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Saturated fat and cholesterol are critical to inducing murine metabolic syndrome with robust nonalcoholic steatohepatitis☆

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Abstract

Nonalcoholic fatty liver disease (NAFLD) is the hepatic manifestation of the metabolic syndrome (MetS). Up to a third of NAFLD subjects are at risk for developing nonalcoholic steatohepatitis (NASH). Many rodent models fail to replicate both MetS and NASH. The purpose of this study was to develop a reliable mouse model of NASH and MetS using a diet containing cholesterol, saturated fat and carbohydrate that is reflective of Western diets of North Americans. Experimental design: We used adult male C57BL/6 J 4- to 5-week-old mice and administered a solid diet containing 0.2% cholesterol, 45% of its calories from fat, with 30% of the fat in the form of partially hydrogenated vegetable oil. We also provided carbohydrate largely as high-fructose corn syrup equivalent in water. In a separate cohort, we gave the identical diet in the absence of cholesterol. Glucose and insulin tolerance testing was conducted throughout the feeding period. The feeding was conducted for 16 weeks, and the mice were sacrificed for histological analysis, markers of MetS, liver inflammation, circulating lipids, as well as liver staining for fibrosis and alpha smooth muscle actin (α-SMA). Results: We found that cholesterol significantly increased serum leptin, interleukin-6, liver weight and liver weight/body weight ratio, fibrosis and liver α-SMA. Conclusions: Mice administered a diet accurately reflecting patterns associated with humans afflicted with MetS can reliably replicate features of MetS, NASH and significant liver fibrosis. The model we describe significantly reduces the time by several months for development of stage 3 hepatic fibrosis.

Keywords: Fibrosis; NASH; Adipocytokine; Cholesterol; Fructose

Nonalcoholic fatty liver disease (NAFLD) has increased in concordance with rates of obesity and is a key component of the metabolic syndrome (MetS). According to the Centers for Disease Control, the United States has seen a sharp increase in the prevalence of obesity from 14%–17% in 1994 to ≥26.0% in 2007 of the adult population. The data collected from the National Health and Nutrition Examination Survey reveal that the number of Americans classified as obese (body mass index ≥30) continues to increase, with the greatest increases occurring in adult men (30.4%) [1]. At present, targeted treatment for NAFLD-related liver disease or the fibrosis that leads to cirrhotic changes is not available. In part, our inability to target treatment for these significant clinical issues is related to our inability to replicate MetS and nonalcoholic steatohepatitis (NASH) with hepatic fibrosis in mice.

Nonalcoholic fatty liver disease is a spectrum of liver disorders classified on histological criteria. These include NASH as well as the more benign lesion, nonalcoholic fatty liver (NAFL), a bland steatosis, or fatty infiltration of hepatocytes. Roughly 20% of NAFLD patients may progress to have hepatic lesions including mixed lobular inflammation, hepatocyte degeneration or "ballooning," and pericentral deposition of fibrillar collagen [2–4]. These features are hallmarks of NASH, along with Mallory bodies. The histological classification of NASH has been standardized by the National Institutes of Health NAFLD Clinical Research Network and is referenced [5]. Data from population-based studies have consistently demonstrated that most cases of NAFL in humans do not progress, although the ability to predict in which patients progression does occur is limited at present [6–8]. Together, these findings represent increased risk for cirrhosis, clinical end-stage liver disease and hepatocellular carcinoma [3,6,9]. In patients with the diagnosis of NASH, a significantly greater proportion (perhaps up to one third) will develop cirrhosis. NASH-related cirrhosis is currently the third leading indication for liver transplantation in the United States; however, NASH-related cirrhosis will likely surpass chronic alcoholism and chronic hepatitis C infection as the major indication for orthotopic liver transplantation.

There is a growing body of evidence linking a Western diet [high in saturated fat, trans-saturated fatty acids (trans-fat) and table sugar] with the increasing incidence of NASH [10]. In addition, diets

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containing trans-fat and high-fructose corn syrup (HFCS) have been shown independently to increase insulin resistance, total body fat mass, and liver lipid accumulation in rodent and human livers [10–14]. A major stumbling block for investigators, however, is to recapitulate a murine model which fulfills three major criteria. The first is that the rodent develops MetS. That is, the animal develops hypertension, dyslipidemia and, most importantly, insulin resistance. NAFLD is the hepatic manifestation of MetS. The second is a rodent model that precipitates hepatic fibrosis in the setting of MetS. Finally, the diet needs to mimic what Westerners, in this case Americans, eat. Many investigators have studied hepatic fibrosis using the methionine–choline deficiency diet, but in so doing fail to provide the most common phenotypic scenario in human NAFLD, which is insulin resistance. Nonetheless, creating a NASH phenotype in a mouse model which concomitantly develops MetS has been exceedingly challenging and required using supraphysiologic quantities of fat in rodent chow for 6 or more months, making studies to elucidate NASH-related mechanisms of hepatic fibrosis time consuming, inefficient and costly for investigators.

We recently reported data in a mouse model on a diet high in saturated fat and trans-fat: the American Lifestyle-Induced Obesity Syndrome feeding model, introduced by Tetri et al., which mimicked the Western-diet-induced pathophysiology of MetS observed in humans [15–17]. In our previous studies, we demonstrated that administering this diet to 4- to 5-week-old male mice induced hepatic insulin resistance within 6 weeks and systemic insulin resistance within 8 weeks, and recapitulated the features of MetS including dyslipidemia, significant weight gain, hepatic steatosis and high blood pressure [16,17]. However, we failed to induce hepatic fibrosis in studies as long as 16 weeks. Recent studies by Min et al. showed that increased hepatic synthesis and dysregulation of cholesterol are associated with the severity of NASH [18]. Additional studies by Savard et al. utilized a 30-week feeding model with 1% cholesterol to examine the effect cholesterol in developing NASH; their results showed a significant increase in inflammation and perisinusoidal fibrosis [19]. Consequently, the aim of this study was to develop a mouse model that included all of the components of a diet typical of Americans, and induced MetS and NASH with induced fibrosis by 16 weeks while maintaining a diet that was in proportion (in terms of fat and cholesterol) to what average Americans consume on a daily basis.

1. Research design and methods

1.1. Animal studies

Seventy-two male C57BL/6 J mice were obtained from Jackson Laboratories (Bar Harbor, ME, USA). Four- to 5-week-old male mice were cared for in accordance with protocols approved by the Animal Care and Use Committee of Emory University. Animals were housed in laboratory cages at 23°C under a 12-h light/dark cycle. Mice were fed either standard chow, the high-fat, trans-fat (HFTF) diet as described by Tetri et al. or the HFTF diet plus 0.2% cholesterol (HFTFX) (Harlan Teklad custom diet TD.120330) [15–17]. The HFTF and HFTFX diets derive 45% of their calories from fat, with 30% of the fat in the form of partially hydrogenated vegetable oil (28% saturated, 57% monounsaturated fatty acids, 13% polyunsaturated fatty acids; HFTF custom diet TD06303, Harlan Teklad) [15]. The HFTF and HFTFX mice were also given HFCS equivalents in rodent drinking water at 42 g/L (55% fructose, 45% glucose w/w). The HFTFX diet also contained 2.0 g/kg of cholesterol. Food and water consumption was measured by weighing new and remaining food and water three times weekly. At the onset and throughout the study, fasting blood samples were obtained. At necropsy, liver and fat samples were snap-frozen in liquid nitrogen and stored at −80°C.

1.2. Pathology

Tissues were prepared as described previously [9,16,17]. Briefly, liver was removed, weighed and divided into three samples for cryosection, formalin fixation and frozen samples. Hematoxylin and eosin stain (formalin fixed embedded in paraffin), Sirius red (Sigma-Aldrich, St. Louis, MO, USA), Masson Trichrome (Thermo Scientific) and immunohistochemistry were performed on the cryosections [9,16,17,20]. Frozen samples were used for oil red O staining and performed as described Mehlem et al. [21]. Hydroxyproline colorimetric assay (BioVision, Milpitas, CA, USA) was performed as described by the manufacturer on frozen samples. Visceral fat was removed, weighed and stored at −80°C.

1.3. Glucose and insulin tolerance testing

For the glucose tolerance test, mice were fasted for 8 h. Glucose (2 g/kg) was then administered intraperitoneally using a 31-gauge insulin syringe. Glucose levels were measured at 0, 15, 30, 60, 90 and 120 min by tail vein sampling with portable glucometer. Insulin tolerance was measured as described previously [15]. Briefly, mice were fasted for 6 h and injected intraperitoneally with 0.6 U/kg human regular insulin at a concentration of 0.2 U/ml with a 31-gauge insulin syringe. Glucose levels were measured by tail vein sampling with a portable glucometer at 0, 15, 30, 45 and 60 min.

1.4. Blood chemistry

Blood drawn from the submandibular vein was used to measure alanine aminotransferase (ALT), aspartate aminotransferase (AST), total cholesterol and serum triglycerides. Lipids were measured on the CX7 chemistry autoanalyzer (Beckman Coulter Diagnostics, Miami, FL, USA). Adipokines were measured using the Milliplex Map Mouse Serum Adipokine panel (EMD Millipore, Billerica, MA, USA).

1.5. Statistical analysis

The data are presented as means±standard error of the mean (S.E.M.). Statistical analysis was performed using JMP v.8.01 (SAS Institute, Cary, NC, USA). All data were initially analyzed using one-way analysis of variance. The Student's *t* test was also used to determine difference between groups.

2. Results

2.1. Both diets result in obesity and insulin resistance in mice

C57BL6 mice fed for 16 weeks on either the HFTF or HFTFX diet were significantly heavier (33%–38%; $P<.05$) than mice fed the standard laboratory chow (Fig. 1A and Table 1). Both cohorts also had significantly higher fasting blood glucose than mice fed standard chow (Fig. 1B–C). Additionally, both glucose tolerance and insulin tolerance tests demonstrated that both the HFTF and HFTFX mice were insulin resistant; both groups had significantly larger area under the curve values (AUC) than the control group (Fig. 1B–C). There was no significant difference in AUC values between the high-fat-fed groups. The addition of cholesterol was shown to have no appreciable effect on body weight, insulin sensitivity or glucose disposal.

2.2. Cholesterol — independent of visceral adiposity — significantly impacts liver weight and morphology

Mice fed either the HFTF or HFTFX diet had significantly more visceral fat than mice fed standard chow ($P<.0001$), and this trend persisted even when adjusted for body weight (Table 1).