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RESEARCH ARTICLES

Isocaloric manipulation of macronutrients within a high-carbohydrate/moderate-fat diet induces unique effects on hepatic lipogenesis, steatosis and liver injury

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Abstract

Diets containing excess carbohydrate and fat promote hepatic steatosis and steatohepatitis in mice. Little is known, however, about the impact of specific carbohydrate/fat combinations on liver outcome. This study was designed to determine whether high-energy diets with identical caloric density but different carbohydrate and fat composition have unique effects on the liver. Four experimental diets were formulated with 60% kcal carbohydrate and 20% kcal fat, each in nearly pure form from a single source: starch-oleate, starch-palmitate, sucrose-oleate and sucrose-palmitate. The diets were fed to mice for 3 or 12 weeks for analysis of lipid metabolism and liver injury. All mice developed hepatic steatosis over 12 weeks, but mice fed the sucrose-palmitate diet accumulated more hepatic lipid than those in the other three experimental groups. The exaggerated lipid accumulation in sucrose-palmitate-fed mice was attributable to a disproportionate rise in hepatic *de novo* lipogenesis. These mice accrued more hepatic palmitate and exhibited more evidence of liver injury than any of the other experimental groups. Interestingly, lipogenic gene expression in mice fed the custom diets did not correlate with actual *de novo* lipogenesis. In addition, *de novo* lipogenesis rose in all mice between 3 and 12 weeks, without feedback inhibition from hepatic steatosis. The pairing of simple sugar (sucrose) and saturated fat (palmitate) in a high-carbohydrate/moderate-fat diet induces more *de novo* lipogenesis and liver injury than other carbohydrate/fat combinations. Diet-induced liver injury correlates positively with hepatic *de novo* lipogenesis and is not predictable by isolated analysis of lipogenic gene expression.

Keywords: Fatty liver; Sugar; Sucrose; Saturated fat; Palmitate; Steatohepatitis; Triglyceride

1. Introduction

Chronic overconsumption of calories is well known to result in obesity, insulin resistance and end-organ damage to the pancreas, heart and liver [1]. Obesity-related health complications, however, can vary widely among individuals despite the presence of similar risk factors. This is certainly true of obesity-related fatty liver disease, in which hepatic steatosis and steatohepatitis do not always correlate directly with body mass index or insulin resistance [2]. Although some of this variability in liver outcome has been attributed to genetic differences, there is also evidence that the composition of the diet can have a unique influence on the liver. The latter point has been made in experimental animals in which diets enriched in specific macronutrients such as fructose, saturated fat, transfat or cholesterol cause more severe liver disease than diets without these ingredients [3–7]. Importantly, animal studies that have compared different diets side by side to address the effect of specific nutrients on the pathogenesis of fatty liver disease have not always been carefully controlled for energy content. This makes it difficult to dissect the impact of diet composition from that of overall energy intake.

One experimental model of fatty liver disease that has been used as a platform for evaluating the hepatotoxicity of individual macronutrients is the methionine-choline-deficient (MCD) diet model. MCD diets are completely devoid of methionine and choline, which leads to hepatic steatosis by inhibiting phospholipid synthesis, impairing VLDL assembly and preventing the normal export of hepatic lipids [8,9]. MCD feeding also causes steatohepatitis, characterized by severe hepatocellular injury and inflammation; histologically, MCD-mediated liver disease

Abbreviations: ALT, alanine aminotransferase; CPT1, carnitine palmitoyltransferase 1; DAG, diacylglycerol; DNL, *de novo* lipogenesis; FAS, fatty acid synthase; FFA, free fatty acid; GC-MS, gas chromatography-mass spectrometry; MCD, methionine-choline-deficient; MIDA, mass isotopomer distribution analysis; MUFA, monounsaturated fatty acids; NASH, nonalcoholic steatohepatitis; PUFA, polyunsaturated fatty acids; SCD1, stearoyl-CoA desaturase 1; SFA, saturated fatty acids; TAG, triacylglycerol; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

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resembles nonalcoholic steatohepatitis (NASH) in humans [10,11]. Isocaloric manipulation of individual nutrients within the MCD diet has revealed that the type of dietary carbohydrate has a profound impact on liver disease. Specifically, simple sugar must be present in the MCD formula for it to induce steatohepatitis. When sugar is replaced with complex carbohydrate, the ability of the diet to induce liver injury is markedly reduced [12,13].

Experiments have shown that dietary sugar promotes MCD-mediated liver injury by stimulating *de novo* lipogenesis (DNL) and the production of palmitate, a cytotoxic long-chain saturated fatty acid [12]. Because MCD-fed mice cannot efficiently export hepatic lipid, this newly synthesized palmitate accumulates in the liver and causes hepatocellular injury. Whether dietary sugar is more hepatotoxic than complex carbohydrate under non-MCD conditions has not been rigorously tested. Also uncertain is how the adverse effects of dietary sugar on the liver are influenced by the accompanying type of dietary fat. The objective of this study was to address these questions by feeding mice diets similar in macronutrient content to the MCD formula (60% kcal carbohydrate, 20% kcal fat) but with methionine and choline repleted. The carbohydrate and fat composition of these diets was then customized to enable direct comparisons of liver outcome in response to four unique macronutrient pairs (two different carbohydrates×two different fats). The results indicate that, in the presence of adequate methionine and choline, all four custom diets induced hepatic steatosis, but the formula that paired simple sugar (sucrose) with saturated fat (palmitate) promoted the most hepatic lipid accumulation and liver injury. As in the MCD model, steatosis and liver injury correlated positively with the amount of hepatic DNL induced by the diet.

2. Materials and methods

2.1. Animals and diets

Adult male C3H/HeOuJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed in the University of California San Francisco vivarium for a minimum of 2 weeks before starting experimental diets. The mean weight of the mice at the start of the diet study was 25.7±0.3 g. In the study protocol, mice were fed either chow (PicoLab 5053, LabDiet, Inc., St. Louis, MO) or custom high-energy diets comprising 60% kcal carbohydrate, 20% kcal fat and 20% kcal protein (Dyets Inc., Bethlehem, PA). The detailed composition of the custom diets is shown in Table 1. Mice were maintained on experimental diets for 3 or 12 weeks. Animals were weighed and food consumption was measured twice weekly. Mice were fasted for 4 h prior to killing. Procedures for animal care and euthanasia followed the guidelines set by the American Veterinary Association, and all protocols were approved by the Committee on Animal Research at the University of California San Francisco.

2.2. Serum chemistries

Alanine aminotransferase (ALT), glucose and lipids were measured in mouse serum using an ADVIA 1800 autoanalyzer (Siemens Healthcare Diagnostics, Deerfield, IL) in the clinical chemistry laboratory at San Francisco General Hospital.

2.2. Histology and immunohistochemistry

Paraffin sections of liver tissue were stained with hematoxylin and eosin. Slides were viewed blindly by a pathologist and scored for steatosis, ballooning and inflammation as described by Kleiner *et al.*[14]. Cell death was evaluated in liver sections by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) (ApopTag Plus Peroxidase *In Situ* Apoptosis Detection Kit; Millipore, Billerica, MA). Counting of TUNEL-positive cells was performed manually in 10 microscopic fields per liver, each measuring 0.4 mm^2. Data were reported as the average number of cells per microscopic field. Hepatocyte ballooning was demonstrated qualitatively by immunoperoxidase staining for cytokeratin-8 (Thermo Fisher Scientific, Rockford, IL) [15].

2.3. Quantitation of hepatic lipids

Lipids were extracted from fresh liver tissue by the Folch method [16]. Total triglyceride was measured spectrophotometrically as previously described (TR0100; Sigma Chemical Co., St. Louis, MO) [17]. Adjacent pieces of liver tissue were flash-frozen in liquid nitrogen for fatty acid analysis by the Mouse Metabolic Phenotyping Center at Vanderbilt University. Fatty acid profiles were analyzed in the free fatty acid (FFA), diacylglycerol (DAG) and triacylglycerol (TAG) compartments by gas–liquid chromatography.

Table 1
Composition of the four custom dietary formulas

	Starch-Oleate	Starch-Palmitate	Sucrose-Oleate	Sucrose-Palmitate
Protein (g/kg)				
L-Arginine (free base)	**6.3**	**6.3**	**6.3**	**6.3**
L-Histidine (free base)	**4.5**	**4.5**	**4.5**	**4.5**
L-Lysine	**16.1**	**16.1**	**16.1**	**16.1**
L-Methionine	**2.0**	**2.0**	**2.0**	**2.0**
L-Tyrosine	**9.2**	**9.2**	**9.2**	**9.2**
L-Tryptophan	**2.1**	**2.1**	**2.1**	**2.1**
L-Phenylalanine	**8.7**	**8.7**	**8.7**	**8.7**
L-Cysteine	**3.7**	**3.7**	**3.7**	**3.7**
L-Threonine	**6.6**	**6.6**	**6.6**	**6.6**
L-Leucine	**15.3**	**15.3**	**15.3**	**15.3**
L-Isoleucine	**8.4**	**8.4**	**8.4**	**8.4**
L-Valine	**9.9**	**9.9**	**9.9**	**9.9**
Glycine	**3.1**	**3.1**	**3.1**	**3.1**
L-Proline	**20.4**	**20.4**	**20.4**	**20.4**
L-Glutamic acid	**36.2**	**36.2**	**36.2**	**36.2**
L-Alanine	**4.5**	**4.5**	**4.5**	**4.5**
L-Aspartic acid	**11.3**	**11.3**	**11.3**	**11.3**
L-Serine	**9.4**	**9.4**	**9.4**	**9.4**
Carbohydrate (g/kg)				
Cornstarch	**587.9**	**587.9**	**0.0**	**0.0**
Dyetrose	**50.0**	**50.0**	**50.0**	**50.0**
Sucrose	**0.0**	**0.0**	**587.9**	**587.9**
Cellulose	**30.0**	**30.0**	**30.0**	**30.0**
Fat (g/kg)				
Tripalmitin	**0.0**	**100.0**	**0.0**	**100.0**
High-oleate (85%) sunflower oil	**100.0**	**0.0**	**100.0**	**0.0**
Additives (g/kg)				
Salt mix #210030	**35.0**	**35.0**	**35.0**	**35.0**
Sodium bicarbonate	**7.4**	**7.4**	**7.4**	**7.4**
Vitamin mix #310025	**10.0**	**10.0**	**10.0**	**10.0**
Choline chloride	**2.0**	**2.0**	**2.0**	**2.0**
Total (g/kg)	**1000.0**	**1000.0**	**1000.0**	**1000.0**
Protein	**18%**	**18%**	**18%**	**18%**
Carbohydrate	**64%**	**64%**	**64%**	**64%**
Fat	**10%**	**10%**	**10%**	**10%**
Fiber	**3%**	**3%**	**3%**	**3%**

2.4. Measurement of DNL

DNL was measured in the liver and adipose tissue *in vivo* by the use of ^2H_2O labeling combined with mass isotopomer distribution analysis (MIDA) as described previously [18–20]. Mice were injected with 99.8% ^2H_2O in saline (30 ml/kg ip) and placed on drinking water containing 8% ^2H_2O for 3 days before killing. This yielded ^2H_2O enrichment in plasma of 4.2±0.04%, a value that was constant across all dietary groups. At the time of killing, lipids were extracted from tissues and separated by thin-layer chromatography. Triglycerides were retrieved and transesterified by incubation with 3 N methanolic HCl. Fatty acid methyl esters were separated from the glycerol fraction and analyzed by gas chromatography-mass spectrometry (GC-MS) using Agilent (Santa Clara, CA) DB-225ms fused silica columns (30 m length, 0.25 mm ID, 0.25 μm film thickness) with helium carrier gas at 1 ml/min.

Methyl-palmitate and its isotopes (m/z 270–272 representing M_0–M_2) were quantified using electron ionization mode with selected ion monitoring and MIDA calculations were based on 22 possible sites for deuterium incorporation [21,22]. The proportion of deuterium-labeled palmitate in hepatic triglyceride was reported as fractional DNL. The absolute amount of palmitate retained in the tissue over the labeling period was calculated by multiplying fractional DNL by the total amount of palmitate in the triglyceride compartment of the tissue.

2.5. Incorporation of dietary palmitate into hepatic lipid

Twenty-four hours before killing, mice were gavaged with 10 mg $^2H_{31}$-palmitic acid (Cambridge Isotope Laboratories, Tewksbury, MA) dissolved in 200 μl olive oil and returned to their experimental diets. At the time of killing, liver triglycerides were extracted and processed as described above for DNL. $^2H_{31}$-Labeled methyl-palmitate was measured by GC-MS as a percent of total palmitate [23]; m/z 301 was monitored to calculate the proportion of $^2H_{31}$-palmitate in the sample. The $^2H_{31}$-palmitate peak exhibited a shift in GC retention time relative to the natural palmitate-methyl ester and had to be monitored accordingly. Abundance-matched standard curves of $^2H_{31}$-palmitate were run concurrently to quantify true isotopic enrichment. The raw data were then used to calculate the amount of $^2H_{31}$-labeled palmitate that would have been recovered from