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Ezetimibe ameliorates cardiovascular complications and hepatic steatosis in obese and type 2 diabetic db/db mice

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A list of nonstandard abbreviations:

NADPH oxidase; nicotinamide adenine dinucleotide phosphate oxidase

SOD; superoxide dismutase

IGTT; intraperitoneal glucose tolerance test

IITT; intraperitoneal insulin tolerance test

DHE; dihydroethidium

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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 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-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 downregulation of Cu/Zn-SOD and EC-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 provided the first evidence that ezetimibe prevented cardiovascular injury and hepatic steatosis in diabetic mice and 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) show that ezetimibe inhibits the development and the progression of atherosclerosis in ApoE-knockout mice, improves vascular endothelial function, and attenuates oxidative stress in ApoE-knockout mice.   As well as hypercholesterolemia, diabetes also 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 is a popular model of obesity and type 2 diabetes and 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.

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 3 groups, and were fed (1) normal diet (MF, Oriental Yeast, Japan) , (2) Western diet (450kcal/100g, 16% energy as protein [primarily casein], 40% energy as fat [primarily butterfat], and 44% energy as carbohydrate [primarily sucrose]) (Oriental Yeast, Japan), or (3) Western diet containing 0.005% ezetimibe which was supplied by Schering-Plough Co Ltd..   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 start of the experiment.   Intraperitoneal glucose tolerance test (IGTT) was performed on each group 4 and 7 weeks after start of the experiment, and intraperitoneal insulin tolerance test (IITT) was performed 5 and 8 weeks after 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 (PBS), 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)

Intraperitoneal glucose tolerance test (IGTT) and intraperitoneal insulin tolerance test (IITT)

For IGTT, db/db mice were intraperitoneally injected with glucose (1 g/kg body weight) after 6 hr fasting. Blood samples were collected from tail vein at 0, 30, 60, and 120 min after glucose administration, to measure blood glucose. For IITT, db/db mice were intraperitoneally injected with human regular insulin (2 units/kg body weight) after 6 hr fasting. Blood samples were collected from 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 previously described. (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 % O_2 and 5 % CO_2 at 37 °C. The preparations were attached to a force transducer, and isometric tension was recorded on a polygraph. Vessel rings were primed with KCl (50 mmol/L), and then precontracted with L-phenylephrine (10^{-7} mol/L) producing a sub-maximal (70-80% of maximum) contraction. After the plateau was attained, the rings were exposed to increasing concentrations of acetylcholine (Ach) (10^{-9} mol/L to 10^{-4} mol/L) to obtain cumulative concentration-response curves.

Measurement of tissue superoxide

Heart, thoracic aorta, and liver, removed from mice, were immediately frozen in Tissue-Tek O.C.T. embedding medium (Sakura Finetek). Dihydroethidium (DHE) was used to evaluate superoxide levels of tissue in situ, as described in detail. (Yamamoto et al., 2007a) DHE fluorescence of tissue sections was quantified using Lumina Vision version 2.2, analysis software.

Cardiac and hepatic NADPH oxidase activity

Cardiac and hepatic tissues were homogenized with an Ultraturrax T8, 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 by us. (Yamamoto et al., 2007a)   Protein concentrations were

measured by the method of Bradford.

Histological examination and immunohistochemistry

The heart and liver were fixed with 4% formalin overnight, embedded in paraffin,

sectioned 5-μm slices. The heart sections were stained with Sirius Red F3BA (0.5% in

saturated aqueous picric acid, Aldrich Chemical Company) 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 previously described. (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; x 500) at 4 °C overnight.

After incubation with the primary antibody, sections were reacted with horseradish

peroxidase-conjugated anti-rat IgG secondary antibody (BioSource), and visualized with

3,3'-diaminobenzidine (Dako Cytomation).

Preparation of aortic protein extracts and Western blot analysis

The detailed method was previously described. (Yamamoto et al., 2007b)   Briefly, after

aortic protein extracts were subjected to sodium-dodecyl sulfate polyacrylamide gel

electrophoresis (SDS-PAGE) and electric transfer to polyvinylidene difluoride membrane, the

membranes were probed with specific antibodies. Antibodies used were as follows:

anti-phospho-eNOS (x 2000, BD Transduction Laboratories), anti-total eNOS (x 2000, BD

Transduction Laboratories), anti-phospho-Akt (x 1000, Cell Signaling Technology), anti-total

Akt (x 2000, Cell Signaling Technology), anti-Cu/Zn-SOD (x 5000, Stressgen

Biotechnologies), anti-extracellular (EC)-SOD (x 2000, Upstate Cell Signaling Solutions),

anti-gp91[phox] (x 2000, BD Transduction Laboratories), Nox4 (x 2000, Abcam), and

anti-β-actin (x 2000, Cell Signaling Technology).   The antibody was visualized using an

enhanced chemiluminescence method (ECL Plus; Amersham Biosciences).   The intensity of

the bands was quantified using NIH Image analysis software v1.61. In individual samples,

each value was corrected for that of β-actin.

Measurement of hepatic total cholesterol and triglyceride

Hepatic total cholesterol and triglyceride was measured with a kit (Wako Pure Chemical,

Osaka Japan).

Analysis of plasma biochemistry

Plasma biochemistry analysis was entrusted to SRL Inc. (Tokyo, Japan)

Statistics

Results were expressed as mean ± SEM.   The data on time course experiments were

analyzed by two-way ANOVA, followed by Fisher's PLSD test, using StatView for Windows

(SAS Institute, Inc. Cary, U.S.A.).   For pairwise comparisons, statistical significance was

determined with Student's t test.   For multiple comparisons, statistical significance was

determined with one-way ANOVA, followed by Fisher's PLSD 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 between db/db mice on standard diet and western diet.   As shown in Figure 1, 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 and ezetimibe treatment completely suppressed the increase in all these parameters in db/db mice fed western diet.   Furthermore, ezetimibe significantly reduced triglyceride in db/db mice fed western diet (P<0.01).

Ezetimibe also prevented the increase in plasma GPT in western diet fed db/db mice (P<0.01) (Figure 1 (F)).

Effects on glucose tolerance and insulin resistance of db/db mice

Supplementary Figure 1 indicates IGTT and IITT in 3 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 western diet.

Effects on blood pressure of db/db mice

Supplementary Figure 2 indicates that western diet did not affect blood pressure of db/db mice.   Ezetimibe treatment did not change 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 Figure 2 (A), western diet significantly impaired acetylcholine-induced endothelium-dependent relaxation in db/db mice, indicating the significant vascular endothelial dysfunction in db/db mice by western diet.   Ezetimibe treatment almost completely restored the impairment of vascular endothelial function induced by western diet in db/db mice.   As shown in Figure 2 (B), 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).　Western diet also caused the reduction of phospho-Akt in db/db mice (P<0.01), and this downregulation of phosphor-Akt was restored by ezetimibe treatment (P<0.01) (Figure 2 (C)).

Figure 3 shows the effect of ezetimibe on vascular oxidative stress in db/db mice fed western diet.　Western diet significantly increased vascular superoxide in db/db mice, and this increase in superoxide was associated with the upregulation of NADPH oxidase subunits, gp91^{phox} and Nox 4, and the downregulation 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 upregulation of gp91^{phox} and Nox4 and downregulation of Cu/Zn-SOD and EC-SOD in db/db mice fed western diet.

Effects on cardiac fibrosis, coronary remodeling, inflammation, and oxidative stress of db/db mice

Western diet significantly enhanced cardiac interstitial fibrosis and coronary arterial thickening in db/db mice, and these cardiac lesions induced by western diet were completely prevented by ezetimibe treatment (Figure 4).

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

Effects on hepatic steatosis, inflammation, and oxidative stress of db/db mice

As shown in Figure 6, western diet significantly increased hepatic total cholesterol and triglyceride, and enhanced hepatic macrophage infiltration, superoxide, and NADPH oxidase activity.　Ezetimibe treatment prevented all the above mentioned hepatic injury in western diet fed db/db mice.

Discussion

We have previously reported that compared with non-diabetic 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 downregulation of eNOS phosphorylation and SOD. (Dong et al., 2010; Fukuda et al., 2010)   In the present work, we found that western diet exacerbated the above mentioned cardiovascular injury observed in db/db mice, indicating additive detrimental effects of high fat diet on diabetic cardiovascular complications. Furthermore, we found that the exacerbation of diabetic cardiovascular complications by 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 downregulation.   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 as well as eNOS dysfunction is involved in the normalization of vascular endothelial function by ezetimibe in diabetic mice.   Moreover, ezetimibe treatment normalized the increase in vascular gp91^{phox} and Nox4 which are major NADPH oxidase subunits (Griendling et al., 2000), and also normalized the downregulation of Cu/Zn-SOD

and EC-SOD. Therefore, the normalization of NADPH oxidase and SOD appears to be responsible for the attenuation of vascular superoxide by ezetimibe in diabetic mice.

Diabetes is well known to enhance cardiac remodeling and heart failure. (Kenchaiah et al., 2002; Van Gaal et al., 2006) However, to our knowledge, 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 the beneficial effect on diabetic cardiomyopathy.

Previous reports show 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). Being 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, findings 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 IGTT 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 beneficial effect of ezetimibe on cardiovascular injury and hepatic steatosis in diabetic db/db mice seems to be mainly attributed to cholesterol lowering effect of ezetimibe. However, further study using other animal models is needed to define more detailed effects of ezetimibe on diabetic dyslipidemia, since 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 impairment of vascular endothelial function, coronary arterial remodeling, cardiac interstitial fibrosis and inflammation, and hepatic steatosis in type 2 diabetic mice, and 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. Interestingly, recent human studies suggest that ezetimibe can improve dyslipidemia in diabetic patients (Bardini et al., ; Conard et al.) and suppress hepatic injury in patients with nonalcoholic fatty liver disease (Enjoji et al., ; Abel et al., 2009). However, large scale clinical trial is necessary to warrant the efficacy of ezetimibe on diabetic cardiovascular complications and hepatic steatosis.

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Footnotes

Figure Legends

Figure 1. Effects of ezetimibe on plasma total cholesterol (Total-Chol) (A), LDL-cholesterol (LDL-C) (B), HDL-cholesterol (HDL-C) (C), triglyceride (TG) (D), free fatty acid (FFA) (E), and GPT (F) in western diet fed db/db mice

Abbreviations used: S, standard diet; W, western diet; W+E, western diet plus ezetimibe.

Values are means $\pm$ SEM (n=6-8). ** P<0.01

Figure 2. Effects of ezetimibe on vascular endothelial function (A), eNOS phosphorylation (B), and Akt phosphorylation (C) of db/db mice

Upper panels in (B) and (C) indicate representative Western blots. The lanes were run on the same gel but were noncontiguous. Abbreviations used are the same as in Figure 1.

Values are means $\pm$ SEM (n=6-8). * P<0.05, ** P<0.01

Figure 3. Effects of ezetimibe on aortic superoxide (A), gp91 ^{phox} (B), Nox4 (C), Cu/Zn-SOD (D), and EC-SOD (E) of db/db mice

Upper panels in (A) indicate representative photomicrographs of aortic sections stained with dihydroethidium. Bar = 100 μm in (A). Upper panels in (B), (C), (D), and (E) indicate representative Western blots. The lanes were run on the same gel but were noncontiguous. Abbreviations used are the same as in Figure 1. Values are means $\pm$ SEM (n=6-10).

* P<0.05, ** P<0.01

Figure 4. Effects of ezetimibe on cardiac interstitial fibrosis (A) and coronary arterial thickening (B) of db/db mice

Upper panels in (A) and (B) indicate representative photomicrographs of cardiac sections stained with Sirius Red. Abbreviations used are the same as in Figure 1. Values are means $\pm$ SEM (n=8-9). Bar = 50 μm. * P<0.05, ** P<0.01

Figure 5. Effects of ezetimibe on cardiac macrophage infiltration (A), superoxide (B), and NADPH oxidase activity (C) in db/db mice
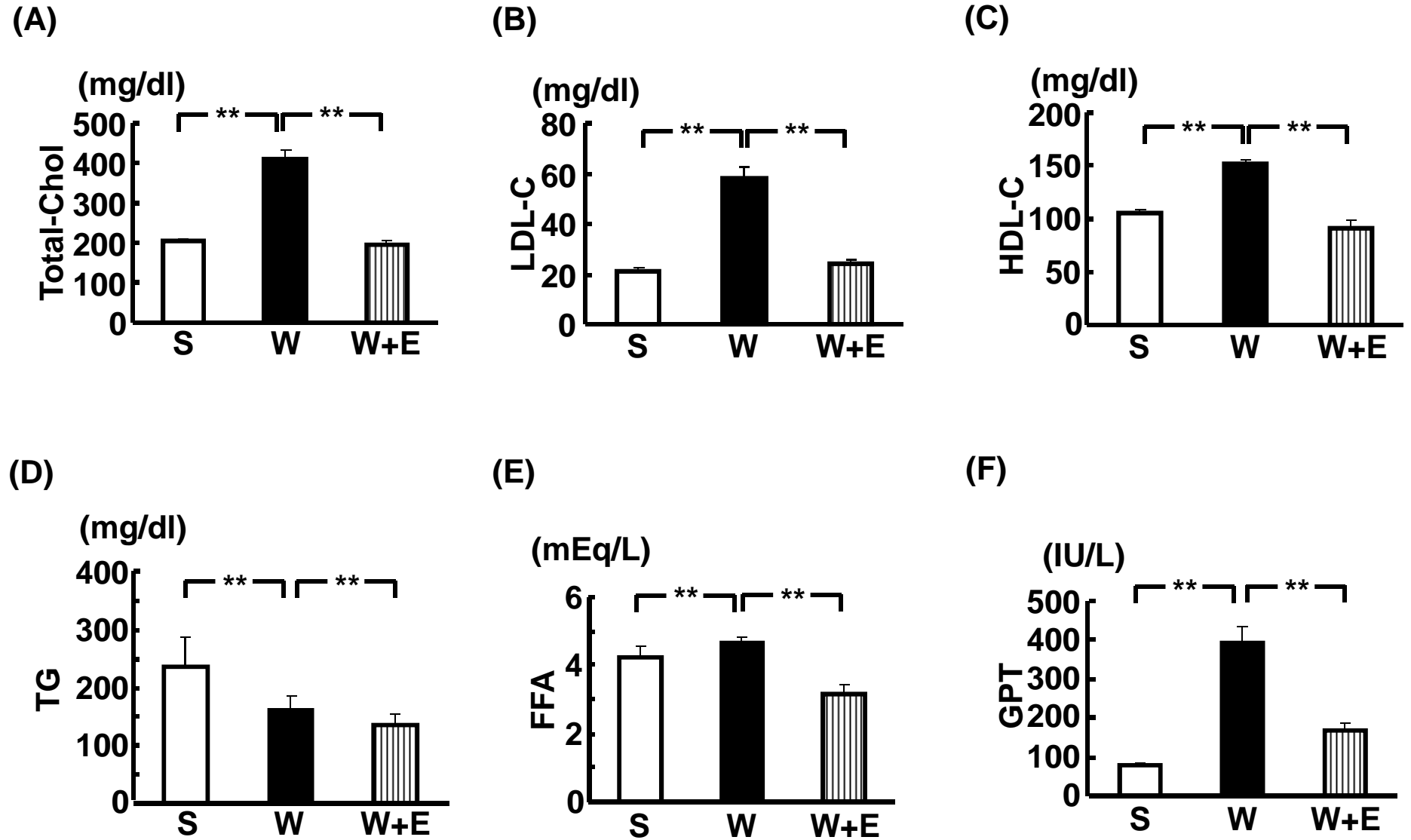
Upper panels in (A) and (B) indicate representative photomicrographs of cardiac sections stained with CD68 antibody and dihydroethidium, respectively.   Abbreviations used are the same as in Figure 1.   Values are means $\pm$ SEM (n=6-9).
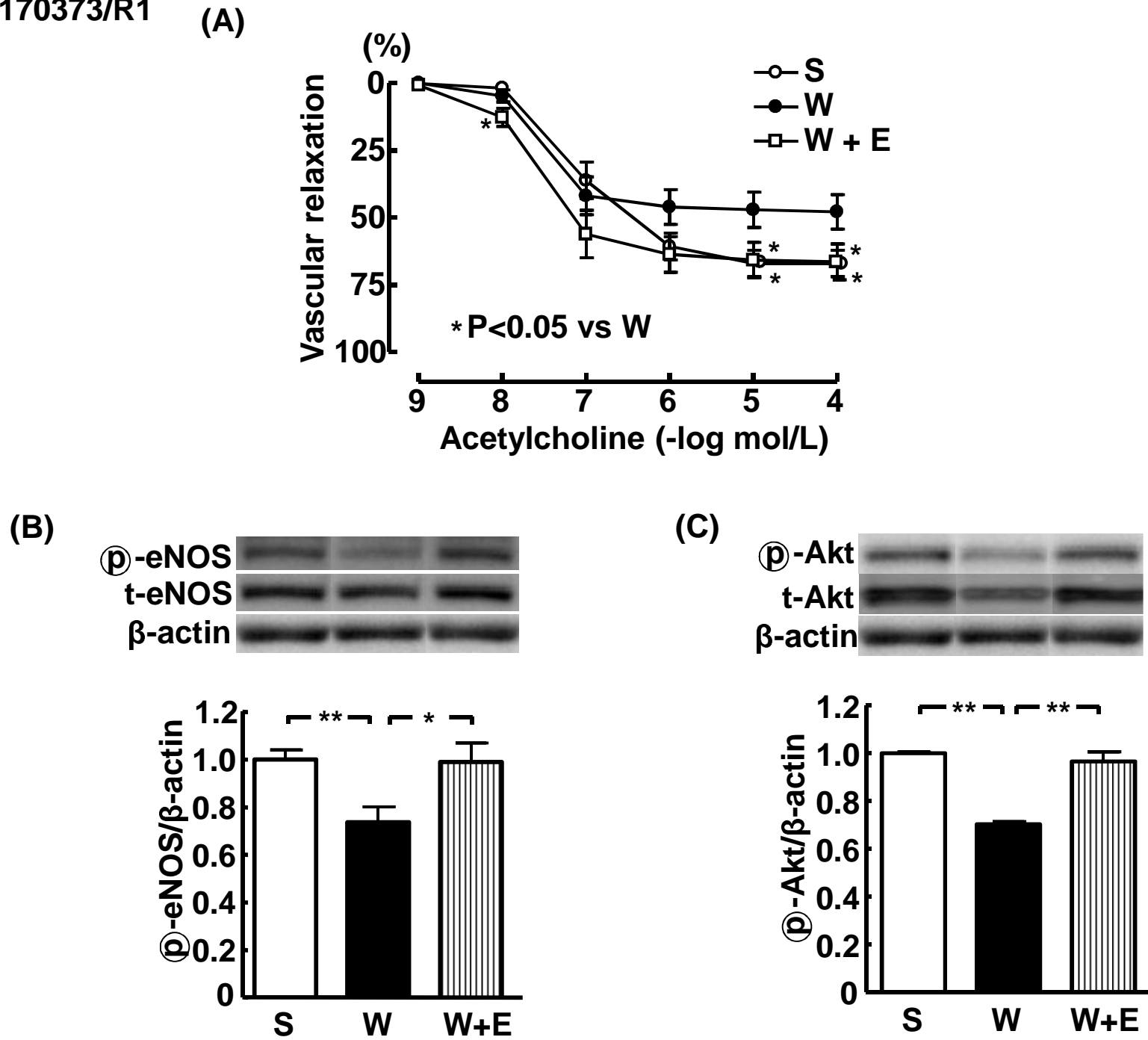
Bar = 100 μm.   * P<0.05, ** P<0.01

Figure 6. Effects of ezetimibe on hepatic total cholesterol (A), triglyceride (B), macrophage infiltration (C), superoxide (D), and NADPH oxidase activity (E) of db/db mice

Upper panels in (C) and (D) indicate representative photomicrographs of hepatic sections stained with CD68 antibody and dihydroethidium, respectively.   Abbreviations used are the same as in Figure 1.   Values are means $\pm$ SEM (n=7-11).
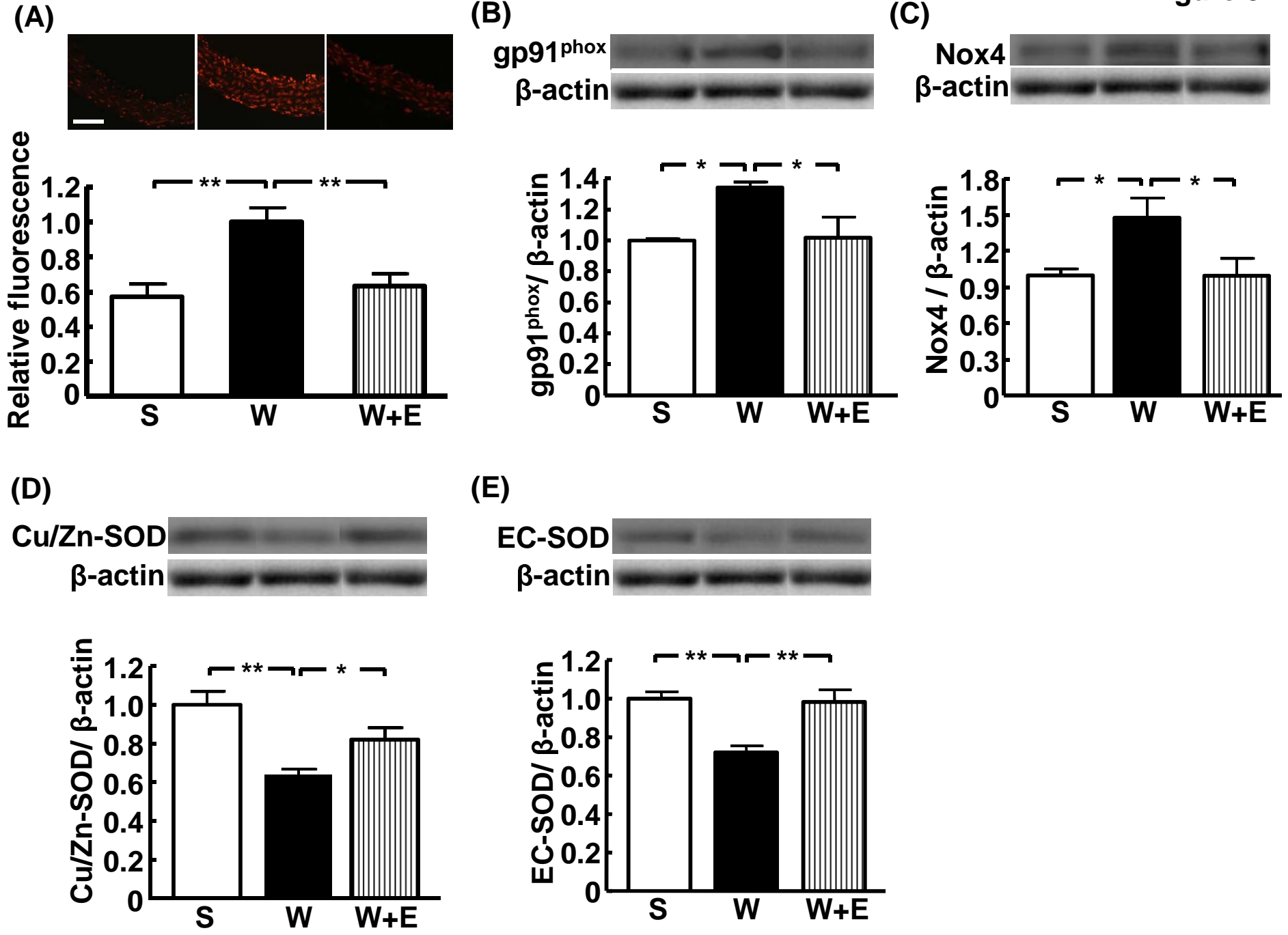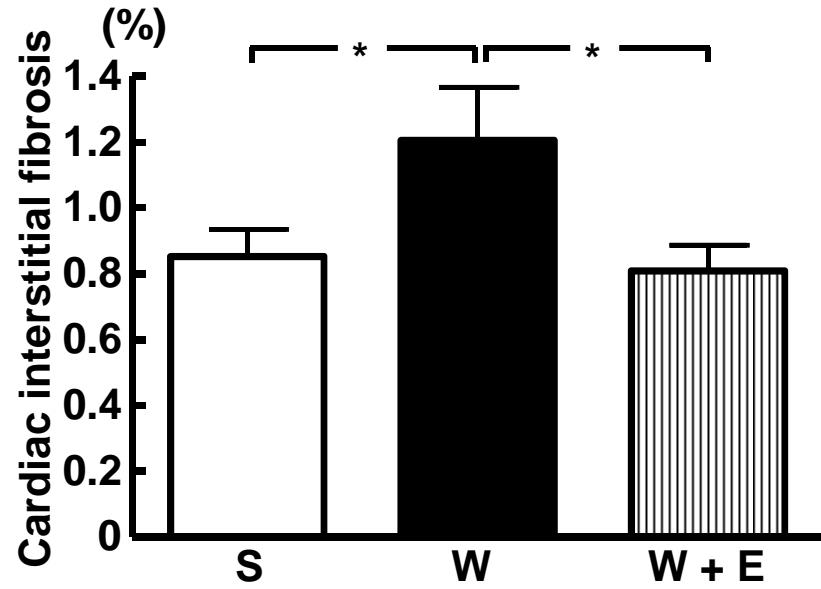
Bar = 100 μm.   ** P<0.01

Figure 1

Figure 2

(A)

(B)

(C)

Figure 3

(A)

(B)

gp91^{phox}

β-actin

(C)

Nox4

β-actin

(D)

Cu/Zn-SOD

β-actin

(E)

EC-SOD

β-actin

Figure 4

(A)

(B)

(A)

(B)

(C)

Figure 6