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

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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 endoplasmic reticulum (ER) stress and one-carbon metabolism in the mechanism of interindividual variability in alcoholic liver injury. We administered alcohol (up to 27 mg/kg/d) in a high-fat diet using an 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 interstrain differences (more than 3-fold) in alcohol-induced steatohepatitis were observed among the strains in spite of consistently high levels of urine alcohol that were monitored throughout the study. We found that ER stress genes were induced only in strains with the most liver injury. Liver glutathione and methyl donor levels were affected in all strains, albeit to a different degree. The 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. (HEPATOLOGY 2012;56:130-139)
studies suggested that polymorphisms in genes for alcohol (e.g., \( ADH \) [alcohol dehydrogenase] and \( ALDH \) [aldehyde dehydrogenase], etc.) and folate metabolism (e.g., \( MTHFR \) [methyleneetetrahydrofolate reductase]), as well as oxidative stress (e.g., \( MNSOD \)) and immune response (e.g., \( CD14 \), tumor necrosis factor \( \alpha \)), 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 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 nonalcoholic fatty liver disease\(^9\) and alcohol-related cirrhosis.\(^10\)

This study evaluated key molecular events postulated to play a role in alcoholic liver injury: endoplasmic reticulum (ER) stress, lipid, and one-carbon metabolism. Specifically, we tested the hypothesis that a panel of genetically diverse mouse strains may be used to examine the role of one-carbon metabolism in the mechanism of interindividual 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.\(^11\)-\(^13\)

**Materials and Methods**

**Animals, Diets, and Treatment**

Male mice (8-14 weeks 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 intragastric 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 by way of gastric cannula a high-fat liquid diet prepared as detailed elsewhere.\(^16\) Animals had free access to water and nonnutritious cellulose pellets throughout the study. Control groups received high-fat diet (HFD) supplemented with isocaloric maltose-dextrin and lipotropes,\(^15\) whereas alcohol groups received HFD 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 four times daily and sacrificed after 28 days of treatment. All animals were given humane care in compliance with National Institutes of Health (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 \( -80^\circ C \). 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 \( -80^\circ C \).

Formalin-fixed/paraffin-embedded liver sections were stained with hematoxylin/eosin (H&E). Liver pathology was evaluated in a blind manner by a certified veterinary pathologist and scored\(^18\) as follows: steatosis (% of hepatocytes containing fat): \( <25\% = 1\), \( >25\% = 2\), \( >50\% = 3\), \( >75\% = 4\); 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 described.\(^20\)

**Molecular Assays**

Tissues collected from three 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. Primary antibodies against actin, glucose-regulated protein 78 (Grp78), C/EBP-homologous protein (Chop), betaine-homocysteine methyltransferase (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 (NIH, Bethesda, MD). 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 the Supporting Methods. Genes assayed and their primer information is included in the Supporting Methods.

**Statistical Analyses**

Results are presented as mean ± SD. 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**

**Interstrain Variability in the Severity of Alcohol-Induced Liver Injury in the Mouse.** The intragastric subchronic infusion model was used to study the population-wide effects of alcohol on the liver because 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 are available for individual animals as Supporting 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 with the strain-matched animals on a high-fat corn oil-based diet (Fig. 1A, B; see Supporting Fig. 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 macrovesicular fat accumulation in the liver was exacerbated by alcohol feeding in all strains except for WSB/EiJ, MOLF/EiJ, and DBA/2J (Fig. 2A), data which are supported by measurements of liver triglyceride content in select strains (Fig. 2B). Accordingly, we examined several pathways for hepatic fat metabolism. Microsomal triglyceride transfer protein (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 and its expression was induced in strains that were resistant to alcohol-induced fatty liver (Fig. 2C). Alcohol is also known to decrease peroxisomal lipid metabolism and we found decreased expression of acyl-coenzyme A oxidase 1, palmitoyl (Acox1) in strains with severe fatty liver (Fig. 2D). Finally, the fat-derived hormone adiponectin alleviates alcoholic fatty liver disease in mice and liver adiponectin receptor 2 (Adipor2) expression was...
decreased by alcohol treatment in mice, an effect that was not observed in alcoholic liver injury-resistant strains (Fig. 2E).

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

**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. 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 sterol regulatory element binding transcription factor 1 (Srebf1) and CCAAT/enhancer-binding protein alpha (Cebpa), key transcription factors involved in fatty acid metabolism, were observed. In some strains that exhibited the greatest degree of alcohol-induced liver injury, a concordant induction of ER stress factors Grp78 (Fig. 3A) and Chop (Fig. 3B,C), and dysregulation of Cebpa (Fig. 3D) and Srebf1 (Fig. 3E), as well as a decrease activated cleaved Srebp1 (Fig. 3F), was observed.

**Changes in Hepatic Reduced Glutathione (GSH) and Oxidized Glutathione (GSSG).** Oxidative stress and lipid peroxidation are well-established hallmarks of alcohol-induced liver injury. Hepatic GSH depletion after chronic alcohol consumption was shown both in experimental animals and in humans. We evaluated the content of GSH and GSSG in livers of alcohol- and HFD-fed mice (Fig. 4). GSH depletion was observed in most of the strains (Fig. 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. Although in most strains a modest increase in GSSG was observed (Fig. 4B), the effect was not significant and no correlation with liver injury was observed. Reduction in the GSH/GSSG ratio (Fig. 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. 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, S-adenosylmethionine (SAM), S-adenosylhomocysteine (SAH), and homocysteine were measured in the liver (Fig. 5). Overall, little interstrain difference in hepatic levels of methionine and SAM, or the effect of alcohol feeding (with the exception of a nonsignificant, yet consistent decrease in SAM) was observed (Fig. 5A,B). Liver SAH (Fig. 5C) and homocysteine (Fig. 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 (Fig. 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 an alcohol-containing diet. In addition, hyperhomocysteinemia has been associated with the degree of liver injury. We observed that plasma levels of homocysteine are elevated in alcohol-fed mice (Fig. 6A) and that the degree of hyperhomocysteinemia is correlated significantly with both overall liver injury (Fig. 6B).
and steatosis (Fig. 6C). These correlations remained significant when only alcohol-fed animals were considered (Supporting Table 2).

**Interstrain Differences in Alcohol-Induced Alterations in the One-Carbon Metabolism Pathway.** Homocysteine metabolism is dependent on the concordant action by a number of enzymes in the one-carbon metabolism pathway. To evaluate the mechanisms of interstrain differences in hyperhomocysteinemia, we evaluated the expression of genes or protein levels of major enzymes responsible for the maintenance of the methyl donor pool in the liver (Fig. 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 (Fig. 7A). There was a significant decreasing nonlinear relationship between alcohol-induced change in liver Bhmt and plasma homocysteine (Supporting Table 2).

Changes in other regulators of one-carbon metabolism were assessed using gene expression, as messenger RNA (mRNA), protein, and activity levels of these enzymes correlate closely.31 Genes encoding 5-methyltetrahydrofolate-homocysteine methyltransferase (Mtr), an enzyme that catalyzes the final step in methionine biosynthesis, and Mthfr, an enzyme that is involved in homocysteine-methionine transition, were generally

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**Fig. 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).

**Fig. 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).
down-regulated in alcohol-fed mice, especially in strains that exhibited higher liver injury (Fig. 7B,C). Methionine adenosyltransferase 1 alpha (Mat1a), an enzyme that converts methionine into SAM, was markedly induced in strains with low liver injury (Fig. 7D). Glycine N-methyltransferase (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 the most severe injury (Fig. 8A). Similar trends were observed in the expression of adenosylhomocysteinase (Ahcy) (Fig. 8B), cystathionine-beta-synthase (Cbs) (Fig. 8C), and cystathionase (Cth) (Fig. 8D), enzymes responsible for SAH-homocysteine conversion and downstream homocysteine catabolism, respectively.

**Discussion**

Alcoholic liver disease is a complex pathological condition that depends on both parenchymal and non-parenchymal cells and involves multiple pathways. Much is known about the roles of alcohol metabolism, oxidative stress and inflammation, ER stress, apoptosis, as well as disruptions in lipid, glutathione, and methionine metabolism. 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. 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); 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. Genetic variation across the inbred mouse strains is at least as large, if not greater than, as the variation observed in the human population, 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 because it (1) closely mirrors the pathophysiology of human alcoholic steatohepatitis; (2) is amenable to multistrain studies of liver injury independent of alcohol preference; and (3) allows control of the dose and animal’s nutrition.

The 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 interstrain 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.

![Fig. 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).](image)

![Fig. 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).](image)
Furthermore, the interstrain 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. 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 evidence of failure to adapt to chronic unfolded protein response, thus serving as a prodeath factor that exacerbates liver injury caused by alcohol.

ER stress has also been implicated as one of the regulatory mechanisms in hepatocyte lipid metabolism. 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. In this study we observed an unexpected decrease in the SAM/SAH ratio in ER membrane that could either lead to increased processing of SREBP1 or ER stress response. In Caenorhabditis 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. However, the latter compensatory attempt to correct SAM/SAH may be impaired by the effects of alcohol.

Although the precise mechanism of alcohol-induced effects on 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 point to the genetic factors that may control adaptation to ER stress as one of the key events in the predisposition to alcoholic liver disease.

A 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 intragastic feeding. 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, the 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. These results are strongly suggestive that hyperhomocysteinemia is a common event and a potential biomarker of the severity of liver disease. The observation of hyperhomocysteinemia in rodents 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. 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.

Impairment in remethylation secondary to folate deficiency was suggested as the mechanism for hyperhomocysteinemia in chronic alcoholics. Indeed, interstrain 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, 129S1/SvImJ,
and AKR/J, were characterized by a significant up-regulation of Matla, Abcy, 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, expression of Matla, Abcy, and Cth, whereas 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 transsulfuration pathway. Therefore, a decreased expression of the Cbs gene may consequently lead to a lower protein level and activity of Cbs, substantially altering the biosynthesis of glutathione by way of transsulfuration 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 by way of 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 examined only one timepoint 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 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 that 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 homocysteinylaton, redox effects, or mass effect on SAH to lower SAM/SAH in mediating effects on ER stress or other epigenetic effects requires additional investigation.

References


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