Ezetimibe prevents hepatic steatosis induced by a high-fat but not a high-fructose diet

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1Division of Endocrinology and Metabolism, Department of Medicine, Shiga University of Medical Science, Seta, Otsu, Shiga, Japan; 2Diabetes, Metabolism and Endocrinology, Kagoshima University Graduate School of Medical and Dental Sciences, Sakuragaoka, Kagoshima, Japan; 3Division of Endocrinology and Metabolism, St. Marianna University Hospital, Kawasaki, Kanagawa, Japan; and 4Division of Endocrinology and Metabolism, National Hospital Organization Shiga Hospital, Oumi, Shiga, Japan

Submitted 27 September 2012; accepted in final form 28 May 2013

Ezetimibe prevents hepatic steatosis induced by a high-fat but not a high-fructose diet. Am J Physiol Endocrinol Metab 305: E293–E304, 2013. First published May 28, 2013; doi:10.1152/ajpendo.00442.2012.—Nonalcoholic fatty liver disease is the most frequent liver disease. Ezetimibe, an inhibitor of intestinal cholesterol absorption, has been reported to ameliorate hepatic steatosis in human and animal models. To explore how ezetimibe reduces hepatic steatosis, we investigated the effects of ezetimibe on the expression of lipogenic enzymes and intestinal lipid metabolism in mice fed a high-fat or a high-fructose diet. CBA/JN mice were fed a high-fat diet or a high-fructose diet for 8 wk with or without ezetimibe. High-fat diet induced hepatic steatosis accompanied by hyperinsulinemia. Treatment with ezetimibe reduced hepatic steatosis, insulin levels, and glucose production from pyruvate in mice fed the high-fat diet, suggesting a reduction of insulin resistance in the liver. In the intestinal analysis, ezetimibe reduced the expression of fatty acid transfer protein-4 and apoB-48 in mice fed the high-fat diet. However, treatment with ezetimibe did not prevent hepatic steatosis, hyperinsulinemia, and intestinal apoB-48 expression in mice fed the high-fructose diet. Ezetimibe decreased liver X receptor-α binding to the sterol regulatory element-binding protein-1c promoter but not expression of carbohydrate response element-binding protein and fatty acid synthase in mice fed the high-fructose diet, suggesting that ezetimibe did not reduce hepatic lipogenesis induced by the high-fructose diet. Elevation of hepatic and intestinal lipogenesis in mice fed a high-fructose diet may partly explain the differences in the effect of ezetimibe.

ezetimibe; NAFLD; hepatic and intestinal lipogenesis

THE NUMBER OF PATIENTS with nonalcoholic fatty liver disease (NAFLD) (43), or nonalcoholic steatohepatitis (NASH), has been increasing worldwide (1), resulting in NAFLD becoming the most frequent disease of the liver. NAFLD is characterized by two steps of liver injury (27). The first is intrahepatic fat accumulation, and the second is inflammatory changes leading to NASH (19). Although many factors are associated with the causes of hepatic fat accumulation, the main factor may be overintake of nutrition, especially overintake of fat or nutrients such as fructose or sucrose, leading to an increase in hepatic lipogenesis (18, 39). Indeed, animals fed a high-fat or high-fructose diet show steatosis in the liver (29, 30, 42). NAFLD is significant because it is strongly associated with hepatic insulin resistance and may progress to liver cirrhosis (4). To prevent or ameliorate NAFLD, a reduction of energy intake and/or an increase in energy expenditure by exercise are essential. No other treatment for NAFLD has been established to date.

Ezetimibe is an effective LDL-cholesterol-lowering drug that blocks Niemann-Pick C1-Like 1 (NPC1L1) transporters mediating cholesterol absorption in the apical brush border membrane of jejunal enterocytes (2). Recently, it was reported that treatment with ezetimibe ameliorated hepatic steatosis and insulin resistance in both human and animal models (9, 10, 28).

Treatment with ezetimibe attenuated the intestinal production of chylomicrons in mice fed a high-fat diet, resulting in reduced delivery of fatty acids from the intestine to the liver (38). The reduced production of chylomicrons from the intestine after ezetimibe treatment may be a mechanism that ameliorates hepatic steatosis. However, whether ezetimibe has direct or indirect effects on the liver, and prevention of hepatic steatosis, cannot be delineated from an animal model showing an increase in hepatic lipogenesis. We have reported that a high-fructose diet causes hepatic steatosis and induces hepatic sterol regulatory element-binding protein-1c (SREBP-1c) gene expression, which is a master transcriptional regulator of the expression of lipogenic enzyme genes (29, 30, 40). In contrast to animals fed with a high-fructose diet, the elevation of lipogenesis in the liver is a main cause of hepatic steatosis in animals fed with a high-fructose diet (5, 23). There are no data on whether ezetimibe ameliorates hepatic steatosis induced by a high-fructose diet.

In the present study, to explore how ezetimibe reduces hepatic steatosis, we investigated the effects of treatment with ezetimibe on fat accumulation, the expression of lipogenic enzymes in the liver, and lipid metabolism in the intestine by using two animal models of fatty liver induced by two different diets.

MATERIALS AND METHODS

Materials. All materials were reagent grade and purchased from Nacalai Tesque (Kyoto, Japan) or Sigma Chemical (St. Louis, MO) unless otherwise indicated.

Animals. Four-week-old male CBA/JN mice were purchased from CLEA Japan (Tokyo, Japan). The mice were housed in an environmentally controlled room on a 12:12-h light-dark cycle. The animals were divided into the following six groups: 1) a control diet, 2) a control diet with ezetimibe treatment, 3) a high-fructose diet, 4) a high-fructose diet with ezetimibe treatment, 5) a high-fat diet, and 6) a high-fat diet with ezetimibe treatment. The mice of each group...
were pair fed, and each diet totaled 150 kcal/wk and was supplemented with 0.008% wt/wt ezetimibe treatment (Kemprotec, UK) for 4 and 8 wk. The control diet (Oriental Yeast, Tokyo, Japan) consisted of 58% carbohydrate (starch, no fructose), 12% fat, and 30% protein (energy percent of diet). The high-fructose diet (Oriental Yeast) contained 63% carbohydrate (100% of which was fructose), 14.5% fat, and 22.5% protein. The high-fat diet (Oriental Yeast) contained 36.5% carbohydrate (starch, no fructose), 46% fat, and 17.5% protein. Four days before the onset of experiments, food was withdrawn from all animals at 20:00. For the subsequent 3 days, all animals were fed the control diet, the high-fructose diet, or the high-fat diet in the dark from 0600 to 0800. On the final day, one-half of the animals were fed in the dark from 0600 to 0800 before the experiment, and the other half were kept in a fasting state. At 1000, after a 10 mg/kg ip pentobarbital sodium injection and under deep anesthesia, the liver, intestine, and epididymal fat were excised. Blood samples were also taken for the analysis of triglyceride, total cholesterol, blood sugar, and insulin levels. All experiments were approved by the Shiga University of Medical Science Animal Care Committees.

**Western blot analysis.** Western blot analysis was performed using a commercial kit (Cayman Chemical, Ann Arbor, MI) (Takara Bio, Otsu, Japan). cDNA was synthesized using reverse transcript reagents extracted from liver samples using the RNeasy Kit (Qiagen, Tokyo, Japan). cDNA was further adjusted with the levels of mRNA expression of 36B4 as an internal control. Primer sequences used were as follows: m36B4 Forward AGCAGGGTTTTGAAACGGCAGC, Reverse ACACCTGGACACACGCGGC; SREBP-1c Forward GGAGCCATGGATTGAG, Reverse GGAGCCATGGATTGAG; CD36 Forward GAGCAACTGGTGCTCCACCA, Reverse GAGACGTGTCACTCCTGGACTTG; fatty acid transfer protein-4 (FATP4) Forward ATCAACACCAACCTTAGGCG, Reverse AGGAAGGCTTCCAGAGAGGA; fatty acid-oxidase-linked secondary antibodies followed by chemiluminescence detection.

**Electrophoretic mobility shift assays.** EMSAs were performed using radiolabeled double-stranded oligonucleotides corresponding to the following pairs of oligonucleotides from the promoter regions: LXRE in SREBP-1c promoter: 5’-CAGTGACCGCGAGTAACCCCAGC-3’ and 5’-GCTGGGGTTAAGTGGCGTC3’-. The probes were labeled with [γ-32P]dATP using T4-polynucleotide kinase (Takara Bio). The reaction mixture with nuclear extract was loaded onto 6% polyacrylamide gels in 0.25× Tris borate-EDTA buffer and run at 150 V. The gels were dried and exposed to film. A nonlabeling oligonucleotide for LXRE and the LXR antibody was used to evaluate competition or a mobility shift of the target band, respectively.

**Oil red O stain.** Liver tissue sections (20 μm thickness) were fixed for 24 h in 4% paraformaldehyde, 0.2% picric acid, and 0.5% glutaraldehyde in 0.2 mol/l phosphate buffer (pH 7.4) at 4°C. After being washed for 1 day with 15% sucrose at 4°C, the sections were incubated for 1 h in an Oil red d ye bath to stain for triglyceride.

**Pyruvate tolerance tests.** Mice were fasted for 24 h, followed by injection of 2 g/kg ip sodium pyruvate (Sigma-Aldrich). Blood glucose was determined 15, 30, 45, 60, 75, 90, 105, and 120 min after injection.

**Insulin tolerance tests or glucose tolerance tests.** Mice were injected with 0.5 U/kg ip insulin or fasted for 6 h before injection with 1 g/kg ip glucose, respectively. Blood glucose was determined 15, 30, 60, 90, and 120 min after injection.

**Primary small intestinal enterocytes.** The small intestine was extracted and cut into 2 × 2 mm fragments. The fragments obtained were transferred to a plastic culture dish containing 7 ml of ice-cold Matrisperse and incubated at 4°C for 8 h without agitation. The dish was then gently shaken to separate the villi, and the villi suspension was washed twice in ice-cold PBS (–) (180 g, 5 min). After the final spin, the villi were resuspended in DMEM with 10% FBS and 0.2 U/ml insulin in the culture dishes (1 × 106 cells/well).

**Isolation of triacylglycerol-rich lipoproteins and assessment of intestinal lipoprotein production.** Intestinal lipoprotein production was measured using poloxamer-407 (P-407), which inhibits endogenous low-density lipoprotein lipase (LPL) activity with few adverse effects on lipoprotein metabolism compared with Triton WR-1339. The mice were given a 200-μl olive oil load via oral gavage after a 12-h fast, and P-407 solution [0.1 ml/kg body wt of a 10% (wt/vol) prepared in saline] was injected into mice via the tail vein. At 90 min after P-407 injection, blood samples were collected. To isolate the triacylglycerol-rich lipoprotein (TRL) fraction, blood samples were first centrifuged for 15 min at 4°C and 2,500 g to separate the plasma layer. Plasma (150 μl) was overlaid with 3 ml of potassium bromide solution (density 1.006 g/ml in a 5-ml ultracentrifuge tube and centrifuged for 70 min at 4°C and 116,140 g (46,000 rpm) and at 15°C using a rotor (TLA100.4). The TRL fractions were collected as the top 300 μl of the tube content. Immunoblotting using apoB-48 antibody was performed on the TRL fractions by SDS-PAGE analysis.

**Statistical analysis.** The data were expressed as means ± SE unless otherwise stated. Tukey’s multiple comparison test was used to compare the means.

### Table 1. Effects of ezetimibe on fasting characteristics of CBA/JN mice fed for 4 wk

<table>
<thead>
<tr>
<th>Diet</th>
<th>Ezetimibe</th>
<th>Body Weight, g</th>
<th>Epididymal fat weight, g</th>
<th>Glucose, mg/dl</th>
<th>Cholesterol, mg/dl</th>
<th>Triglyceride, mg/dl</th>
<th>Insulin, μU/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>(−)</td>
<td>21.2 ± 0.2</td>
<td>0.26 ± 0.03</td>
<td>91 ± 11</td>
<td>85 ± 5</td>
<td>108 ± 10</td>
<td>3.8 ± 0.6</td>
</tr>
<tr>
<td>Control</td>
<td>(+)</td>
<td>20.5 ± 0.2</td>
<td>0.21 ± 0.02</td>
<td>84 ± 9</td>
<td>67 ± 10</td>
<td>107 ± 24</td>
<td>3.0 ± 0.3</td>
</tr>
<tr>
<td>High-fructose</td>
<td>(−)</td>
<td>22.4 ± 0.5</td>
<td>0.35 ± 0.04</td>
<td>115 ± 23</td>
<td>103 ± 7</td>
<td>141 ± 23</td>
<td>5.8 ± 1.4</td>
</tr>
<tr>
<td>High-fructose</td>
<td>(+)</td>
<td>23.0 ± 0.5</td>
<td>0.36 ± 0.03</td>
<td>93 ± 7</td>
<td>82 ± 7</td>
<td>147 ± 34</td>
<td>3.5 ± 0.4</td>
</tr>
<tr>
<td>High-fat</td>
<td>(−)</td>
<td>29.5 ± 0.7</td>
<td>1.31 ± 0.09</td>
<td>93 ± 11</td>
<td>93 ± 5</td>
<td>123 ± 10</td>
<td>6.4 ± 0.6</td>
</tr>
<tr>
<td>High-fat</td>
<td>(+)</td>
<td>26.2 ± 1.0d</td>
<td>0.78 ± 0.09</td>
<td>81 ± 5</td>
<td>68 ± 3d</td>
<td>113 ± 5</td>
<td>5.2 ± 0.9</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE for 5–7 mice in each group.

E294 IS EZETIMIBE EFFECTIVE ON NAFLD IN THE HIGH-FRUCTOSE DIET?
**RESULTS**

**Characteristics of the experimental animals.** The metabolic characteristics of the experimental animals at 4 and 8 wk after starting feeding are shown in Tables 1 and 2. At 4 wk, ezetimibe had significantly decreased serum cholesterol levels in mice fed the high-fat diet (by 26%, \( P < 0.05 \)) compared with the control mice. Compared with mice fed the control diet, we observed significant increases in body weight in mice fed the high-fructose diet (by 11% \( P < 0.05 \)) for 8 wk or the high-fat diet (by 284%, \( P < 0.01 \)) for 8 wk. Treatment with ezetimibe significantly decreased the insulin levels (by 43%, \( P < 0.05 \)) in mice fed the high-fat diet for 8 wk, suggesting a reduction in hepatic insulin resistance in these mice. Although ezetimibe significantly increased the body weight, epididymal fat weight, serum cholesterol, and plasma insulin of in the mice fed the high-fructose diet for 8 wk compared with mice fed the regular diet, there were no significant differences between mice treated with or without ezetimibe when fed the high-fructose diet.

**Insulin sensitivity in the liver and peripheral tissues.** To evaluate hepatic glucose production in the experimental mice, pyruvate tolerance tests were performed. As shown in Fig. 1, the blood glucose response to an intraperitoneal pyruvate load was upregulated only in mice fed the high-fat diet (\( P < 0.01 \)), suggesting a decrease in hepatic insulin activity. Ezetimibe significantly reduced the upregulated response to the pyruvate load in mice fed the high-fat diet (at 90 and 105 min, \( P < 0.05 \), and at 120 min, \( P < 0.01 \), respectively), but not in mice fed the control or high-fructose diets.

Insulin tolerance tests and glucose tolerance tests were also performed. As shown in Fig. 2, the changes in blood glucose concentrations after the intraperitoneal insulin load were significantly lower, and the area under the curve (AUC) was

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**Table 2. Effects of ezetimibe on fasting characteristics of CBA/JN mice fed for 8 wk**

<table>
<thead>
<tr>
<th>Diet</th>
<th>Ezetimibe</th>
<th>Body Weight, g</th>
<th>Epididymal fat weight, g</th>
<th>Glucose, mg/dl</th>
<th>Cholesterol, mg/dl</th>
<th>Triglyceride, mg/dl</th>
<th>Insulin, uIU/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>(-)</td>
<td>25.7 ± 0.4</td>
<td>0.42 ± 0.05</td>
<td>87 ± 7</td>
<td>77 ± 2</td>
<td>114 ± 19</td>
<td>9.3 ± 2.2</td>
</tr>
<tr>
<td>Control</td>
<td>(+)</td>
<td>25.6 ± 0.7</td>
<td>0.38 ± 0.05</td>
<td>91 ± 7</td>
<td>77 ± 4</td>
<td>139 ± 28</td>
<td>7.7 ± 1.2</td>
</tr>
<tr>
<td>High-fructose</td>
<td>(-)</td>
<td>28.5 ± 0.8</td>
<td>0.68 ± 0.11</td>
<td>93 ± 4</td>
<td>108 ± 4b</td>
<td>113 ± 7b</td>
<td>16.3 ± 4.2</td>
</tr>
<tr>
<td>High-fructose</td>
<td>(+)</td>
<td>28.9 ± 0.7a</td>
<td>0.71 ± 0.07a</td>
<td>92 ± 7</td>
<td>113 ± 7b</td>
<td>110 ± 28</td>
<td>30.5 ± 9.2b</td>
</tr>
<tr>
<td>High-fat</td>
<td>(-)</td>
<td>35.7 ± 1.6a</td>
<td>1.82 ± 0.15a</td>
<td>115 ± 8</td>
<td>100 ± 3b</td>
<td>129 ± 23</td>
<td>35.8 ± 6.7a</td>
</tr>
<tr>
<td>High-fat</td>
<td>(+)</td>
<td>30.9 ± 1.4a,d</td>
<td>1.30 ± 0.19a,d</td>
<td>89 ± 6</td>
<td>101 ± 5b</td>
<td>110 ± 23</td>
<td>20.3 ± 0.9b,d</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE; 7–9 mice in each group. \(^a P < 0.01\) vs. control diet. \(^b P < 0.05\) vs. control diet. \(^c P < 0.01\) vs. without ezetimibe. \(^d P < 0.05\) vs. without ezetimibe.

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Fig. 1. Effects of ezetimibe (EZ) on blood glucose levels during the pyruvate tolerance test in mice fed the control diet (A), the high-fructose diet (B), or the high-fat diet (C). Mice were fasted for 24 h before the injection of 2 g/kg ip of sodium pyruvate. Data are means ± SE for 4 mice per group. *\( P < 0.01\).
Fig. 2. Effects of ezetimibe on blood glucose levels during insulin (A–D) and glucose (E–H) tolerance tests in mice fed a control diet (A and E), high-fructose diet (B and F), or high-fat diet (C and G). D and H: areas under the curve (AUC) for blood glucose concentrations in insulin and glucose tolerance tests. Mice were intraperitoneally injected with 0.5 U/kg ip insulin (A–D) or fasted for 6 h before injection with 1 g/kg ip of glucose (E–H). Data are means ± SE for 7–8 mice per group. **P < 0.05.
significantly decreased (by 74%) in mice fed the high-fat diet ($P < 0.01$) compared with mice fed the control diet. The changes in blood glucose concentrations after the intraperitoneal glucose load were significantly greater, and the AUC was significantly increased (by 186%) in mice fed the high-fat diet ($P < 0.01$) compared with mice fed the control diet, suggesting a decrease in insulin sensitivity in the high-fat diet-fed mice. Ezetimibe significantly reduced the changes in blood glucose levels during the insulin tolerance test ($P < 0.05$) and the glucose tolerance test ($P < 0.05$) in mice fed the high-fat diet but not in mice fed the control or high-fructose diets. These results suggest that ezetimibe improved insulin resistance in the liver and peripheral tissues induced by the high-fat diet but not by the high-fructose diet.

**Hepatic fat accumulation.** Hepatic steatosis was evaluated after 4 or 8 wk of feeding with the high-fat or high-fructose diet. Quantitative analysis of hepatic triglyceride and cholesterol content after 4 or 8 wk of feeding with each diet is shown in Fig. 3, A and B. At 4 wk, hepatic triglyceride content was twofold and fourfold greater in mice fed the high-fructose ($P < 0.05$) and high-fat diets ($P < 0.01$, respectively), compared with that in mice fed the control diet. At 8 wk, hepatic triglyceride and cholesterol content was twofold greater in mice fed the high-fructose diet ($P < 0.01$ and $P < 0.05$, respectively) and sixfold greater in mice fed the high-fat diet (both $P < 0.01$) compared with that in mice fed the control diet. Ezetimibe significantly decreased hepatic triglyceride and cholesterol content by 27% in mice fed the high-fat diet ($P < 0.01$ and $P < 0.05$, respectively), but not in mice fed the high-fructose diet. These results were confirmed by measuring lipid droplets deposited in the liver using Oil red O staining (Fig. 3C). Ezetimibe reduced hepatic lipid deposition in mice fed the high-fat diet but not in mice fed the high-fructose diet.

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**Fig. 3.** A and B: Quantitative analysis of hepatic triglyceride (A) and cholesterol (B) content in mice fed the experimental diets for 4 or 8 wk. C: representative histological staining of liver sections obtained from mice after 8 wk of feeding. Samples were stained with Oil red O. Data are means ± SE for 6 mice for 4 wk and 4 mice for 8 wk per group. *$P < 0.05$ and **$P < 0.01$.**
Expression of de novo lipogenic genes in the liver.

As shown in Fig. 4A, the mRNA expression of SREBP-1c in the liver was increased in mice fed the high-fructose diet (by 132%, *P < 0.01) but not in mice fed the high-fat diet. The induction of SREBP-1c expression by the high-fructose diet was confirmed by analyzing the nuclear protein expression level (Fig. 4, B and C; by 80%, **P < 0.01). Ezetimibe significantly reduced the hepatic mRNA expression of SREBP-1c in mice fed the high-fructose (by 35%, *P < 0.01) and high-fat diets (by 10%, **P < 0.01; Fig. 4A). Ezetimibe decreased the nuclear protein expression of SREBP-1c in the liver from mice fed the high-fructose (by 42%, *P < 0.01) and high-fat diets (by 49%, **P < 0.01). Similarly to the effects of ezetimibe on hepatic mRNA expression of SREBP-1c, the nuclear expression of LXRα, a major transcriptional regulator of the SREBP-1c gene, was partly decreased by ezetimibe in mice fed the high-fructose or high-fat diets (Fig. 4, D and E). The results of the EMSA suggested that ezetimibe reduced the binding activity of LXRα to the promoter of the SREBP-1c gene in the livers of mice fed the high-fructose or high-fat diets (Fig. 4F).

Fig. 4. Expression of lipogenic enzymes in the liver. A: total mRNA was isolated from fed liver and quantified using primers for sterol regulatory element-binding protein-1c (SREBP-1c). B–E: Western blotting analysis of SREBP-1 (B and C) and LXRα (D and E). F: EMSA of liver nuclear protein using a radiolabeled probe containing the LXRE consensus sequence. A nonlabeling oligonucleotide for LXRE and the LXR antibody was used to evaluate competition or a mobility shift of the target band, respectively. G: total mRNA was isolated from the liver and quantified using primers for fatty acid synthase (FAS). mRNA expression levels were normalized for the expression of 36B4. Data are means ± SE for 8 mice per group. *P < 0.05 and **P < 0.01.
Next, we measured the mRNA expression of FAS, a target of SREBP-1c in the liver. As shown in Fig. 4G, the mRNA expression of FAS was remarkably increased at refeeding in mice fed the high-fructose diet (by 380%, \( P < 0.01 \)), whereas the high-fat diet did not affect the hepatic mRNA expression of FAS. Ezetimibe did not affect the hepatic mRNA expression of FAS, unlike that of SREBP-1c.

**Nuclear expression of ChREBP in the liver.** To investigate the mechanism involved in the upregulation of hepatic FAS gene expression in mice fed the high-fructose diet, we measured the nuclear protein expression of ChREBP, another transcriptional regulator of FAS. As shown in Fig. 5, A and B, the nuclear expression of ChREBP was increased after refeeding (\( P < 0.01 \)). The high-fructose diet induced a greater increase in nuclear expression of ChREBP compared with the control diet (by 158%, \( P < 0.01 \); Fig. 5B). Ezetimibe did not affect nuclear expression of ChREBP in the liver in mice fed any of the diets.

**Effects of ezetimibe on expression of genes controlling lipid trafficking and metabolism in the intestine.** Intestinal gene expression of the major fatty acid transporters FATP4 and CD36 is shown in Fig. 6, A and B. The high-fructose and high-fat diets did not affect intestinal mRNA expression of FATP4. However, ezetimibe decreased intestinal mRNA expression of FATP4 in mice fed the high-fat diet (by 18%, \( P < 0.01 \)). By contrast, intestinal mRNA expression of CD36 was increased in mice fed the high-fructose (by 68%, \( P < 0.01 \)) or high-fat diets (by 135%, \( P < 0.01 \)). Ezetimibe did not affect CD36 expression. As shown in Fig. 6C, intestinal mRNA expression of GLUT5, the major fructose transporter in the intestine, was increased in mice fed the high-fructose diet (by 495%, \( P < 0.01 \)) but not in mice fed the high-fat diet. Furthermore, as shown in Fig. 6D, intestinal mRNA expression of FAS was significantly increased by ezetimibe in mice fed the high-fructose or high-fat diets (by 60%, \( P < 0.01 \)). As shown in Fig. 6E, intestinal mRNA expression of HMG-CoAR, a rate-limiting enzyme in cholesterol biosynthesis, was also increased by ezetimibe in mice fed the high-fat diet. apoB-48 is produced in the liver and intestine of mice, where it plays an important role in the formation of chylomicrons by interacting with cholesterol and triglyceride. However, intestinal mRNA expression of apoB-48 was not affected by ezetimibe in mice fed either diet (Fig. 6F).

**apoB-48 immunoblotting on TRL fractions.** Based on these results, the apoB-48 content in the TRL fraction, after injection with P-407 to inhibit endogenous LPL, was used as a marker of de novo lipogenesis in the liver and intestine. As shown in Fig. 7, A and B, apoB-48 content in the TRL plasma fraction was increased in mice fed the high-fructose (by 30%, \( P < 0.05 \)) and high-fat diets (by 51%, \( P < 0.01 \)). Ezetimibe significantly reduced apoB-48 content in the TRL fraction in mice fed the high-fat diet (by 48%, \( P < 0.01 \)) but not in mice fed the high-fructose diet.
apoB-48 immunoblotting of primary small intestinal enterocytes. Since villus enterocytes synthesize and secrete apoB-48, we measured the amount of apoB-48 secreted from primary cultured enterocytes into media for 180 min after resuspension. As shown in Fig. 7, C and D, apoB-48 secretion was increased in mice fed the high-fructose (by 300%, \(P < 0.01\)) and high-fat diets (by 700%, \(P < 0.01\)). Ezetimibe significantly reduced the secretion of apoB-48 from enterocytes isolated from mice fed the high-fat diet (by 73%, \(P < 0.01\)) but not from enterocytes isolated from mice fed the high-fructose diet.

**DISCUSSION**

In this study, we determined the effects of ezetimibe on NAFLD, using two mouse models of NAFLD that was established using distinct diets. We found that ezetimibe improved serum cholesterol, hepatic fat accumulation, and insulin resistance in the liver and peripheral tissues in mice fed the high-fat diet. However, ezetimibe did not improve these parameters in mice fed the high-fructose diet. Furthermore, we found that ezetimibe reduced the gene expression of FATP4, the major transporter of long-chain fatty acids, and reduced enterocyte...
Ezetimibe - + - + - +

A

Fig. 7. apoB-48 protein expression. * and ** indicate p < 0.05 and **p < 0.01.

apoB-48 secretion in mice fed the high-fat diet but not in mice fed the high-fructose diet. These observations clearly suggest that the effects of ezetimibe on fat accumulation and insulin resistance in the liver differ according to the type of diet that was responsible for NAFLD.

In this experiment, we used CBA/JN mice because we previously had demonstrated that these mice are highly susceptible to the high-fructose diet, which accelerates metabolic disorders. We believe that studies using these mice are appropriate to evaluate the effects of ezetimibe because these mice show many of the typical characteristics of NAFLD, including reduced blood cholesterol levels, enhanced intrahepatic lipid accumulation, and altered apoB-48 secretion.

The effects of ezetimibe on hepatic steatosis are probably associated with improvements in hepatic insulin resistance. It was previously reported that insulin resistance is strongly correlated with hepatic steatosis (7). However, it is unclear whether hepatic steatosis is a cause or a consequence of insulin resistance. Insulin resistance contributes to the elevated activity of hormone-sensitive lipase and increased free fatty acid flux from adipose tissue into the liver (37). Furthermore, it was reported that hyperinsulinemia, because of insulin resistance, fails to suppress hepatic glucose genesis but can promote hepatic de novo lipogenesis (13), which accelerates intrahepatic fat accumulation. However, intrahepatic fat accumulation and elevated SREBP-1c expression can cause hepatic insulin resistance (40). Additionally, intrahepatic cholesterol accumulation, caused by diet-derived intestinal cholesterol absorption, increases reactive oxygen species (ROS) production and endoplasmic reticulum (ER) stress, which reduce insulin sensitivity in hepatocytes (24, 34, 35). In Zucker obese fatty (ZOF) rats, ezetimibe improved hepatic insulin signaling and hepatic steatosis by reducing hepatic ROS generation, JNK activation, and ER stress (32). Our findings indicate that ezetimibe improves hepatic insulin resistance by reducing intrahepatic accumulation of cholesterol or triglyceride, particularly in mice fed the high-fat diet.

In our experiments, the mice fed the high-fructose diet did not show pronounced hepatic insulin resistance in the pyruvate tolerance test or systemic insulin resistance in the insulin and glucose tolerance tests, even though the high-fructose diet significantly increased hepatic fat accumulation. We speculate that the extent of hepatic insulin resistance in mice fed the high-fructose diet in our experiments was mild, despite obvious intrahepatic fat accumulation. These findings may imply that intrahepatic fat accumulation induces hepatic insulin resistance.

The gene expression of SREBP-1c is regulated by LXRα, a member of the nuclear receptor family of transcription factors (20, 25). In the current study, we found that ezetimibe reduced the binding activity of LXRα to the SREBP-1c promoter in mice fed the high-fructose or the high-fat diet, partially by reducing nuclear protein expression of LXRα in the liver. Ezetimibe was also reported to reduce the content of hepatic oxysterol, an LXRα ligand, by inhibiting intestinal sterol absorption through intestinal NCP1L1 (3). These findings suggest that ezetimibe inhibited the binding activity of LXRα to the SREBP-1c promoter by inhibiting intestinal sterol absorption and ultimately reduced SREBP-1c gene expression.

However, ezetimibe did not appear to inhibit hepatic mRNA expression of FAS. These results differ from those of previous reports, in which FAS gene expression was linked to the gene expression of SREBP-1c (11, 12). It was reported that the transcription of FAS is upregulated by both SERBP-1c and ChREBP in the liver (8). Thus, in the current study, refeeding with a high-fructose diet remarkably activated FAS gene expression in association with an increase of nuclear expression of ChREBP and an increase in SREBP-1c expression, which increased de novo lipogenesis in the liver. Additionally, the expression level of L-type pyruvate kinase, another target of ChREBP (21), was strongly increased in mice fed the high-fructose diet, indicating the induction of ChREBP in the liver. These results suggest that ezetimibe does not affect the tran-
scriptional activation of ChREBP involved in de novo lipogenesis, despite inhibiting hepatic SREBP-1c expression. Unlike its effects in mice fed the high-fructose diet, ezetimibe markedly ameliorated hepatic steatosis induced by the high-fat diet. In the present study, the hepatic mRNA expression of SREBP-1c and FAS was not increased in mice fed the high-fat diet, even though hepatic steatosis was apparent. These findings suggest that an increase in influx of chy-
ome of the intestine into the liver is responsible for the induction of hepatic steatosis in mice fed the high-fat diet. Indeed, it was reported that ezetimibe blocks NPC1L1 and thus inhibits cholesterol transport and attenuates the increase in chylomicrons in plasma and lymph fluid induced by a high-fat diet (38). Consistent with these reports, we found that ezetimibe decreased the gene expression of FATP4 and apoB-48 secretion in the intestine of mice fed the high-fat diet but not in mice fed the high-fructose diet. Although the importance of intestinal FATP4 in fat absorption is not fully understood, some studies have reported that the knockdown of FATP4 in cultured enterocytes decreases their uptake of long-chain fatty acids (16, 41). Furthermore, it was recently reported that intestinal FATP4 expression was decreased in ezetimibe-treated mice and in Npc1l1−/− mice (22). Clinical and animal studies have shown that ezetimibe decreases postprandial apoB-48 levels (31). The inhibition of cholesterol absorption and long-chain fatty acid uptake may attenuate chylomicron formation and reduce apoB-48 stability in the intestine (14–15, 17). By contrast, the significant upregulation of de novo lipogenesis in mice fed the high-fructose diet was likely due to increased FAS expression and inadequate amelioration of apoB-48 secretion following treatment with ezetimibe. We also found that ezetimibe increased intestinal mRNA expression of FAS and HMG-CoAR in mice fed the high-fat diet. This might be due to increased compensatory endogenous production of fatty acids and cholesterol in the intestine. However, in mice fed the high-fat diet, the inhibition of apoB-48 secretion in the postprandial state by ezetimibe will suppress lipid production and thus reduce lipid flux to the liver. By contrast, in mice fed the high-fructose diet, ezetimibe did not inhibit intestinal apoB-48 secretion, probably because apoB-48 stability was unchanged in association with overproduction of endogenous lipids. Increased de novo lipogenesis in enterocytes, such as that seen in fructose-fed hamsters (15), may provide further substrates for the production and secretion of chylomicrons.

In human studies, postprandial triglyceride and apoB-48, which are involved in chylomicron production, were increased in subjects with obesity, dyslipidemia, or insulin resistance (6, 33). Conversely, improvements in metabolic control in patients with type 2 diabetes reduce the number of postprandial apoB-48-containing particles (36). Improvements in insulin sensitivity were reported to reduce TRL overproduction in fructose-fed hamsters (26). We speculate that the reduction in intestinal apoB-48 production and stability in mice treated with ezetimibe may be due to improvements in insulin resistance, hepatic steatosis, or body weight in mice fed the high-fat diet but not in those fed the high-fructose diet. Further studies are needed to determine how ezetimibe reduces intestinal FATP4 expression and apoB-48 secretion in mice fed a high-fat diet but not in those fed a high fructose-diet.

In conclusion, as shown in Fig. 8, ezetimibe ameliorated hepatic steatosis mainly by inhibiting extrahepatic lipid trafficking via the small intestine with overtake of exogenous lipids provided by a high-fat diet. However, ezetimibe did not influence hepatic steatosis associated with overnutrition associated with a high-fructose diet, which increased endogenous lipogenesis in the liver and small intestine. Our results suggest that the effects of ezetimibe on improving lipid metabolism are highly dependent on dietary content, and that fatty liver is dependent on lipid trafficking and metabolism in the intestine, in which case it is important to inhibit intestinal lipoprotein secretion.

ACKNOWLEDGMENTS

We thank Keiko Kosaka (Shiga University of Medical Science, Shiga, Japan) and the Central Research Laboratory of Shiga University of Medical Science for technical assistance.

GRANTS

This work was supported in part by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: M.U., Y. Nishio, O.S., Y. Nagai, Y.M., S.U., T.Y., K.M., S.K., A.K., and H.M. conception and design of research; M.U. and O.S. performed experiments; M.U. analyzed data; M.U., Y. Nishio, O.S., Y. Nagai, Y.M., S.U., T.Y., K.M., S.K., A.K., and H.M. interpreted results of experiments; M.U. prepared figures; M.U. drafted manuscript; M.U. edited and revised manuscript; Y. Nishio, O.S., A.K., and H.M. approved final version of manuscript.

REFERENCES

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