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Ezetimibe improves liver steatosis and insulin resistance in obese rat model of metabolic syndrome

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Abstract **Non-alcoholic fatty liver disease (NAFLD) is associated with the metabolic syndrome characterized by dislipidemia and insulin resistance. We hypothesized that ezetimibe, an inhibitor of NPC1L1, improves these metabolic disorders in Zucker obese fatty rats (ZOF). Ezetimibe significantly lowered total cholesterol and triglycerides in ZOF with prominent reduction in the remnant lipoprotein fraction and small dense low density lipoprotein fraction. Moreover, lipid deposition and fibrosis of liver were decreased by ezetimibe. Interestingly, ezetimibe improved insulin and plasma glucose response after intraperitoneal glucose injection. Further, ezetimibe enhanced insulin signaling in cultured hepatocytes. Our results indicate the potential of ezetimibe in treating the metabolic syndrome and NAFLD.**

Keywords: Metabolic syndrome; Lipid metabolism; Liver steatosis; Insulin resistance; NPC1L1; Ezetimibe

1. Introduction

Dyslipidemia including hypercholesterolemia is a major risk factor for coronary heart disease. The level of cholesterol is tightly regulated by endogenous synthesis in the liver and dietary absorption/biliary reabsorption in the small intestine [1]. Niemann-Pick C1 Like 1 (NPC1L1) has been shown to play a pivotal role in cholesterol incorporation in the enterocytes [2–4]. Genetically engineered NPC1L1 null mouse exhibited a significant reduction of atherosclerosis compared to wild type under high-fat diet [2,5]. In addition to changes in plasma lipid levels, NPC1L1 null mouse were significantly protected from fatty liver formation, which occurred in wild type mouse with high-fat diet [2,6], suggesting a potential role for NPC1L1 in regulation of lipid metabolism in liver. Ezetimibe, a potent inhibitor of cholesterol absorption, has been shown to inhibit the NPC1L1-dependent cholesterol transport at the brush borders of intestines [3,7]. Ezetimibe selectively inhibits intestinal cholesterol absorption [8]. In humans, ezetimibe has been shown to lower serum LDL cholesterol levels and triglycerides (TG) [9]. Moreover, recent studies suggest its potential effect on liver steatosis [10] and insulin resistance [11] which comprise the metabolic syndrome (MetS). Non-alcoholic fatty liver disease (NAFLD) including non-alcoholic steatohepatitis (NASH) is characterized by varying degrees of progressive steatosis, lobular inflammation and fibrosis of the liver [12,13]. Recent reports suggest type 2 diabetes mellitus, obesity and dyslipidemia often coexists with NAFLD [14]. In particular, hyperlipidemia and insulin resistance importantly contribute to the initiation and progression of NAFLD [15]. Recent clinical studies show that NAFLD is one of the main common liver diseases that lead to the liver cirrhosis and hepatocellular carcinoma [13]. However, the molecular mechanisms responsible for progression of NAFLD have not been fully understood. Further, intestinal cholesterol absorption is elevated in those with type 2 diabetic patients with coronary heart diseases [16], and low cholesterol absorption associates with fewer recurrent cardiovascular events [17]. Zucker obese fatty (ZOF) rat is good metabolic syndrome model [18] and has hepatic steatosis [19]. The present study investigated the potential effect of ezetimibe on the development of NAFLD using Zucker obese fatty (ZOF) rats fed with high-fat diet.

2. Materials and methods

2.1. Cell culture and reagents

HepG2 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% FBS. Monoclonal Abs (mAb) used in this study were as follows: Anti-α smooth muscle actin (SMA) (from Sigma), monoclonal anti-TGFβ antibody (from Sigma), anti-IR-β (from Santa Cruz), anti-phospho-IR-β (from Santa Cruz), anti-IRS-1 (from Santa Cruz), anti-phospho-IRS-1 (from Santa Cruz), anti-Akt-1 (from Santa Cruz), and anti-phospho-Akt-1 (from Santa Cruz).

2.2. Animal study

The experiments were approved by the Ethical Committee for Animal Experimentation of Tokyo Medical and Dental University. Male Zucker fatty (fa/fa) rats (7 week of age) (Japan SLC, Inc., Shizuoka, Japan) were housed with a regular 12-h/12-h light/dark cycle. Rats were fed a low fat diet for 1 week, and following 4 weeks, one group ($n = 6$) were fed Rodent Diet with 60 kcal% fat (soy bean oil:lard = 1:10), protein 20 kcal%, and carbohydrate 20 kcal% (RESEARCH DIETS, Inc., NJ) as high-fat diet (HF) and the other group ($n = 6$) were fed HF containing 0.008% w/w ezetimibe (HF + Ez) which was supplied by Schering-Plough K.K. The concentration of ezetimibe

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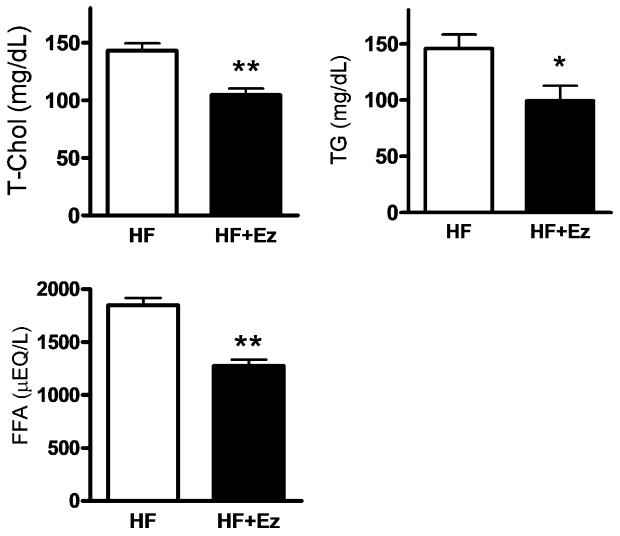

Fig. 1. Total serum cholesterol (T-Chol), triglyceride (TG), and free fatty acid (FFA) in ZOF rats fed with high-fat diet (HF) or high-fat diet with ezetimibe (HF + Ez) for 4 weeks. $^{*}P < 0.05$ HF vs. HF + Ez, $^{**}P < 0.001$ HF vs. HF + Ez.

(2.7–6.4 mg/kg/day) was determined based on previous experiments using rats [8,20]. Rats were free access to water and the foods until the experiments. Some results were pooled and a subset was analyzed.

2.3. Metabolic measurement

Plasma lipoproteins were analyzed by on-line dual enzymatic method for simultaneous quantification of cholesterol and TG by HPLC as described previously [21].

2.4. TG secretion rate

TG secretion rate (TGSR) was measured as previously described [22]. Briefly, 500 mg/kg body weight of Triton WR-1339 (Sigma–Aldrich) was injected via the tail vein into rats fasted for 5 h, and triglyceride concentrations were measured in plasma samples taken 90 min after injection. The secretion rate expressed in milligrams per minute was calculated from the increment in triglyceride concentration per minute multiplied by the plasma volume of the rats (estimated as 3.5% of body weight in grams).

2.5. Tissue morphology

Liver samples were fixed overnight in 4% paraformaldehyde for cryo-sections, and 10% buffered formalin for embedding in paraffin. Paraffin sections of livers were stained with hematoxylin and eosin. Cryo-sections were stained with Oil red O. For the Fast green/Sirius red staining, 5 μm sections were stained with 0.04% Fast green/0.1% Sirius red for 15 min. Samples were dehydrated and mounted using

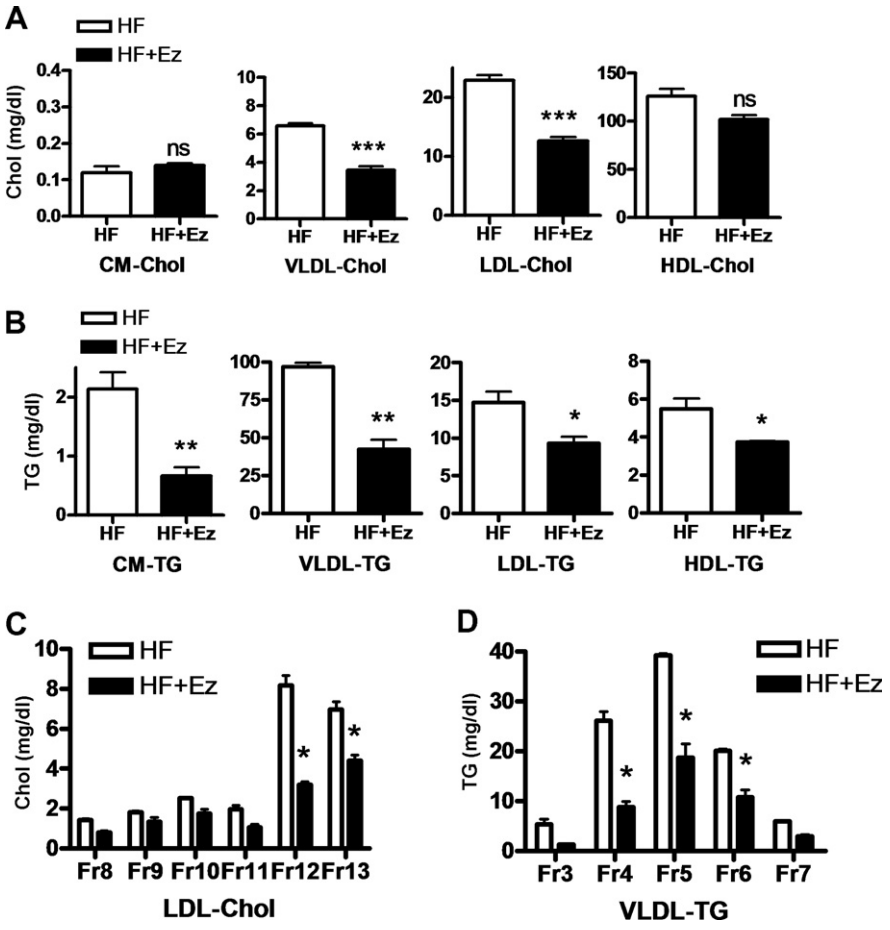

Fig. 2. Lipoprotein profile by HPLC in ZOF rats fed with high-fat diet (HF, open bars) or high-fat diet with ezetimibe (HF + Ez, closed bars) for 4 weeks. Cholesterol (A) and triglycerides (B) content in chylomicron (CM), very low-density lipoprotein (VLDL), low-density lipoprotein (LDL), and high-density lipoprotein (HDL) were determined using enzymatic reagents. $^{*}P < 0.05$, $^{**}P < 0.005$ vs. HF. (C) Cholresterol content (Chol) in LDL was further analyzed in 6 subfractions according to the particle size. $^{*}P < 0.001$ vs. HF. (D) Triglycerides content (TG) in VLDL was further analyzed in 6 subfractions according to the particle size. $^{*}P < 0.001$ vs. HF.

malinol. To quantitate Sirius red intensity, the color was eluted form the slide with 1 ml of 0.1% NaOH:absolute methanol (50:50) and the absorbance was measured at 540 nm (λmax for Sirius red corresponding to collagenous proteins).

2.6. Liver lipid analysis

The liver was rapidly removed and lipids were extracted from these tissues (50 mg) according to methods modified from Folch et al. [23]. Briefly, snap frozen liver was homogenized and extracted with chloroform/methanol (2:1 v/v) solution. The organic phase was dried and resolubilized in 2-propanol containing 10% Triton X-100. Total cholesterol levels and TG were determined by enzymatic kits as described [24,25].

2.7. Preparation of HepG2 cells lysate

HepG2 cells were grown in 6 well plates up to 80% confluence prior to any treatment. Cells were washed with PBS and treated with ezetimibe (25 μM) or DMSO in serum-free medium for 48 h, followed by the treatment of insulin (10 nM) or PBS for 5 min. Cells were lysed and subjected to Western blot analysis.

2.8. Western blot analysis

Western blot analysis was performed using lysates prepared from HepG2 cells and liver sample as described previously [26]. An equal amount of protein (20 μg) from each condition was subjected to 10% SDS-PAGE. Immunoreactive proteins were detected using an enhanced chemiluminescence (ECL) kit (Amersham Bioscience).

2.9. Intraperitoneal glucose tolerance test (IPGTT)

ZOF rats were given an intraperitoneal glucose tolerance test (IPGTT) (2 g/kg body weight of glucose) after 16 h of fasting. Glucose and insulin levels were measured at 0, 30, and 120 min using an enzymatic method (SRL Inc., Tokyo, Japan) for glucose and an Ultra Sensitive ELISA kit (Morinaga, Kanagawa, Japan) for insulin.

2.10. Statistical analysis

Data are expressed as mean values ± S.E.M. One-way ANOVA with Tukey post-hoc test or two-tailed unpaired *t*-test was used to analyze statistical significance. Values are expressed as means ± S.E. with a value of $P < 0.05$ considered statistically significant.

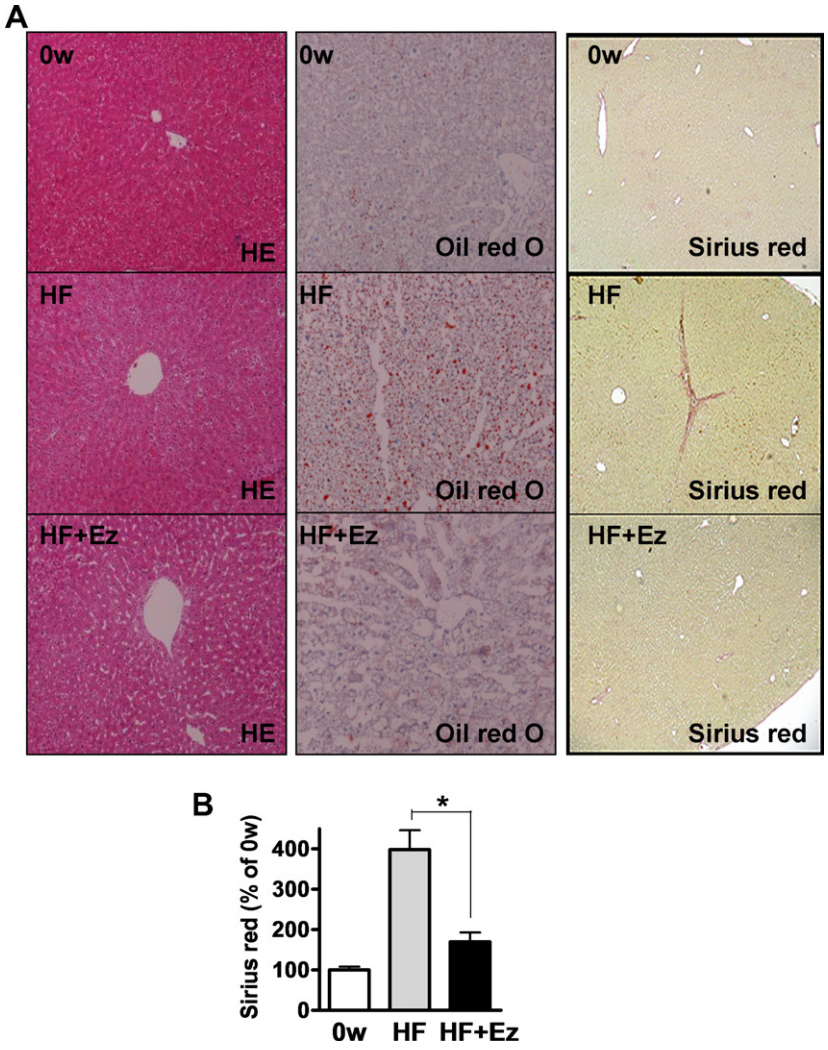

Fig. 3. (A) Histological analysis of liver samples stained with hematoxylin + eosin (HE, 100×), Oil red O (200×), Sirius Red/Fast green staining (40×) before (0w) and after 4 weeks with high-fat diet (4w HF) or high-fat diet with ezetimibe (4w HF + Ez). (B) Sirius Red-positive collagenous proteins in livers of ZOF rats fed a high-fat diet (HF, gray bars) or high-fat diet with ezetimibe (HF + Ez, closed bars) for 4 weeks as compared to control (0w, open bars) were quantified as described in Section 2. $^{*}P < 0.01$ vs. HF.

3. Results

3.1. Food consumption and body weights

We monitored the food consumption and body weight of both groups throughout the observation period. Food consumption was not affected by ezetimibe treatment, and both groups continuously gained weight. Finally, the mean body weight was not significantly changed in both groups (data not shown).

3.2. Ezetimibe improves HF-induced dyslipidemia in ZOF

We measured plasma lipid and lipoprotein profiles of both groups after 4 weeks of HF diet. HF diet induced hyperlipidemia characterized by elevated serum cholesterol level. Ezetimibe treatment reduced plasma cholesterol, TG, and free fatty acid (FFA) by 27%, 32%, and 31%, respectively (Fig. 1) and significantly decreased cholesterol in VLDL and LDL fractions (Fig. 2A). Decrease in TG content was prominent for chylomicron and VLDL fractions (Fig. 2B). As shown in Fig. 2C, reduction of cholesterol was attributed to smaller size particles, Fr12 and Fr13 (the average diameters are 18.6 nm and 16.7 nm, respectively), which correspond to small dense LDL. In contrast, reduction of TG was prominent in larger size particles, Fr4 and Fr5 (the average diameters are 53.6 nm and 44.5 nm, respectively), which correspond to VLDL1 (Fig. 2D).

3.3. Ezetimibe improves HF-induced hepatic steatosis and fibrosis in ZOF

We examined whether ezetimibe affects HF-induced steatosis in ZOF. Liver histology showed that HF diet induced lipid accumulation in the liver determined by Oil Red O staining, though we did not find significant infiltration of inflammatory cells by HE staining (HE and Oil Red O in Fig. 3A). Ezetimibe treatment improved HF-induced hepatic fibrosis of ZOF judged from Sirius Red staining (Sirius Red in Fig. 3A) and its quantity (Fig. 3B). In fact, ezetimibe treatment improved HF increased hepatic cholesterol and TG content (Fig. 4A and B). Interestingly, there was no significant difference in TG secretion rate between ezetimibe treated and control groups (Fig. 4C). Further, immunoblotting showed increased expressions of SMA and TGFβ in the liver by HF diet (Fig. 4D). Taken together, ZOF rats had liver steatosis, which did not reach to cirrhosis, and ezetimibe could inhibit progression of liver steatosis.

3.4. Ezetimibe improves HF-induced insulin resistance in ZOF

Hepatic steatosis as well as hyperlipidemia closely associates with insulin resistance. Since NPC1L1 is expressed in liver as well as intestine in humans, we examined whether ezetimibe affects glycemic metabolism in the liver [2]. When IPGTT was carried out in ZOF, serum glucose level was strongly peaked at 30 min and gradually decreased at 120 min after glucose loading in HF-fed ZOF as shown in Fig. 5A. In contrast, ezetimibe treatment significantly reduced peak glucose level at 30 min as well as that at 120 min, though fasting glucose level was not significantly different from control. When insulin levels were measured, its fasting level in HF diet with ezetimibe treatment group was lower than without ezetimibe treatment group, though not statistically significant. The glucose-induced insulin secretion was significantly decreased in ezetimibe treatment group at 30 min and 120 min after glucose loading (Fig. 5B). These results indicate that ezetimibe improves insulin resistance of ZOF rats.

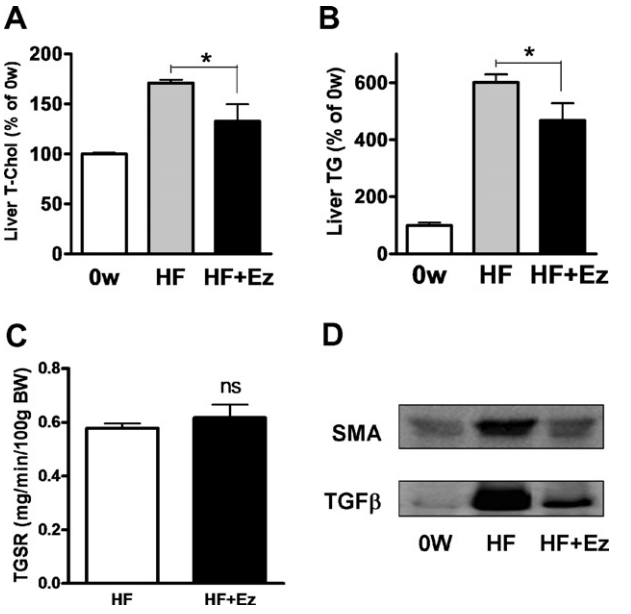

Fig. 4. Biochemical analysis of liver samples taken from ZOF with high-fat diet (4w HF) or high-fat diet with ezetimibe (4w HF + Ez). (A) Total cholesterol levels (Liver T-Chol) and (B) triglycerides (Liver TG) were measured from lipids extracted from livers of ZOF rats fed a high-fat diet (HF, gray bars) or high-fat diet with ezetimibe (HF + Ez, closed bars) for 4 weeks as compared to control (0w, open bars). $^{*}P < 0.01$ vs. HF. (C) TG secretion rate (TGSR) of liver of ZOF rats fed a high-fat diet (HF, open bars) or high-fat diet with ezetimibe (HF + Ez, closed bars). (D) Expressions of SMA and TGFβ in the liver samples before (0w) and after 4 weeks with high-fat diet (4w HF) or high-fat diet with Ezetimibe (4w HF + Ez). Blots represent 3 independent sets of experiments with similar results.

To further investigate an effect of ezetimibe on insulin signaling, phosphorylation of insulin receptor-β (IR-β), insulin receptor substrate-1(IRS-1), and Akt-1 was measured in HepG2 cells. Ezetimibe treatment augmented insulin-induced-phosphorylation of IR-β, IRS-1, and Akt-1 (Fig. 5C), suggesting its direct effect on insulin resistance in hepatocytes.

4. Discussion

In the present study, we demonstrated favorable effects of ezetimibe on liver steatosis and fibrotic change in addition to lipid profile in ZOF rats fed with high-fat diet. Feeding high-fat diet to ZOF rats led to obesity accompanied by hypercholesterolemia, hypertriglyceridemia, fasting hyperglycemia, and hyperinsulinemia, which are characteristic of the profile often observed in patients with MetS. Ezetimibe reduced TG and FFA as well as cholesterol in ZOF rats without affecting food consumption. HPLC analysis revealed that ezetimibe treatment lowered preferentially VLDL fraction that corresponds to VLDL1 sub fraction (Sf 60–400) in humans [27], that reflects a significant reduction in plasma TG. Though the precise mechanisms are yet to be elucidated, we speculated that reduction of chylomicron synthesis in enterocytes may account for its effect in TG improvement [28]. Moreover, Davis et al.

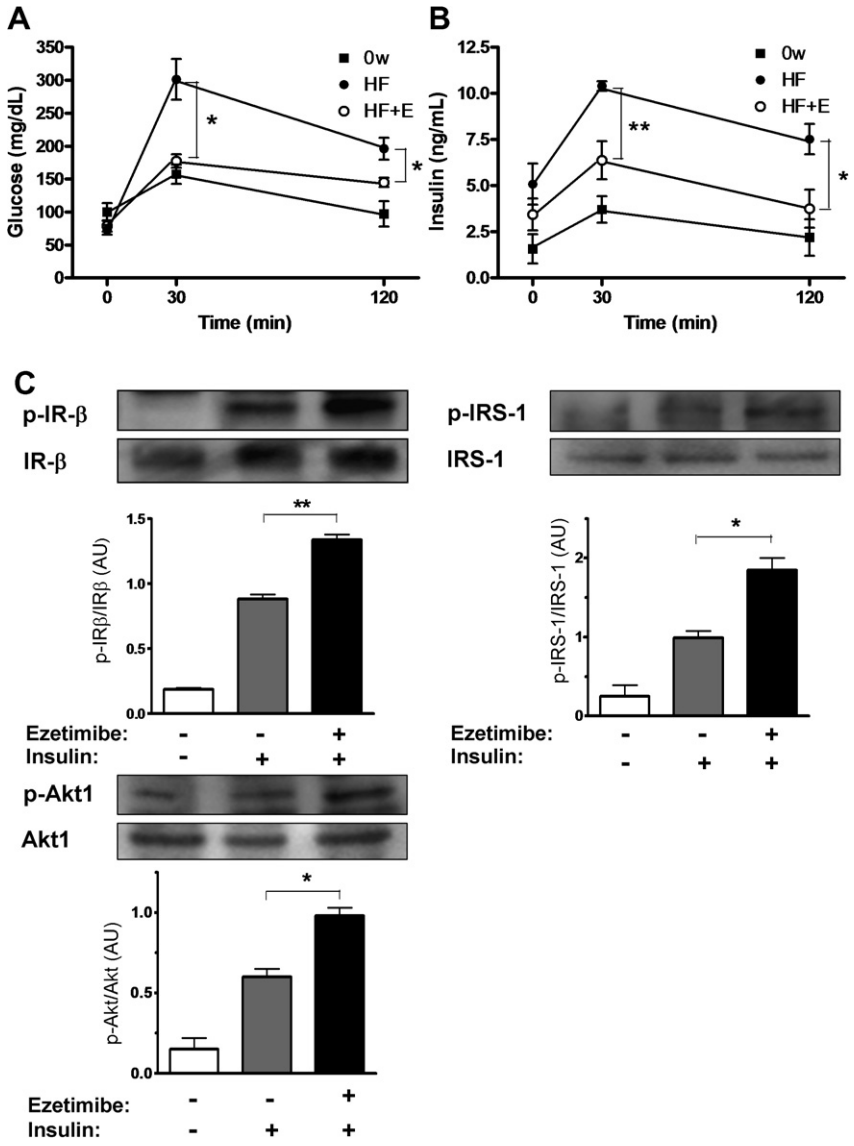

Fig. 5. (A) Glucose responses to IPGTT after ezetimibe treatment in ZOF. Ezetimibe treatment (HF + Ez) significantly improved glucose. $^{*}P < 0.05$ vs. HF. (B) Insulin responses to IPGTT after ezetimibe treatment in ZOF. Ezetimibe treatment (HF + Ez) significantly improved insulin. $^{*}P < 0.05$ vs. HF, $^{**}P < 0.01$ vs. HF. (C) Effects of ezetimibe on insulin-induced-phosphorylation of IR-β, IRS-1 and Akt in HepG2 cells. HepG2 cells were incubated with or without ezetimibe (25 μM) for 48 h followed by stimulation with insulin (10 nM) for 5 min. Immunoblotting analysis was performed as described in methods. $^{*}P < 0.05$ vs. HF, $^{**}P < 0.01$ vs. HF. AU, arbitrary units.

showed chylomicron remnant/VLDL cholesterol levels were reduced 80–90% in NPC1L1/apoE-double deficient mice relative to apoE-deficient mice [5]. Future experiments will require to elucidate the effect of ezetimibe in TG-rich lipoprotein metabolism. Large VLDL1 particles are converted to atherogenic small dense LDL (sdLDL) particles by cholesteryl ester transport protein (CETP) and hepatic lipase (HL), which are commonly increased in type 2 diabetes [29]. Indeed, ezetimibe treatment lowered LDL subfractions 12 and 13, which suggests the favorable effect of the compound on artherogenic lipid profile.

Interestingly, ezetimibe also improved HF-induced hepatic steatosis or lipid accumulation in addition to systemic dyslipidemia. Fat content of the liver reflects the balance between FFA flux, fatty acid oxidation, de novo lipogenesis and VLDL secretion. Taking into consideration its primary effect, ezetimibe seemed to ameliorate liver steatosis by decreasing lipid absorption and subsequently decreased lipoprotein synthesis. On the other hand, lipid secretion from the liver determined by the TGSR was not affected by ezetimibe treatment. This finding indicates that ezetimibe may affect clearance or uptake but not secretion of triglyceride [30]. HF also induced mild fibrotic changes in the liver, which were inhibited by ezetimibe treatment. In chronic liver diseases, TGFβ plays a pivotal role in the progression of hepatic fibrosis. TGFβ produced from Kupffer cell and inflammatory cells activates hepatic satellite cell (HSC). In fact, Ezetimibe abolished upregulation of TGFβ further suggesting its protective role against liver steatosis.

Unlike mouse NPC1L1 protein, which is predominantly expressed in intestine, human and rat NPC1L1 is abundantly expressed in liver [2]. Thus contribution of NPC1L1 protein, expressed in the liver, to the formation of steatosis can be

postulated. It requires further investigation as to whether the effect of ezetimibe on liver steatosis and the subsequent inflammatory burden is dependent on lipid-lowering or the direct effect ezetimibe has on hepatocytes.

We also found that ezetimibe ameliorated insulin resistance in ZOF rats. Although ezetimibe did not significantly change fasting glucose, IPGTT data strongly indicate that ezetimibe improved insulin sensitivity. As reported, the expression level of NPC1L1 is higher in liver compared to other insulin-sensitive organs such as muscle and adipose tissue [2]. Therefore we hypothesized that ezetimibe could directly affect insulin signaling in liver, and showed that ezetimibe dramatically enhanced insulin signaling (i.e., phosphorylation of IR, IRS-1, and Akt-1) in HepG2 cells in vitro (Fig. 5C). Insulin resistance increases TG lipolysis and FFA release from adipocytes, which in turn augment insulin resistance. Boden et al. reported that FFA not only induces insulin resistance but also activates the proinflammatory NF-κB pathway in rat liver, resulting in the development of steatohepatitis [31]. Hyperinsulinemia and high glucose resulting from insulin resistance also independently accelerate the progression of hepatic steatosis through de novo lipogenesis [10,32]. Thus, the break down of this vicious circle by ezetimibe may also contribute to inhibition of hepatic steatosis. Further investigation will be need to elucidate the effect of ezetimibe on hepatic insulin signaling.

The limitation of our study is that we used ZOF rats fed with high-fat diets for the model of NAFLD with MetS. Since Leptin-dependent pathway plays a role in liver fibrosis and insulin sensitivity, our findings could be tested in a different animal model with intact leptin-dependent pathway. Though González-Ortiz et al. reported that ezetimibe treatment did not affect insulin sensitivity in obese subjects with dyslipidemia [11,33], these subjects are not diabetic and their plasma levels of FFA and insulin are not described. Therefore further study will be necessary to critically access the effect of ezetimibe on insulin sensitivity in NAFLD with MetS.

In conclusion, ezetimibe treatment improved hepatic steatosis via both NPC1L1 pathway and recovery of insulin resistance, as well as dyslipidemia in ZOF rats. This indicates that ezetimibe can be an effective therapy for ameliorating NAFLD with MetS. Large-scale clinical trials will provide us with more definite answer as to whether ezetimibe treatment can improve fatty liver, and resultantly reduce the risk of progression of liver diseases in patients with the MetS.

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