

JPET #203448

## **EZETIMIBE INCREASES HEPATIC IRON LEVELS IN MICE FED A HIGH-FAT DIET**

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JPET #203448

**Short Title:** Effects of ezetimibe on iron metabolism

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**Abbreviations:** Acox: acyl-CoA oxidase, C: the control group, CE: the control plus ezetimibe group, CI: the control plus iron group, CIE: the control plus iron plus ezetimibe group, Dcytb: duodenal cytochrome b, DMT1: divalent metal transporter1, Fas: fatty acid synthase, H: the high-fat diet group, HE: the high-fat diet plus ezetimibe group, HI: the high-fat diet plus iron group, HIE: the high-fat diet plus iron plus ezetimibe group, Hmg-CoA-R: 3-hydroxy-3-methylglutaryl coenzyme A-reductase, Ldl: low-density lipoprotein, Mdr: Multidrug resistance, Mrp: Multidrug resistance-associated protein, NPC1L1: Niemann-Pick C1-Like 1, NAFLD: non-alcoholic fatty liver disease, NASH: non-alcoholic steatohepatitis, PCR: polymerase chain reaction, Srebp: sterol regulatory element-binding protein, transferrin %: transferrin saturation, Tfr: transferrin receptor, UIBC: unsaturated iron

JPET #203448

binding capacity.

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JPET #203448

## Abstract

Accumulating evidence suggests that ezetimibe may be a promising agent for treatment of non-alcoholic fatty liver disease and steatohepatitis (NAFLD/NASH). Phlebotomy and dietary iron restriction reduces serum transaminase in NAFLD/NASH patients. Recent studies showed that mutual effects exist between lipid metabolism and iron metabolism. Accordingly, the effects of ezetimibe on iron metabolism were examined in mice fed a high-fat diet with or without iron. C57BL/6 mice were fed the following diets for 12 weeks. Experiment 1: 1) a control diet; C 2) C plus ezetimibe (0.3mg/day; 4 weeks); CE 3) a high-fat diet; H 4) H plus ezetimibe; HE. Experiment 2: 1) C containing carbonyl iron (average; 22.4mg/day; 6 weeks); CI 2) CI plus ezetimibe; CIE 3) H containing carbonyl iron; HI 4) HI plus ezetimibe; HIE. Blood, livers, and duodenum were removed after 12 weeks. In Experiment 1, hepatic iron levels were higher in HE than H, whereas there was no difference between C and CE. Hepatic mRNA expression of transferrin receptor 1 and 2, ferritins, and hepcidin were increased more in CE than C, and in HE than H. In duodenum, DMT1, ferritinH, and hephaestin mRNA levels were increased in CE compared to C. In Experiment 2, hepatic iron concentrations were higher in HIE than HI. Hepatic mRNA expression of ferritinL and hepcidin were increased in HIE compared to HI. In duodenum, ferritinL mRNA was increased in HIE compared to CIE. Ezetimibe induced hepatic iron uptake transporter expression in mice fed a high-fat diet, causing increased hepatic

JPET #203448

iron concentrations.

JPET #203448

## Introduction

Cholesterol is taken into cells by Niemann-Pick C1-Like 1 (NPC1L1), which is a cholesterol transporter residing in the small intestinal epithelial cells, and then is incorporated into Apo-B48 to form chylomicrons, which are transported into the circulation via lymphatic vessels (Tomkin, 2008; Iqbal J and Hussain MM, 2009). Ezetimibe, an inhibitor of NPC1L1, is known to suppress cholesterol intake via the intestine (Altmann et al., 2004; Garcia-Calvo et al., 2005). This agent influences fatty acid supply to the body not only through suppression of cholesterol absorption but also by influencing the synthesis and secretion of chylomicrons (Bari et al., 2012). It has been reported that the agent reduces postprandial hyperlipidemia (Yunoki et al., 2011), improves insulin resistance, and reduces low-density lipoproteins (Tamaki et al., 2012).

Non-alcoholic fatty liver disease (NAFLD) represents the hepatic manifestation of the metabolic syndrome, characterized by insulin resistance, dyslipidemia, and hypertension. The term NAFLD encompasses a broad spectrum of liver diseases ranging from simple steatosis to non-alcoholic steatohepatitis (NASH). Although simple steatosis usually follows a benign course, NASH is a progressive disease that can evolve into cirrhosis and hepatocarcinoma (Krawczyk et al., 2010; Smith and Adams, 2011). It has been assumed that simple steatosis progresses stepwise to NASH as a result of excess generation of reactive oxygen species which trigger lipoperoxidation as well as mitochondrial dysfunction, leading to oxidative stress-induced liver tissue damage (Berson et al., 1998; Day and James, 1998; Koek et al., 2011).

NAFLD patients often have iron deposition in the liver (George et al., 1998; Nelson et al., 2011), which underscores the relationship between hepatic iron overload and tissue damage. Experiments in rodents have shown that iron overload

JPET #203448

stimulates the synthesis of cholesterol (Graham et al., 2010) and reduces triglyceride levels (Kirsch et al., 2006) in the liver. In addition, several epidemiologic surveys have demonstrated that a correlation exists between hyperferritinemia and insulin resistance (Jiang et al., 2004; Jehn et al., 2004; Forouhi et al., 2007). It has also been shown that mice fed a high-fat diet undergo sustained hepatic inflammation, which influences hepatic hepcidin expression, resulting in a reduction of the hepatic iron level (Chung et al., 2011). These reports suggest the possibility that lipid metabolism interacts with iron metabolism. Bile duct ligation was found to reduce the hepatic iron level but pravastatin administration reversibly increased the level, affecting expression of iron metabolism-related genes in the rat (Kolouchova et al., 2011). This report suggests the possibility that pravastatin, a cholesterol-lowering agent, may influence hepatic iron metabolism. Accordingly, we explored the effects of ezetimibe, which corrects hypercholesterolemia by mechanisms different from those of statins, on iron metabolism in the liver as well as the duodenum of mice fed either a high-fat or a control diet. It was found that ezetimibe influenced the hepatic iron level and expression of iron metabolism-related genes, and thus we proceeded to explore the effects of ezetimibe on iron metabolism in mice that ingested excessive amounts of iron.

## Materials and Methods

**Materials and chemicals.** Milk casein, corn starch,  $\alpha$ -corn starch, mineral AIN-93 mixture and vitamin AIN-93 VX mixture were purchased from CLEA Japan (Osaka Japan). Soybean oil and lard were purchased from Oriental Yeast (Tokyo, Japan) and Yamakei (Osaka Japan), respectively. Ezetimibe was kindly provided by Merck and Co., Inc. (Whitehouse Station, NJ). Carbonyl iron was purchased from Sigma-Aldrich (St.Louis, MO) and all other chemicals were purchased from Wako Pure Chemical Industries (Osaka, Japan), unless noted.

**Animals and diets.** Seven-week old male C57BL/6 mice (CLEA Japan, Shizuoka, Japan) were housed in the same animal care facility controlling for temperature ( $22 \pm 2$  °C), humidity ( $55 \pm 5\%$ ), and light (lights on; 07:00–19:00 h). Two separate experiments were conducted. In Experiment 1, mice ( $n = 6/\text{group}$ ) were divided into four groups fed the following diets : 1) a control diet (AIN-93, containing 4% soybean oil) for 12weeks; the control group (C), 2) a control diet for 8 weeks followed by a control diet containing ezetimibe (0.3 mg/day) for 4 weeks; the control plus ezetimibe group (CE), 3) a high-fat diet for 12weeks; the high-fat diet group (H), 4) a high-fat diet for 8 weeks followed by a high-fat diet containing ezetimibe (0.3 mg/day) for 4 weeks; the high-fat diet plus ezetimibe group (HE). In Experiment 2, animals ( $n= 5$  or  $6/\text{group}$ ) were divided into four groups fed the following diets : 1) a control diet for 6 weeks followed by a control diet containing carbonyl iron (21.4mg/day) for 6 weeks;



JPET #203448

the control plus iron group (CI), 2) the same diet as the CI group for 12 weeks (iron; 21.1mg/day), but ezetimibe (0.3 mg/day) was added for the last four weeks, similarly to Experiment 1; the control plus iron plus ezetimibe group (CIE), 3) a high-fat diet for 6 weeks followed by a high-fat diet containing carbonyl iron (24.3mg/day) for 6 weeks; the high-fat diet plus iron group (HI), 4) the same diet as the HI group for 12 weeks (iron; 22.7mg/day), but ezetimibe (0.3 mg/day) was added for the last four weeks; the high-fat diet plus iron plus ezetimibe group (HIE). The composition of the control and experimental diets is shown in Supplemental Table. As carbonyl iron and ezetimibe were fine powder, ezetimibe and/or carbonyl iron were thoroughly mixed with other compositions of each diet using a food mixer to obtain the desired concentrations in the feed. The doses of ezetimibe and carbonyl iron were chosen with reference to previous reports (Zheng et al., 2008; Paraskevas et al., 2011). Individual body weights and food intake were recorded once or twice a week, respectively. Based on these data, the amount of ezetimibe and carbonyl iron mixed in diets was adjusted each time to administer approximately same total amount of ezetimibe and/or carbonyl iron between groups when diets were prepared. After 12 weeks, food was removed two hours prior to collecting tissues and blood samples were collected by heart puncture after anesthesia, and livers and duodenum were harvested and stored at -80°C until use. A mouse of the HI group was dropped out of Experiment 2 because he was injured and extremely lost his weight. Studies were approved by

JPET #203448

Kinki University Faculty of Medicine Animal Care and Use Committee.

**Quantification of serum biochemical markers , hepatic lipids, and hepatic iron.**

Serum biochemical markers were quantified using biochemistry autoanalyzer Labospect 008 (Hitach High-Technologies Corporation, Ibaragi, Japan). Liver lipid content was extracted according to the method of previous report (Folch et al., 1957). Hepatic cholesterol and triglyceride concentrations were determined by standard enzymatic-colorimetric assays using commercially available kits (Wako Pure Chemical Industries, Osaka, Japan). Hepatic iron concentrations were quantified by the method of previous report (Torrance and Bothwell, 1980).

**RNA isolation and real-time PCR.** Total RNA was isolated using TRIzol reagent (Life Technologies, Tokyo, Japan) according to the manufacturer's protocol. The concentration of total RNA in each sample was quantified spectrophotometrically at 260nm. The mRNA expression of lipid and iron metabolism-related genes and 18s rRNA were quantified by SYBR real-time polymerase chain reaction (PCR) (SYBR Premix Ex Taq ; Takara bio Inc. , Shiga, Japan). Primers were used from previous reports (Tanaka et al., 2012; Dupic et al., 2002; Wallace et al., 2005; Vokurka et al., 2006; Lin et al., 2006; Wang et al., 2009; Kiessling et al., 2009). The amplification reactions were carried out in an ABI Prism 7900 HT sequence detection system (Applied Biosystems, Foster City, CA). The amount of mRNA was calculated using the comparative CT method which determines the amount of target normalized to an

JPET #203448

endogenous reference. Each gene was normalized to 18s rRNA.

**Statistical analysis.** Statistical analysis was performed using the software package, SYSTAT, version 11 (Systat Inc., Evanston, IL). Differences among individual groups were analyzed by two-way analysis of variance (ANOVA) with treatment and diet as main factors, followed by Tukey's multiple comparison test. All results are expressed as means  $\pm$  S.E.M. Significance was set at  $p < 0.05$ .

## Results

### Experiment 1

**Body weight, liver weight, liver weight to body weight, and serum and hepatic lipid profiles.** After 12 weeks, HE tended to gain less body weight compared to H. Liver weight was lower in HE than H. The final ratio of liver weight to body weight was lower in HE than CE as well as H. At 12 weeks, serum triglycerides were lower in H than C. Although ezetimibe was administered to animals of two groups in Experiment 1, serum total cholesterol was not changed in any group. However, ezetimibe tended to decrease HDL-cholesterol levels in the control-diet groups (data not shown). After 12 weeks, hepatic total cholesterol was decreased in CE compared to C. Hepatic triglyceride concentrations were lower in HE than H (Table 1).

**Serum ALT and ALP.** Hepatobiliary toxicity was determined after feeding each diet for 12 weeks. However, there were no significant differences in serum ALT (marker for hepatocyte toxicity) or ALP (marker for biliary toxicity) between groups (data not shown).

**mRNA expression of cholesterol-related genes in liver.** After 12 weeks, sterol regulatory element-binding protein (Srebp) 2 mRNA expression was increased more in H than C, HE than CE, CE than C, and in HE than H. At 12 weeks, the mRNA expression of 3-hydroxy-3-methylglutaryl-CoA reductase(HMGCoAR) and Ldl

JPET #203448

receptor were increased more in CE than C, and in HE than H (Table 1).

**mRNA expression of fatty acid-related genes in liver.** Srebp-1c was not changed between groups. After 12 weeks, fatty acid synthase (Fas) was increased in CE compared to C. Acyl-CoA oxidase 1 (Acox1) was increased more in CE than C, and in HE than H (Table 1).

**Serum iron and hepatic iron concentrations.** There was no significant difference in the serum iron levels, unsaturated iron binding capacity (UIBC), and transferrin saturation (transferrin %) among the groups. Hepatic iron concentrations were higher in the HE group compared to the H group, whereas there was no significant difference between the C group and the CE group (Fig. 1).

**mRNA expression of iron metabolism-related genes in liver.** Hepatic mRNA expression of iron metabolism related genes, including transferrin receptor1(Tfr1), divalent metal transporter1(DMT1), ferritinH, ferritinL, hepcidin, hemojuvelin, neogenin, and transferrin receptor2 (Tfr2) were increased in the CE group compared to the C group, and in the HE group compared to the H group. Furthermore, ferritinH, ferroportin1, hepcidin, neogenin, and Tfr2 mRNA levels were increased in the HE group compared to the CE group (Fig. 2).

**mRNA expression of iron metabolism-related genes in duodenum.** In duodenum, the mRNA expression of iron metabolism-related genes, including DMT1, FerritinH, and hephaestin were increased in the CE group compared to the C group (Table 2). No change was observed in iron metabolism-related genes expression between the H group and the HE group.

JPET #203448

## Experiment 2

**Body weight, liver weight, liver weight to body weight, and serum and hepatic lipid profiles.** After 12 weeks, HIE tended to gain less body weight compared to HI. Liver weight was lower in HIE than HI as well as CIE. The final ratio of liver weight to body weight was lower in HIE than HI. Similar to Experiment 1, the administration of ezetimibe did not alter serum total cholesterol concentrations in any group. However, ezetimibe decreased HDL-cholesterol levels in the high-fat diet groups (data not shown). At 12 weeks, serum triglycerides were lower in the CIE and HI groups compared to the CI group. Hepatic total cholesterol was decreased in the CIE group compared to the CI group. Hepatic triglycerides were decreased in the HIE group compared to the HI group (Table 3).

**Serum ALT and ALP.** Similar to the results of Experiment 1, serum ALT and ALP were not statistically significant differences between groups (data not shown).

**mRNA expression of cholesterol-related genes in liver.** At 12 weeks, Srebp2 mRNA expression was increased more in HI than C, HIE than CIE, and in HIE than HI. After 12 weeks, HMGCoAR mRNA was increased in the HIE group compared to the CIE group and tended to be increased in the HIE group compared to the HI group. The mRNA expression of Ldl receptor was not changed in any group (Table 3).

**mRNA expression of fatty acid-related genes in liver.** Srebp-1c was not changed between groups. At 12 weeks, Fas mRNA was not changed in any group, but the mRNA expression of Acox1 was increased in the HI group compared to the CI

JPET #203448

group (Table 3).

**Serum iron and hepatic iron concentrations.** After 12 weeks, serum iron concentrations, UIBC, and transferrin % were not changed among the groups. At 12 weeks, hepatic iron concentrations were higher in the HIE group compared to the HI group, whereas there was no significant difference between the CI group and the CIE group. Furthermore, hepatic iron levels were higher in HI than CI, and in HIE than CIE (Fig. 3).

**mRNA expression of iron metabolism-related genes in liver.** There was no significant difference in any mRNA expression of iron metabolism-related genes in liver between the CI group and the CIE group. However, hepatic mRNA expression of ferritinL and hepcidin were increased in the HIE group compared to the HI group. The mRNA expression of hepcidin and Tfr2 were increased in the HI group compared to the CI group. Ferritin H, ferritin L, ferroportin1, hepcidin and Tfr2 mRNA levels were increased in the HIE group compared to in the CIE group (Fig. 4).

**mRNA expression of iron metabolism-related genes in duodenum.** In duodenum, only ferritin L mRNA expression was increased in the HIE group compared to the CIE group (Table 4). No change was observed in other iron metabolism-related genes expression among the groups.

## Discussion

It is known that excessive iron deposition in the body injures various organs including the liver (Papanikolaou and Pantopoulos, 2005; Weinberg, 2010) and it has been reported that a relationship exists between hepatic iron deposition and disease progression in NAFLD/NASH patients (Sumida et al., 2009; Nelson et al., 2012). Since several studies have indicated that a relationship exists between lipid metabolism and iron metabolism, we assumed that a cholesterol-lowering agent could potentially influence iron metabolism. The current study reported for the first time that ezetimibe, an inhibitor of cholesterol transport, affected iron metabolism. Although the dosage of ezetimibe was determined with reference to previous reports (5mg/Kg/day to 10mg/Kg/day) (Zheng et al., 2008; Paraskevas et al., 2011), this dose was much higher compared to the ordinary dose for humans. Thus, further studies should be conducted to clarify whether ezetimibe increases hepatic iron in humans.

In Experiment 1, the hepatic iron level did not differ significantly between C and CE, whereas it was higher in the HE group versus the H group. Regarding expression of genes related to hepatic iron metabolism, the levels of Tfr1, Tfr2, DMT1, ferritin H, and ferritin L mRNA were increased by ezetimibe administration in both the control-diet groups and high-fat diet groups. These results suggest that ezetimibe stimulated iron uptake into the liver in the control-diet groups and high-fat diet groups and it also enhanced mRNA expression of hepcidin, hemojuvelin, and neogenin.



JPET #203448

Hepcidin mRNA expression is assumed to be increased and controlled by hemojuvelin and neogenin, respectively (Zhang et al., 2009). The expression of mRNA for ferritin H, ferroportin 1, hepcidin, neogenin, and Tfr2 was enhanced to a significantly greater degree in HE than in CE. This observation was assumed to reflect secondary changes whereby ezetimibe stimulated hepatic iron deposition to a greater degree in the high-fat diet groups than the control-diet groups. Enhanced iron intake or suppressed iron excretion can be assumed to be responsible for increased iron deposition in the liver. In general, Tfr1 plays an important role in hepatic iron uptake (Herbison et al., 2009), but Tfr2 as well as Tfr1 may have contributed to an increase in hepatic iron levels in Experiment 1. In the duodenum, mRNA levels of DMT1, ferritin H and hephaestin were more significantly elevated in CE than C, while DMT1 mRNA expression tended to be increased in HE compared to H. It was therefore suggested that ezetimibe enhanced duodenal iron absorption.

In Experiment 2, we explored the effects of ezetimibe on iron metabolism under conditions of iron overload. Whereas hepatic iron levels did not differ between CI and CIE, the levels were higher in HIE than HI similar to the effects observed in Experiment 1. The expression of iron metabolism-related genes in the liver was generally not altered as much as in Experiment 1. Ferritin L and hepcidin mRNA were only increased more in HIE than in HI. Thus, the mechanisms underlying the increase in hepatic iron levels seen in Experiment 2 are unclear, because neither Tfr1

JPET #203448

mRNA nor Tfr2 mRNA expression was enhanced by ezetimibe administration in that experiment. In the duodenum, there was no change in the expression of iron metabolism-related genes in the iron-overload experiment. An excessive amount of iron administered orally into the duodenum seemed to mitigate the effect of ezetimibe on those genes. Experiment 2 showed that hepatic iron levels were higher in HI than CI, and higher in HIE than CIE. Furthermore, the expression of mRNA for ferritin H/L, ferroportin 1, hepcidin, and Tfr2 was enhanced to a significantly greater degree in HIE than CIE. It was therefore assumed that ezetimibe-induced hepatic iron deposition was greater in the high-fat diet groups than the control-diet groups in Experiment 2, similarly to Experiment 1. Tfr2 mRNA expression, but not Tfr1mRNA expression, was enhanced by high-fat diet feeding. Under conditions of high-fat diet feeding with iron overload, Tfr2 may play a more important role than Tfr1 in increasing hepatic iron levels (Fleming et al., 2000; Tsuchiya et al., 2010a). It was demonstrated in both Experiments 1 and 2 that ezetimibe increased hepatic iron levels in mice fed a high-fat diet in the presence or absence of hepatic iron overload. Recent study showed that a high-fat diet increased hepatic iron and Tfr2 mRNA expression in rats (Ahmed and Oates, 2013). Another study demonstrated that hepatic steatosis in mice fed a choline-deficient diet correlated with increased total amount of hepatic iron, suggesting that there exists a link between hepatic lipid and iron metabolism (Tsuchiya et al., 2010b). Taken together, some components of the high-fat diet,

JPET #203448

including saturated fatty acids, and/or increased hepatic lipid accumulation may be involved in the effects of ezetimibe on iron metabolism, leading to a synergistic increase in hepatic iron levels. In our study, mice were fed a high-fat diet for six weeks and then were fed either a high-fat diet or a high-fat diet with iron until 8 weeks. At 8 weeks, hepatic steatosis was considered enough to be developed. Thus, it might be interesting to determine whether ezetimibe administration on a control diet increases hepatic iron concentrations in genetic obese mice with hepatic steatosis. If ezetimibe could enhance hepatic iron levels in these mice, hepatic steatosis rather than the components of the high-fat diet would be involved in the effects of ezetimibe on the regulation of iron metabolism genes. Indeed, it is not known why the high-fat diet or steatosis modulates the effects of ezetimibe on iron metabolism. Previous studies showed that multidrug resistance (Mdr) 1 and multidrug resistance-associated protein (Mrp) 2 deficiency increased serum and hepatic ezetimibe glucuronide levels (Oswald et al., 2006; Oswald et al., 2010). Recent study demonstrated that serum levels of ezetimibe glucuronide were increased after either oral or intravenous administration of ezetimibe in rats fed a methionine- and choline-deficient diet (Hardwick et al., 2012). Previous report showed that Mrp2 mRNA levels were reduced in livers of male rats fed a high-fat diet (Lickteig et al., 2007). Furthermore, Mrp2 protein expression was decreased in fatty liver of the obese Zucker rats, an established model for NAFLD (Geier et al., 2005). Taken together, these data

JPET #203448

suggest that the high-fat diet or fatty liver-induced changes in efflux transporter expression may affect the disposition and pharmacological effects of ezetimibe. Thus, it might be of interest to determine whether serum and hepatic levels of ezetimibe and hepatic Mrp2 expression are different between the control-diet groups and the high-fat diet groups in our study.

In regard to iron excretion, ferroportin1, which is the only iron exporter that has been identified to date, plays a crucial role. Ferroportin1 was originally identified as an iron exporter that transports iron from the duodenal epithelial cells to the circulation. The circulating peptide hormone hepcidin inhibits cellular iron efflux by binding to duodenal ferroportin1. Previous studies demonstrated that hepatic ferroportin1 is also down-regulated by hepcidin produced by hepatocytes (Aigner et al., 2008; Ramey et al., 2010). In Experiment 1, however, the present study showed that hepatic ferroportin1 mRNA expression was enhanced despite an increase in hepcidin mRNA, which is consistent with another report (Theurl et al., 2005). Although the reason for this discrepancy is not clear, it may be explained by between the species studied. Ezetimibe stimulated hepatic iron uptake into the liver, leading to increased hepatic iron levels in our current experiments. It can be inferred that hepatic ferroportin1 mRNA expression was induced in a compensatory manner to maintain a constant level of hepatic iron.

In terms of hepatic lipid metabolism and deposition, feeding a high-fat diet

JPET #203448

increased hepatic triglycerides in H compared to C. However, ezetimibe reduced hepatic triglycerides in HE and HIE compared to H and HI, respectively, in agreement with previous reports (Zheng et al., 2008; Muraoka et al., 2011; de Bari et al., 2012). Furthermore, recent studies demonstrated the efficacy of ezetimibe treatment on NAFLD in humans (Park et al., 2011; Tamaki et al., 2012). Previous reports showed that ezetimibe ameliorates hepatic steatosis by reducing SREBP-1c mRNA expression in livers of mice fed a high-fat diet and by inducing hepatic mRNA expression of microsomal triglyceride transfer protein (MTTP), which secretes VLDL-triglyceride into blood, in human NASH patients (Muraoka et al., 2011; Yoneda et al., 2011). In our study, hepatic triglycerides were decreased in HI compared to H, suggesting that hepatic iron reduces hepatic triglycerides in mice fed a high-fat diet with iron. In addition, the present study showed that ezetimibe increased hepatic iron in mice fed a high-fat diet with or without iron. Taken together, increased hepatic iron by ezetimibe might partially contribute to decrease hepatic triglycerides in mice fed a high-fat diet with or without iron. Concerning markers for liver injury, serum ALT and ALP were quantified in our study. Serum ALT and ALP were not statistically significant differences between groups. Although the increased hepatic iron by ezetimibe did not cause liver injury, it may induce oxidative stress in livers of mice. Therefore, it might be of interest to quantify marker for oxidative stress, including malondialdehyde (MDA), in liver. In

JPET #203448

regard to cholesterol metabolism, iron supplemented control diet (CI group) increased hepatic cholesterol levels, in agreement with the aforementioned report (Graham et al., 2010). Besides, ezetimibe decreased hepatic cholesterol in the only control-diet groups. Conversely, neither ezetimibe nor iron reduced hepatic cholesterol in the high-fat diet groups. Summary of high-fat diet with ezetimibe and/or iron-induced changes in hepatic triglyceride, cholesterol, and iron levels was shown in Table 5.

In conclusion, ezetimibe tended to increase DMT1 mRNA expression in the duodenum of mice fed a high-fat diet and significantly enhanced hepatic Tfr1 mRNA expression, leading to an increase in hepatic iron levels. Because hepatic iron levels were not increased in mice fed a control diet, it was possible that some components of the high-fat diet and/or the steatosis induced by high-fat diet feeding modified the effects of ezetimibe on iron metabolism in the liver.

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JPET #203448

## **Authorship Contributions**

*Participated in research design:* Kishino, Tanaka.

*Conducted experiments:* Kishino, Tanaka, Ikeda, Kamisako, Yamamoto

*Contributed new reagents or analytic tools:* Kamisako, Ogawa, Iwatani, Ikeda,

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*Performed data analysis:* Kishino

*Wrote or contributed to the writing of the manuscript:* Kishino, Tanaka

JPET #203448

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JPET #203448

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JPET #203448

## Footnotes

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JPET #203448

## Figure Legends

### **Fig. 1. Serum iron-related markers and hepatic iron concentrations in mice after 12 weeks of each diet in Experiment 1.**

Data are presented as mean  $\pm$  S.E.M. (each group, n = 6 animals). C, the control group; CE, the control plus ezetimibe group; H, the high-fat diet group; HE, the high-fat diet plus ezetimibe group. \* represents statistically significant difference (p < 0.05) between H and HE.

### **Fig. 2. mRNA expression of iron-regulatory genes in mouse liver after 12 weeks of each diet in Experiment 1.**

Data are presented as mean  $\pm$  S.E.M. (each group, n = 6 animals). C, the control group; CE, the control plus ezetimibe group; H, the high-fat diet group; HE, the high-fat diet plus ezetimibe group. \* represents statistically significant difference (p < 0.05) between two groups as indicated by connected lines in figure; \*\* similarly represent a statistically significant difference (p < 0.01) between two groups.

### **Fig. 3. Serum iron-related markers and hepatic iron concentrations in mice after 12 weeks of each diet in Experiment 2.**

JPET #203448

Data are presented as mean  $\pm$  S.E.M. (each group, n = 5 or 6 animals). CI, the control plus iron group; CIE, the control plus iron plus ezetimibe group; HI, the high-fat diet plus iron group; HIE, the high-fat diet plus iron plus ezetimibe group. \* represents statistically significant difference ( $p < 0.05$ ) between two groups as indicated by connected lines in figure; \*\* similarly represent a statistically significant difference ( $p < 0.01$ ) between two groups.

**Fig. 4. mRNA expression of iron-regulatory genes in mouse liver after 12 weeks of each diet in Experiment 2.**

Data are presented as mean  $\pm$  S.E.M. (each group, n = 5 or 6 animals). CI, the control plus iron group; CIE, the control plus iron plus ezetimibe group; HI, the high-fat diet plus iron group; HIE, the high-fat diet plus iron plus ezetimibe group. \* represents statistically significant difference ( $p < 0.05$ ) between two groups as indicated by connected lines in figure; \*\* similarly represent a statistically significant difference ( $p < 0.01$ ) between two groups.

JPET #203448

**Table 1. Body weight, liver weight, liver weight to body weight, serum and hepatic lipid profiles, and mRNA expression of lipid metabolism related genes in liver in Experiment 1**

Data are presented as mean  $\pm$  S.E.M. (each group, n = 6 animals). C, the control group; CE, the control plus ezetimibe group; H, the high-fat diet group; HE, the high-fat diet plus ezetimibe group. \*p < 0.05, significant difference from C; \*\*p < 0.01, significant difference from C; ††p < 0.01, significant difference from CE; ‡‡p < 0.01, significant difference from H.

	C	CE	H	HE
Starting body weight (g)	22.5 $\pm$ 0.5	22.6 $\pm$ 0.4	22.2 $\pm$ 0.2	22.1 $\pm$ 0.1
Body weight increase (g)	14.1 $\pm$ 1.1	12.5 $\pm$ 0.8	17.5 $\pm$ 1.9	13.3 $\pm$ 1.0
Liver weight (g)	1.55 $\pm$ 0.06	1.45 $\pm$ 0.05	1.68 $\pm$ 0.14	1.21 $\pm$ 0.05 <sup>††</sup>
Liver / body weight (%)	4.21 $\pm$ 0.06	4.13 $\pm$ 0.09	4.20 $\pm$ 0.18	3.42 $\pm$ 0.08 <sup>††, ‡‡</sup>
Serum total cholesterol (mg/dl)	122 $\pm$ 5	92 $\pm$ 8	152 $\pm$ 14	126 $\pm$ 10
Serum triglyceride (mg/dl)	43 $\pm$ 6	36 $\pm$ 3	26 $\pm$ 3 <sup>*</sup>	25 $\pm$ 2
Hepatic total cholesterol (mg/g tissue)	2.66 $\pm$ 0.13	2.09 $\pm$ 0.06 <sup>**</sup>	3.41 $\pm$ 0.13 <sup>**</sup>	3.04 $\pm$ 0.10 <sup>††</sup>
Hepatic triglyceride (mg/g tissue)	40.7 $\pm$ 4.9	30.9 $\pm$ 3.5	89.0 $\pm$ 15.0 <sup>**</sup>	38.1 $\pm$ 5.3 <sup>††</sup>
Hepatic mRNA expression of lipid metabolism-related genes (fold change of C)				
Srebp2	1.00 $\pm$ 0.09	3.22 $\pm$ 0.24 <sup>**</sup>	1.85 $\pm$ 0.17 <sup>*</sup>	4.20 $\pm$ 0.19 <sup>††, ‡‡</sup>
HMGCoAR	1.00 $\pm$ 0.19	4.48 $\pm$ 0.76 <sup>**</sup>	1.30 $\pm$ 0.10	6.46 $\pm$ 0.90 <sup>††</sup>
Ldl receptor	1.00 $\pm$ 0.07	2.36 $\pm$ 0.21 <sup>**</sup>	1.21 $\pm$ 0.07	2.95 $\pm$ 0.41 <sup>††</sup>
Srebp-1c	1.00 $\pm$ 0.12	1.11 $\pm$ 0.12	1.16 $\pm$ 0.15	0.98 $\pm$ 0.15

JPET #203448

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Fas	1.00±0.14	2.24±0.56 *	0.71±0.07	1.14±0.21
Acox1	1.00±0.08	1.80±0.18 *	1.52±0.12	2.79±0.28 **,‡‡

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JPET #203448

**Table 2. mRNA expression of iron metabolism-related genes in duodenum in Experiment 1**

Data are presented as mean  $\pm$  S.E.M. (each group, n = 6 animals). C, the control group; CE, the control plus ezetimibe group; H, the high-fat diet group; HE, the high-fat diet plus ezetimibe group. \* p < 0.05, significant difference from C; \*\* p < 0.01, significant difference from C.

	Fold change of C			
	C	CE	H	HE
Dcytb	1.00 $\pm$ 0.47	3.84 $\pm$ 1.62	0.25 $\pm$ 0.05	1.26 $\pm$ 0.30
DMT1	1.00 $\pm$ 0.15	2.62 $\pm$ 0.64 *	1.19 $\pm$ 0.06	2.34 $\pm$ 0.24
FerritinH	1.00 $\pm$ 0.12	2.35 $\pm$ 0.38 **	1.33 $\pm$ 0.09	1.76 $\pm$ 0.23
FerritinL	1.00 $\pm$ 0.11	1.39 $\pm$ 0.10	1.71 $\pm$ 0.14 **	1.67 $\pm$ 0.14
Ferroportin1	1.00 $\pm$ 0.27	3.67 $\pm$ 1.33	1.12 $\pm$ 0.28	2.91 $\pm$ 0.56
Hephaestin	1.00 $\pm$ 0.11	1.85 $\pm$ 0.22 *	1.52 $\pm$ 0.09	1.74 $\pm$ 0.27

JPET #203448

**Table 3. Body weight, liver weight, liver weight to body weight, serum and hepatic lipid profiles, and mRNA expression of lipid metabolism related genes in liver in Experiment 2**

Data are presented as mean  $\pm$  S.E.M. (each group, n = 5 or 6 animals). CI, the control plus iron group; CIE, the control plus iron plus ezetimibe group; HI, the high-fat diet plus iron group; HIE, the high-fat diet plus iron plus ezetimibe group. \*p < 0.05, significant difference from CI; \*\*p < 0.01, significant difference from CI; †p < 0.05, significant difference from CIE; ††p < 0.01, significant difference from CIE; ‡p < 0.05, significant difference from HI; ‡‡p < 0.01, significant difference from HI.

	CI	CIE	HI	HIE
Starting body weight (g)	22.6 $\pm$ 0.3	22.2 $\pm$ 0.2	21.7 $\pm$ 0.2	22.4 $\pm$ 0.1
Body weight increase (g)	12.4 $\pm$ 0.8	10.7 $\pm$ 1.1	10.7 $\pm$ 0.9	6.8 $\pm$ 1.1 <sup>†</sup>
Liver weight (g)	1.70 $\pm$ 0.07	1.53 $\pm$ 0.05	1.63 $\pm$ 0.04	1.29 $\pm$ 0.06 <sup>†,‡‡</sup>
Liver / body weight (%)	4.85 $\pm$ 0.10	4.67 $\pm$ 0.12	5.03 $\pm$ 0.14	4.43 $\pm$ 0.09 <sup>‡‡</sup>
Serum total cholesterol (mg/dl)	111 $\pm$ 6	108 $\pm$ 9	125 $\pm$ 4	98 $\pm$ 6
Serum triglyceride (mg/dl)	55 $\pm$ 5	35 $\pm$ 3 <sup>**</sup>	29 $\pm$ 1 <sup>**</sup>	28 $\pm$ 1
Hepatic total cholesterol (mg/g tissue)	3.33 $\pm$ 0.12	2.51 $\pm$ 0.11 <sup>**</sup>	3.52 $\pm$ 0.19	2.95 $\pm$ 0.15
Hepatic triglyceride (mg/g tissue)	45.1 $\pm$ 4.5	42.3 $\pm$ 8.7	49.5 $\pm$ 5.8	20.0 $\pm$ 4.9 <sup>‡</sup>
Hepatic mRNA expression of lipid metabolism-related genes (fold change of CI)				
Srebp2	1.00 $\pm$ 0.10	1.27 $\pm$ 0.08	1.63 $\pm$ 0.20 <sup>*</sup>	2.57 $\pm$ 0.13 <sup>††,‡‡</sup>
HMGCoAR	1.00 $\pm$ 0.13	1.39 $\pm$ 0.14	2.51 $\pm$ 0.72	3.71 $\pm$ 0.35 <sup>††</sup>
Ldl receptor	1.00 $\pm$ 0.12	1.15 $\pm$ 0.09	1.18 $\pm$ 0.09	1.55 $\pm$ 0.14

JPET #203448

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Srebp-1c	1.00±0.11	0.68±0.03	1.36±0.19	1.26±0.22
Fas	1.00±0.15	0.98±0.10	1.08±0.26	0.90±0.05
Acox1	1.00±0.14	1.14±0.10	1.83±0.26 **	1.70±0.08

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JPET #203448

**Table 4. mRNA expression of iron metabolism-related genes in duodenum in Experiment 2**

Data are presented as mean  $\pm$  S.E.M. (each group, n = 5 or 6 animals). CI, the control plus iron group; CIE, the control plus iron plus ezetimibe group; HI, the high-fat diet plus iron group; HIE, the high-fat diet plus iron plus ezetimibe group. <sup>†</sup>p < 0.05, significant difference from CIE.

	Fold change of CI			
	CI	CIE	HI	HIE
Dcytb	1.00 $\pm$ 0.68	1.23 $\pm$ 0.59	0.95 $\pm$ 0.43	1.22 $\pm$ 0.93
DMT1	1.00 $\pm$ 0.11	0.94 $\pm$ 0.12	1.29 $\pm$ 0.18	1.07 $\pm$ 0.23
FerritinH	1.00 $\pm$ 0.06	1.07 $\pm$ 0.11	1.16 $\pm$ 0.18	1.15 $\pm$ 0.19
FerritinL	1.00 $\pm$ 0.08	0.78 $\pm$ 0.05	1.49 $\pm$ 0.26	1.53 $\pm$ 0.24 <sup>†</sup>
Ferroportin1	1.00 $\pm$ 0.41	0.93 $\pm$ 0.21	1.21 $\pm$ 0.45	1.09 $\pm$ 0.45
Hephaestin	1.00 $\pm$ 0.19	0.99 $\pm$ 0.09	1.32 $\pm$ 0.20	1.49 $\pm$ 0.21

JPET #203448

**Table 5. Summary of high-fat diet with ezetimibe and/or iron-induced changes in hepatic triglyceride, cholesterol, and iron concentrations**

↔, no significant change in hepatic levels; ↑/↓, increased (or decreased) hepatic levels; ↑↑/↓↓, increased (or decreased) more hepatic levels; ↑↑↑, increased much more hepatic levels; and e.g., CE vs. C, CE compared to C.

	<b>Effect of Ezetimibe</b>	change	<b>Effect of Iron</b>	change	<b>Effect of High-fat diet</b>	change
<b>Hepatic Triglycerides</b>	CE vs. C	↔	CI vs. C	↔	H vs. C	↑↑
	CIE vs. CI	↔	CIE vs. CE	↔	HE vs. CE	↔
	HE vs. H	↓↓	HI vs. H	↓	HI vs. CI	↔
	HIE vs. HI	↓	HIE vs. HE	↔	HIE vs. CIE	↔
<b>Hepatic Cholesterol</b>	CE vs. C	↓	CI vs. C	↑	H vs. C	↑↑
	CIE vs. CI	↓	CIE vs. CE	↔	HE vs. CE	↑
	HE vs. H	↔	HI vs. H	↔	HI vs. CI	↔
	HIE vs. HI	↔	HIE vs. HE	↔	HIE vs. CIE	↔
<b>Hepatic Iron</b>	CE vs. C	↔	CI vs. C	↑↑↑	H vs. C	↔
	CIE vs. CI	↔	CIE vs. CE	↑↑↑	HE vs. CE	↔
	HE vs. H	↑	HI vs. H	↑↑↑	HI vs. CI	↑
	HIE vs. HI	↑↑	HIE vs. HE	↑↑↑	HIE vs. CIE	↑↑

### Serum iron concentrations

	C	CE	H	HE
Iron ( $\mu\text{g}/\text{dl}$ )	123 $\pm$ 2.0	152 $\pm$ 14.9	148 $\pm$ 10.9	121 $\pm$ 7.5
UIBC ( $\mu\text{g}/\text{dl}$ )	200 $\pm$ 5.0	186 $\pm$ 6.5	192 $\pm$ 7.2	180 $\pm$ 8.7
Transferrin%	38.1 $\pm$ 0.47	44.4 $\pm$ 1.79	43.3 $\pm$ 2.00	40.3 $\pm$ 1.74

### Hepatic iron concentrations

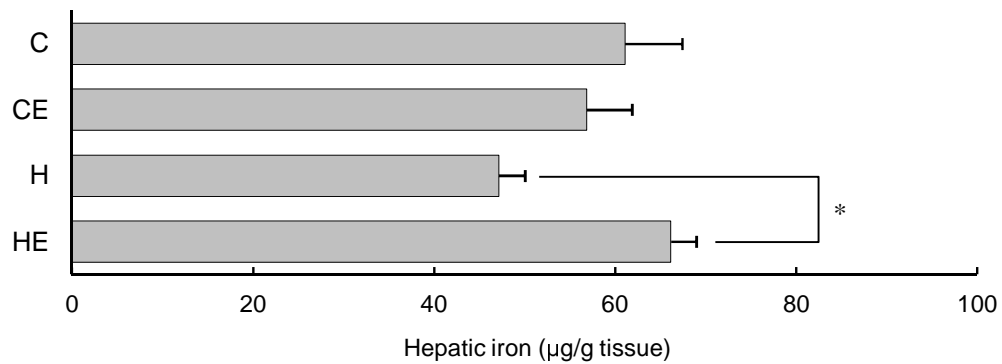


Fig.1

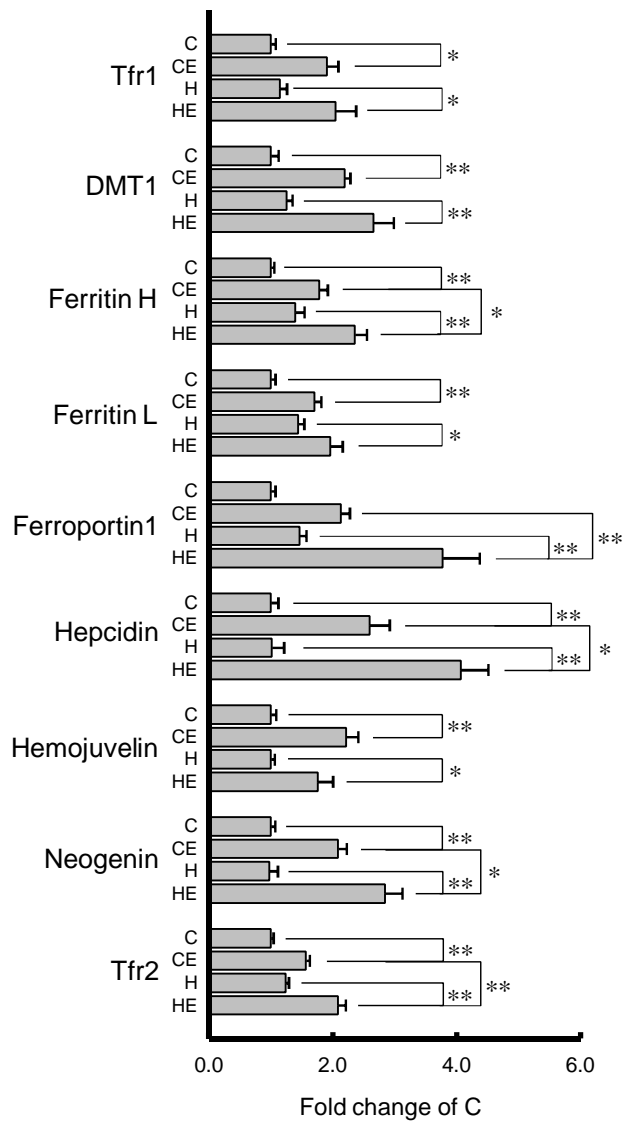


Fig.2

### Serum iron concentrations

	CI	CIE	HI	HIE
Iron ( $\mu\text{g}/\text{dl}$ )	225 $\pm$ 24.7	216 $\pm$ 39.6	215 $\pm$ 23.2	236 $\pm$ 21.2
UIBC ( $\mu\text{g}/\text{dl}$ )	81 $\pm$ 27.0	65 $\pm$ 37.5	100 $\pm$ 37.5	20 $\pm$ 5.9
Transferrin%	74.2 $\pm$ 7.78	77.3 $\pm$ 13.06	70.3 $\pm$ 10.76	91.5 $\pm$ 2.74

### Hepatic iron concentrations

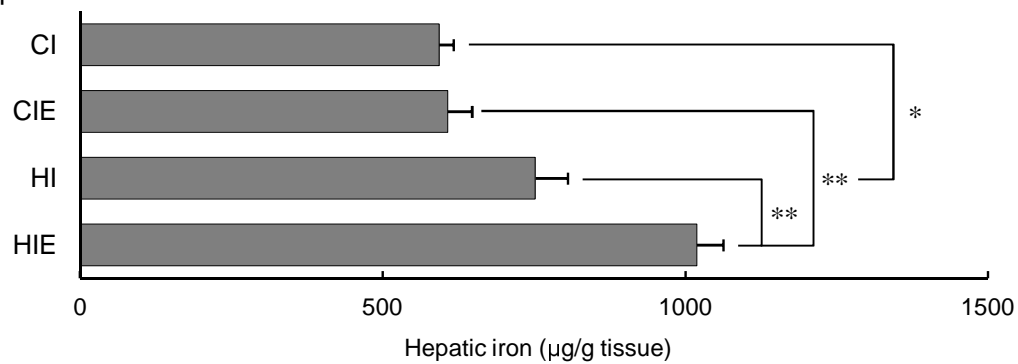


Fig.3

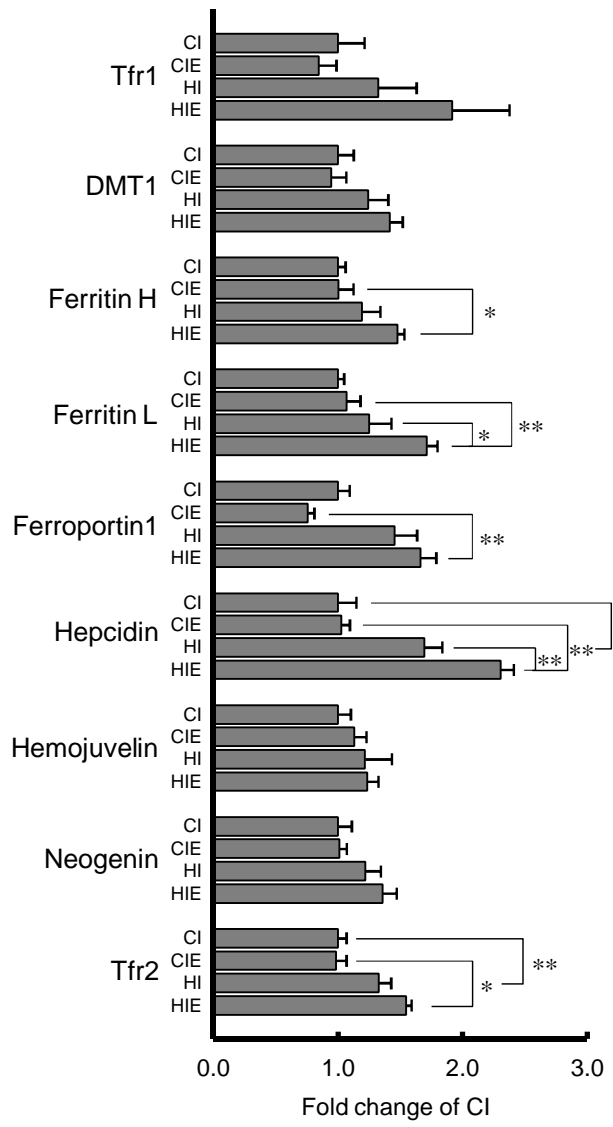


Fig.4

**Supplemental Table. “Composition of the control and experimental diets (g/100g)”**

by Yoshizumi Kishino, Yuji Tanaka, Takanori Ikeda, Kazuo Yamamoto, Hiroshi Ogawa, Yoshinori Iwatani and Toshinori Kamisako. *Journal of Pharmacology and Experimental Therapeutics*. Abbreviations: C, the control group; CE, the control plus ezetimibe group; H, the high-fat diet group; HE, the high-fat diet plus ezetimibe group; CI, the control plus iron group; CIE, the control plus iron plus ezetimibe group; HI, the high-fat diet plus iron group; HIE, the high-fat diet plus iron plus ezetimibe group.

## Experiment 1

	C	CE	H	HE
Lard	—	—	20.00	20.00
Ezetimibe	—	0.007	—	0.013
Daily ezetimibe intake	—	0.3mg/day	—	0.3mg/day
Soybean oil	4.00	4.00	4.00	4.00
Milk casein	14.00	14.00	14.00	14.00
L-Cystine	0.18	0.18	0.18	0.18
AIN-93G mineral mixture	3.50	3.50	3.50	3.50
AIN-93VX vitamin mixture	1.00	1.00	1.00	1.00
Cellulose powder	5.00	5.00	5.00	5.00
Corn starch	46.57	46.57	26.57	26.57
$\alpha$ -Corn starch	15.50	15.50	15.50	15.50
Sucrose	10.00	10.00	10.00	10.00
Choline bitartrate	0.25	0.25	0.25	0.25

	C	CE	H	HE
Energy (kJ/g diet)	15.95	15.95	20.13	20.13
Energy (kcal/g diet)	3.81	3.81	4.81	4.81

## Experiment 2

	CI	CIE	HI	HIE
Lard	—	—	20.00	20.00
Ezetimibe	—	0.007	—	0.015
Daily ezetimibe intake	—	0.3mg/day	—	0.3mg/day
Carbonyl iron	0.54	0.54	1.00	1.00
Daily carbonyl iron intake	21.3mg/day	21.1mg/day	24.3mg/day	22.7mg/day
Soybean oil	4.00	4.00	4.00	4.00
Milk casein	14.00	14.00	14.00	14.00
L-Cystine	0.18	0.18	0.18	0.18
AIN-93G mineral mixture	3.50	3.50	3.50	3.50
AIN-93VX vitamin mixture	1.00	1.00	1.00	1.00
Cellulose powder	5.00	5.00	5.00	5.00
Corn starch	46.04	46.04	25.57	25.57
$\alpha$ -Corn starch	15.50	15.50	15.50	15.50

Sucrose	10.00	10.00	10.00	10.00
Choline bitartrate	0.25	0.25	0.25	0.25
	CI	CIE	HI	HIE
Energy (kJ/g diet)	15.87	15.87	19.97	19.97
Energy (kcal/g diet)	3.79	3.79	4.77	4.77