Steatohepatitis develops rapidly in transgenic mice overexpressing *Abcb11* and fed a methionine-choline-deficient diet

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Sundaram, Shikha S., Peter F. Whitington, and Richard M. Green. Steatohepatitis develops rapidly in transgenic mice overexpressing *Abcb11* and fed a methionine-choline-deficient diet. Am J Physiol Gastrointest Liver Physiol 288: G1321–G1327, 2005. First published January 13, 2005; doi:10.1152/ajpgi.00455.2004.—Nonalcoholic fatty liver disease is the most common reason for abnormal liver chemistries in the United States. The factors that lead from benign steatosis to nonalcoholic steatohepatitis are poorly understood. Transferritin-Abcb11 (TTR-Abcb11) transgenic mice overexpress the bile salt transporter *Abcb11* and hypersecrete biliary lipids. Thus the aim of this study is to employ feeding of the methionine-choline-deficient (MCD) diet to TTR-Abcb11 transgenic mice to further determine the mechanisms responsible for the development of steatohepatitis. FVB/NJ and TTR-Abcb11 mice were fed control or MCD diets for up to 30 days. Serum aminotransferase levels, serum and hepatic triglyceride content, cytokines, markers of oxidative stress, and expression of selective genes were examined. MCD diet-fed TTR-Abcb11, but not wild-type, mice have elevated serum aminotransferase levels when compared after 7 days. They also have significantly lower hepatic triglyceride levels at all time points studied. After 14 days on the MCD diet, TTR-Abcb11 mice have 3-fold increases in TNF-α mRNA and 3.9-fold increases in IL-6 mRNA compared with FVB/NJ mice. TTR-Abcb11 mice also had a greater increase in cytochrome *P*-450 2E1 expression. A greater decrease in sterol regulatory element binding protein-1c and fatty acid synthase mRNA expression was also seen in TTR-Abcb11 compared with wild-type mice fed an MCD diet. They also have enhanced TNF-α, IL-6, and cytochrome *P*-450 2E1 expression. We conclude that TTR-Abcb11 mice develop a more rapid hepatitis with less steatosis.

Second hit has been suggested to be toxic lipid peroxidation and oxidative stress due to reactive oxygen species accumulation (7). Inflammatory cytokine-mediated liver injury has also been implicated as a second hit (7, 33, 49). NASH, however, is most likely a disease of multifactorial etiology, in which disease pathogenesis involves “multiple hits” or pathogenic factors.

Mice fed a diet deficient in methionine and choline (MCD) have been shown to develop steatosis, necroinflammation, and eventually progressive fibrosis. This MCD dietary model of NASH has been extensively used in previous studies since this nutritional model creates progressive, fibrosing steatohepatitis in rodents (19, 21, 23, 32, 33). Both methionine and choline are essential precursors of hepatic phosphatidylcholine (PC) synthesis, and the hepatic steatohepatitis induced by this diet may be due, in part, to impairments in PC synthesis. Figge et al. (13) recently developed the transferritin-Abcb11 (TTR-Abcb11) transgenic mouse model, which overexpresses the hepatic canalicular ATP-dependent bile salt transporter *Abcb11*. Hepatobiliary secretion of bile salt is the primary driving force for bile formation, and enhanced canalicular expression of *Abcb11* [often termed bile salt export pump (BSEP)] results in increased bile flow and increased biliary lipid secretion. In particular, TTR-Abcb11 mice exhibit an increase in biliary secretion of bile salts, cholesterol, and PC. In addition, when fed a lithogenic diet (high in fat, cholesterol, and cholic acid), these mice demonstrate significantly less hepatic steatosis than wild-type FVB/NJ controls (13).

Hepatobiliary secretion of PC is high, and it has been estimated that the entire hepatic PC content is secreted into bile each day (42, 43). Thus mice with enhanced biliary secretion of PC, such as TTR-Abcb11 mice, may be more susceptible to rapidly develop hepatic injury induced by the MCD diet. Steatosis, however, is likely an essential precursor for the development of steatohepatitis. Mice that are more resistant to the development of hepatic steatosis may be more resistant to oxidative stress, hepatocyte injury, and steatohepatitis. Therefore, the primary aim of this study was to employ TTR-Abcb11 transgenic mice to further determine the mechanisms responsible for the development of steatohepatitis.

**MATERIALS AND METHODS**

Animal protocol. Male FVB/NJ mice were purchased from Jackson Laboratories (Bar Harbor, ME). Male TTR-Abcb11 transgenic mice, in an FVB/NJ background, were bred from founder lines at Northwestern University or the Lakeside Veterans Affairs Medical Center.

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An investigator (R. M. Green) blinded to experimental conditions paraffin, and 5-formalin for histology. Blood was collected by cardiac puncture and centrifuged at 7,000 rpm for 6 min to collect serum. Livers were rapidly excised, rinsed in ice-cold saline, and weighed, and aliquots were snap-frozen in liquid nitrogen and kept at −80°C until analyzed. A portion of each liver was fixed in 10% formalin for histology.

Histological evaluation. Formalin-fixed liver was embedded in paraffin, and 5-µm sections were stained with hematoxylin and eosin. An investigator (R. M. Green) blinded to experimental conditions examined sections for steatosis and inflammation as follows: 1) steatosis: grade 0, none present; grade 1, steatosis of <25% of parenchyma; grade 2, steatosis of 25–50% of parenchyma; grade 3, steatosis of 51–75% of parenchyma; grade 4, steatosis of >76% of parenchyma; 2) inflammation: grade 0, no inflammatory foci; grade 1, 1 inflammatory foci/high-powered field (hpf); grade 2, 2–3 inflammatory foci/hpf; grade 3, >4 inflammatory foci/hpf.

Measurement of serum and liver triglyceride levels and chemistries. Liver samples were homogenized in 50 mM Tris-HCl buffer, pH 7.4, containing 150 mM NaCl, 1 mM EDTA, and 1 mM 1,1, trichloro-2,2-bis(p-chlorophenyl)ethane. Liver triglyceride and cholesterol content was measured using a spectrophotometric assay from Sigma Diagnostics (St. Louis, MO) as per instructions from the manufacturer and was expressed as milligrams of lipid per milligrams of liver protein. Liver thiobarbituric acid reactive substance (TBARS) content was measured by spectrophotometric assay using a kit from Zeptomexrix (Buffalo, NY) and expressed as nanomoles of TBARS per milligrams of protein. Reduced glutathione levels were measured in liver homogenate prepared in 5% metaphosphoric acid using a spectrophotometric assay purchased from Calbiochem (San Diego, CA) per instructions from the manufacturer and expressed as micromoles of glutathione per milligrams of liver protein. Serum alanine aminotransferase (ALT) was determined using spectrophotometric assay kits purchased from Sigma Diagnostics as per instructions from the manufacturer. Protein concentration of liver homogenate was measured, employing a Coomassie assay reagent (Pierce, IL).

Western blot analysis. Liver samples were homogenized in a lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 25 mM EDTA, 5 mM EGTA, 0.25% sodium deoxycholate, 1% Nonidet P-40, and 1 mM DTT) containing a protease inhibitor cocktail (Calbiochem). Homogenates were centrifuged at 12,000 rpm for 5 min at 4°C. Samples were mixed with 5× reducing electrophoresis buffer (50 mM Tris-HCl, pH 6.8, containing 10% glycerol, 2% SDS, 1% β-mercaptoethanol, and 0.02% bromophenol blue) and heated at 95°C for 5 min. Samples containing 10–50 µg of protein were separated by 10% SDS polyacrylamide gel electrophoresis. Proteins were then transferred onto a nitrocellulose membrane overnight by electrophoresis. Protein detection was performed using a polyclonal rabbit anti-human cytochrome P-450 2E1 (CYP2E1) antibody (1:2,000 dilution, Chemicon, Temecula, CA) or rabbit polyclonal γ-glutamyl cysteine synthetase (γ-GCS) antibody (1:200 dilution, Neomarkers, Fremont, CA). Bound primary antibody was detected using a horseradish peroxidase conjugated secondary antibody (1:1,000 dilution, Amersham, Arlington Heights, IL) and enhanced chemiluminescence method. Blots were also stripped and reprobed for tubulin using anti-tubulin antibody (1:200 dilution, Sigma-Aldrich, St. Louis, MO). Quantification of protein levels was performed by densitometric analysis using an Eagle Eye II system (Stratagene, La Jolla, CA) and normalized for tubulin expression.

Statistical analysis. Data were presented as means ± SE. Comparision between groups was performed using Student’s t-test or ANOVA analysis.

RESULTS

The effect of the MCD diet on serum ALT levels. After 7 days on the MCD diet, TTR-Abcb11 mice had significantly elevated serum ALT levels (82.6 ± 11.2 IU), which were higher than those observed in FVB/NJ mice (38.9 ± 7.3 IU), P < 0.005. After 14 days on the MCD diet, serum ALT levels were significantly elevated to a comparable degree in both the TTR-Abcb11 mice (144.2 ± 13.9 IU) and FVB/NJ (161.2 ± 13.9 IU) controls. Histologically, TTR-Abcb11 mice also showed more inflammation after 14 days on the MCD diet (1.3 ± 0.5 IU), compared with FVB/NJ mice (0.8 ± 0.5 IU), P = 0.05. Elevated ALT levels persisted through 30 days in both TTR-Abcb11 (111.9 ± 13.9 IU) and FVB/NJ (102.3 ± 9.0 IU) mice. When fed a control diet for up to 30 days, both
TTR-Abcb11 and FVB/NJ wild-type controls maintained normal serum ALT levels (39.6 ± 8.1 and 37.3 ± 7.2 IU, respectively) (see Fig. 1).

The effect of the MCD diet on hepatic triglyceride and cholesterol levels. TTR-Abcb11 mice fed an MCD diet had lower hepatic triglyceride levels than FVB/NJ mice after 7 (0.16 ± 0.03 vs. 0.33 ± 0.06 mg/mg protein), 14 (0.2 ± 0.03 vs. 0.37 ± 0.06 mg/mg protein), and 30 days (0.34 ± 0.08 vs. 0.58 ± 0.2 mg/mg protein), P = 0.001. Consistent with this finding, histologically FVB/NJ mice also demonstrated more steatosis than TTR-Abcb11 mice at 7 (2.3 ± 0.5 vs. 1.7 ± 0.5 mg/mg protein) and 14 (3.0 ± 0.6 vs. 1.5 ± 0.5 mg/mg protein) days, P < 0.05 (see Fig. 2). As illustrated in Fig. 3, both mouse strains demonstrate progressive hepatic triglyceride deposition over time, results that we confirmed histologically (P < 0.05). Hepatic triglyceride content in mice fed a control chow diet for up to 30 days was minimal and similar in both TTR-Abcb11 (0.04 ± 0.006 mg/mg protein) and FVB/NJ (0.06 ± 0.02 mg/mg protein) mice. Serum triglyceride levels were unchanged.

FVB/NJ mice showed progressive increases in hepatic cholesterol content from baseline levels (0.03 ± 0.01 mg/mg protein) after both 7 days (0.05 ± 0.01 mg/mg protein) and 14 days (0.06 ± 0.01 mg/mg protein) on an MCD diet, P < 0.05. Hepatic cholesterol levels in TTR-Abcb11 mice, however, remained similar to baseline levels (0.04 ± 0.02 mg/mg protein) after either 7 days (0.05 ± 0.01 mg/mg protein) or 14 days (0.05 ± 0.01 mg/mg protein) on the MCD diet. Serum cholesterol levels were similar in chow-fed FVB/NJ (152.9 ± 33.1 mg/dl) and TTR-Abcb11 mice (175.3 ± 11.6 mg/dl). Serum cholesterol levels decreased after 7 days (127.1 ± 20.7 mg/dl).

Fig. 1. Serum aminotransferase (ALT) levels in mice fed the methionine-choline-deficient (MCD) diet. Transthyretin (TTR)-Abcb11 and wild-type FVB/NJ mice were fed an MCD diet for 2 wk, and serum ALT levels were measured. TTR-Abcb11 mice developed markedly elevated serum ALT levels after 7 days on the MCD diet. Values are means ± SE. *P = 0.005 compared with FVB/NJ mice.

Fig. 2. Hepatic histology in mice fed control and MCD diet. A: normal hepatic architecture in FVB/NJ mice fed a control diet. B: normal hepatic architecture in TTR-Abcb11 mice fed a control diet. C: severe steatosis and mild inflammation in FVB/NJ mice fed an MCD diet for 14 days. D: mild steatosis and more severe inflammation in TTR-Abcb11 mice fed an MCD diet for 14 days.

Fig. 3. Hepatic triglyceride content of mice fed the MCD diet. TTR-Abcb11 and FVB/NJ mice were fed an MCD diet for 2 wk, and hepatic triglyceride levels were measured. TTR-Abcb11 mice had lower hepatic triglyceride levels than wild-type mice fed the MCD diet. Values are means ± SE. *P = 0.001 compared with FVB/NJ mice.
mg/dl) and 14 days (79.4 ± 12.7 mg/dl) on the MCD diet in FVB/NJ mice, P < 0.05. Similarly, serum cholesterol levels also decreased after 7 days (93.3 ± 13.3 mg/dl) and 14 days (95.7 ± 26.0 mg/dl) on the MCD diet in TTR-Abcb11 mice, P < 0.05.

Cytokine analysis using real-time PCR. Expression of TNF-α mRNA was assayed using real-time PCR. After 14 days on the MCD diet, TTR-Abcb11 mice demonstrated a threefold increase in TNF-α mRNA expression compared with FVB/NJ mice (P = 0.03). A 3.9-fold increase in IL-6 mRNA expression was also seen in TTR-Abcb11 compared with FVB/NJ mice after 14 days on the MCD diet. There was no significant difference in gene expression of both TNF-α and IL-6 mRNA at baseline in control diet-fed mice and in mice fed an MCD diet for only 7 days (see Table 1).

The effect of the MCD diet on oxidative stress. Hepatic TBARS, glutathione levels, and expression of CYP2E1 were analyzed as markers of oxidative stress. Minimally elevated TBARS occurred at 7 days on the MCD diet in both FVB/NJ (0.76 ± 0.24 nM/mg protein) and TTR-Abcb11 (1.34 ± 0.68 nM/mg protein) mice (P = not significant). By 14 days on the MCD diet, however, FVB/NJ mice had significantly greater TBARS compared with both control-fed mice and TTR-Abcb11 mice fed an MCD diet. TBAR levels were 4.05 ± 0.32 nM/mg protein in FVB/NJ mice vs. 1.79 ± 0.4 nM/mg protein in TTR-Abcb11 mice (see Fig. 4). After 30 days on the MCD diet, both FVB/NJ and TTR-Abcb11 mice had significantly elevated TBARS with no strain-specific differences. TBARS in control diet-fed mice were similar and minimal in both FVB/NJ and TTR-Abcb11 mice fed a chow diet.

Both mouse strains showed dramatic increases in CYP2E1 protein expression over time (P < 0.0005). After 14 days on the MCD diet, FVB/NJ mice showed a twofold increase in CYP2E1 protein expression, whereas this was increased fourfold in TTR-Abcb11 mice. This pattern persisted at 30 days on the MCD diet. On the chow diet, hepatic CYP2E1 protein expression showed no strain-specific differences (see Fig. 5).

Baseline hepatic glutathione levels in TTR-Abcb11 mice were higher (6.87 mM/mg protein) than in FVB/NJ mice (5.01 mM/mg protein), P < 0.05. Both mouse strains demonstrated minimal increases, rather than decreases, in hepatic glutathione over 30 days (see Fig. 6). The TTR-Abcb11 mice maintained higher levels of hepatic glutathione at all time points up to 30 days (P < 0.0001). Although increased glutathione production
could account for these effects, we observed no corresponding increases of γ-GCS enzymatic activity or protein expression.

The effect of the MCD diet on SREBP-1c and FAS gene expression. SREBP-1c regulates many hepatic metabolic factors, including the rate of hepatic FAS (10, 16, 17, 34). After 7 days on the MCD diet, TTR-Abcb11 mice had a fourfold decrease in SREBP-1c mRNA expression ($P < 0.001$), and this effect persisted at 14 days ($P = 0.002$). Similar decreases of SREBP-1c mRNA expression were not observed in the FVB/NJ mice fed the MCD diet for up to 14 days. TTR-Abcb11 mice fed an MCD diet for 7 days had significantly lower SREBP-1c mRNA expression than FVB/NJ mice ($P < 0.001$). This difference between strains was not noted after 14 days of feeding of the MCD diet. Baseline expression of hepatic SREBP-1c mRNA was similar in FVB/NJ and TTR-Abcb11 mice fed a chow diet (see Table 1).

We also examined the expression of the SREBP-1c downstream target gene FAS. Consistent with SREBP-1c expression, after 7 days on the MCD diet, TTR-Abcb11 mice had a strong trend toward a decrease in FAS gene expression, with gene expression being reduced almost twofold ($P = 0.07$). By 14 days of MCD diet feeding, TTR-Abcb11 mice had a highly significant 2.6-fold decrease in FAS expression ($P < 0.01$). Baseline expression of hepatic FAS mRNA was similar in FVB/NJ and TTR-Abcb11 mice fed a chow diet (see Table 1).

**DISCUSSION**

The MCD diet induces the rapid development of hepatitis in TTR-Abcb11 transgenic mice, despite relative steatosis resistance. Both FVB/NJ strain controls and TTR-Abcb11 mice eventually manifest progressive hepatic triglyceride deposition, marked elevation of serum ALT, and histologically evident inflammation. A more pronounced increase in mRNA expression of both TNF-α and IL-6 was also observed in the TTR-Abcb11 mice at 1 and 2 wk compared with the FVB/NJ mice. In contrast, FVB/NJ mice exhibited elevated markers of oxidative stress sooner than the TTR-Abcb11 mice. A disassoc-

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**Fig. 5.** Cytochrome P-450 2E1 (CYP2E1) expression in TTR-Abcb11 and FVB/NJ mice fed an MCD diet. Protein densitometry was performed on Western blots examining CYP2E1 expression and was normalized for tubulin expression. Both mouse strains showed dramatic increases in CYP2E1 protein expression over time, although it was greater in TTR-Abcb11 mice. $P < 0.001$ by ANOVA.

**Fig. 6.** Hepatic glutathione (GTH) levels in TTR-Abcb11 and FVB/NJ mice fed the MCD diet. TTR-Abcb11 and FVB/NJ mice were fed an MCD diet, and hepatic GTH levels were measured. FVB/NJ mice had lower GTH levels than TTR-Abcb11 mice at all time points. Values are means ± SE. $P < 0.001$ by ANOVA.
Hepatic steatosis is likely affected by SREBPs, transcription factors that regulate enzymes needed for fatty acid, triglyceride, and cholesterol synthesis (16). SREBP-1c controls the rate of hepatic FAS (10, 16, 17, 34). Overexpression of SREBP-1c enhances FAS and induces lipogenic enzymes like FAS that may control triglyceride accumulation and result in fatty liver disease (36, 37–39, 48). FAS expression is downregulated by higher levels of triglyceride accumulation. The hepatic steatosis and elevated hepatic triglyceride levels evident in the TTR-Abcb11 mice in our study correlate well with downregulation of the regulatory genes SREBP-1c and FAS mRNA. This suggests the physiological importance of a negative feedback system. This negative feedback likely works in conjunction with insulin to affect SREBP-1c expression (4).

Multiple studies have underscored the importance of inflammatory cytokine signaling and stimulation in the pathogenesis of NASH through mediation of hepatic inflammation, apoptosis, and necrosis (6, 9, 12, 15, 27, 29, 50). Patients with NASH demonstrate increased hepatic and serum TNF-α expression, a proinflammatory cytokine (8, 22). Experimental treatments against TNF-α or receptor deletions are known to attenuate the NASH disease process (18, 24, 50). Additionally, rodents on the MCD diet have higher TNF-α levels and are more sensitive to endotoxin-mediated injury (6). In our experimental model, TTR-Abcb11 mice fed the MCD diet demonstrated threefold increases in TNF-α RNA expression compared with wild-type controls. TNF-α may also trigger the production of other cytokines, such as IL-6 (15). The higher levels of cytokine expression resulting from 7 days compared with 14 days of MCD diet feeding may occur because, in this nutritional model, cytokines may be primarily elevated during the initial phases of inflammation. The upregulation of hepatic TNF-α and IL-6 mRNA seen in the TTR-Abcb11 mouse likely reflects the importance of this cytokine-mediated injury.

The substantial rise in TBARS seen after 14 days in the FVB/NJ mice and both strains after 30 days on the MCD diet emphasizes the role of lipid peroxidation in hepatic injury. It is not surprising that the TTR-Abcb11 mice initially manifest lower TBARS than their wild-type strain controls because they have less substrate (triglyceride) available for lipid peroxidation. In addition, there may be disassociation between the degree of steatosis and the severity of hepatitis (19). The TTR-Abcb11 mice, however, manifest significant hepatitis without concurrent elevations in TBARS after 7 days on the MCD diet. These data demonstrate a novel disassociation between the severity of hepatitis and the degree of oxidative stress.

NASH shares many pathophysiological characteristics with alcoholic steatohepatitis. In alcoholic steatohepatitis, evidence exists that upregulation of CYP2E1 results in lipid peroxidation and resultant accumulation of reactive oxygen species (7, 11, 30). Several studies have underscored the role of CYP2E1 in the pathogenesis of steatohepatitis (7, 23, 45, 46). Leclercq et al. (23) have further emphasized the importance of microsomal enzymes in the development of NASH by demonstrating their induction in a dietary model of NASH. The significant increase in CYP2E1 observed in TTR-Abcb11 mice fed an MCD diet is consistent with the role of CYP2E1 in the pathogenesis of steatohepatitis.

As reactive oxygen species are generated, antioxidant defense by glutathione may occur through scavenging of free radicals, and removing lipid peroxides (47). The imbalance of prooxidants, like microsomal CYP2E1, ROS release from mitochondria, and antioxidants like glutathione, results in hepatic damage (7). A reduction in glutathione may contribute to hepatic damage caused by oxidative stress, abrogated by secondary insults from cytokines such as TNF-α and IL-6. In our MCD dietary model of NASH, the expected decrease in glutathione activity or functional protein expression did not occur. The known antioxidant properties of glutathione could be responsible for the attenuated inflammation and injury seen in this model.

The TTR-Abcb11 transgenic mouse hypersecretes biliary lipids and is resistant to steatosis. It provides an animal model with which to investigate the pathogenic mechanisms of NASH. Our study is consistent with the observation in humans that the absolute amount of fat is not primarily responsible for the transition from bland hepatic steatosis to steatohepatitis. Rather, beyond a certain threshold of steatosis required for injury, the second hit becomes central to NASH development and progression. Therapy directed toward these latter pathological mechanisms could enhance the care of patients with NASH.

GRANTS
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REFERENCES


