Ezetimibe Ameliorates Cardiovascular Complications and Hepatic Steatosis in Obese and Type 2 Diabetic db/db Mice

Masaya Fukuda, Taishi Nakamura, Keiichiro Kataoka, Hisato Nako, Yoshiko Tokutomi, Yi-Fei Dong, Osamu Yasuda, Hisao Ogawa, and Shokei Kim-Mitsuyama

Departments of Pharmacology and Molecular Therapeutics (M.F., T.N., K.K., H.N., Y.T., Y.-F.D., S.K.-M.) and Cardiovascular Medicine (H.O.), Kumamoto University Graduate School of Medical Sciences, Kumamoto, Japan; and Department of Clinical and Translational Research, Kumamoto University Hospital, Kumamoto, Japan (O.Y.)

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ABSTRACT

Type 2 diabetes plays a major role in the development of cardiovascular diseases. The present study was undertaken to investigate the effect of ezetimibe, a potent cholesterol absorption inhibitor, on cardiovascular injury of obese and type 2 diabetic db/db mice. Diabetic db/db mice fed a Western diet were given ezetimibe for 9 weeks, and the effects on cardiovascular injury and hepatic steatosis were examined. Ezetimibe treatment of db/db mice significantly improved vascular endothelial function, which was associated with the restoration of the decreased phospho-Akt and phospho-endothelial nitric-oxide synthase (eNOS). Moreover, ezetimibe also reduced vascular superoxide levels in db/db mice, accompanied by the attenuation of NADPH oxidase subunit gp91phox and Nox4 and the prevention of down-regulation of Cu/Zn-superoxide dismutase (SOD) and extracellular SOD. Thus, the improvement of vascular endothelial function by ezetimibe in diabetic mice seems to be attributed to the improvement of eNOS function and the attenuation of oxidative stress. Ezetimibe treatment also significantly attenuated cardiac interstitial fibrosis and coronary arterial thickening of diabetic mice and ameliorated cardiac macrophage infiltration. This improvement of cardiac injury was also related to the attenuation of NADPH oxidase-mediated oxidative stress. Furthermore, ezetimibe significantly prevented hepatic steatosis, inflammation, and oxidative stress in diabetic mice. Our work provides the first evidence that ezetimibe prevented cardiovascular injury and hepatic steatosis in diabetic mice. These beneficial effects were attributed to the attenuation of oxidative stress and inflammation and the improvement of eNOS function. Therefore, we propose that ezetimibe may be a promising therapeutic drug for obese and type 2 diabetes.

Introduction

Hypercholesterolemia is a major risk factor for ischemic heart disease. Endogenously synthesized cholesterol, absorption of dietary cholesterol, and the absorption of biliary cholesterol in the small intestine all contribute to the regulation of plasma cholesterol levels. Ezetimibe, a potent cholesterol absorption inhibitor, lowers plasma cholesterol by selectively inhibiting dietary and biliary cholesterol uptake at the brush border of the small intestine (Rosenblum et al., 1998; Knopp et al., 2003; Garcia-Calvo et al., 2005; Temel et al., 2007). Therefore, ezetimibe is expected to be a useful drug for the prevention of cardiovascular events. Previous reports (Davis et al., 2001; Kuhlencordt et al., 2009; Nakagami et al., 2009) showed that ezetimibe inhibits the development and progression of atherosclerosis in ApoE-knockout mice, improves vascular endothelial function, and attenuates oxidative stress in ApoE-knockout mice. Like hypercholesterolemia, diabetes plays a causative role in the development of ischemic heart disease (Kenchaiah et al., 2002; Van Gaal et al., 2006). However, to our knowledge, there is no available report investigating the effect of ezetimibe on type 2 diabetic animals.

db/db mice, a popular model of obesity and type 2 diabetes, is well characterized by not only obesity and type 2 diabetes but also diabetic cardiovascular and renal complications. (Coleman, 1982; Dong et al., 2010; Fukuda et al., 2010). Therefore, in this study, to elucidate the potential beneficial effects of ezetimibe on cardiovascular injury in type 2 diabetes, we investigated the effect of ezetimibe on cardiovascular injury in db/db mice. We obtained the
first evidence that ezetimibe improved cardiovascular complications and hepatic steatosis in type 2 diabetic mice.

Materials and Methods

Animals and Experimental Protocol. All procedures were in accordance with institutional guidelines for the care and use of laboratory animals. Male C57BL/KsJ db/db mice were purchased from Charles River (Kanagawa, Japan). Six-week-old db/db mice were randomly assigned to three groups and fed either a standard diet (MF; Oriental Yeast, Tokyo, Japan), a Western diet [450 kcal/100 g, 16% energy as protein (primarily casein)], 40% energy as fat (primarily butterfat), and 44% energy as carbohydrate (primarily sucrose)] (Oriental Yeast), or a Western diet containing 0.005% ezetimibe that was supplied by Schering Plough (Kenilworth, NJ). Drug treatment was performed for 9 weeks (from 6 to 15 weeks of age). Mice had free access to water and food during the experimental periods.

Blood pressure and heart rate were measured before and 4 and 8 weeks after the start of the experiment. An intraperitoneal glucose tolerance test (IGTT) was performed on each group 4 and 7 weeks after the start of the experiment, and an intraperitoneal insulin tolerance test (ITT) was performed 5 and 8 weeks after the start of the experiment.

After 9 weeks of drug treatment, db/db mice were anesthetized with ether, the blood was collected by cardiac puncture, and plasma was collected by centrifugation and stored at −80°C until use. After perfusion with phosphate-buffered saline, heart, thoracic aorta, and liver were rapidly excised from mice for measurement of various parameters as described below in detail.

Measurement of Blood Pressure. Blood pressure of conscious mice was measured by the tail cuff method (BP-98A; Softron Co, Tokyo, Japan) (Fukuda et al., 2010).

IGTT and ITT. For IGTT, db/db mice were intraperitoneally injected with glucose (1 g/kg body weight) after 6 h of fasting. Blood samples were collected from the tail vein at 0, 30, 60, and 120 min after glucose administration to measure blood glucose. For ITT, db/db mice were intraperitoneally injected with human regular insulin (2 units/kg body weight) after 6 h of fasting. Blood samples were collected from the tail vein at 0, 20, 40, and 60 min after insulin administration to measure blood glucose.

Vessel Ring Preparation and Organ Chamber Experiments. Isometric tension studies were performed as described previously (Dong et al., 2010). In brief, thoracic aortas from mice were cut into 5-mm rings with special care to preserve the endothelium and mounted in organ baths, filled with modified Tyrode buffer aerated with 95% O2 and 5% CO2 at 37°C. The preparations were attached to a force transducer, and isometric tension was recorded on a polygraph. Vessel rings were pruned with KCl (50 mM), and then precontracted with 1-phenylephrine (10−7 M), producing a submaximal (70–80% of maximum) contraction. After the plateau was attained, the rings were exposed to increasing concentrations of acetylcholine (10−10 to 10−4 M) to obtain cumulative concentration-response curves.

Measurement of Tissue Superoxide. Heart, thoracic aorta, and liver removed from mice were immediately frozen in Tissue-Tek OCT embedding medium (Sakura Finetek Europe, Zoeterwoude, The Netherlands). Dihydroethidium was used to evaluate superoxide levels of tissue in situ as described in detail (Yamamoto et al., 2007a). Dihydroethidium fluorescence of tissue sections was quantified by using Lumina Vision version 2.2 analysis software (Mitani Corporation, Tokyo, Japan).

Cardiac and Hepatic NADPH Oxidase Activity. Cardiac and hepatic tissues were homogenized with an Ultra Turrax T8 homogenizer (IKA Japan Y.K., Yamato Koriyama Shi, Japan) and centrifuged, and NADPH oxidase activity of the resulting supernatant was measured by lucigenin chemiluminescence in the presence of 10 μM NADPH and 10 μM lucigenin as electron acceptor as described in detail (Yamamoto et al., 2007a). Protein concentrations were measured by the method of Bradford (1976).

Histological Examination and Immunohistochemistry. The heart and liver were fixed with 4% formalin overnight, embedded in paraffin, and sectioned into 5-μm slices. The heart sections were stained with Sirius red F3BA (0.5% in saturated aqueous picric acid; Sigma-Aldrich, St. Louis, MO) for assessment of cardiac interstitial fibrosis and coronary remodeling. To evaluate coronary remodeling, coronary arterial thickening was assessed by calculating the wall-to-lumen ratio (the medial thickness to the internal diameter) as described previously (Izumiya et al., 2003). The area of fibrosis was assessed by using Lumina Vision version 2.2 analysis software.

For detection of cardiac and hepatic macrophage infiltration, frozen tissue sections were stained with the primary antibodies (rat anti-mouse CD68; Serotec, Oxford, UK; ×500) at 4°C overnight. After incubation with the primary antibody, sections were reacted with horseradish peroxidase-conjugated anti-rat IgG secondary antibody (BioSource International, Camarillo, CA) and visualized with 3,3′-diaminobenzidine (Dako Japan Inc., Tokyo, Japan).

Preparation of Aortic Protein Extracts and Western Blot Analysis. Detailed methods have been described previously (Yamamoto et al., 2007b). In brief, after aortic protein extracts were subjected to SDS-polyacrylamide gel electrophoresis and electrophoretic transfer to polyvinylidene difluoride membrane, the membranes were probed with specific antibodies. Antibodies used were as follows: anti-phospho-eNOS (×2000; BD Transduction Laboratories, Lexington, KY), anti-total eNOS (×2000; BD Transduction Laboratories), anti-phospho-Akt (×1000; Cell Signaling Technology, Danvers, MA), anti-total Akt (×2000, Cell Signaling Technology), anti-Cu/Zn-SOD (×5000; AKELA Pharma Inc., Montreal, QC, Canada), anti-extracellular SOD (EC-SOD) (×2000; Millipore Corporation, Billerica, MA), anti-ig9p1phox (×2000, BD Transduction Laboratories), Nox4 (×2000; Abcam Inc., Cambridge, MA), and anti-beta-actin (×2000, Cell Signaling Technology). The antibody was visualized by using an enhanced chemiluminescence method (ECL Plus; GE Healthcare Little Chalfont, Buckinghamshire, UK). The intensity of the bands was quantified by using National Institutes of Health (Bethesda, MD) Image analysis software version 1.61. In individual samples, each value was corrected for that of beta-actin.

Measurement of Hepatic Total Cholesterol and Triglyceride. Hepatic total cholesterol and triglyceride were measured with a kit (Wako Pure Chemicals, Osaka Japan).

Analysis of Plasma Biochemistry. Plasma biochemistry analysis was done at SRL Inc. (Tokyo, Japan).

Statistics. Results are expressed as mean ± S.E.M. The data on time course experiments were analyzed by two-way analysis of variance, followed by Fisher's Protected Least Significant Difference test, using StatView for Windows (SAS Institute, Cary, NC). For pairwise comparisons, statistical significance was determined with Student’s t test. For multiple comparisons, statistical significance was determined with one-way analysis of variance, followed by Fisher’s Protected Least Significant Difference test. In all tests, differences were considered statistically significant at a value of P < 0.05.

Results

Effects on Body Weight, Fat Weight, and Biochemical Parameters of db/db Mice. As shown in Supplementary Table 1, body weight and fat weight were similar in db/db mice on a standard diet and those on a Western diet. As shown in Fig. 1, the Western diet increased total cholesterol by 2.0-fold (P < 0.01), LDL cholesterol by 2.7-fold (P < 0.01), HDL cholesterol by 1.4-fold (P < 0.01), and free fatty acid by 1.1-fold (P < 0.01) in db/db mice. Ezetimibe treatment completely suppressed the increase in all of these parameters in db/db mice fed a Western diet. Furthermore,
ezetimibe significantly reduced triglyceride in db/db mice fed a Western diet ($P < 0.01$).

Ezetimibe also prevented the increase in plasma GPT in Western diet-fed db/db mice ($P < 0.01$) (Fig. 1F).

**Effects on Glucose Tolerance and Insulin Resistance of db/db Mice.** Supplementary Fig. 1 indicates IGTT and IITT in three groups of db/db mice. Western diet did not apparently affect IGTT or IITT in db/db mice. Ezetimibe treatment did not alter IGTT or IITT in db/db mice fed a Western diet.

**Effects on Blood Pressure of db/db Mice.** Supplementary Fig. 2 indicates that a Western diet did not affect the blood pressure of db/db mice. Ezetimibe treatment did not change the blood pressure of Western diet-fed db/db mice.

**Effects on Vascular Endothelial Function, eNOS, Akt, and Oxidative Stress of db/db Mice.** As shown in Fig. 2A, a Western diet significantly impaired acetylcholine-induced endothelium-dependent relaxation in db/db mice, indicating significant vascular endothelial dysfunction in db/db mice on a Western diet. Ezetimibe treatment almost completely restored the impairment of vascular endothelial function induced by Western diet in db/db mice. As shown in Fig. 2B, a Western diet significantly decreased phospho-eNOS in db/db mice ($P < 0.01$), and this decreased phospho-eNOS was reversed by treatment with ezetimibe ($P < 0.05$). A Western diet also caused the reduction of phospho-Akt in db/db mice ($P < 0.01$), and this down-regulation of phospho-Akt was restored by ezetimibe treatment ($P < 0.01$) (Fig. 2C).

Figure 3 shows the effect of ezetimibe on vascular oxidative stress in db/db mice fed a Western diet. A Western diet significantly increased vascular superoxide in db/db mice, and this increase in superoxide was associated with the up-regulation of NADPH oxidase subunits, gp91phox and Nox 4, and the down-regulation of Cu/Zn-SOD and EC-SOD. Ezetimibe treatment significantly attenuated vascular superoxide in Western diet-fed db/db mice ($P < 0.01$). Moreover, ezetimibe significantly prevented the up-regulation of gp91phox and Nox 4 and down-regulation of Cu/Zn-SOD and EC-SOD in db/db mice fed a Western diet.

**Effects on Cardiac Fibrosis, Coronary Remodeling, Inflammation, and Oxidative Stress of db/db Mice.** A Western diet significantly enhanced cardiac interstitial fibrosis and coronary arterial thickening in db/db mice, and these cardiac lesions induced by a Western diet were completely prevented by ezetimibe treatment (Fig. 4).

A Western diet significantly increased cardiac macrophage infiltration and increased cardiac superoxide and NADPH oxidase activity (Fig. 5). Ezetimibe treatment normalized the increase in cardiac inflammation and oxidative stress in db/db mice fed a Western diet.

**Effects on Hepatic Steatosis, Inflammation, and Oxidative Stress of db/db Mice.** As shown in Fig. 6, a Western diet significantly increased hepatic total cholesterol and triglyceride and enhanced hepatic macrophage infiltration, su-
In the present study, lipid lowering with ezetimibe in diabetic mice significantly improved vascular endothelial function, which was associated with the prevention of phospho-eNOS and phospho-Akt down-regulation. Therefore, the improvement of vascular endothelial function by ezetimibe in diabetic mice seems to be at least partially attributed to the improvement of eNOS function. Furthermore, we also examined the effects of ezetimibe on vascular oxidative stress in db/db mice, because we have previously shown that the attenuation of oxidative stress by tempol, a SOD mimetic, in vivo leads to the improvement of cardiovascular injury in db/db mice, indicating the causative role of oxidative stress in cardiovascular injury in db/db mice. Of note are the observations that ezetimibe significantly attenuated vascular superoxide in db/db mice, showing that the attenuation of oxidative stress and eNOS dysfunction is involved in the normalization of vascular endothelial function by ezetimibe in diabetic mice. Moreover, ezetimibe treatment normalized the increase in vascular gp91phox and Nox4, which are major NADPH oxidase subunits (Griendling et al., 2000), and also normalized the down-regulation of Cu/Zn-SOD and EC-SOD. Therefore, the normalization of NADPH oxidase and SOD seems to be responsible for the attenuation of vascular superoxide by ezetimibe in diabetic mice.

Diabetes is well known for enhance cardiac remodeling and heart failure (Kenchaiah et al., 2002; Van Gaal et al., 2006). However, to our knowledge, the cardiac effect of ezetimibe remains to be elucidated. We obtained the evidence that ezetimibe significantly suppressed the development of cardiac interstitial fibrosis and inflammation and coronary arterial remodeling. As in the case of vascular tissues, ezetimibe also attenuated cardiac superoxide through the inhibition of NADPH oxidase. Our result supports the notion that lipid lowering with ezetimibe in diabetes may exert a beneficial effect on diabetic cardiomyopathy.

**Discussion**

We reported previously that compared with nondiabetic control db/m mice db/db mice display vascular endothelial dysfunction, cardiac interstitial fibrosis and inflammation, and coronary arterial remodeling and exhibit the enhancement of cardiovascular oxidative stress and NADPH oxidase and the down-regulation of eNOS phosphorylation and SOD (Dong et al., 2010; Fukuda et al., 2010). In the present work, we found that a Western diet exacerbated the above-mentioned cardiovascular injury observed in db/db mice, indicating additive detrimental effects of a high-fat diet on diabetic cardiovascular complications. Furthermore, we found that the exacerbation of diabetic cardiovascular complications by a high-fat diet was associated with further impairment of eNOS and the enhancement of oxidative stress.

In the present study, lipid lowering with ezetimibe in diabetic mice significantly improved vascular endothelial function, which was associated with the prevention of phospho-eNOS and phospho-Akt down-regulation. Therefore, the improvement of vascular endothelial function by ezetimibe in diabetic mice seems to be at least partially attributed to the improvement of eNOS function. Furthermore, we also examined the effects of ezetimibe on vascular oxidative stress in db/db mice, because we have previously shown that the attenuation of oxidative stress by tempol, a SOD mimetic, in vivo leads to the improvement of cardiovascular injury in db/db mice, indicating the causative role of oxidative stress in cardiovascular injury in db/db mice. Of note are the observations that ezetimibe significantly attenuated vascular superoxide in db/db mice, showing that the attenuation of oxidative stress and eNOS dysfunction is involved in the normalization of vascular endothelial function by ezetimibe in diabetic mice. Moreover, ezetimibe treatment normalized the increase in vascular gp91phox and Nox4, which are major NADPH oxidase subunits (Griendling et al., 2000), and also normalized the down-regulation of Cu/Zn-SOD and EC-SOD. Therefore, the normalization of NADPH oxidase and SOD seems to be responsible for the attenuation of vascular superoxide by ezetimibe in diabetic mice.

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Previous reports showed that ezetimibe significantly suppresses hepatic steatosis in high fat-fed C57BL/6J mice (Zheng et al., 2008), Zucker obese fatty rats (Deushi et al., 2007), and ApoE-deficient mice (Nakagami et al., 2009). Also consistent with the previous reports (Deushi et al., 2007; Zheng et al., 2008; Nakagami et al., 2009), we found that ezetimibe significantly reduced hepatic lipid accumulation, inflammation, and NADPH-mediated oxidative stress in type 2 diabetic mice, confirming the beneficial effect of ezetimibe on hepatic steatosis.

*db/db* mice are established to be a useful model of not only human type 2 diabetes but also diabetic dyslipidemia (Kobayashi et al., 2000). In this study, ezetimibe significantly reduced total cholesterol, LDL cholesterol, triglyceride, and free fatty acid in Western diet-fed *db/db* mice, and our present findings are in good agreement with previous reports on the effects of ezetimibe on dyslipidemia in Western diet-fed hamsters (van Heek et al., 2001) and mice (Davis et al., 2004). Thus, our present data confirmed the significant suppression of intestinal cholesterol absorption by ezetimibe. On the other hand, as shown by IGTTT and IITT, ezetimibe did not apparently affect glucose tolerance and insulin resistance in *db/db* mice. Furthermore, blood pressure in *db/db* mice was not altered by ezetimibe treatment. Therefore, our present data provided no evidence for the role of hyperglycemia and blood pressure in the beneficial effects of ezetimibe on cardiovascular injury. Collectively, the underlying mechanism of the beneficial effect of ezetimibe on cardiovascular injury and hepatic steatosis in diabetic *db/db* mice seems to be attributed mainly to the cholesterol-lowering effect of ezetimibe. However, further study using other animal models is needed to define more detailed effects of ezetimibe on diabetic dyslipidemia, because *db/db* mice are characterized by severe leptin resistance.

In conclusion, our present study provided the first evidence that lipid lowering with ezetimibe ameliorated the impair-
ment of vascular endothelial function, coronary arterial remodeling, cardiac interstitial fibrosis and inflammation, and hepatic steatosis in type 2 diabetic mice. These beneficial effects of ezetimibe in db/db mice were attributed to the attenuation of oxidative stress, through the normalization of eNOS, NADPH oxidase, and SOD. We propose that lipid lowering with ezetimibe is a promising strategy for the prevention of diabetic cardiovascular complications. It is noteworthy that recent human studies suggest that ezetimibe can improve dyslipidemia in diabetic patients (Bardini et al., 2010; Conard et al., 2010) and suppress hepatic injury in patients with nonalcoholic fatty liver disease (Abel et al., 2009; Enjoji et al., 2010). However, a large-scale clinical trial is necessary to warrant the efficacy of ezetimibe on diabetic cardiovascular complications and hepatic steatosis.

References


Shokei Kim-Mitsuyama, Department of Pharmacology and Molecular Therapeutics, Kumamoto University Graduate School of Medical Sciences, 1-1-1 Honjiyo, Kumamoto 860-8556, Japan. E-mail: kimmitsu@gpm.kumamoto-u.ac.jp

Address correspondence to: Dr. Shokei Kim-Mitsuyama, Department of Pharmacology and Molecular Therapeutics, Kumamoto University Graduate School of Medical Sciences, 1-1-1 Honjiyo, Kumamoto 860-8556, Japan.