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ANIMAL MODELS

Dietary Linoleic Acid and Its Oxidized Metabolites Exacerbate Liver Injury Caused by Ethanol via Induction of Hepatic Proinflammatory Response in Mice

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Alcoholic liver disease is a major human health problem leading to significant morbidity and mortality in the United States and worldwide. Dietary fat plays an important role in alcoholic liver disease pathogenesis. Herein, we tested the hypothesis that a combination of ethanol and a diet rich in linoleic acid (LA) leads to the increased production of oxidized LA metabolites (OXLAMs), specifically 9- and 13-hydroxyoctadecadienoic acids (HODEs), which contribute to a hepatic proinflammatory response exacerbating liver injury. Mice were fed unsaturated (with a high LA content) or saturated fat diets (USF and SF, respectively) with or without ethanol for 10 days, followed by a single binge of ethanol. Compared to SF+ethanol, mice fed USF+ethanol had elevated plasma alanine transaminase levels, enhanced hepatic steatosis, oxidative stress, and inflammation. Plasma and liver levels of 9- and 13-HODEs were increased in response to USF+ethanol feeding. We demonstrated that primarily 9-HODE, but not 13-HODE, induced the expression of several proinflammatory cytokines *in vitro* in RAW264.7 macrophages. Finally, deficiency of arachidonate 15-lipoxygenase, a major enzyme involved in LA oxidation and OXLAM production, attenuated liver injury and inflammation caused by USF+ethanol feeding but had no effect on hepatic steatosis. This study demonstrates that OXLAM-mediated induction of a proinflammatory response in macrophages is one of the potential mechanisms underlying the progression from alcohol-induced steatosis to alcoholic steatohepatitis. *(Am J Pathol 2017, 187: 2232–2245; http://dx.doi.org/10.1016/j.ajpath.2017.06.008)*

Alcohol-associated health problems, including alcoholic liver disease (ALD), are major global health problems. ALD progresses through the course of several pathologies, including steatosis, alcoholic hepatitis, cirrhosis, and potentially hepatocellular carcinoma. Alcoholic hepatitis occurs in approximately 10% to 35% of chronic heavy drinkers, and severe alcoholic hepatitis accounts for significant morbidity and mortality, approaching 35% to 45%.[1] Approximately 10% to 20% of heavy drinkers will eventually develop cirrhosis.[2,3] The specific mechanisms responsible for ALD development and progression are not fully understood, and there is no Federal Drug Administration–approved therapy for any stage of ALD.

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The link between diet, specifically dietary fat, and alcohol consumption in ALD pathogenesis has been increasingly appreciated. Findings from an epidemiological study that analyzed dietary fat intake in individuals from countries with similar per capita alcohol consumption revealed that intake of saturated fat (SF) was associated with lower mortality rates, whereas dietary intake of unsaturated fat (USF) was associated with a higher mortality from alcoholic cirrhosis.[4] The beneficial effects of dietary SF (predominantly rich in medium- or long-chain saturated fatty acids) and the damaging effects of dietary USF [primarily enriched in linoleic acid (LA), an ω-6 polyunsaturated fatty acid (PUFA)] on alcohol-induced liver injury have been demonstrated in numerous preclinical studies using rodent models of ALD.[5–9] LA is required for the development of experimental ALD, with the severity of liver pathology correlating to the amount of LA in the diet.[10] On a mechanistic level, the deleterious effects of USF in comparison to the protective effects of SF are thought to result from alterations in hepatic lipid homeostasis,[6,11–14] induction of hepatic lipid peroxidation and oxidative stress,[6,15] changes in the gut microbiota, impaired intestinal barrier integrity, and elevated endotoxemia with subsequent hepatic macrophage activation and increased production of hepatic proinflammatory cytokines.[7,8,16,17] LA is the most abundant ω-6 PUFA in human diets and in human plasma and membrane lipids.[18] The consumption of LA, which has dramatically increased during the past several decades,[19] is positively correlated with increasing prevalence of several pathological conditions, including obesity.[20] LA can be enzymatically converted to bioactive oxidized LA metabolites (OXLAMs), primarily via the actions of cellular lipoxygenases [arachidonate 15-lipoxygenase (ALOX15) and ALOX15B in humans, and ALOX15 (12/15-LO) in rodents] or nonenzymatically via free radical–mediated oxidation in response to oxidative stress. OXLAMs are involved in various intracellular signaling pathways and may induce a proinflammatory response in different cell types.[21–23] OXLAMs play a role in the development of different pathological conditions, such as inflammatory hyperalgesia and the metabolic syndrome.[24,25] OXLAMs, specifically 9- and 13-hydroxyoctadecadienoic acids (9- and 13-HODEs, respectively), were elevated in patients with nonalcoholic fatty liver disease,[26] and a decrease in plasma OXLAM levels was correlated with hepatic histological improvement in these patients.[27] Increased levels of 9- and 13-HODEs were found in patients with alcoholic liver cirrhosis in parallel with the induction of hepatic ALOX15 and ALOX15B.[28] Elevated 9- and 13-HODE levels were also observed in experimental rodent models of ALD and were associated with ethanol-induced liver injury, steatosis, and inflammation.[29,30] Based on these observations, we hypothesized that OXLAMs may play a pathogenic role in ALD development and/or progression; however, the underlying mechanisms remain to be determined.

In the present study, we tested the hypothesis that the combination of ethanol and a diet with a high content of LA contributes to and exacerbates ethanol-induced liver injury through increased OXLAM production and OXLAM-mediated induction of hepatic proinflammatory responses. We postulated that both increased substrate availability (LA-rich dietary fat) and activation of metabolic pathways of OXLAM production, in particular the 12/15-LO–mediated pathway, contribute to an ethanol-mediated increase in OXLAMs. Therefore, we further examined whether genetic ablation of *Alox15* (the gene encoding 12/15-LO) would decrease OXLAM levels and attenuate liver injury caused by ethanol and an LA-enriched diet. For our studies, we used a chronic-binge ethanol exposure animal model of ALD (NIH National Institute on Alcohol Abuse and Alcoholism model).[31] This model recapitulates the drinking pattern in humans and induces a moderately severe alcoholic liver injury with increased hepatic neutrophil infiltration,[31] which is often observed in alcoholic hepatitis patients.[32]

Materials and Methods

Animals and Treatments

Eight-week-old genetically unaltered wild-type (WT; C57BL/6J) and *Alox15* gene knockout (B6.129S2-*Alox15*^{tm1Fun}/J, 11th backcross generation) male mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Animals were housed in a specific pathogen-free barrier facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. A 10-day ethanol feeding plus a single binge of ethanol administration was used as an experimental model of ALD (NIH National Institute on Alcohol Abuse and Alcoholism model).[31] Briefly, the mice were provided free access to a Lieber-DeCarli control or ethanol (5% w/v)-containing diet for 10 days. On day 11, the mice were gavaged with a single dose of ethanol solution (20% v/v) prepared in ultrapure water; the gavage volume was calculated to deliver 5 g ethanol/kg body weight. The mice in control groups received an isocaloric/isovolumetric maltodextrin solution. The mice were euthanized 9 hours after the gavage. The experimental paradigm is outlined in Figure 1A. Two separate experiments were performed: WT mice were provided a USF- or SF-enriched diet with or without ethanol; and WT and *Alox15* knockout mice were fed a USF-enriched diet with or without ethanol. The USF diet was supplemented with corn oil, a rich source of LA, and the SF diet was supplemented with beef tallow and medium-chain triglycerides (Research Diets, New Brunswick, NJ) (Table 1). The detailed composition of these diets has been described previously.[33] In the control group, the levels of protein, carbohydrate, and fat were held constant at 17%, 43%, and 40% of total energy, respectively. In the alcohol-containing diets, ethanol (35% of total calories) was substituted for carbohydrate energy. The diets were prepared fresh each day, and the food consumption was monitored daily. The control groups