Dietary sucrose is essential to the development of liver injury in the methionine-choline-deficient model of steatohepatitis

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Abstract Methionine-choline-deficient (MCD) diets cause steatohepatitis in rodents and are used to study the pathophysiology of fatty liver disease in human beings. The most widely used commercial MCD formulas not only lack methionine and choline but also contain excess sucrose and fat. The objective of this study was to determine whether dietary sucrose in the MCD formula plays a role in the pathogenesis of MCD-related liver disease. We prepared two custom MCD formulas, one containing sucrose as the principal carbohydrate and the other substituting sucrose with starch. Mice fed the sucrose-enriched formula developed typical features of MCD-related liver disease, including hepatic steatosis, hepatocellular apoptosis, alanine aminotransferase elevation, lipid peroxidation, and hepatic inflammation. In contrast, mice fed MCD-starch were significantly protected against liver injury. MCD-sucrose and MCD-starch mice displayed identical diet-related abnormalities in hepatic fatty acid uptake and triglyceride secretion. Hepatic de novo lipogenesis and triglyceride synthesis, however, were 2 times higher in MCD-sucrose mice than MCD-starch mice (P < 0.01). Hepatic lipid analysis revealed accumulation of excess saturated fatty acids in MCD-sucrose mice that correlated with hepatocellular injury.‡ Overall, the results indicate that dietary sucrose is critical to the pathogenesis of MCD-mediated steatohepatitis. They suggest that saturated fatty acids, which are products of de novo lipogenesis, are mediators of hepatic toxicity in this model of liver disease.—Pickens, M. K., J. S. Yan, R. K. Ng, H. Ogata, J. P. Grenert, C. Beysen, S. M. Turner, and J. J. Maher. Dietary sucrose is essential to the development of liver injury in the methionine choline-deficient model of steatohepatitis. J. Lipid Res. 2009. 50: 2072–2082.

Supplementary key words fatty acid • fatty liver • apoptosis • de novo lipogenesis

Diet deprived of methionine and choline [methionine-choline-deficient (MCD)] cause hepatic steatosis and inflammation that mimics nonalcoholic steatohepatitis in human beings (1–3). MCD feeding reportedly induces hepatic steatosis through a dual process involving enhanced uptake of fatty acids by the liver as well as impaired secretion of hepatic triglyceride (TG) (2). Stimulation of fatty acid uptake in MCD-fed animals correlates with hepatic upregulation of fatty acid transport proteins. Suppression of hepatic TG secretion is due to the reduced availability of methionine and choline for phospholipid synthesis, which is critical to the formation of TG-rich VLDL particles (4, 5). Notably, commercial MCD formulas (MP Biomedicals, Harlan Teklad, and Dyets) not only lack methionine and choline but are also enriched in sucrose and fat. These nutrients can themselves stimulate hepatic lipid accumulation (6–8); thus, they may accentuate the hepatic lipid accumulation caused by methionine and choline deprivation alone.

The role of dietary fat as a determinant of liver injury in the MCD model has been the subject of recent investigation. Somewhat unexpectedly, studies have shown that the fat content of the MCD formula can be varied over a wide range (10% to 40% of calories) without any impact on the amount of TG that accumulates in the liver (9, 10). Even more surprising is that dietary fat does not affect MCD-mediated hepatocellular injury based on biochemical or

Abbreviations: ALT, alanine aminotransferase; ChREBP, carbohydrate responsive element binding protein; CYP4a10, cytochrome P450 4a10; DNL, de novo lipogenesis; DAG, diacylglycerol; MCD, methionine-choline-deficient; MCS, methionine choline-sufficient; SCD-1, stearoyl-CoA desaturase-1; SFA, saturated fatty acid; SREBP-1, sterol regulatory element binding protein-1; TAG, triacylglycerol; TRARS, thiobarbituric acid-reactive substances; TG, triglyceride; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; XBP-1, X-box protein-1.

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The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of one figure.
histologic criteria (10). Dietary fat does influence MCD-mediated hepatic lipid peroxidation and inflammatory cytokine induction. As would be expected, MCD formulas enriched in polyunsaturated fat cause the highest levels of lipid peroxidation and provoke hepatic inflammation (10).

The role of dietary sucrose in the pathogenesis of MCD-mediated liver disease is unknown. Sucrose stimulates de novo lipogenesis (11–13) and thus has the potential to contribute to hepatic steatosis and steatohepatitis in the MCD model. The objective of this study was to determine the importance of dietary sucrose to the hepatotoxicity of the MCD formula. We addressed this by feeding mice two different MCD formulas, one that contained sucrose as the principal source of carbohydrate and the other containing starch in place of sucrose. Mice fed the sucrose-based MCD formula developed the characteristic MCD-related features of hepatic steatosis and steatohepatitis. By contrast, mice fed the starch-based MCD formula exhibited some of the features of MCD-fed mice but developed no steatosis or steatohepatitis. The principal difference between the two groups was that the starch-based diet suppressed hepatic lipogenesis and TG synthesis. This was accompanied by reduced hepatic accumulation of saturated fatty acids and significant protection against hepatocellular injury.

### MATERIALS AND METHODS

#### Dietary studies

Adult male C3H/HeOuJ mice (The Jackson Laboratory, Bar Harbor, ME) were fed methionine-choline-sufficient (MCS) or MCD formulas as described in Table 1 for 21 days. All formulas were custom manufactured to include 18% protein, 64% carbohydrate, and 10% fat by weight (Dyets, Bethlehem, PA). Two of the four formulas used sucrose as the principal dietary carbohydrate (590 g/kg; designated MCS-sucrose and MCD-sucrose); the other two used cornstarch in place of sucrose (590 g/kg; designated MCS-starch and MCD-starch). Paired MCS and MCD formulas were matched in all nutrients except L-methionine and choline chloride. Mice were fed the custom formulas ad libitum or steatohepatitis. The principal difference between the two groups was that the starch-based diet suppressed hepatic lipogenesis and TG synthesis. This was accompanied by reduced hepatic accumulation of saturated fatty acids and significant protection against hepatocellular injury.

#### Serum chemistries

Alanine aminotransferase (ALT), glucose, cholesterol, and TG 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. Serum insulin, leptin, and adiponectin were measured by ELISA (Millipore-Linco, St. Charles, MO).

#### TG and fatty acid analysis

Lipids were extracted from fresh liver tissue using the Folch method (14). Extracts were evaporated under a stream of nitrogen and resuspended in chloroform:methanol (2:1) containing 0.01% butyraldehyde hydroxytoluene. Aliquots were dried and resuspended in 1-butanol containing 0.01% butyraldehyde hydroxytoluene for measurement of total TG (TR0100; Sigma Chemical, St. Louis, MO). Results were reported as milligrams of TG per gram liver.

Fatty acid analysis was performed on flash-frozen liver tissue. Lipid extraction and TrueMass® neutral lipid analysis were performed by Lipomics Technologies (West Sacramento, CA). Tissue samples were subjected to a combination of liquid- and solid-phase extraction procedures to separate neutral lipids from phospholipids, followed by thin-layer chromatography to separate neutral lipid classes and gas chromatography to quantitate individual fatty acids. All samples were processed in the presence of internal standards to monitor extraction efficiency and verify measurement accuracy.

#### Measurement of hepatic lipid peroxidation

Lipid peroxidation was evaluated by measuring thiobarbituric acid-reactive substances (TBARS) in liver homogenates. Liver tissue was homogenized in 1.15% potassium chloride containing 2 mM deferroxamine; TBARS were measured as described by Jozwik et al. (15). Results were expressed as nanomoles of TBARS per milligram of liver.

#### Measurement of hepatic fatty acid uptake

Fatty acid uptake by the liver was monitored in vivo using radiolabeled oleic acid as a tracer. After an overnight fast with free access to drinking water, mice were gavaged with 200 μl olive oil containing 2 μCi 1-14C-oleic acid (57 mCi/mmol; GE Healthcare, Piscataway, NJ). Blood was collected at 0, 30, and 240 min to verify absorption. At 240 min, the mice were killed for collection of liver tissue. Radioactivity was measured in serum and liver homogenates by scintillation counting. Uptake was expressed as cpm 14C/liver/4 h.

#### Measurement of hepatic TG secretion

Hepatic TG secretion was measured by monitoring the rise in serum TG resulting from administration of Triton WR-1339, which blocks lipoprotein lipase (16). After an overnight fast with free access to drinking water, mice were injected with 500 mg/kg Triton WR-1339 [10% (w/v), 5 ml/kg IV]. Blood was collected at 0 and 6 h. TG was measured in serum using an ADVIA autoanalyzer as described above.

#### Measurement of de novo lipogenesis, hepatic TG synthesis, and glycolytic contribution to hepatic TG-glycerol

De novo lipogenesis (DNL), TG synthesis, and the contribution of glycolysis to TG-glycerol synthesis were measured in the liver in vivo by the use of 3H2O labeling combined with mass isotopomer distribution analysis as described previously (17–19). Mice were injected with saline prepared with 99.8% 2H2O (30 ml/kg IP) and maintained at steady state by administration of drinking water containing 8% 3H2O. After 3 days of heavy water labeling, mice were fasted for 4 h and euthanized. Lipids were extracted from fresh liver tissue by the Folch method (14). TGs were separated by TLC and transesterified by incubation with 3 N methanolic HCl. Fatty acid methyl esters were separated from the glycerol fraction, and both fractions were analyzed by GC-MS.

Methyl palmitate and its isotopes (m/z 270–272, representing M0–M2) were quantified under the selected ion-monitoring mode, and mass isotopomer distribution analysis calculations were based on 22 possible sites for deuterium incorporation (20, 21). The proportion of deuterium-labeled palmitate in hepatic TG was reported as fractional DNL. The absolute amount of palmitate retained in the liver over the 3-day labeling period was...
Neutralization of glucose-derived triacylglycerols by 5-deoxy-5-(1,2-di-O-hexadecanoyl)-D-erythro-pentrose, a potent inhibitor of fat accumulation.

Paraffin sections of liver tissue were stained with hematoxylin and eosin. Slides were blindly evaluated and scored for steatosis, ballooning, and inflammation. Steatosis (0–4): 0 = absent; 1 = mild (focal involving fewer than three hepatocytes); 2 = moderate (focal involving more than three hepatocytes or multifocal); 3 = severe (multifocal with more than two foci of three or more hepatocytes). Inflammation (0–4): 0 = absent; 1 = minimal (zero to one focus per 20× field); 2 = mild (two foci); 3 = moderate (three foci); 4 = severe (four or more foci). A combined activity score was calculated as the arithmetic sum of all individual scores.

Liver histology and quantitative scoring system

Paraffin sections of liver tissue were stained with hematoxylin and eosin. Slides were blindly evaluated and scored for steatosis, ballooning, and inflammation. Steatosis (0–4): 0 = absent; 1 = mild (focal involving fewer than three hepatocytes); 2 = moderate (focal involving more than three hepatocytes or multifocal); 3 = severe (multifocal with more than two foci of three or more hepatocytes). Inflammation (0–4): 0 = absent; 1 = minimal (zero to one focus per 20× field); 2 = mild (two foci); 3 = moderate (three foci); 4 = severe (four or more foci). A combined activity score was calculated as the arithmetic sum of all individual scores.

The results showed that the MCD-sucrose formula did not affect its influence on body weight, serum glucose, or serum triglycerides. Commercial MCS and MCD formulas (e.g., MP Biomedicals #960439 and Harlan Teklad #90262) typically contain 65% carbohydrate by weight, provided as a 70:30 mixture of sucrose and cornstarch (46% sucrose and 19% starch). In this experiment, we prepared custom MCS and MCD formulas in which nearly the entire carbohydrate fraction (59%) was composed of either pure sucrose or pure cornstarch. A small amount of complex carbohydrate was retained in each formula to permit compounding into pellets (Table 1). Mice fed the sucrose- or starch-enriched formulas exhibited many typical responses to MCS and MCD feeding. Specifically, MCS-fed mice gained weight and MCD-fed mice lost weight, respectively, and exhibited serum leptin levels that paralleled their adipose tissue mass (Table 2). In addition, MCS-fed mice developed hyperglycemia and hyperlipidemia, whereas MCD-fed mice remained normoglycemic and developed hypolipidemia, and MCD-fed mice were more insulin sensitive than MCS controls (1, 26, 27). For the most part, the biochemical abnormalities caused by the MCS and MCD formulas were comparable regardless of their carbohydrate content. The only exception was serum cholesterol, which was diminished to a lesser degree in MCD-starch mice than MCD-sucrose mice. Serum cholesterol was lower in starch-fed control (MCS) mice than sucrose-fed control mice, consistent with previous reports documenting the hypocholesterolemic nature of complex dietary carbohydrate (28–30). Why this same effect was not observed in the MCD groups with different dietary carbohydrate is unknown.

Quantitation of apoptotic cells in mouse liver sections

Apoptotic cells were identified in tissue sections by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) (ApopTag Plus peroxidase in situ apoptosis detection kit; Millipore, Billerica, MA). Sections were counterstained with methyl green for viewing and photog-
controls ($P < 0.0001$). In striking contrast to the liver disease that developed in mice fed the MCD-sucrose formula, hepatic abnormalities were much less prominent in mice fed the starch-enriched MCD formula for 21 days. MCD-starch mice accumulated more hepatic fat than MCD-starch controls, but much less than MCD-sucrose mice. Serum ALT was only mildly elevated in MCD-starch mice, and they displayed almost no hepatocellular ballooning, apoptosis, or inflammation, achieving a combined histologic activity score of only $0.8 \pm 0.3$ ($P < 0.0001$ vs. MCD-sucrose).

**Hepatic steatosis in MCD-sucrose mice is attributable to de novo lipogenesis**

In an effort to explain why the MCD-sucrose and MCD-starch formulas led to such marked differences in hepatic lipid accumulation, we investigated whether the two diets have divergent effects on hepatic lipogenesis. As expected, MCD feeding stimulated fatty acid uptake by the liver, but there was no difference in uptake between the expected, MCD feeding stimulated fatty acid uptake by the liver, but there was no difference in uptake between the MCD-sucrose and MCD-starch groups (Fig. 2A). MCD feeding also suppressed hepatic TG secretion (Fig. 2B); again, there was no difference between the sucrose- or starch-enriched MCD groups. We then explored whether MCS-sucrose mice and MCS-starch formulas have distinct effects on hepatic lipogenesis. Previous studies have shown that methionine and choline deprivation does not stimulate lipogenic genes in the liver, and, in some instances, suppresses lipogenic genes (27). To test whether dietary carbohydrate influences the effect of MCD feeding on lipogenic gene expression, we measured mRNA encoding carbohydrate-responsive element binding protein (ChREBP), sterol regulatory element binding protein-1 (SREBP-1), X-box protein-1 (XBP-1), FAS, and stearoyl-CoA desaturase-1 (SCD-1) in the livers of MCD-fed mice and their respective controls (Fig. 2C–G) (32–34). Each of these genes was less abundant in MCD mice than MCS controls, and some (SREBP-1, XBP-1, and SCD-1) were suppressed even below the levels measured in Chow-fed mice. Notably, these genes were expressed similarly in MCD-sucrose mice and MCD-starch mice despite their difference in dietary carbohydrate composition. This was true for all except ChREBP, which paradoxically was less abundant in MCD-sucrose than MCD-starch mice. In contrast to lipogenic gene expression, hepatic DNL, measured as the amount of newly synthesized palmitate accumulating in the liver over a 3-day period, was significantly higher in MCD mice than controls (Fig. 2H). The high values for DNL in MCD-fed mice may be due in part to their defect in hepatic lipid export, which promotes the retention of newly synthesized palmitate in the liver and contributes to an elevation in the measurement over the labeling interval. This does not affect comparisons between MCD-sucrose and MCD-starch mice because both groups share the same abnormality in hepatic lipid secretion. Despite

**TABLE 1. Composition of the custom MCS and MCD formulas**

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>MCS-Sucrose</th>
<th>MCS-Starch</th>
<th>MCD-Sucrose</th>
<th>MCD-Starch</th>
</tr>
</thead>
<tbody>
<tr>
<td>t-Amino acids (g/kg)</td>
<td>175.7</td>
<td>175.7</td>
<td>175.7</td>
<td>175.7</td>
</tr>
<tr>
<td>t-Methionine (g/kg)</td>
<td>2.0</td>
<td>2.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Choline chloride (g/kg)</td>
<td>2.0</td>
<td>2.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Sucrose (g/kg)</td>
<td>587.9</td>
<td>0.0</td>
<td>591.9</td>
<td>0.0</td>
</tr>
<tr>
<td>Dextromaltose (g/kg)</td>
<td>30.0</td>
<td>30.0</td>
<td>30.0</td>
<td>30.0</td>
</tr>
<tr>
<td>Cornstarch (g/kg)</td>
<td>0.0</td>
<td>100.0</td>
<td>0.0</td>
<td>100.0</td>
</tr>
<tr>
<td>Cellulose (g/kg)</td>
<td>30.0</td>
<td>30.0</td>
<td>30.0</td>
<td>30.0</td>
</tr>
<tr>
<td>Corn oil (g/kg)</td>
<td>100.0</td>
<td>0.0</td>
<td>100.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Salt mix (g/kg)</td>
<td>35.0</td>
<td>35.0</td>
<td>0.0</td>
<td>35.0</td>
</tr>
<tr>
<td>Sodium bicarbonate (g/kg)</td>
<td>7.4</td>
<td>7.4</td>
<td>7.4</td>
<td>7.4</td>
</tr>
<tr>
<td>Vitamin mix (g/kg)</td>
<td>10.0</td>
<td>10.0</td>
<td>10.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Total (g/kg)</td>
<td>1000.0</td>
<td>1000.0</td>
<td>1000.0</td>
<td>1000.0</td>
</tr>
</tbody>
</table>

**TABLE 2. Clinical and biochemical data from mice fed MCS and MCD formulas**

<table>
<thead>
<tr>
<th>Nutrient</th>
<th>MCS-Sucrose</th>
<th>MCS-Starch</th>
<th>MCD-Sucrose</th>
<th>MCD-Starch</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight change (%)</td>
<td>21.8 ± 2.4</td>
<td>15.0 ± 2.5</td>
<td>-29.1 ± 0.7b</td>
<td>-23.4 ± 0.8c,d</td>
</tr>
<tr>
<td>Liver weight/body weight (%)</td>
<td>4.8 ± 0.2</td>
<td>4.3 ± 0.1</td>
<td>6.3 ± 0.3c</td>
<td>4.3 ± 0.5d</td>
</tr>
<tr>
<td>Adipose weight/body weight (%)</td>
<td>4.6 ± 0.2</td>
<td>4.3 ± 0.3</td>
<td>0.5 ± 0.1c</td>
<td>0.5 ± 0.1c</td>
</tr>
<tr>
<td>Serum TG (mg/dl)</td>
<td>138.3 ± 12.2</td>
<td>135.9 ± 11.2</td>
<td>75.4 ± 2.1bc</td>
<td>81.4 ± 6.7bc</td>
</tr>
<tr>
<td>Serum cholesterol (mg/dl)</td>
<td>261.7 ± 18.6</td>
<td>162.8 ± 5.3ab</td>
<td>85.7 ± 2.8b</td>
<td>101.6 ± 3.0c,d</td>
</tr>
<tr>
<td>Serum glucose (mg/dl)</td>
<td>385.6 ± 42.3</td>
<td>284.3 ± 11.1</td>
<td>173.8 ± 20.0b</td>
<td>209.2 ± 9.7bc</td>
</tr>
<tr>
<td>Serum insulin (ng/ml)</td>
<td>1.04 ± 0.28</td>
<td>1.07 ± 0.23</td>
<td>0.2 ± 0.1b</td>
<td>0.1 ± 0b</td>
</tr>
<tr>
<td>QUICKI</td>
<td>0.18 ± 0.0</td>
<td>0.19 ± 0.01</td>
<td>0.23 ± 0.01b</td>
<td>0.23 ± 0.01b</td>
</tr>
<tr>
<td>Serum leptin (ng/ml)</td>
<td>26.68 ± 5.96</td>
<td>16.88 ± 2.65</td>
<td>0.3 ± 0.1b</td>
<td>0.2 ± 0.1b</td>
</tr>
<tr>
<td>Serum adiponectin (µg/ml)</td>
<td>4.2 ± 0.2</td>
<td>4.5 ± 0.2</td>
<td>5.3 ± 0.4c</td>
<td>4.7 ± 0.5</td>
</tr>
<tr>
<td>Hepatic TG (mg/g liver)</td>
<td>42.9 ± 8.9</td>
<td>29.2 ± 3.3</td>
<td>115.2 ± 11.3b</td>
<td>34.4 ± 2.9c,d</td>
</tr>
<tr>
<td>Serum ALT (IU/l)</td>
<td>56.2 ± 6.2</td>
<td>59.6 ± 7.0</td>
<td>571.3 ± 7.4b</td>
<td>93.0 ± 8.8b,c,d</td>
</tr>
</tbody>
</table>

Values represent mean ± SEM for n = 10. QUICKI, qualitative insulin sensitivity check index = (log insulin [ng/ml] + log glucose [mg/dl])⁻¹.  
* $P < 0.05$ for MCS-starch versus MCS-sucrose.  
* $P < 0.05$ for MCD-sucrose versus MCS-sucrose.  
* $P < 0.05$ for MCD-starch versus MCD-sucrose.  
* $P < 0.05$ for MCD-starch versus MCD-sucrose.
MCD-sucrose mice accumulate more saturated fatty acids in the liver than MCD-starch mice

DNL yields palmitate, a long-chain saturated fatty acid that has been implicated as a mediator of hepatic lipotoxicity (35–38). This is particularly relevant in the MCD model because MCD feeding also suppresses SCD-1, the enzyme that converts long-chain saturated fatty acids to less toxic monounsaturated species (39). The combination of enhanced DNL and suppressed SCD-1 should result in significant accumulation of saturated fatty acid in the liver, an outcome that may be exacerbated even further by the inability of MCD-fed mice to effectively export hepatic TG. This buildup of saturated fatty acid could be the cause of hepatocellular injury in the MCD model. MCD-starch mice are similar to MCD-sucrose mice in SCD-1 expression and hepatic lipid secretion (Fig. 2B, G). DNL in MCS-starch mice, however, is significantly reduced (Fig. 2H). Consequently, MCD-starch mice may exhibit a less saturated hepatic lipid profile than MCD-sucrose mice, which may be an important factor in their protection

TUNEL-positive cells. Mice fed the MCD-sucrose formula displayed prominent hepatic steatosis and inflammatory foci, as well as scattered TUNEL-positive cells. MCD-starch mice showed no significant steatosis, slight inflammation, and rare TUNEL-positive cells. Original magnification: ×10. B: Histograms depict the quantitative histology scores from mice in the four treatment groups. Steatosis, ballooning, and inflammation were all increased in MCD-sucrose mice compared with MCD-starch mice and MCS controls, yielding a high composite histology score. TUNEL-positive cells were significantly more numerous in MCD-sucrose mice than in the other three treatment groups. Values represent mean ± SEM for n = 10. * P < 0.05 for MCD versus carbohydrate-matched MCS. † P < 0.05 for MCD-starch versus MCD-sucrose.
Sucrose is essential to MCD-mediated liver disease

Commercial MCD formulas and has been postulated to play a role in MCD-mediated liver injury (27). The saturated-to-monounsaturated fatty acid ratios were no higher in MCD-sucrose mice than MCD-starch mice (Fig. 3C). This suggests that an absolute, rather than relative, increase in long-chain SFAs in the liver coincides with the development of MCD-mediated liver injury.

Polyunsaturated fat, oxidative stress, and cytokine induction in MCD-sucrose and MCD-starch mice

Our fatty acid analysis demonstrated that in addition to SFA, MCD-sucrose mice accumulated excess PUFA in the liver (FFA 160%, DAG 140%, and TAG 250%) (Fig. 3B). The individual SFAs that accumulated in these livers were the long-chain species palmitate (C16:0) and stearate (C18:0) (see supplementary Fig. 1). Lastly, we calculated the ratio of saturated to monounsaturated long-chain fatty acids in the liver, which increases in mice fed commercial MCD formulas and has been postulated to play a role in MCD-mediated liver injury (27). The saturated-to-monounsaturated fatty acid ratios were no higher in MCD-sucrose mice than MCD-starch mice (Fig. 3C). This suggests that an absolute, rather than relative, increase in long-chain SFAs in the liver coincides with the development of MCD-mediated liver injury.

Fig. 2. Hepatic lipid metabolism in MCS and MCD mice after 21 days on experimental diets. A: Histogram depicts hepatic uptake of 14C-oleate 4 h after oral administration as outlined in Materials and Methods. Uptake was increased in MCD-sucrose and MCD-starch mice compared with control mice, with no difference between the two groups. B: Serum TG levels at 0 and 6 h after IV administration of Triton WR-1339. TG levels increased as expected in control mice but rose much less in MCD mice with no difference between MCD-sucrose and MCD-starch mice. C–G: Hepatic expression of mRNAs encoding SREBP-1, ChREBP, XBP-1, FAS, and SCD-1. All genes were less abundant in MCD-fed mice than MCS controls. There was no difference in expression between MCD-sucrose and MCD-starch except for ChREBP, which was paradoxically lower in MCD-sucrose than MCD-starch mice. H, I: Hepatic DNL and TG synthesis. DNL and TG synthesis were measured in the liver during the final 3 days of experimental feeding as described in Materials and Methods. Both were significantly higher in MCD mice than controls. DNL was reduced by 53% in MCD-starch mice compared with MCD-sucrose mice. TG synthesis was reduced by 46% in MCD-sucrose mice compared with MCD-sucrose mice. J: Origin of TG-glycerol. The relative contribution of glycolysis to hepatic glycerol synthesis was measured as described in Materials and Methods. MCD-sucrose mice displayed the highest value among all dietary groups, 38% higher than MCD-starch mice. K: Serum β-hydroxybutyrate. Mice were fasted for 4 h prior to blood collection. β-Hydroxybutyrate levels were higher in MCD-sucrose mice than all other treatment groups. Values represent mean ± SEM for n = 4 (A, B, and H–J), n = 5 (C–G), or n = 10 (K). * P < 0.05 for MCD versus carbohydrate-matched MCS. ‡ P < 0.05 for MCD-starch versus MCD-sucrose.
on these processes, we measured the hepatic expression of cytochrome P450 4a10 (CYP4a10) in mice fed MCD and control diets as well as hepatic lipid peroxidation and the expression of several pro-inflammatory genes (Fig. 4).

CYP4a10 mRNA was strongly induced in the livers of all MCD mice, without any difference resulting from modulation of dietary carbohydrate. Lipid peroxidation (measured as TBARs), however, was much more evident in MCD-sucrose mice than MCD-starch mice, presumably due to their abundance of hepatic PUFA. These fatty acids could come either from the diet or from adipose tissue, although the diet is more likely, due to the high linoleate content of corn oil used as fat in the MCS and MCD formulas. PUFA may play a role in MCD-related liver disease because of their ability to act as substrates for cytochrome P450-mediated lipid peroxidation. PUFA metabolism causes oxidant stress in the liver, which in turn can stimulate the local production of pro-inflammatory cytokines. To determine the influence of MCD-sucrose and MCD-starch formulas

(see supplementary Fig. 1). These fatty acids could come either from the diet or from adipose tissue, although the diet is more likely, due to the high linoleate content of corn oil used as fat in the MCS and MCD formulas. PUFA may play a role in MCD-related liver disease because of their ability to act as substrates for cytochrome P450-mediated lipid peroxidation. PUFA metabolism causes oxidant stress in the liver, which in turn can stimulate the local production of pro-inflammatory cytokines. To determine the influence of MCD-sucrose and MCD-starch formulas
Sucrose is essential to MCD-mediated liver disease

...the reason why MCD diets provoke significant liver injury has remained elusive. Emerging data indicate that specific dietary nutrients, when coupled with methionine and choline deprivation, can influence MCD-mediated liver disease. This study demonstrates that MCD-mediated steatohepatitis is absolutely dependent on the presence of sucrose in the MCD formula. Dietary sucrose is in fact more important than dietary fat, which can modulate hepatic lipid peroxidation and hepatic inflammation in the MCD model but has no impact on MCD-mediated steatosis or hepatocellular injury (10).

DISCUSSION

Although methionine and choline deprivation is known to cause a number of disturbances in hepatic lipid metabolism (2, 5, 27), the reason why MCD diets provoke significant liver injury has remained elusive. Emerging data indicate that specific dietary nutrients, when coupled with methionine and choline deprivation, can influence MCD-mediated liver disease. This study demonstrates that MCD-mediated steatohepatitis is absolutely dependent on the presence of sucrose in the MCD formula. Dietary sucrose is in fact more important than dietary fat, which can modulate hepatic lipid peroxidation and hepatic inflammation in the MCD model but has no impact on MCD-mediated steatosis or hepatocellular injury (10).

The importance of sucrose to the pathogenesis of MCD-mediated steatohepatitis was established by removing it from the MCD formula. Substituting this simple sugar with...
complex carbohydrate significantly diminished all the features of MCD-mediated liver disease, including hepatic steatosis, hepatocellular apoptosis, lipid peroxidation, and inflammation. This was true even though the starch-enriched MCD formula still provoked several characteristic features of MCD-fed animals, including weight loss and hypolipidemia. Most importantly, mice fed MCD-starch diet never developed overt hepatic steatosis. In the absence of steatosis, there was little additional evidence of liver injury or inflammation.

Substituting sucrose with starch in the MCD formula did not prevent hepatic steatosis by interrupting any of the derangements in hepatic lipid metabolism that are known to be precipitated by MCD feeding. Specifically, dietary starch did not suppress MCD-mediated stimulation of hepatic fatty acid uptake nor did it reverse MCD-related impairment of hepatic TG secretion. Starch also caused no apparent increase in hepatic fatty acid oxidation that could potentially reduce steatosis. Instead, the principal consequence of including starch in the MCD formula was a reduction in hepatic DNL. Starch is a much more potent stimulus to DNL than starch (40–42); thus, the difference in hepatic DNL between MCD-sucrose mice and MCD-starch mice is not surprising. More noteworthy is that the difference was not accompanied by a disparity in lipogenic gene expression between the two dietary groups. This discordance between the metabolic and molecular data indicates that flux through the lipogenic pathway in the liver cannot be predicted solely by gene expression, as noted recently in a study involving adipose tissue (43). Importantly, our metabolic experiments revealed that DNL plays a dominant role in MCD-mediated liver disease. Substituting sucrose with starch in the MCD formula decreased DNL by 53% and hepatic TG synthesis by 55%, resulting in a 66% reduction in hepatic lipid content.

The reduced hepatic DNL in MCD-starch mice coincided with a significant reduction in the amount of long-chain saturated fatty acid present in the liver. This, in turn, was accompanied by a significant diminution of hepatocyte apoptosis and ALT release. These findings point to long-chain saturated fatty acids, produced from dietary sucrose via DNL, as mediators of hepatocyte injury in the MCD model. Long-chain saturated fatty acids are highly toxic to hepatocytes. They kill cells by inducing endoplasmic reticulum stress (38) and activating mitochondrial apoptosis pathways (35–37, 44). Given the prominent hepatocyte apoptosis and the striking ALT elevation that occurred after 21 days of MCD-sucrose feeding, these abnormalities are likely due to saturated fatty acid-induced lipotoxicity. It should be noted that long-chain saturated fatty acids were not the only fatty acids to accumulate in MCD-sucrose livers. PUFAs were also disproportionately increased. Although we cannot entirely exclude a contribution of PUFAs to hepatocyte injury based on the current experiments, other studies from our laboratory suggest that this is not the case. We previously employed dietary means to reduce the PUFAs content of MCD livers to the levels found in MCS controls. This did not result in any improvement in MCD-mediated ALT release or apoptosis (10). Taken together, these findings suggest that excess SFAs rather than excess PUFAs are responsible for hepatocellular injury in mice fed sucrose-enriched MCD formulas. PUFAs may play a separate role in MCD-mediated liver injury, specifically as inducers of cytokines and inflammation. In this study as well as others, the induction of pro-inflammatory cytokines in the livers of MCD mice parallels hepatic lipid peroxidation, which is directly related to hepatic PUFA content (10). Overall, these findings suggest that dietary sucrose is the driving force behind MCD-mediated hepatic lipid accumulation and liver injury, whereas dietary PUFA regulate MCD-mediated hepatic inflammation. In combination, the two nutrients produce the complete picture of MCD-mediated steatohepatitis.

Although the suppression of DNL in MCD-starch mice can explain why they accumulated less hepatic SFA than MCD-sucrose mice, there is no ready explanation why MCD-starch mice also accumulated less hepatic PUFA. This observation suggests that in MCD-sucrose mice, the accumulation of SFA and PUFA is somehow connected, and both are downstream of DNL. One can envision the following scenario: because the saturated fatty acids produced by DNL in MCD-sucrose livers cannot be desaturated due to low SCD-1 activity, the mice are at high risk of hepatocellular lipotoxicity. Esterification into TG could reduce this toxicity (45), but SFA cannot effectively combine with each other to form TG due to fluidity constraints (46). Unsaturated fatty acid is needed to package the SFA into TG; dietary PUFA can be used for this purpose. If dietary PUFAs are in fact used to shunt newly synthesized SFA into the TG compartment, the end result would be TG that contains excess SFA as well as excess PUFA. This fits our experimental observations but requires further study for confirmation. In support of this theory, hepatic TG synthesis has already been shown to be an important defense against MCD-mediated hepatotoxicity. When mice fed a sucrose-rich MCD formula were prevented from producing hepatic TG by suppressing the enzyme diacylglycerol acyltransferase 2, they developed an unusually severe form of MCD-mediated steatohepatitis characterized by extremely high ALT levels (47).

Noteworthy in our experiments was that MCD-sucrose and MCD-starch mice exhibited markedly different outcomes despite similar degrees of methionine and choline deprivation. This indicates that methionine and choline deprivation by itself is not hepatotoxic, at least over a 3-week interval. Indeed, it is remarkable that hepatic phospholipids in MCD mice remain at near-normal levels despite a complete cutoff of exogenous precursors. This implies the existence of strong homeostatic mechanisms to preserve cellular phospholipids under conditions of stress, which could be achieved by regulation of VLDL secretion, biliary phospholipid secretion, or both (48, 49). Ip et al. (50) made a similar observation previously. They noted that MCD-fed mice treated with a peroxisome proliferator-activated receptor α agonist to stimulate fatty acid oxidation did not develop hepatic steatosis or steatohepatitis. In this situation as well, all features of steatohepatitis were eliminated from MCD-fed animals without influencing the...
underlying methionine and choline deficiency. Together, these studies confirm that methionine and choline deficiency is necessary, but not sufficient, to promote steatohepatitis in the MCD model. A second insult in the form of excess sucrose represents the essential first step to the development of hepatic steatosis and ultimately more severe liver disease in the MCD model.

In summary, our experiments demonstrate that dietary sucrose is essential to the pathogenesis of steatohepatitis in the MCD model of liver disease. Sucrose provokes DNL, which yields saturated fatty acids that become trapped in the liver due to an MCD-related defect in hepatic lipid secretion. The accumulation of excess SFA coincides with hepatic steatosis, manifest as ALT release and histologic apoptosis. Dietary PUFA also accumulate in the liver, possibly in an effort to reduce SFA-induced lipotoxicity. They contribute to MCD-mediated liver disease by promoting lipid peroxidation and pro-inflammatory cytokine induction. Substituting sucrose with starch in the MCD formula prevents all the features of MCD-mediated liver disease, including steatosis, apoptosis, lipid peroxidation, and cytokine induction. Starch likely reduces hepatocellular injury in MCD-fed mice by reducing hepatic DNL. The mechanism by which it prevents PUFA accumulation and hepatic inflammation is currently unknown. In human beings with nonalcoholic fatty liver disease, as much as 26% of hepatic lipid is produced by DNL. This study emphasizes the harmful potential of DNL, particularly when the saturated fatty acids produced by this process cannot be detoxified or exported from the liver. It also demonstrates that eliminating simple sugar from the diet can reduce DNL and its toxic consequences in the liver.

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REFERENCES