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Dietary fructose exacerbates hepatocellular injury when incorporated into a methionine-choline-deficient diet

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Abstract

Background—Methionine-choline-deficient (MCD) diets cause steatohepatitis in rodents and are used to model fatty liver disease in human beings. Recent studies have identified sucrose as a major contributor to MCD-related liver disease through its ability to promote hepatic *de novo* lipogenesis.

Aims—To determine whether glucose and fructose, the two constituents of sucrose, differ in their capacity to provoke steatohepatitis when incorporated individually into MCD formulas.

Materials & Methods—MCD and control formulas prepared with either glucose or fructose as the sole source of carbohydrate were fed to mice for 21 days. Liver injury was assessed biochemically and histologically together with hepatic gene expression and fatty acid analysis.

Results—Mice fed MCD formulas developed similar degrees of hepatic steatosis whether they contained glucose or fructose. By contrast, mice fed MCD-fructose developed significantly more hepatocellular injury than mice fed MCD-glucose, judged by histology, apoptosis staining and serum alanine aminotransferase. Liver injury in MCD-fructose mice coincided with an exaggerated rise in the ratio of long-chain saturated to unsaturated fatty acids in the liver. Notably, hepatic inflammation was not enhanced in mice fed MCD-fructose, correlating instead with hepatic lipid peroxidation, which was equivalent in the two MCD groups.

Discussion—Fructose is more cytotoxic than glucose when used as the source of carbohydrate in MCD formulas.

Conclusion—The data suggest the enhanced cytotoxicity of fructose in the MCD model is related to its ability to stimulate *de novo* lipogenesis, which yields harmful long-chain saturated fatty acids.

Keywords

apoptosis; choline; *de novo* lipogenesis; fatty acid; fatty liver; methionine

Diets devoid of methionine and choline (methioninecholine deficient; MCD) cause hepatic steatosis and inflammation that mimics non-alcoholic steatohepatitis in human beings (1–3). MCD feeding induces steatosis because methionine and choline deprivation stimulates the hepatic uptake of fatty acids from the circulation (2) while at the same time preventing the physiological export of triglyceride from the liver in the form of VLDL particles (4, 5). Commonly used commercial MCD formulas not only lack methionine and choline, but also are enriched in sucrose and fat. These nutrients themselves stimulate hepatic lipid accumulation, and thus they have the potential to amplify the fatty liver disease induced by methionine and choline deprivation alone.

Recent studies have shown that dietary sucrose is critical to the hepatotoxicity of the MCD diet (6). Excess sucrose stimulates hepatic lipogenesis, which yields long-chain saturated fatty acids that are directly toxic towards hepatocytes (7–10). Under normal circumstances, toxic saturated fatty acids are rapidly converted to non-toxic monounsaturated fatty acids by the enzyme stearoyl- CoA desaturase-1 (SCD-1) (11). In MCD-fed mice, however, hepatic SCD-1 activity is profoundly suppressed (12), and thus these toxic species are not readily eliminated. Hepatic triglyceride is also inefficiently exported from the livers of MCD-fed mice because of the diet-related defect in hepatic VLDL secretion. This combination of abnormalities results in the accumulation of saturated fatty acids in the liver and the development of liver injury. The importance of dietary sucrose as a trigger to these pathophysiological events was demonstrated by eliminating it from the MCD formula. When sucrose was substituted with complex carbohydrate, hepatic lipogenesis was reduced, saturated fatty acids did not accumulate in the liver and steatohepatitis was prevented (6).

Glucose and fructose, the monosaccharide components of sucrose, both have the capacity to stimulate hepatic lipogenesis (13, 14). Given the importance of lipogenesis to liver injury in the MCD model of steatohepatitis, we wished to determine the comparative effects of each monosaccharide on hepatic outcome. Accordingly, we fed mice MCD formulas containing either glucose or fructose as the sole source of carbohydrate. Mice fed both formulas developed hepatic steatosis and steatohepatitis, but hepatocellular injury was significantly worse in mice fed the fructose-containing formula.

Materials and methods

Dietary studies

Adult male C3H/HeO_uJ mice (The Jackson Laboratory, Bar Harbor, ME, USA) were fed methionine-choline-sufficient (MCS) or methionine-choline-deficient (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, Inc., Bethlehem, PA, USA). Two of the four formulas utilized glucose as the principal dietary carbohydrate (590 g/kg, designated MCS-glucose and MCD-glucose); the other two utilized fructose (590 g/kg, designated MCS-fructose and MCD-fructose). Paired MCS and MCD formulas were matched in all nutrients except L-methionine and choline chloride. Mice were fed the custom formulas *ad libitum* and had free access to drinking water for the 21-day study period. At the end of the experiment, mice were fasted for 4 h before killing. All animals received humane care according to guidelines set forth by the US Public Health Service. All experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of California, San Francisco.

Serum chemistries

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

Triglyceride and fatty acid analysis

Lipids were extracted from fresh liver tissue using the Folch method (15). Extracts were evaporated under a stream of nitrogen and resuspended in chloroform: methanol (2:1) containing 0.01% butyrate hydroxytoluene. Aliquots were dried and resuspended in 1-butanol containing 0.01% BHT for measurement of total triglyceride (TR0100; Sigma Chemical Co., St Louis, MO, USA). Results were reported as mg triglyceride 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 Inc. (West Sacramento, CA, USA). 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 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 deferoxamine; TBARS were measured as described by Jozwik *et al.* (16). Results were expressed as nanomoles of TBARS per mg liver.

Evaluation of gene expression by real-time quantitative polymerase chain reaction

Total RNA was extracted from mouse liver by homogenization in TRI reagent (Molecular Research Center, Cincinnati, OH, USA), followed by chloroform extraction and ethanol precipitation. RNA was incubated with DNase (Qiagen, Inc., Valencia, CA, USA) to remove contaminating DNA; the enzyme was then inactivated and removed according to the manufacturer's specifications (RNeasy, Qiagen). cDNA was synthesized from 1 µg RNA in a reaction mixture containing 2.5 U/µl M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and 5 µM random hexamer primers (Invitrogen).

Real-time PCR analysis was performed using an AB Prism 7900 sequence detection system (Applied Biosystems, Foster City, CA, USA). Assays-on-Demand[®] primer and probe sets (Applied Biosystems) were used for all the genes of interest. The expression of each test gene was normalized to that of mouse β -glucuronidase. Quantitative detection of specific nucleotide sequences was based on the fluorogenic 5' nuclease assay (17). Relative gene expression was calculated using the method of Livak and Schmittgen (18).

Liver histology and quantitative scoring system

Paraffin sections of liver tissue were stained by haematoxylin and eosin. Slides were blindly evaluated and scored for steatosis, ballooning, and inflammation. Steatosis (0–4): 0 = <5%; 1 = 5–25%; 2 = 25–50%; 3 = 50–75%; 4 = 75–100%. Ballooning (0–3): 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 (0–1 focus per 20X field); 2 = mild (two foci); 3 = moderate (three foci); 4 = severe (four or more foci).

Quantitation of apoptotic cells in mouse liver sections

Apoptotic cells were identified in tissue sections by terminal deoxynucleotide transferase-mediated deoxyuridine triphosphate nick end-labelling (TUNEL) (ApopTag Plus Peroxidase *In Situ* Apoptosis Detection Kit; Millipore, Billerica, MA, USA). Sections were counterstained with methyl green for viewing and photography. TUNEL-positive cells were counted in five randomly selected $\times 10$ microscopic fields per liver. The results for each liver were reported as the average number of positive cells in the five fields.

Statistical methods

Experiments included five to 20 mice per study group, with specifics noted in the tables and figure legends. Mean values were compared by two-way analysis of variance with Bonferroni's correction. *P* values < 0.05 were considered statistically significant.

Results

MCD-glucose and MCD-fructose formulas induce similar biochemical and molecular alterations in mice but MCD-fructose causes more hepatocellular injury

To maximize our ability to discern the influence of individual sugars on MCD-related liver disease, we prepared custom MCS and MCD formulas in which 92% of all carbohydrate was composed of either glucose or fructose. A small amount of dextromaltose was retained in each formula to permit compounding into pellets (Table 1). Mice fed the custom formulas for 21 days exhibited many typical responses to MCS and MCD feeding: specifically, those in the two control (MCS) groups gained weight and developed hyperglycaemia and hyperlipidaemia, whereas those in the two MCD groups lost weight and became hypolipidaemic (Table 2) (1, 12, 19). As noted previously in human beings (20), fructose in the control diet caused slightly more hyperglycaemia than glucose. The small increment in glucose between the two groups, as well as the lack of any difference in serum cholesterol and triglyceride between fructose- and glucose-fed control mice, may be related to the short duration of the study. Among MCD-fed mice, both the MCD-fructose and MCD-glucose groups developed hepatic steatosis, as demonstrated by a significant increase in the liver weight/body weight ratio and a significant increase in hepatic triglyceride content relative to carbohydrate-matched MCS controls (Table 2). Notably, there was no difference in hepatic triglyceride content between MCD-glucose and MCD-fructose mice, nor were any differences in circulating lipids resulting from the use of glucose or fructose in the MCD formula.

Commercial (sucrose-based) MCD formulas typically suppress lipogenic gene expression in the liver compared with MCS controls, while having little or no effect on the expression of genes involved in fatty acid oxidation (12). To determine whether this same pattern is observed with glucose- and fructose-based MCD formulas, we compared mRNA levels for several genes involved in lipid metabolism in mice fed our custom MCS and MCD diets (Table 3). Genes pertaining to fatty acid oxidation (long-chain acyl dehydrogenase) (carnitine palmitoyltransferase-1) were unaltered by MCD-glucose or MCD-fructose feeding compared with MCS controls, mimicking the findings reported previously with sucrose-rich MCD formulas. Similarly, genes involved in fatty acid and triglyceride synthesis [fatty acid synthase (FAS)] [stearoyl-CoA desaturase-1 (SCD-1)] were downregulated in MCD-glucose and MCD-fructose mice compared with MCS control mice, as they are in mice fed sucrose-based MCD formulas. Notably in the current experiments, mRNA encoding FAS and SCD-1 were suppressed less dramatically in MCD-fructose mice than they were in MCD-glucose mice (Table 3). This is consistent with a stimulatory effect of fructose relative to glucose on the expression of lipogenic genes (MCS fructose vs. MCS glucose, Table 3) (21). Even so,

the stimulatory effect of fructose could not counterbalance the strong suppressive effect of the MCD diet on lipogenic gene expression, which maintained FAS gene expression below 50% of control levels and SCD-1 expression below 15% of control levels. Although low compared with MCS controls, lipogenic gene expression in MCD mice is sufficient to support fatty acid and triglyceride synthesis in the liver, as has been shown in previously published studies (6, 12).

As predicted by their abundant hepatic triglyceride content, MCD-glucose and MCD-fructose mice displayed marked hepatic steatosis by histology (Fig. 1). Quantitative histological scoring confirmed the equivalent degree of steatosis in the two MCD groups, both exceeding a score of 3 on a scale from 0–4 (Table 4). Unlike the amount of fat, the pattern of fat accumulation differed between MCD-glucose and MCD-fructose mice. MCD-glucose mice exhibited primarily macrovesicular steatosis (80% of animals), whereas MCD-fructose mice exhibited a mixture of macrovesicular and microvesicular steatosis (65% of animals). Hepatocyte ballooning, a marker of cell injury, was slightly more prominent in MCD-fructose than MCD-glucose mice. To better characterize cell death in MCS and MCD livers, we performed TUNEL staining of tissue sections. TUNEL-positive cells were much more abundant in MCD-fructose livers than MCD-glucose livers ($P=0.0006$) (Fig. 2A, B). This was reflected in the serum ALT levels, which were twice as high in MCD-fructose mice as MCD-glucose mice ($P=0.0003$) (Fig. 2C).

The methionine-choline-deficient fructose formula causes disproportionate accumulation of long-chain saturated fatty acids in the liver

Knowing that *de novo* lipogenesis (DNL) is critical to the pathogenesis of liver injury in MCD-fed mice (6), we reasoned that a fructose-enriched MCD formula may be more toxic than a glucose-enriched formula because fructose is a more potent inducer of hepatic DNL than sucrose (22). To test this hypothesis, we analysed the fatty acid content of hepatic lipids in MCS- and MCD-fed mice, paying particular attention to the concentration of long-chain saturated fatty acids that are the immediate products of DNL. Examination of all the neutral lipids in MCS and MCD livers confirmed that hepatic lipid accumulation was equivalent in MCD-glucose and MCD-fructose mice (Fig. 3A) and that the overall profiles of saturated, monounsaturated and polyunsaturated fatty acids were similar (Fig. 3B). The ratio of toxic C16:0 to non-toxic C16:1 fatty acids, however, was markedly increased in all MCD mice relative to MCS controls and was even higher in the MCD-fructose mice than the MCD-glucose mice ($P=0.007$) (Fig. 3C). High saturated-to-monounsaturated C16 fatty acid ratios were observed in every neutral lipid compartment (Fig. 3D). The greatest differences between MCD-glucose and MCD-fructose mice were in diacylglycerols, triacylglycerols and phospholipids. The reason this discrepancy was not apparent in the overall hepatic profile of saturated fatty acids is that other saturated fatty acid species (ranging from C14:0 through C22:0) were not significantly different between MCD-glucose and MCD-fructose mice (data not shown).

The methionine-choline-deficient fructose formula causes the same amount of hepatic inflammation as the methionine-choline-deficient glucose formula

Despite evidence of increased hepatocyte injury in mice fed MCD-fructose compared with MCD-glucose, the amount of inflammation in the liver was similar or even lower in MCD-fructose mice than MCD-glucose mice (Fig. 1 and Table 3). The inflammatory score in MCD-fructose mice was 1.45 compared with 1.90 in MCD-glucose mice ($P=0.07$), which coincided with a milder induction of pro-inflammatory genes in the MCD-fructose group. In an earlier report, we demonstrated that hepatic inflammation in MCD-fed mice correlates with oxidant stress and lipid peroxidation in the liver rather than hepatocyte injury (23). To investigate this relationship in MCD-glucose and MCD-fructose mice, we measured hepatic

expression of the gene that encodes cytochrome P450 4a10 (CYP4a10), a microsomal enzyme that promotes the production of oxidants in the liver. CYP4a10 mRNA was markedly induced in both groups of MCD mice, but to a slightly lesser degree in MCD-fructose mice. (Fig. 4A). Lipid peroxidation products were also readily identified in the livers of MCD-glucose- and MCD-fructose-fed mice with no significant difference between the two groups (Fig. 4B). When lipid peroxidation products were plotted as a function of the histological inflammatory score in all MCD-fed mice regardless of dietary sugar, a linear relationship was obtained ($P < 0.05$) (Fig. 4C).

Discussion

Dietary sugar is a critical mediator of liver damage in the MCD model of fatty liver disease (6, 23). The toxic potential of sugar lies in its conversion within the liver to long-chain saturated fatty acids, via DNL (6). Long-chain saturated fatty acids are lethal to hepatocytes in culture (9, 10); they are also hepatotoxic *in vivo*, particularly when they cannot be efficiently packaged into triglyceride molecules (24–26). Given the importance of DNL to the hepatotoxicity of dietary sugar, it follows that the ability of any individual dietary sugar to cause liver injury should coincide with its potency as an inducer of DNL. The current experiments confirm this by demonstrating that fructose, a robust inducer of DNL, is significantly more hepatotoxic than glucose.

The close connection between DNL and liver injury in the MCD model is noteworthy because MCD diets do not stimulate lipogenic gene expression in the liver (6, 12). Instead, MCD feeding prevents the products of hepatic lipogenesis (saturated fatty acids) from being exported from the liver, which permits their accumulation over time. Superimposed upon this MCD-mediated derangement in hepatic lipid secretion, the type of carbohydrate incorporated into the MCD formula can influence lipogenic gene expression. This can independently affect the hepatic content of saturated fatty acids and influence the severity of liver injury. Among the dietary monosaccharides, fructose has been shown to induce lipogenic gene expression in the liver more strongly than glucose (21). Our experiments confirmed this, by demonstrating that SCD-1 and FAS mRNA levels were significantly higher in MCD-fructose mice than MCD-glucose mice even though the absolute level of expression of both genes was low in the setting of methionine and choline deprivation. Importantly, the incremental increase in hepatic lipogenesis stimulated by dietary fructose in MCD-fed mice explained the disproportionate rise in hepatic saturated fatty acid content in the MCD-fructose group as well as the increased liver injury in this group compared to mice fed MCD-glucose.

Glucose is a less potent stimulus to lipogenic gene expression than fructose, but it still promotes hepatic lipogenesis (21) and acts as a substrate for hepatic DNL (27). Thus, it was not surprising that mice fed MCD formulas containing glucose also developed hepatic steatosis. Based on their differential ability to stimulate lipogenesis, however, we predicted that glucose-enriched MCD formulas would cause less steatosis than MCD-fructose formulas. Contrary to our expectation, MCD-glucose-fed mice developed just as much hepatic steatosis as MCD-fructose-fed mice. The comparable degree of steatosis between the two groups suggests that any differences in hepatic fat accumulation that may have resulted from diet-induced variations in hepatic lipogenesis were obscured by fat accumulating in the liver from other sources. In support of this theory, the majority of fatty acids in the livers of MCD-fed mice were polyunsaturated species (Fig. 3B). These presumably derived from the polyunsaturate-rich corn oil (17% saturated fatty acids; 24% monounsaturated fatty acids; 59% polyunsaturated fatty acids) that was used as the dietary fat in the MCD formula. The lack of a perceptible difference in hepatic steatosis between the two groups despite a significant difference in liver-related outcome supports the notion that hepatic triglyceride

per se is not a major determinant of liver injury. Instead, hepatocellular damage is likely to result from fatty acids that cannot be incorporated into triglyceride (24, 25).

Importantly in our study, the sugar composition of the MCD formula did not significantly influence hepatic inflammation even though it affected hepatocellular injury. This is intriguing, because the death of liver cells is considered an important harbinger of hepatic inflammation and even fibrosis (28–32). Previous work from our laboratory has shown that in MCD-fed mice, hepatic inflammation correlates with hepatic lipid peroxidation; lipid peroxidation, in turn, is a function of the polyunsaturated fat content of the MCD formula (23). In the current study, the MCD-sucrose and MCD-fructose formulas contained identical amounts of polyunsaturated fat and provoked equivalent degrees of hepatic lipid peroxidation. Minor variations in hepatic inflammation between the two groups were attributable to minor variations in lipid peroxidation among individual animals (Fig. 4C). The fact that the degree of hepatic inflammation did not correlate with the severity of hepatocellular injury in MCD-sucrose and MCD-fructose mice does not completely discount a contribution of liver cell death to hepatic inflammation; indeed, when hepatocyte death is prevented in MCD-fed mice, hepatic inflammation also decreases (6). It is possible in the case of sucrose and fructose that both sugars cause sufficient hepatocellular injury to provoke some hepatic inflammation, but the difference in cell death between the two groups is inadequate to prompt a significant difference in inflammation. Whether dietary fructose, through its ability to provoke excess hepatocellular injury, stimulates excess liver fibrosis could not be addressed in our study. The reason is that MCD-mediated weight loss precludes experimentation beyond three weeks, which is essential for the evaluation of fibrosis. Nevertheless, data from the Non-Alcoholic Steatohepatitis Clinical Research Network suggest a link between dietary fructose and hepatic fibrosis. They indicate that fructose consumption is a risk factor for liver cell death (ballooning) and hepatic fibrosis in human beings (33). Regarding hepatic steatosis as a potential marker of disease severity, glucose and fructose did induce unique patterns of hepatic steatosis in MCD-fed mice with mixed macrovesicular and microvesicular fat predominating in MCD-fructose-fed mice. Whether the smaller hepatic lipid droplets observed in mice consuming MCD-fructose are indicators of disease severity is uncertain. In support of this theory, microvesicular steatosis has been reported to portend severe hepatocyte injury in many clinical settings (34). Moreover, in adipose tissue, small lipid droplets often coincide with active lipolysis, which yields free fatty acids and diacylglycerols that can cause cellular dysfunction and death (35–37). Further study will be required to determine whether microvesicular steatosis is indicative of enhanced triglyceride lipolysis in MCD-fructose-fed mice and whether lipolysis contributes to the pathogenesis of liver injury.

Studies addressing glucose and fructose metabolism in human beings have yielded results similar to those in experimental animals, specifically, that fructose is a more potent inducer of hepatic lipogenesis than glucose (38–40). In healthy individuals, newly synthesized hepatic lipids are efficiently exported as VLDL, and thus fructose feeding typically causes hyperlipidaemia and weight gain before it promotes hepatic steatosis (20). Still, fructose can rapidly cause fatty liver when consumed in great excess (two to three times the average daily consumption of Americans) (41). Our data indicate that fructose-induced hepatic steatosis has serious adverse effects on liver cell survival. These findings provide a scientific rationale for the epidemiological observation that fructose overconsumption as an important risk factor for the development of fatty liver disease in human beings (33, 42).

In summary, fructose is more hepatotoxic than glucose when incorporated isocalorically into an MCD diet that causes retention of hepatic lipids. The enhanced liver injury in fructose-fed mice coincides with enhanced accumulation of long-chain saturated fatty acids in the liver, which is commensurate with the fact that fructose is a more potent inducer of DNL

than glucose. Although the results of this study are correlative, they supplement a growing body of evidence that the products of DNL can cause significant liver disease (6, 24, 43). They suggest that limiting fructose consumption will be a critical strategy in the effort to avert fatty liver disease in human beings.

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Abbreviations

ALT	alanine aminotransferase
CE	cholesterol ester
CYP4a10	cytochrome P450 4a10
DAG	diacylglycerol
DNL	<i>de novo</i> lipogenesis
FAS	fatty acid synthase
FFA	free fatty acid
MCD	methionine-choline deficient
MCS	methionine-choline sufficient
MUFA	monounsaturated fatty acid
PL	phospholipid
PUFA	polyunsaturated fatty acid
SCD-1	stearoyl-CoA desaturase-1
SFA	saturated fatty acid
TAG	triacylglycerol
TBARS	thiobarbituric acid-reactive substances
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labelling

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Fig. 1.

Liver histology in mice fed methionine-choline-sufficient (MCS) or methionine-choline-deficient (MCD) formulas for 21 days. (A) Haematoxylin and eosin-stained liver sections from mice fed MCS or MCD formulas for 21 days. Livers from mice fed the two control formulas (MCS-glucose, MCS-fructose) appear normal. Livers from mice fed the two MCD formulas (MCD-glucose, MCD-fructose) display hepatic steatosis and inflammation. MCD-glucose livers have predominantly macrovesicular steatosis (M), whereas MCD-fructose livers have both macrovesicular (M) and microvesicular (m) fat. Clusters of inflammatory cells are marked by arrows. (B) Oil red O stains of liver sections from the four dietary groups as shown in (A). The photomicrographs highlight the macrovesicular steatosis in

MCD-glucose mice and the mixed macro- and microvesicular steatosis in MCD-fructose mice. Original magnification $\times 10$.

Fig. 2.

Markers of hepatocellular injury in mice fed methionine-choline-sufficient (MCS) or methionine-choline-deficient (MCD) formulas for 21 days. (A) Photomicrographs illustrate TUNEL staining of liver sections from mice fed glucose- or fructose-enriched formulas for 21 days. MCD-fructose livers displayed more terminal deoxynucleotide transferase-mediated deoxyuridine triphosphate nick end-labelling (TUNEL)-positive cells than MCD-glucose livers, and MCS-control livers had no TUNEL-positive cells (arrowheads highlight TUNEL-positive cells in MCD-glucose livers). Original magnification $\times 10$. (B) Histogram shows quantitation of TUNEL-stained cells in liver sections from the four dietary groups. TUNEL-positive cells were found almost exclusively in MCD-fed mice, with a significantly

greater number of stained cells in MCD-fructose livers than MCD-glucose livers. (C) Histogram depicts serum alanine aminotransferase (ALT) levels in the four dietary groups. ALT levels were normal in MCS control mice, but markedly abnormal in MCD-fed mice. ALT was two times higher in MCD-fructose mice than MCD-glucose mice. Values represent mean $\pm$ SE for $n = 10$ (MCS-glucose, MCS-fructose, MCD-glucose) or $n = 20$ (MCD-fructose). * $P < 0.05$ for MCD vs. carbohydrate-matched MCS. ‡ $P < 0.05$ for MCD-glucose vs. MCD-fructose.

Fig. 3.

Hepatic lipid analysis in MCS and MCD mice at 21 days. (A) Histogram depicts hepatic neutral lipid content in mice fed glucose- or fructose-enriched formulas for 21 days. Total hepatic lipid was markedly increased in MCD-fed mice but was unaffected by dietary sugar composition. (B) Histogram depicts total hepatic fatty acids categorized by degree of saturation: saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA). All species were elevated in MCD-fed mice regardless of dietary sugar composition. (C) Histogram shows the ratio of saturated to monounsaturated long-chain fatty acids (C16:0-C16:1) in the liver among the 4 dietary groups. The ratio is significantly increased in MCD mice compared with MCS controls, and higher in MCD-fructose mice than MCD-glucose mice. (D) Histogram shows the ratio of C16:0-C16:1 fatty acids in the liver categorized by hepatic lipid compartment. The ratios in MCD-fructose mice exceeded those in MCD-glucose mice in DAG, TAG and PL. Values represent mean $\pm$ SE for $n=5$. * $P < 0.05$ for MCD vs. carbohydrate-matched MCS controls. ‡ $P < 0.05$ for MCD-glucose vs. MCD-fructose. CE, cholesterol esters; DAG, diacylglycerols; FFA, free fatty acids; MCD, methionine–choline deficient; MCS, methionine–choline sufficient; PL, phospholipids; TAG, triacylglycerols.

Fig. 4.

Oxidant stress and inflammation in the livers of methionine-choline sufficient (MCS) and methionine-choline deficient (MCD) mice at 21 days. Histograms illustrate hepatic cytochrome P450 4a10 (CYP 4a10) mRNA expression (A) and thiobarbituric acid-reactive substances (TBARS) (B) after 21 days on glucose- or fructose-enriched diets. Legend is identical to that in Figures 2 and 3. CYP4a10 expression was markedly induced in MCD-fed mice compared with MCS controls, but there was no difference between the MCD-glucose and MCD-fructose groups. Coincident with the upregulation of CYP4a10, hepatic TBARS were markedly elevated in MCD-glucose and MCD-fructose mice. Again, no difference was noted between the two MCD groups with different dietary carbohydrate composition. Values represent mean $\pm$ SE for $n=5$ (mRNA) and $n=10$ (TBARS). $\ddagger P < 0.05$ for MCD vs. carbohydrate-matched MCS. (C) Graph depicts hepatic TBARS as a function of hepatic inflammation, judged by histological inflammatory score. The relationship is linear ($P < 0.05$).

Table 1

Composition of the custom methionine-choline-sufficient and methionine-choline-deficient formulas

Nutrient	MCS-glucose	MCS-fructose	MCD-glucose	MCD-fructose
L-amino acids (g/kg)	175.7	175.7	175.7	175.7
L-methionine (g/kg)	2.0	2.0	0.0	0.0
Choline chloride (g/kg)	2.0	2.0	0.0	0.0
Glucose (g/kg)	587.9	0.0	591.9	0.0
Fructose (g/kg)	0.0	587.9	0.0	591.9
Dextromaltose (g/kg)	50.0	50.0	50.0	50.0
Cellulose (g/kg)	30.0	30.0	30.0	30.0
Corn oil (g/kg)	100.0	100.0	100.0	100.0
Salt mix (g/kg)	35.0	35.0	35.0	35.0
Sodium bicarbonate (g/kg)	7.4	7.4	7.4	7.4
AIN-93 vitamin mix (g/kg)	10.0	10.0	10.0	10.0
Total (g/kg)	1000.0	1000.0	1000.0	1000.0

MCD, methionine-choline-deficient; MCS, methionine-choline-sufficient.

Table 2

Clinical and biochemical data from mice fed methionine-choline-sufficient and methionine-choline-deficient formulas

	MCS-glucose, <i>n</i> = 10	MCS-fructose, <i>n</i> = 10	0MCD-glucose, <i>n</i> =10	MCD-fructose, <i>n</i> = 20
Body weight change (%)	+ 22.9 ± 2.5	+23.0 ± 2.9	-25.7 ± 1.0 ^a	-24.3 ± 0.7 ^b
Liver weight /body weight (%)	4.3 ± 0.1	4.5 ± 0.1	6.0 ± 0.2 ^a	6.2 ± 0.1 ^b
Gonadal fat weight/body weight (%)	3.6 ± 0.4	4.1 ± 0.2	0.4 ± 0.1 ^a	0.6 ± 0.1 ^b
Serum triglyceride (mg/dl)	147.4 ± 9.9	119.5 ± 11.1	71.5 ± 5.7 ^a	72.1 ± 2.1 ^b
Serum cholesterol (mg/dl)	168.5 ± 10.0	175.2 ± 9.7	70.0 ± 3.3 ^a	72.3 ± 2.2 ^b
Serum glucose (mg/dl)	286.4 ± 19.9	329.2 ± 19.2	136.9 ± 11.5 ^a	146.5 ± 7.9 ^b
Serum β-hydroxybutyrate (mg/dl)	0.55 ± 0.05	0.38 ± 0.04	0.94 ± 0.09 ^a	1.13 ± 0.11 ^b
Hepatic triglyceride (mg/g liver)	26.3 ± 3.4	24.0 ± 3.2	101.3 ± 8.4 ^a	99.9 ± 5.0 ^b

Values represent mean ± SE.

^a*P*<0.05 for MCD-glucose vs. MCS-glucose.

^b*P*<0.05 for MCD-fructose vs. MCS-fructose.

MCD, methionine-choline-deficient; MCS, methionine-choline-sufficient.

Table 3

Hepatic gene expression in mice fed methionine-choline-sufficient and methionine-choline-deficient formulas

Pathway	Gene	MCS-glucose	MCS-fructose	MCD-glucose	MCD-fructose
Fatty acid oxidation	LCAD	1.00 ± 0.05	0.91 ± 0.06	1.14 ± 0.14	1.09 ± 0.14
	CPT-1	1.00 ± 0.07	0.86 ± 0.05	1.35 ± 0.18	1.01 ± 0.12
Lipogenesis	FAS	1.00 ± 0.18	2.53 ± 0.44 ^a	0.42 ± 0.06 ^b	0.73 ± 0.17 ^b
	SCD-1	1.00 ± 0.19	2.33 ± 0.38 ^a	0.01 ± 0.00 ^b	0.13 ± 0.02 ^{b,c}
Inflammation	TNF	1.00 ± 0.13	1.41 ± 0.25	6.04 ± 0.76 ^b	3.44 ± 0.19 ^{b,c}
	CXCL2	1.00 ± 0.06	1.24 ± 0.41	7.67 ± 0.85 ^b	3.22 ± 0.31 ^{b,c}

Gene expression was normalized to β-glucuronidase and expressed as a relative value compared with the MCS-glucose group as a control. Values represent mean ± SE for *n* = 5. LCAD, long-chain acyl dehydrogenase; CPT-1, carnitine palmitoyltransferase-1; FAS, fatty acid synthase; SCD-1, stearoyl CoA desaturase-1. ND = not determined.

^a *P* < 0.05 for MCS-fructose vs. MCS-glucose.

^b *P* < 0.05 for MCD vs. sugar-matched MCS.

^c *P* < 0.05 for MCD-glucose vs. MCD-fructose.

MCD, methionine-choline-deficient; MCS, methionine-choline-sufficient.

Table 4

Histology scores from mice fed methionine-choline-sufficient and methionine-choline-deficient formulas

	<u>MCS-glucose</u>	<u>MCS-fructose</u>	<u>MCD-glucose</u>	<u>MCD-fructose</u>
	<i>n</i> = 10	<i>n</i> = 10	<i>n</i> = 10	<i>n</i> = 20
Steatosis (0–4)	0.5±0.2	0.7±0.2	3.4±0.3 ^a	3.4±0.3 ^b
Steatosis type (macro, micro, mixed)	NA	NA	Macro (80%) Mixed (20%)	Mixed (65%) Macro (35%)
Ballooning (0–3)	0.0±0.0	0.2±0.2	0.5±0.2	1.2±0.3 ^{b,c}
Inflammation (0–4)	0.00±0.0	0.14±0.14	1.90±0.26 ^a	1.45±0.15 ^b

Values represent mean ± SE.

^a*P* < 0.05 for MCD-glucose vs. MCS-glucose.^b*P* < 0.05 for MCD-fructose vs. MCS-fructose.^c*P* < 0.05 for MCD-glucose vs. MCD-fructose.

MCD, methionine-choline-deficient; MCS, methionine-choline-sufficient; NA, not applicable.