Supplementation of Saturated Long-Chain Fatty Acids Maintains Intestinal Eubiosis and Reduces Ethanol-induced Liver Injury in Mice

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BACKGROUND & AIMS: Alcoholic liver disease is a leading cause of mortality. Chronic alcohol consumption is accompanied by intestinal dysbiosis, and development of alcoholic liver disease requires gut-derived bacterial products. However, little is known about how alterations to the microbiome contribute to pathogenesis of alcoholic liver disease. **METHODS:** We used the Tsukamoto-French mouse model, which involves continuous intragastric feeding of isocaloric diet or alcohol for 3 weeks. Bacterial DNA from the cecum was extracted for deep metagenomic sequencing. Targeted metabolomics assessed concentrations of saturated fatty acids in cecal contents. To maintain intestinal metabolic homeostasis, diets of ethanol-fed and control mice were supplemented with saturated long-chain fatty acids (LCFA). Bacterial genes involved in fatty acid biosynthesis, amounts of lactobacilli, and saturated LCFA were measured in fecal samples of nonalcoholic individuals and patients with active alcohol abuse. **RESULTS:** Analyses of intestinal contents from mice revealed alcohol-associated changes to the intestinal metagenome and metabolome, characterized by reduced synthesis of saturated LCFA. Maintaining intestinal levels of saturated fatty acids in mice resulted in eubiosis, stabilized the intestinal gut barrier, and reduced ethanol-induced liver injury. Saturated LCFA are metabolized by commensal *Lactobacillus* and promote their growth. Proportions of bacterial genes involved in fatty acid biosynthesis were lower in feces from patients with active alcohol abuse than controls. Total levels of LCFA correlated with those of lactobacilli in fecal samples from patients with active alcohol abuse but not in controls. **CONCLUSIONS:** In humans and mice, alcohol causes intestinal dysbiosis, reducing the capacity of the microbiome to synthesize saturated LCFA and the proportion of *Lactobacillus* species. Dietary approaches to restore levels of saturated fatty acids in the intestine might reduce ethanol-induced liver injury in patients with alcoholic liver disease.

Keywords: Metagenomics; Metabolomics; Microbiome; Microbiota.

Liver cirrhosis is the 10th leading cause of mortality in the United States; more than 40% of all cirrhosis-associated deaths are related to alcohol.[1] Alcoholic liver disease can progress from simple hepatic steatosis to cirrhosis and even hepatocellular carcinoma in some individuals.[2]

Chronic alcohol consumption is associated with qualitative and quantitative (overgrowth) dysbiotic changes in the intestinal microbiota.[3–7] Alcoholic dysbiosis is characterized by reduced proportions of commensal probiotic bacteria, such as *Lactobacillus* species in animal models and humans with cirrhosis.[3,8] A common and important feature of disease pathogenesis is leakiness of the intestinal barrier, resulting in translocation of microbial products, such as lipopolysaccharide, from the intestinal lumen to the liver.[9] Development of alcoholic liver disease depends on microbial translocation; mice that express nonfunctional toll-like receptor 4 (TLR4) are resistant to experimental alcoholic liver disease.[10] In addition, mice that are protected from bacterial overgrowth have decreased alcohol-induced liver disease, despite leakier guts.[4,11] Administration of the probiotic *Lactobacillus rhamnosus GG* to alcohol-fed mice restored intestinal integrity, decreased intestinal translocation of microbial products, and reduced features of alcoholic liver disease,[12,13] providing evidence that targeting dysbiosis improves disease.

Although alcohol-associated changes in the enteric microbiome and subsequent disruption of the intestinal

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Abbreviations used in this paper: LCFA, long chain fatty acids; SCFA, short chain fatty acid; SF, saturated fatty acid; TLR, Toll-like receptor; USF, unsaturated fatty acid.

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barrier are required for development and progression of liver disease in animal models and humans,[14,15] little is known about the mechanisms of this process. We used metagenomic and metabolomic analyses to investigate the effects of chronic ethanol administration on metabolism by the intestinal microbiota.

Materials and Methods

Alcohol Feeding

Male C57BL/6J mice (age 8 weeks) were purchased from Jackson Laboratory (Bar Harbor, ME). All mice received humane care in compliance with institutional guidelines. Continuous intragastric infusion of ethanol was performed as described previously.[3,4,16] In brief, mice were anesthetized by injection of ketamine and xylazine, and underwent surgical implantation of a long-term gastrostomy catheter made of Tygon and Silastic tubings with Dacron felt under sterile conditions. The use of a swivel allows free movement of the mouse in a micro-isolator cage. After a 1-week acclimatization period during which mice were given intragastric infusions of a control diet, ethanol infusion was initiated at 22.7 g/kg/d on days one and two, 24.3 g/kg/d on days three and four, 26 g/kg/d on days five and six, and 27.5 g/kg for day 7. For mice fed ethanol for 3 weeks, the dose increased to 29.2 g/kg/d for days 9–14 and 30.9 g/kg/d for week 3. At the initial ethanol dose, total caloric intake was set at 533 cal/kg; the percentages of calories from ethanol, dietary carbohydrate (dextrose), protein (lactalbumin hydrolysate), and fat (corn oil) were 29%, 13%, 23%, and 35%, respectively. A vitamin, salt, and trace mineral mix was included at the recommended amounts by the Committee on Animal Nutrition of the National Research Council (AIN-76A, 4.42 g/L and 15.4 g/L, respectively; Dyets Inc., Bethlehem, PA). Mice were individually caged and control mice received an isocaloric amount of dextrose instead of ethanol.

For fatty acids supplementation experiments, the total caloric intake was reduced by 30%, to 373 cal/kg; the percentage of calories from ethanol, dietary carbohydrate (dextrose), and protein (lactalbumin hydrolysate) were 29%, 13%, and 23%, respectively. Liquid diets were also supplemented with a small amount of corn oil (5% of total calories) to avoid deficiency in essential fatty acids. Most of the dietary fat component was fed via pellets in an isocaloric amount because saturated fatty acids have a wax-like consistency at room temperature and cannot be provided as liquid diet. The unsaturated fatty acid (USF) group received pellets made of corn oil (#401150; Dyets Inc.), and the saturated fatty acid (SF) group received pellets made of hydrogenated soy glyceride (#D404363; Dyets Inc.). Corn oil contains predominately USF (60% polyunsaturated fatty acids [linoleic acid] and 26% monounsaturated fatty acids [oleic acid]; Supplementary Table 1). 80-S hydrogenated soya glyceride contains mostly SF (12% palmitic acid and 85% stearic acid; Supplementary Table 2). Comparable amounts of USF and SF pellets were consumed by mice in both groups (Supplementary Figure 1). After 3 weeks of intragastric alcohol feeding and dietary supplementation with fatty acids, single-caged mice were sacrificed for analysis.

Other materials and methods are described in the Supplementary Materials and Methods.

Results

Chronic Administration of Ethanol Inhibits Biosynthesis of Saturated Fatty Acids by the Intestinal Microflora

We first determined the effect of chronic alcohol intake on the intestinal metagenome. We used individually housed mice given intragastric infusion of ethanol-containing diet as a model of alcohol-induced liver disease. This model is ideal for well-controlled microbiomic and metabolic studies, because the same liquid diet is administered to all mice at the same rate, with the same amount of calories and volume. Control mice received infusion of an isocaloric amount of diet with dextrose instead of ethanol. Mice continuously fed ethanol for 3 weeks developed severe liver injury and steatosis as described previously.[3]

The cecal microbiota from mice fed ethanol for 3 weeks had a lower proportion, compared with controls, of bacterial genes involved in the biosynthesis of saturated fatty acids including malonyl CoA:ACP acyltransferase (FabD; [EC:2.3.1.39]), 3-oxoacyl-[acyl-carrier-protein] synthase II (FabF; [EC:2.3.1.179]) and 3-oxoacyl-[acyl-carrier protein] reductase (FabG; [EC:1.1.1.100]), determined by metagenomic sequencing (Figure 1*A*). Quantitative polymerase chain reaction confirmed these results (Figure 1*B*). To further explore whether ethanol reduces SF biosynthetic gene abundance independently from the host, we performed ex vivo analysis by incubating cecal contents from control mice with ethanol under anaerobic conditions. Ethanol decreased the abundance of *fabF*, *fabG*, and *fabD* genes (Supplementary Figure 2*A*), suggesting that ethanol exerts a direct effect on the microbiota. We further assessed bacterial gene expression on the transcriptional level. The messenger RNA level of all 3 *fab* genes showed a similar reduction as the genomic DNA after ethanol incubation (Supplementary Figure 2*B*), suggesting that ethanol changes microbial communities rather than having a direct inhibitory effect on bacterial gene transcription. Phylogenetic analysis of *fab* genes derived from metagenomic assemblies lacked representation in certain clades of *Bacteroides*, *Clostridium*, and *Akkermansia* spp, suggesting that some phylotypes were more sensitive to the effects of ethanol, therefore, reducing the copy number of these genes in the intestine (Supplementary Figure 2*C*).

Targeted metabolomic studies using tandem mass spectrometry determined the concentrations of the free SF C2:0 to C18:0 in cecal contents. With the exception of C10:0, which was lower in ethanol-fed mice, the concentrations of saturated short-chain fatty acids (SCFA; C2:0–C6:0) and medium-chain fatty acids (C7:0–C12:0) did not differ significantly between control and alcohol-fed mice. In contrast, concentrations of almost all saturated long-chain fatty acids (LCFA; C13:0–C18:0) were markedly decreased in ethanol-fed mice compared with control mice (Figure 1*C*; Supplementary Figure 3).

In control and ethanol-fed mice, the concentrations of SCFA were the highest, when absolute amounts of all SFs were compared. Although there was a slight but not sig-