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Ezetimibe improves high fat and cholesterol diet-induced non-alcoholic fatty liver disease in mice

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Abstract

Ezetimibe is a novel cholesterol and plant sterol absorption inhibitor that reduces plasma low-density lipoprotein–cholesterol by selectively binding to the intestinal cholesterol transporter, Niemann-Pick C1-Like 1. Mice deficient in Niemann-Pick C1-Like 1 are protected from high fat/cholesterol diet-induced fatty liver as well as hypercholesterolemia. The object of the present study was to determine whether ezetimibe treatment could reduce hepatic steatosis in diet-induced obese mice. C57BL/6J mice were fed a high fat/cholesterol containing semi-purified diet (45% Kcal fat and 0.12% cholesterol) for 7 months after weaning. These mice were not only obese, but also developed hepatomegaly and hepatic steatosis, with varying degrees of liver fibrosis and steatohepatitis. About 87% of the mice on the high fat/cholesterol diet for 7 months had elevated plasma alanine aminotransferase activity, a biomarker for non-alcoholic fatty liver disease. Chronic administration of ezetimibe for 4 weeks significantly reduced hepatomegaly by decreasing hepatic triglyceride, cholesteryl ester and free cholesterol in diet-induced obese mice fed high fat/cholesterol diet for 7 months. Chronic ezetimibe treatment also significantly decreased plasma alanine aminotransferase activity. These results suggest that ezetimibe may be a novel treatment for high fat/cholesterol-induced non-alcoholic fatty liver disease.

Keywords: Triglyceride; Cholesterol; Alanine aminotransferase; Steatosis; Fibrosis and steatohepatitis

1. Introduction

Ezetimibe is a novel sterol absorption inhibitor that blocks Niemann-Pick C1-Like 1-mediated cholesterol/phytosterol absorption in the apical brush border membrane of the jejunal enterocyte (Altmann et al., 2004; Garcia-Calvo et al., 2005). Ezetimibe significantly reduces low-density lipoprotein–cholesterol in mice (Davis et al., 2004), hamsters (van Heek et al., 2001a), and monkeys (van Heek et al., 2001b). In humans, it is used as monotherapy or in combination with statins to manage all cause hyperlipidemia, familial hetero- and homozygous hypercholesterolemia, and sitosterolemia (Lipka, 2003; Salen et al., 2004).

The Niemann-Pick C1-Like 1 gene was identified as a homolog of Niemann-Pick C1, one of the intracellular cholesterol trafficking genes. Genetic variation in Niemann-Pick C1-Like 1 contributes to variability in cholesterol absorption and plasma levels of low-density lipoprotein–cholesterol (Cohen et al., 2006). Niemann-Pick C1-Like 1-deficient mice have a significant decrease in low-density lipoprotein–cholesterol due to reduction in intestinal cholesterol absorption (Altmann et al., 2004). These mice are not only protected against diet-induced hyperlipidemia, but are also protected from chronic high dietary cholesterol-induced increases in hepatic cholesteryl ester and free cholesterol, and steatosis of the liver (Davies et al., 2005; Davis et al., 2004).

Non-alcoholic fatty liver disease represents a spectrum of liver diseases associated with fatty infiltration of the liver (steatosis) in the absence of alcohol abuse and other causes. Once hepatic steatosis is established, other factors, including oxidative stress, mitochondrial dysfunction, gut-derived lipopolysaccharide and adipocytokines, may promote further hepatocellular damage.

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Without treatment, these fatty deposits in the liver can lead to more serious inflammation and fibrosis of the liver and, ultimately, may lead to cirrhosis, liver failure, and death (Farrell and Larter, 2006). Non-alcoholic fatty liver disease occurs more commonly in people with type 2 diabetes, central obesity, dyslipidemia, and insulin resistance (Angulo, 2002). Hepatic steatosis is a growing public health concern in developed nations due to the epidemic prevalence of obesity. The incidence of obesity related liver disease in both children and adults is increasing. Non-alcoholic fatty liver disease is now present in 17 to 33% of American adults and parallels the frequency of central adiposity, obesity, insulin resistance, metabolic syndrome and type 2 diabetes (Farrell and Larter, 2006).

In many previously published studies of non-alcoholic fatty liver disease, either genetic rodent models, such as the leptin-deficient (*ob*/*ob*) or the leptin resistant (*db*/*db*) mouse, or diet-induced rodent models, such as the dietary methionine/choline-deficient model have been utilized (Anstee and Goldin, 2006 for review). Unlike the human non-alcoholic fatty liver disease population, *ob*/*ob* and *db*/*db* mice do not spontaneously progress from steatosis to steatohepatitis or hepatic fibrosis (Ikejima et al., 2005). C57BL/6 mice fed a methionine/choline-deficient diet develop hepatic inflammation and necrosis, in addition to steatosis (Kirsch et al., 2003). However, animals on the methionine/choline-deficient diet lose significant body weight which differs from the pattern seen in obesity-induced non-alcoholic fatty liver disease in humans (Kirsch et al., 2003). Furthermore, the nutritional manipulation to remove the methionine and choline from the diet is an artificial exploitation that does not happen in humans with liver disease.

It has been demonstrated that rodents fed a diet with either high cholesterol/cholate content or a high fat content can develop hepatic steatosis. Jeong et al. have recently reported that high cholesterol (1% cholesterol and 0.3% cholate) diet can induce hepatic necrosis and macrophage infiltration in addition to steatosis (Jeong et al., 2005). Since C57BL/6 mice are susceptible to high fat diet-induced obesity (Van Heek et al., 1997), and develop insulin resistance (Park et al., 2005) and hepatic steatosis (Kirsch et al., 2003), in the present studies C57BL/6J mice were fed a high fat and cholesterol containing diet (with 45% Kcal fat and 0.12% cholesterol) chronically to study the progression of non-alcoholic fatty liver disease. The livers of these mice were enlarged, had evidence of steatosis, and displayed varying degrees of fibrosis and necroinflammation, all of which are hallmarks of human non-alcoholic fatty liver disease. Once the model was established, we then evaluated the effect of ezetimibe on liver lipids and plasma alanine aminotransferase in a rodent model that mimics human non-alcoholic fatty liver disease.

2. Materials and methods

2.1. Animals and diets

Four week old C57BL/6J mice (Jackson Laboratories; Bar Harbor, Maine) were housed in individual cages at 22 °C on a 12:12 h light/dark cycle. Mice were fed a diet containing high fat and cholesterol (45% Kcal fat from lard/soybean oil and 0.12% cholesterol by weight; Research Diets D04012801; New Brunswick, NJ) from 6 weeks of age. After 3, 5 and 7 months of exposure to high fat/cholesterol diet, the mice were bled from the tail and plasma alanine aminotransferase levels were monitored to determine progression of hepatocellular damage. Two separate studies were conducted. Study 1: after 7 months of exposure to the high fat/cholesterol diet, mice (n=12/group) were treated with ezetimibe admixed in the high fat/cholesterol diet at 0, 24 or 80 μg/g in diet for 4 weeks (Table 1). The average doses of ezetimibe consumed (0, 1.8, or 5.8 mg/kg/day) were calculated based on food intake and body weight of each mouse, which was monitored daily. Study 2: mice (n=12/group) were treated with ezetimibe admixed in the high fat/cholesterol diet at 0, 8, 24 or 80 μg/g in diet for 4 weeks. The average doses of ezetimibe consumed (0, 0.5, 1.6 and 5.3 mg/kg/day, in Fig. 3) differed slightly between the two studies due to differences in food consumption. Total body fat was determined by a whole body magnetic resonance analyzer (EchoMRI1200 from Echo Medical Systems; Houston, Texas) before and after the four-week ezetimibe study. Plasma alanine aminotransferase levels were also monitored before and after the four-week study. At the end of the four-week study, non-fasting plasma samples were collected at the end of the study by heart puncture after anesthesia. Samples were centrifuged within 1 h and stored at −20 °C until assayed. All studies were carried out in accordance with the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health and the Animal Welfare Act in a

Table 1
Hepatic lipid profile in chow vs. HFC vs. HFC+ezetimibe treated mice

End points/diet	Chow	HFC	HFC+ezetimibe 1.8 mg/kg/day	HFC+ezetimibe 5.8 mg/kg/day
BW (g)	32.8±1.3	52.6±1.0[a]	50.6±1.2	51.1±0.8
Total food intake (g)	n.d.	91.1±1.9	91.1±2.0	90.9±1.7
Liver weight (g)	1.36±0.06	3.82±0.17[a]	3.08±0.2[b]	2.93±0.13[b]
% Liver/BW	4.16±0.06	7.26±0.27[a]	6.04±0.31[b]	5.70±0.19[b]
Triglyceride (mg/liver)	24.2±5.7	858±49[a]	637±76[b]	505±35[b]
Cholesterylesters (mg/liver)	3.75±0.83	90.1±9.8[a]	20.9±2.6[b]	17.0±2.3[b]
Cholesterol (mg/liver)	3.06±0.10	11.62±0.74[a]	7.05±0.59[b]	6.12±0.38[b]
Alanine aminotransferase (U/l)	29.9±14.9	340.5±37.6[a]	203.9±26.3[b]	211.2±27.8[b]

Data are expressed as mean±S.E.M. (n=10–12/group). HFC: high fat/cholesterol diet.
n.d. not determined.
[a] P<0.05 between the HFC and chow diet groups.
[b] P<0.05 between ezetimibe treated and the HFC control groups.

facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

2.2. Liver lipid extraction

After 4 weeks of treatment, the livers of mice were collected; liver lipids were extracted by chloroform:methanol (2:1) in a scintillation vial containing minced liver samples (200–300 mg). The lipid extraction was considered complete when the minced liver tissue settled on the bottom of the vial after vortexing. Hepatic cholesteryl ester and free cholesterol were analyzed by high performance liquid chromatography (HPLC); hepatic triglyceride content was determined by a modified method (Carr et al., 1993) with the enzymatic diagnostic kit (Catalog NO: TR213) from Randox (UK). The recovery of triglyceride was determined to be 85% using an internal standard.

2.3. Plasma cholesterol and triglyceride content

Non-fasting terminal plasma samples were collected at the end of the four-week treatment (0 or 5.8 mg/kg/day ezetimibe in high fat/cholesterol diet). Plasma lipoprotein profiles of individual mice (0.2 ml plasma) were determined by fast protein liquid chromatography (FPLC) with a Pharmacia Superose 6 column. Total plasma cholesterol and the cholesterol of each fast protein liquid chromatography fraction were determined by the Wako Cholesterol E enzymatic diagnostic kit (Wako Chemicals USA, Inc.; Richmond, VA) and the triglyceride kit from Randox (UK).

2.4. Plasma alanine aminotransferase activity

After 3, 5 and 7 months of exposure to high fat/cholesterol diet followed by 4 weeks of treatment with and without ezetimibe,

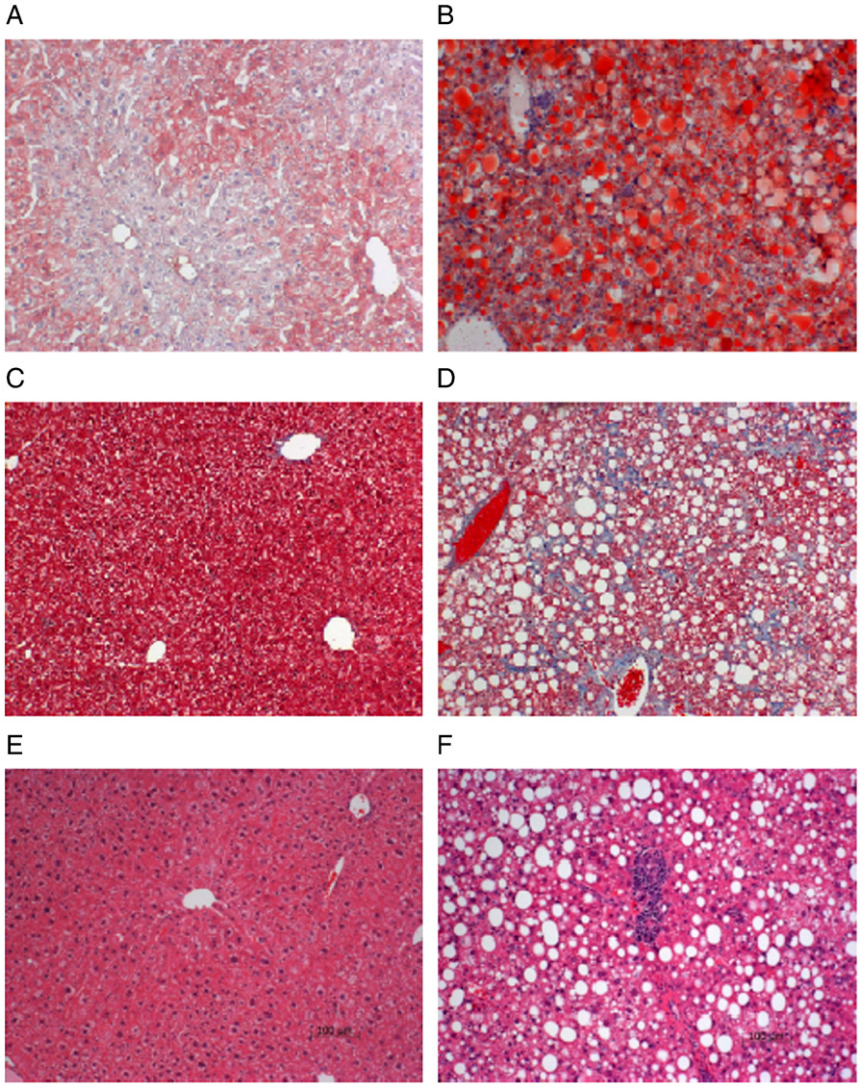

Fig. 1. (A) Livers from chow-fed control mice and (B) high fat/cholesterol fed mice stained for neutral lipids (Oil-red O). Note the large vacuoles of lipid accumulation (red stain) and loss of normal hepatic architecture in the high fat/cholesterol group, compared to the chow-fed control. (C) Livers from chow-fed control and (D) high fat/cholesterol fed mice stained for collagen (Masson's Trichrome). Note the increase in collagen deposition (blue stain) and loss of normal hepatic architecture in the high fat/cholesterol group, compared to the chow-fed control. (E) Liver from chow-fed control and (F) high fat/cholesterol fed mice stained for hemotoxylin and eosin. Note the dark inflammatory infiltrates in the high fat/cholesterol fed liver.

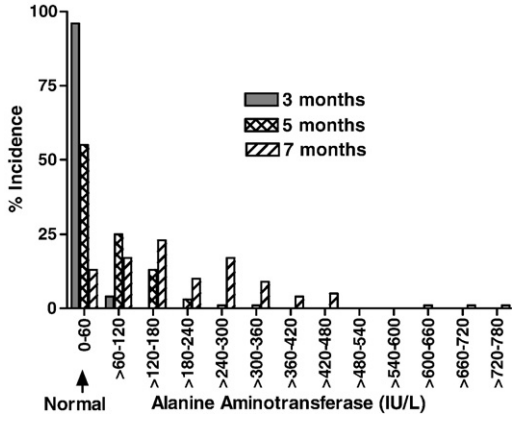

Fig. 2. A histogram showing plasma alanine aminotransferase activities as % incidence in mice fed high fat/cholesterol diet for 3, 5 or 7 months. The grey solid bars represent % incidence from 72 mice fed high fat/cholesterol diet for 3 months. The cross bars represent % incidence from 88 mice fed high fat/cholesterol diet for 5 months. The hatched bars represent % incidence from 73 mice fed high fat/cholesterol diet for 7 months.

plasma alanine aminotransferase activity was measured (Catachem Inc; Bridgeport, CT).

2.5. Histology

Standard samples from each liver were taken and immersion fixed in 10% Neutral buffered formalin. Samples were processed and embedded in paraffin, sectioned at 5 μm using a Zeiss microtome and stained for connective tissue using the Masson Trichrome method. Cryostat sections were cut at 5 μm from the liver samples and stained for neutral lipids using Oil Red O.

2.6. Statistical analysis

Results are reported as means±S.E.M. Comparisons among control and treated groups were analyzed by one-way analysis of variance with Dunnett, Tukey, or Bonferroni multiple comparisons as post-tests. When the groups had significantly different standard deviations or when one of the groups did not pass the normality test, a nonparametric analysis of variance (Kruskal–Wallis test) was used for multiple comparisons. Correlation analyses between 8 months alanine aminotransferase levels vs. body weight and liver lipids were performed using Pearson's linear correlation test. All statistical tests were performed by GraphPad InStat version 3.06 for Windows XP (GraphPad Software; San Diego, CA). Results with P values <0.05 were considered statistically significant.

3. Results

3.1. Development of non-alcoholic fatty liver disease in diet-induced obese mice

Compared to age matched chow-fed mice, C57BL/6J mice chronically fed a high fat and cholesterol diet had significantly higher body weights (+60%), and enlarged livers (+180%) (Table 1). The liver to body weight ratio, a measurement of hepatomegaly, was increased by 75%. Compared to the liver lipid content of the chow-fed mice, the diet-induced obese mice had 35 fold, 24 fold, and 3.8 fold higher levels of hepatic triglyceride, cholesteryl ester and free cholesterol, respectively (Table 1). Histopathological analyses indicated that all the livers of mice fed the high fat/cholesterol diet developed hepatic steatosis (Fig. 1B). A subset of these livers had evidence of hepatic fibrosis (Fig. 1D) and steatohepatitis to varying degrees (Fig. 1F).

Plasma alanine aminotransferase is a plasma biomarker for non-alcoholic fatty liver disease. Age-matched chow-fed C57BL/6J mice had plasma alanine aminotransferase activity averaging 30±15 IU/l (Table 1), which was within the normal range. The incidence and degree of elevated alanine aminotransferase levels augmented with increasing duration of high fat/cholesterol feeding (Fig. 2). After 3 months of high fat/cholesterol feeding, only 4% of the diet-induced obese mice had alanine aminotransferase levels >60 U/l, considered elevated. In contrast, 55% and 87% of the diet-induced obese mice had elevated alanine aminotransferase levels after 5 and 7 months of high fat/cholesterol feeding, respectively. In addition, the plasma alanine aminotransferase levels were positively associated with body weight ($R=0.60$, $P<0.05$) and total body fat content ($R=0.65$, $P<0.05$). The plasma alanine aminotransferase levels were also positively associated with hepatic free cholesterol ($R=0.51$, $P<0.05$), and hepatic cholesteryl ester ($R=0.53$, $P<0.05$). However, no association between alanine aminotransferase and hepatic triglycerides levels ($R=0.25$, $P>0.05$) was observed.

3.2. Ezetimibe treatment for 4 weeks improves plasma and liver lipid profile in diet-induced obese mice

Chronic treatment with ezetimibe (1.8 and 5.8 mg/kg/day) in the high fat/cholesterol diet did not affect either body weight or

Table 2
Plasma lipid profile in HFC vs. HFC+ezetimibe treated mice

End points/diet	Chow	HFC	HFC+ezetimibe 5.8 mg/kg/day
Total triglyceride (mg/dl)	86.3±16.9	84.5±6.3	71.5±4.5
VLDL—cholesterol (mg/dl)	7.5±1.2	2.7±0.8[a]	0.9±0.2
LDL—cholesterol (mg/dl)	16.8±2.0	225.9±10.2[a]	128.4±9.3[b]
HDL—cholesterol (mg/dl)	52.2±5.1	168.7±6.8[a]	153.4±12.2

Data are expressed as mean±S.E.M. ($n=6$/group). HFC: high fat/cholesterol diet.
VLDL: very low-density lipoprotein, LDL: low-density lipoprotein, HDL: high density lipoprotein.
[a] $P<0.05$ between the HFC and chow diet groups.
[b] $P<0.05$ between ezetimibe treated and the HFC control groups.

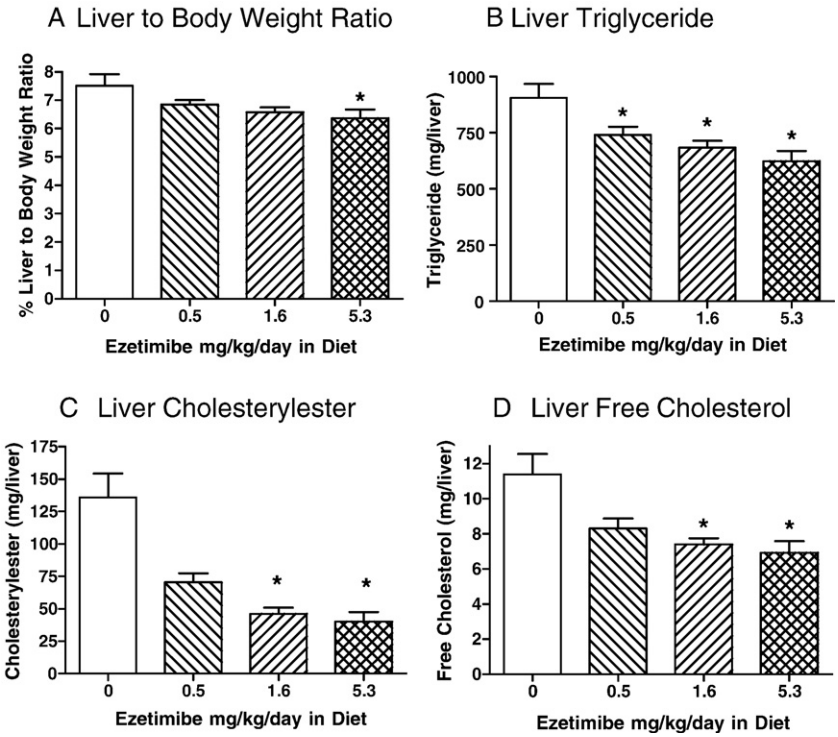

Fig. 3. Dose response of ezetimibe (0, 0.5, 1.6 and 5.3 mg/kg/day) on (A) liver to body weight ratio, (B) hepatic triglyceride content, (C) hepatic cholesteryl ester content and (D) hepatic free cholesterol content after 4 weeks of treatment in high fat/cholesterol diet in mice fed high fat/cholesterol diet for 7 months. Data are expressed as mean ± S.E.M. (n = 11–12/group). [a]P < 0.05 between control and ezetimibe treated groups.

food intake of diet-induced obese mice (Table 1). However, plasma low-density lipoprotein–cholesterol was significantly reduced by 43% by ezetimibe (5.8 mg/kg/day) treatment (Table 2). After 4 weeks of treatment, the liver to body weight ratio (Fig. 3A), hepatic triglyceride (Fig. 3B), hepatic cholesteryl esters (Fig. 3C) and hepatic cholesterol (Fig. 3D) levels were significantly decreased in a dose-dependent manner by ezetimibe. In a separate experiment, plasma alanine aminotransferase levels were significantly reduced after 4 weeks of ezetimibe (1.8 and 5.8 mg/kg/day) treatment (Table 1).

4. Discussion

With the increasing prevalence of obesity, diabetes, and the metabolic syndrome due to the excessive consumption of high fat and cholesterol containing diets and lack of exercise in the global human population, non-alcoholic fatty liver disease is a growing public health concern in developed as well as developing countries. In the United States, it is estimated that over 60 million adults have non-alcoholic fatty liver disease (Adams et al., 2005). Of these, 8.9 million have some degree of non-alcoholic steatohepatitis, which may progress to end stage liver diseases which are associated with a seven- to ten-year liver-related mortality of 12 to 25% (Farrell and Larter, 2006). Various rodent models have been utilized to study hepatic steatosis; however, few replicate the entire human phenotype, ie, steatohepatitis with fibrosis and necroinflammation. In the present studies, C57BL/6J mice were fed a high fat and cholesterol containing "western" diet for 7 months to induce non-alcoholic fatty liver disease which included hepatomegaly, elevated alanine aminotransferase and hepatic steatosis, and in some cases, varying degrees of liver fibrosis and steatohepatitis. Ezetimibe treatment for 4 weeks significantly reduced liver to body weight ratio, indicating an improvement in hepatomegaly. Ezetimibe treatment also improved hepatic steatosis by reducing hepatic levels of triglyceride, cholesteryl ester and free cholesterol. In addition, ezetimibe treatment decreased plasma alanine aminotransferase levels, suggesting that ezetimibe treatment may improve the hepatocellular damage induced by non-alcoholic fatty liver disease.

Serum alanine aminotransferase elevations have been used as a surrogate biomarker for non-alcoholic fatty liver disease and steatohepatitis in the clinical setting. Since alanine aminotransferase is an enzyme mostly produced in hepatocytes, we monitored plasma alanine aminotransferase as a biomarker to evaluate the degree of hepatocellular damage induced by non-alcoholic fatty liver disease in obese mice chronically fed a high fat and cholesterol diet. Alanine aminotransferase activity is normally present at low levels in the circulation in chow-fed mice. In high fat/cholesterol fed mice, the incidence and severity of elevated plasma alanine aminotransferase levels increased significantly the longer the animals were maintained on this diet. Plasma alanine aminotransferase levels in the present study were positively associated with body weight and body fat which is similar to that observed in a national, population-based study (Ruhl and Everhart, 2003). We also observed a positive association of plasma alanine aminotransferase levels with hepatic free cholesterol and cholesteryl ester. Although hepatic steatosis is mainly a result of elevated hepatic triglycerides, we did not

observe a significant association between total hepatic triglycerides and plasma alanine aminotransferase levels. It is known that free cholesterol or saturated fatty acid accumulation can elicit stress in the endoplasmic reticulum and thereby trigger an unfolded protein response which eventually results in activation of hepatocyte apoptosis (Beltroy et al., 2007; Devries-Seimon et al., 2005; Wang et al., 2006). The high fat and cholesterol diet we used in the current study has 40% of the energy source from lard, which contains about 41% saturated fatty acids. Therefore, cholesterol and the saturated fatty acids from the high fat/cholesterol diet may both contribute to the development of hepatocelllular dysfunction that was observed in this model.

Non-alcoholic fatty liver disease is usually caused by two 'hits': the 'first hit' is induced by peripheral insulin resistance, causing hepatic steatosis. The 'second hit' is thought to be caused by reactive oxygen species, inducing vicious cycles of oxidative injury leading to inflammation and fibrosis (Mehta et al., 2002). Using artificial diets to differentially increase triglyceride vs. cholesterol storage in the liver, Mari et al. recently demonstrated that hepatic free cholesterol loading by a sodium cholate (0.5%) supplemented, 2% cholesterol diet led to tumor necrosis factor-α and Fas-induced steatohepatitis through depletion of mitochondrial glutathione (Mari et al., 2006). In contrast, the same group found that triglyceride loading by a choline-deficient Lombardi diet did not have this effect (Mari et al., 2006). In Niemann-Pick C1 knockout mice treated with a high fat diet, hepatic free cholesterol content was positively correlated with plasma alanine aminotransferase activity (Beltroy et al., 2007). These data provide experimental evidence that hepatic free cholesterol may trigger the 'second hit' to induce the progression from simple steatosis to steatohepatitis.

Through blocking intestinal cholesterol absorption, ezetimibe reduces hepatic cholesterol and cholesteryl ester in all species studied (Davis et al., 2004; van Heek et al., 2001a,b). However, most of these studies did not measure hepatic triglyceride levels. The present studies demonstrate that 4 weeks of ezetimibe treatment led to a decrease in hepatic triglyceride, cholesteryl ester and free cholesterol of 40%, 80%, and 50%, respectively. This occurred despite a massive 35-fold, 24-fold and 3.8-fold increase in hepatic triglycerides, cholesteryl ester and free cholesterol after 7 months of high fat/cholesterol feeding. Ezetimibe treatment was associated with a 40% decrease in plasma alanine aminotransferase levels. Recently, Telford et al. reported that ezetimibe treatment in minipigs fed 34% (Kcal) fat and 0.1% cholesterol resulted in a 21% reduction of liver triglycerides (Telford et al., 2007). In addition, ezetimibe (3 mg/kg/day for 6 weeks) improved fatty liver by decreasing hepatic triglycerides, free cholesterol and cholesteryl ester levels in a dietary methionine/choline deficiency rat model (Assy et al., 2006). It was also reported that ezetimibe may have anti-oxidant and anti-inflammatory properties because ezetimibe significantly increased the α-tocopherol/malondialdehyde ratio and decreased plasma tumor necrosis factor-α levels in the methionine/choline-deficient dietary model (Assy et al., 2006). Furthermore, ezetimibe treatment from weaning was able to significantly reduce the high fat diet-induced free cholesterol loading and elevated alanine aminotransferase levels in Niemann-Pick C1 knockout mice (Beltroy et al., 2007). In fact, ezetimibe treatment in dyslipidemic patients with non-alcoholic fatty liver disease caused an unexpected improvement in plasma alanine aminotransferase activity in a few clinical case reports (Hughes et al., 2006).

In conclusion, feeding C57BL/6J mice a high fat and cholesterol containing diet-induced non-alcoholic fatty liver disease, with varying degrees of hepatic fibrosis, hepatic necroinflammation and elevated plasma alanine aminotransferase. Four weeks of ezetimibe treatment was able to significantly reduce the triglyceride, cholesteryl ester and free cholesterol that had accumulated in the liver after 7 months of high fat/cholesterol feeding. The fact that plasma alanine aminotransferase levels were improved in the ezetimibe treated mice suggests that hepatocellular damage can be reversed by ezetimibe treatment. These data suggest that reducing hepatic free cholesterol, cholesteryl ester and triglyceride content by ezetimibe, an intestinal cholesterol absorption inhibitor, may be a novel treatment for non-alcoholic fatty liver disease.

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