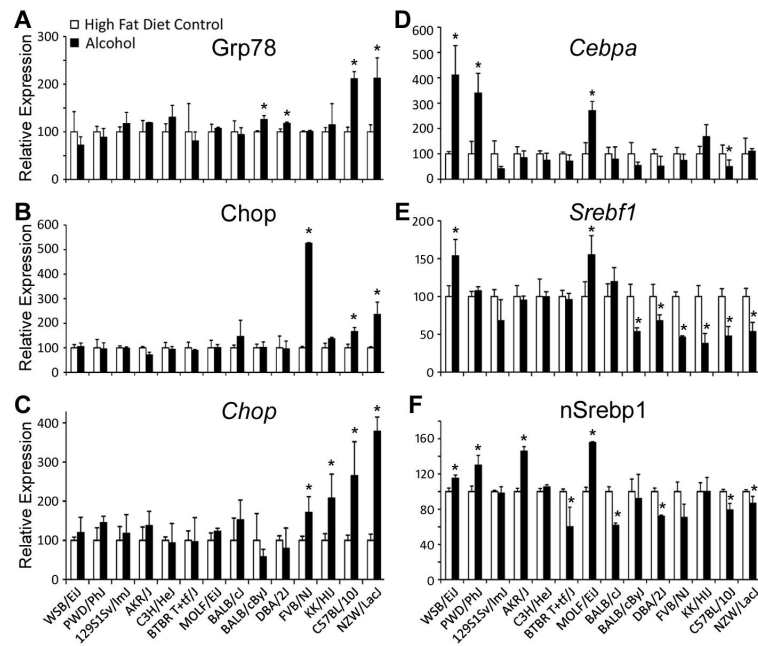
Figure 3. Expression of selective markers of ER stress and fat metabolism in the livers of mice fed intragastrically with control or alcohol-containing diets

Relative levels of Grp78 (A) and Chop (B) proteins. Relative mRNA abundance of *Chop* (C), *Cebpa* (D) and *Srebf1* (E). (F) Relative levels of nuclear (n)Srebp1 protein. *$P<0.05$, compared between control and alcohol groups (mean±SD, n=3).
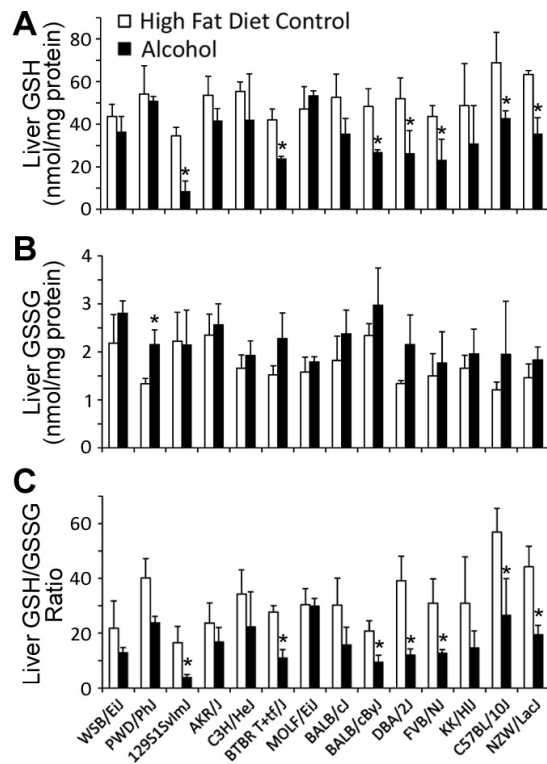
Figure 4. Liver content of glutathione in mice fed intragastrically with control or alcohol-containing diets

Liver GSH (A), GSSG (B) and GSH/GSSG ratio (C). *P<0.05, compared between control and alcohol groups (mean±SD, n=3).
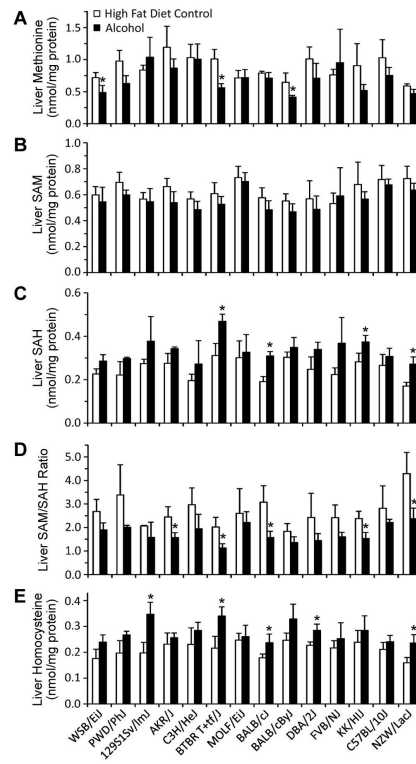
Figure 5. Liver content of methionine and its metabolites in mice fed intragastrically with control or alcohol-containing diets
Liver methionine (A), SAM (B), SAH (C), SAM/SAH ratio (D), and homocysteine (E).
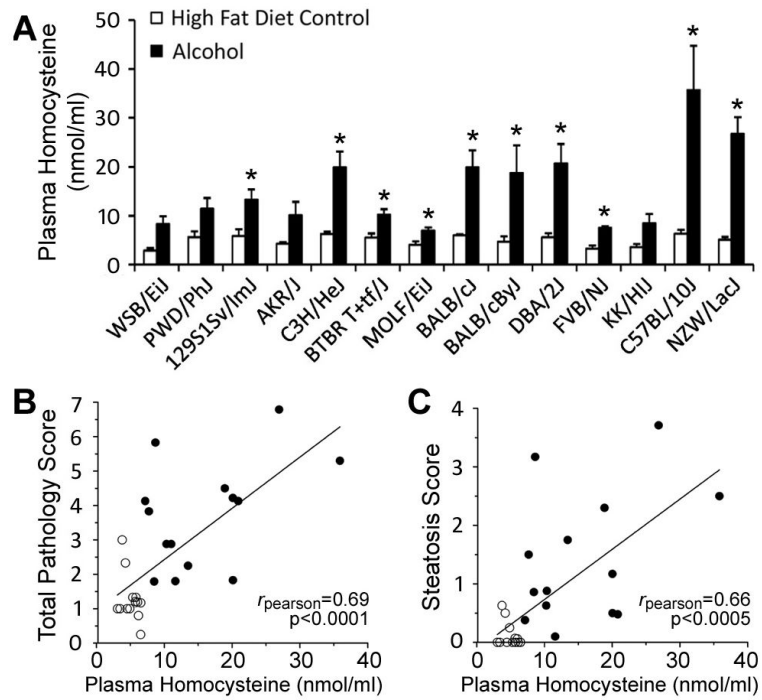*P<0.05, compared between control and alcohol groups (mean±SD, n=3).

Figure 6. Plasma homocysteine (A) in mice fed intragastrically with control or alcohol-containing diets

*$P<0.05$, compared between control and alcohol groups (mean±SD, n=3). Correlation plots of plasma homocysteine and total pathology score (B) or liver steatosis score (C) in control (empty circles) and alcohol-fed (filled circles) animals (strain averages).
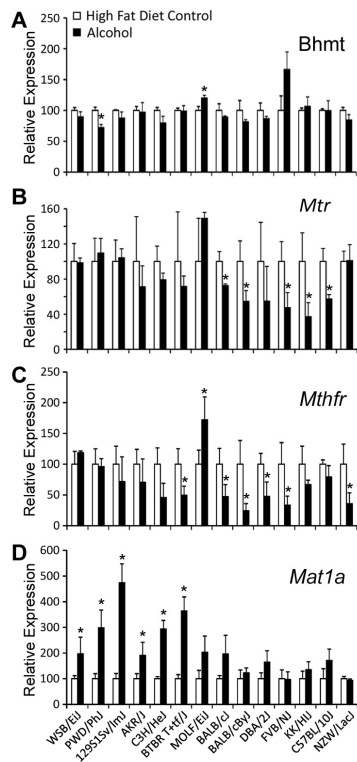
Figure 7. Expression of selective markers of methionine metabolism in the livers of mice fed intragastrically with control or alcohol-containing diets
Relative levels of Bhmt (A) protein. Relative mRNA abundance of *Mtr* (B), *Mthfr* (C), and *Mat1a* (D). *$P<0.05$, compared between control and alcohol groups (mean±SD, n=3).
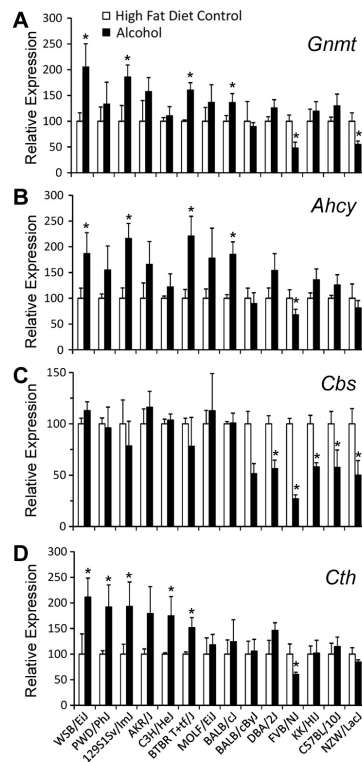
Figure 8. Expression of selective markers of homocysteine metabolism in the livers of mice fed intragastrically with control or alcohol-containing diets
Relative mRNA abundance of *Gnmt* (A), *Ahcy* (B), *Cbs* (C) and *Cth* (D). *P<0.05, compared between control and alcohol groups (mean±SD, n=3).