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BASIC AND TRANSLATIONAL—LIVER

Hepatic Free Cholesterol Accumulates in Obese, Diabetic Mice and Causes Nonalcoholic Steatohepatitis

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BACKGROUND & AIMS: Type 2 diabetes and nonalcoholic steatohepatitis (NASH) are associated with insulin resistance and disordered cholesterol homeostasis. We investigated the basis for hepatic cholesterol accumulation with insulin resistance and its relevance to the pathogenesis of NASH. **METHODS:** *Alms1* mutant (*foz/foz*) and wild-type NOD.B10 mice were fed high-fat diets that contained varying percentages of cholesterol; hepatic lipid pools and pathways of cholesterol turnover were determined. Hepatocytes were exposed to insulin concentrations that circulate in diabetic *foz/foz* mice. **RESULTS:** Hepatic cholesterol accumulation was attributed to upregulation of low-density lipoprotein receptor via activation of sterol regulatory element binding protein 2 (SREBP-2), reduced biotransformation to bile acids, and suppression of canalicular pathways for cholesterol and bile acid excretion in bile. Exposing primary hepatocytes to concentrations of insulin that circulate in diabetic *Alms1* mice replicated the increases in SREBP-2 and low-density lipoprotein receptor and suppression of bile salt export pump. Removing cholesterol from diet prevented hepatic accumulation of free cholesterol and NASH; increasing dietary cholesterol levels exacerbated hepatic accumulation of free cholesterol, hepatocyte injury or apoptosis, macrophage recruitment, and liver fibrosis. **CONCLUSIONS: In obese, diabetic mice, hyperinsulinemia alters nuclear transcriptional regulators of cholesterol homeostasis, leading to hepatic accumulation of free cholesterol; the resulting cytotoxicity mediates transition of steatosis to NASH.**

Keywords: Lipotoxicity; LRH-1; Bsep; Liver Damage.

Nonalcoholic fatty liver disease (NAFLD) is highly prevalent in all contemporary societies. It represents a pathologic spectrum, across which the most common manifestation, simple steatosis, rarely progresses to cirrhosis or hepatocellular carcinoma. However, ~25% of cases also exhibit substantial hepatocellular injury and inflammation, known as nonalcoholic steatohepatitis (NASH), which causes liver fibrosis that can progress to cirrhosis, liver failure, and hepatocellular carcinoma.[1,2] NASH is invariably linked to insulin resistance and hyperinsulinemia and associates strongly with type 2 diabetes (~50% of cases) and metabolic syndrome (85% of cases). The pathogenesis of NASH is now conceptualized as a response to lipotoxicity, but the lipotoxic molecule(s) involved have not been clarified. Currently most in favor are free fatty acids (FFAs),[3] but 2 lipidomic studies have shown increased hepatic cholesterol levels in patients with NASH,[4,5] while mechanistic studies have implicated free cholesterol (FC)[6] or macrophages activated by FC[7] in hepatocyte injury and liver inflammation.

We previously characterized a line of obese, diabetic mice that simulate Alström syndrome, a monozygotic form of childhood obesity associated with type 2 diabetes, NASH, and cirrhosis.[8] After 12 weeks on a high-fat (HF) (0.2% cholesterol) diet, *Alms1* mutant (*foz/foz*) mice on NOD.B10 background develop hyperinsulinemia, diabetes, hypercholesterolemia, and hypoadiponectinemia, in which changes precede or accompany transformation of steatosis to NASH.[9] In preliminary studies, we noted extraordinarily high hepatic total cholesterol levels in *foz/foz* mice with NASH but not in similar lines with simple steatosis. Further, there was no correlation with FFAs, diacylglycerides, or ceramide. We have now used liver samples from our earlier publication[9] to explore the temporal relationships between hepatic cholesterol fractions and pathways of hepatic cholesterol turnover, including a focus on the transcriptional regulators of cholesterol and bile acid (BA) metabolism. We then tested whether insulin was responsible for some or all of the observed changes by direct experiments in primary murine hepatocytes. Finally, we used dietary interventions to deplete or accen-

Abbreviations used in this paper: ABC, adenosine triphosphate–binding cassette; ACAT, acyl-CoA cholesterol/cholesteryl transferase; BA, bile acid; Bsep, bile salt export pump; CE, cholesteryl ester; Cyp, cytochrome P450; FC, free cholesterol; FFA, free fatty acid; FXR, farnesoid X receptor; HF, high-fat; HMGR, 3-hydroxy-3-methyl-glutaryl-CoA reductase; IHC, immunohistochemistry; LDLR, low-density lipoprotein receptor; LRH-1, liver receptor homologue 1; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; Shp, small heterodimer partner; SR-B1, scavenger receptor B1; SREBP, sterol regulatory element binding protein; WT, wild-type.

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tuate hepatic cholesterol stores and showed that hepatic FC accumulation is causally related to the severity of NAFLD/NASH.

Materials and Methods

Animals and Diets

All experiments were approved by the ANU Animal Experimentation Ethics Committee. Only female mice were used. *Foz/foz* (*Alms1* mutant) and wild-type (WT) littermates (8 weeks old) were fed chow (5% fat, 67% carbohydrate, 19% protein, 0% cholesterol) or HF (23% fat, 45% carbohydrate, 20% protein, 0.2% cholesterol) diets (Specialty Feeds, Glen Forrest, WA) ad libitum for 12 or 24 weeks. The first part of the present experiments used tissue from animals reported in an earlier study.[9] Group n values were as follows: 12-week mice, wild-type chow and HF diet (n = 6), *foz/foz* chow (n = 7), and HF diet (n = 10); 24-week mice, n = 5 per group. In a second experiment, female *foz/foz* (n = 8–9) and WT littermates (n = 7–11) were fed HF diet containing 0.0%, 0.2%, or 2.0% (wt/wt) cholesterol for 24 weeks. At experimental end points, mice were fasted (4 hours) and serum/tissues were harvested.

Serum and Hepatic Lipid Analyses

Serum biochemistry was assessed using automated techniques (Clinical Chemistry, ACT Pathology). Hepatic FFA, FC, and neutral (esterified) lipids were quantitated using high-performance liquid chromatography as described[10] and results normalized to wet liver weight (g).

Histologic Analyses

Blinded H&E-stained liver sections were scored by a hepatopathologist (M.M.Y.) using the NAFLD activity score.[11] Sirius red–stained liver sections were used to quantify collagen fibers by image analysis (ImageJ, Bethesda, MD).

Quantitative Analysis of Gene Expression

Gene expression was quantified using real-time polymerase chain reaction as previously described.[9] Primers are presented in Supplementary Table 1.

Protein Quantitation

Nuclear protein was extracted from liver tissue using NE-PER nuclear/cytoplasmic extraction (Thermo, Rockford, IL). Nuclear or whole liver proteins were quantitated using antibodies in Supplementary Table 2. Enhanced chemiluminescence images were captured (LAS-4000; FujiFilm, Tokyo, Japan) and quantitated (MultiGauge V3.0; FujiFilm), and values were expressed relative to heat shock protein 90 or TATA box-binding protein and normalized to WT chow levels.

3-Hydroxy-3-Methyl-Glutaryl-CoA Reductase Activity

3-Hydroxy-3-methyl-glutaryl-CoA reductase (HMGR) activity was assessed in hepatic microsomes by a radiometric assay.[12]

Immunohistochemistry

Tissue sections underwent antigen retrieval (10 mmol/L sodium citrate, pH 6.0) and antibody labeling (Supplementary Table 2) using the IHC Select DAB Kit (Millipore, Billerica, MA). A minimum of 6 random high-power fields was quantitated for each section. Positive staining was normalized to number of hepatocyte nuclei.

Primary Hepatocyte Culture

Primary hepatocytes were isolated from 6-week-old female WT mice[13] and seeded onto rat tail collagen-coated (Gibco, Carlsbad, CA) plates (5 $\mu g/cm^2$). Hepatocytes were cultured in Williams' E medium containing 1% bovine serum albumin (Sigma-Aldrich, St Louis, MO) at $\sim 6.5 \times 10^4$ viable cells/cm^2. For insulin studies, hepatocytes were grown (48 hours) in the presence of bovine pancreatic insulin (Sigma-Aldrich) at concentrations of 0.2, 6.5, and 13.0 ng/mL, which corresponds to previously measured fasting serum insulin concentrations in WT and HF-fed *foz/foz* mice at 12 and 24 weeks, respectively.[9]

Statistical Analyses

Data (mean ± SEM) were analyzed by analysis of variance, with Tukey post hoc testing (version 17.0; SPSS, Inc, Chicago, IL). Histologic assessments were analyzed using Kruskal–Wallis test and group comparisons with Mann–Whitney *U* test. $P < .05$ was considered significant.

Results

Changes in Hepatic Cholesterol Fractions During Development of NASH

As previously reported,[9] hepatomegaly occurs in HF-fed *foz/foz* mice by week 12 of dietary intake, whereas liver weights remain normal in chow-fed *foz/foz* and WT groups. Following onset of diabetes in HF-fed *foz/foz* mice, steatosis evolves to NASH with fibrosis between 12 and 24 weeks of HF intake.[9] Further analysis showed that HF diet (which contains 0.2% cholesterol) increased hepatic cholesteryl ester (CE) fractions ~200-fold at 12 weeks and a further ~50-fold by 24 weeks in *foz/foz* mice compared with diet-matched WT controls ($P < .0001$; Figure 1*A*). Whereas hepatic FC levels increased in both HF-fed *foz/foz* and WT mice at 12 weeks (Figure 1*B*), values in WT mice returned to chow-fed controls by 24 weeks. At this time, when fibrotic NASH was established in HF-fed *foz/foz* mice, hepatic FC level was significantly higher than in HF-fed WT mice ($P = .027$). In this work, oxysterol metabolites could be detected in some samples but generally were below the assay limit for quantitative detection and there were no evident differences between experimental groups.

The Hepatic FC Uptake Pathway, LDLR, Is Increased in HF-Fed foz/foz *Mice*

Three pathways for hepatic cholesterol uptake are the scavenger receptor B1 (SR-B1), the low-density lipoprotein receptor (LDLR), and cluster differentiation protein 36. We earlier reported up-regulation of cluster differentiation protein 36 expression in HF-fed *foz/foz* mice with NASH.[9] In the present studies, SR-B1 was down-regulated in HF-fed *foz/foz* mice compared with HF-fed WT controls ($P = .03$; Supplementary Figure 1*A*), but LDLR, the major transporter responsible for FC uptake, was significantly increased at both 12 and 24 weeks ($P = .001$; Figure 1*C*). Immunohistochemistry (IHC) illustrated dramatic overexpression of LDLR in mice with NASH, characterized by an extension from physiologic vascular

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