

Ezetimibe prevents myocardial remodeling in an obese rat model by inhibiting inflammation

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Inflammation plays an important role in the development of many obesity-related diseases. This study aimed to investigate the effect of ezetimibe on inflammation and myocardial remodeling in obese rats. A rat model of obesity was established, and myocardial damage was examined by transmission electron microscopy and Masson staining. Twenty obese rats were divided into two groups (n=10): obese group and ezetimibe group. Ten SD rats were used as controls. Western blot was performed to monitor the expression of P-p38MAPK and interleukin (IL)-6. Immunohistochemical staining was used to monitor the expression of intercellular adhesion molecule-1 and vascular cell adhesion molecule-1. In the obese rats group, we observed increased inflammatory factors and myocardial hypertrophy. In contrast, the ezetimibe group exhibited decreased expression of inflammatory factors and an improvement in myocardial remodeling compared to the obese group. Mechanistically, we found that ezetimibe decreased P-p38MAPK, IL-6, intercellular adhesion molecule-1, and vascular cell adhesion molecule-1 levels in the hearts of the obese rats. Taken together, these results indicate that ezetimibe may improve myocardial remodeling in obese rats by inhibiting inflammation.

Key words: obese, inflammation, remodeling, ezetimibe, IL-6

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Abbreviations: CVD, cardiovascular disease; CRP, C-reactive protein; ICAM-1, intercellular adhesion molecule-1; EF, ejection fraction; FS, fractional shortening; HF, high fat; HDL-C, high-density lipoprotein-cholesterol; IR, insulin resistance; IRS-1, insulin receptor substrate 1; IL-6, Interleukin 6; LDL-C, low-density lipoprotein-cholesterol; LVEDD, left ventricular end-diastolic diameter; LVESD, left ventricular end-systolic diameter; TEM, transmission electron microscope; TG, triglycerides; MCP-1, macrophage chemoattractant protein 1; VCAM-1, vascular cell adhesion molecule-1

INTRODUCTION

The combination of abdominal obesity and impaired glucose and lipid metabolism place individuals at a significantly increased risk of developing cardiovascular disease (CVD) (Kim *et al.*, 2017; Larsson *et al.*, 2017). Moreover, changes in the macro-vascular structure and myocardial function in obese patients may contribute to the development of CVD. In obese patients, there is increased myocardial remodeling and dysfunction, which are predictive of early pathological changes in the heart (Alpert *et al.*, 2016; Gu *et al.*, 2015). Given the high morbidity and mortality of CVD in obese patients (Liao *et*

al., 2014), it is critically important to understand the mechanisms underlying myocardial remodeling to delay the progression of CVD using multifactorial intervention strategies and decrease cardiovascular end-point events in patients with obesity.

Inflammatory reactions have been shown to contribute to myocardial remodeling, and interleukin 6 (IL-6) is a factor that can function as both a pro-inflammatory cytokine and an anti-inflammatory myokine (González *et al.*, 2015; Pedersen *et al.*, 2006). Previous studies have shown that patients with obesity display increased levels of C-reactive protein (CRP) and IL-6 compared to the controls (Ellulu *et al.*, 2016; Okamoto *et al.*, 2015). In addition, the activation of p38MAPK has been correlated with myocardial remodeling (Yoshida *et al.*, 2001; Baraka *et al.*, 2009; Yu *et al.*, 2012). p38MAPK can be activated by a variety of stimuli, such as elevated levels of free fatty acids, cholesterol, glucose, and proinflammatory mediators (Ragheb *et al.*, 2009; Shalini *et al.*, 2016). Moreover, the activation of p38MAPK can regulate gene expression, which may promote myocardial remodeling (Baraka *et al.*, 2009).

Ezetimibe, a drug that inhibits the absorption of cholesterol from the small intestine and decreases circulating cholesterol levels, attracted recent attention because of its noted anti-inflammatory effects (Kuhlencordt *et al.*, 2009; Gómez-Garre *et al.*, 2009). However, whether the anti-inflammatory properties of ezetimibe can attenuate myocardial remodeling remains unknown. This study aimed to investigate the effects of ezetimibe on improving inflammation and myocardial remodeling. Using obese rats induced by a high fat (HF) diet as an animal model of obesity, we demonstrated that ezetimibe can effectively reduce the expression of inflammatory factors and improve myocardial remodeling in obese rats.

MATERIALS AND METHODS

Animal model. All experimental protocols involving animals were performed in line with the National Institutes of Health and care and use of laboratory animals of Shandong University. An animal housing room was used to house the rats under controlled temperature (23–25°C), humidity, and 14 h light/10 h dark cycle throughout the entire experimental period.

A total of 40 male SD rats (8-week-old) were randomly divided into a control group (n=10) and model group (n=30). The control rats were fed a standard chow and tap water. The rats in the model group were fed a high-fat diet (HF-diet). After 16 weeks, 20 SD rats had developed obesity. The 20 obese rats were further divid-

Table 1. Metabolic variables at the end of the experiment

	NC (n=10)	OB (n=10)	OB+Ezetimibe (n=10)
Body weight (g)	494.96±21.95	654.64±67.54**	514.90±46.75††
Cholesterol (mmol/L)	1.98±0.11	2.93±0.04**	2.07±0.11††
Triglycerides (mmol/L)	0.95±0.08	1.56±0.08**	0.98±0.06††
HDL-C (mmol/L)	1.05±0.07	0.72±0.05*	1.09±0.06††
LDL-C (mmol/L)	0.74±0.04	1.52±0.06**	0.94±0.05††

Values are expressed as the mean ± SEM. * $P < 0.05$; ** $P < 0.01$ compared to the NC group. †† $P < 0.01$ compared to the OB group.

ed into two groups: 1) the obesity (OB) group (n=10), which received a continued HF-diet; 2) the ezetimibe treated (OB+Ezetimibe) group (n=10), which received a continued HF-diet plus treatment with ezetimibe at 10 mg/kg/day by gavage. Rats in both the control and OB groups were given the same volume of saline by gavage. The treatment period lasted for eight weeks.

Body weight (BW) was measured in the morning on a weekly basis throughout the experimental period. Venous blood samples were collected after 12 h fasting and the serum concentration of triglycerides (TG), cholesterol, high-density lipoprotein-cholesterol (HDL-C), low-density lipoprotein-cholesterol (LDL-C), and inflammatory factors were quantified by the Department Clinical Laboratory (Qilu Hospital affiliated with Shandong University, Jinan, China). At the end of the experimental period, rats were sacrificed by an over-dose of pentobarbital and the hearts were aseptically excised for subsequent analysis.

Echocardiographic examination. An echocardiographic examination was performed using a Vevo 770 cardiac system (Visual Sonics Inc., Toronto, Canada) under anesthesia with 10% chloral hydrate (0.3 mL/100 g). Left ventricular (LV) end-diastolic and end-systolic diameters, posterior wall and septum thickness, fractional shortening (FS), and the ejection fraction (EF) were measured according to the American Society of Echocardiography guidelines (Sahn *et al.*, 1978). Doppler measurements included peak early (E) and late (A) mitral valve inflow velocities and E/A ratio. For all echocardiographic measurements, the values of three consecutive cardiac cycles were averaged.

Ultrastructural observations. The left ventricular tissues (approximately 0.5×1×0.5 mm) from each group were fixed overnight in 2% glutaraldehyde and washed with 0.2 mol/L phosphate buffer three times. The samples were then fixed with 1% osmium tetroxide, washed again with 0.2 mol/L phosphate buffer, and dehydrated in different concentrations of ethanol. The ultrastructure of the cardiac muscle was observed using a transmission electron microscope (TEM; H-7000FA, Hitachi, Tokyo, Japan).

Histological analysis. Briefly, the lower part of the left ventricular tissue was fixed in 4% formaldehyde and embedded in paraffin, before being cut into 5 µm thick cross-sections. The sections were dewaxed and rehydrated, and antigen retrieval by microwaving was performed. The sections were stained with hematoxylin-eosin staining and Masson Accustain Trichrome stain (Sigma, St. Louis, MO, USA).

Western blot analysis. The heart tissues were lysed and 50 µg of protein was resolved on 10% SDS-polyacrylamide gels and transferred onto nitrocellulose membranes. Membranes were blocked at 4°C in 5% nonfat milk dissolved in Tris-buffered saline

(25 mM Tris, 137 mM NaCl, and 2.7 mM KCl) containing 0.05% Tween-20 and then incubated with P-p38MAPK (rabbit, 1:400) or IL-6 (rabbit, 1:500) primary antibodies at 4°C overnight. The membranes were washed three times in TBS-T and incubated with a horseradish peroxidase-conjugated goat anti-rabbit secondary antibody at room temperature for 2 h. Immunoreactive bands were visualized using an enhanced chemiluminescence kit and quantified with an image analyzer (AlphaImager 2200, USA). Protein levels were normalized to β-actin.

Immunohistochemical staining. Paraffin-embedded 5 µm thick ventricular tissue sections were rehydrated, and antigen retrieval was performed by an incubation in 1% citrate buffer (pH 6.0) at 92–98°C for 15 min. The sections were then incubated in PBS containing 3% hydrogen peroxide to quench any endogenous peroxidase activity. Next, the sections were blocked in goat serum for 30 min, followed by an incubation with VCAM-1 and ICAM-1 primary antibodies (Abcam, USA) overnight at 4°C. The negative controls were incubated with PBS instead of the primary antibody. A secondary conjugated IgG antibody was then added and the sections were incubated for 1 h at 37°C. DAB substrate kits were applied and the sections were viewed under a confocal FV 1000 SPD Laser Scanning microscope (Olympus, Japan). Three sections per rat and four areas from each ventricular section were analyzed.

Statistical analysis. All values are presented as the mean ± S.E.M. The results were compared using a one-way ANOVA, followed by a Tukey-Kramer post-hoc test. All statistical analyses were performed using SPSS 17.0 software, and a threshold value of $P < 0.05$ was considered significant.

Table 2. Parameters measured by echocardiogram at the end of the study

	NC (n=10)	OB (n=10)	OB+Ezetimibe (n=10)
LVEDD	4.06±0.57	4.43±0.30	4.53±0.75
LVESD	1.82±0.18	1.93±0.22	2.05±0.29
IVS	2.22±0.47	3.40±0.22**	2.89±0.26††
LVPW	3.14±0.44	4.53±0.80**	3.45±0.52††
FS	0.61±0.03	0.59±0.04	0.60±0.05
E/A	2.13±0.19	1.45±0.21**	1.61±0.22†

All data are mean ± S.E.M. LVEDD (mm): left ventricular end-diastolic dimension; LVESD (mm): left ventricular end-systolic dimension; IVS (mm): intraventricular septal wall; LVPW (mm): left ventricular posterior wall thickness; FS: fractional shortening; E/A: ratio of peak early diastolic filling velocity to peak velocity at atrial contractions. ** $P < 0.01$; † $P < 0.05$ compared to the NC group; †† $P < 0.01$, † $P < 0.05$ compared to the OB group.

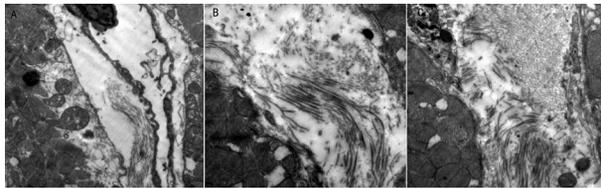


Figure 1. Representative photomicrographs of the transmission electron microscopy analysis of the myocardium of rats.

(A) NC group (n=10): deposition of collagen fibers in the extracellular matrix was clear and normal, and the mitochondria appeared to be of normal size and number; (B) OB group (n=10): cardiac myocytes exhibited an excessive deposition of collagen fibers in the extracellular matrix, and the mitochondria appeared to be swollen; (C) OB+Ezetimibe group (n=10): the mitochondria appeared to be of normal size, and deposition of collagen fibers in the extracellular matrix was decreased compared to the OB group. (TEM $\times 10000$).

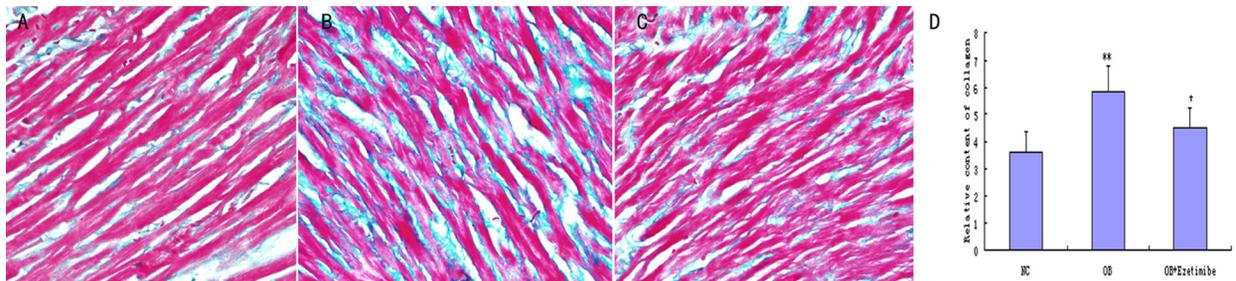


Figure 2. Masson's Trichrome staining of myocardial tissues.

(A) Normal SD rats showed normal quantity and the distribution of collagen fibers (400 \times). (B) OB rats displayed hyperplastic and disorganized collagen fibers (400 \times). (C) OB rats treated with Ezetimibe exhibited almost normal appearance of collagen fibers (400 \times). (D) Comparison of the relative content of collagen in three groups of rats. ** $P < 0.01$ vs. NC group; † $P < 0.05$ vs. OB group. NC group (n=10); OB group (n=10); OB+Ezetimibe group (n=10).

RESULTS

Ezetimibe affected baseline metabolism in obese rats

An obese rat model was established by feeding male SD rats with a HF diet for 16 weeks. After 16 weeks, the obese rats were found to develop hypercholesterolemia, lower HDL-C, and obesity compared to the normal control (NC) group ($P < 0.01$). After eight weeks of treatment with ezetimibe, cholesterol, TG, and BW were significantly decreased in OB+Ezetimibe group compared to the OB rats (Table 1).

Ezetimibe improved cardiac function in obese rats

After 16 weeks, interventricular septal (IVS) and LV posterior wall (LVPW) thickness in the OB rats were substantially increased, indicating the presence of LV

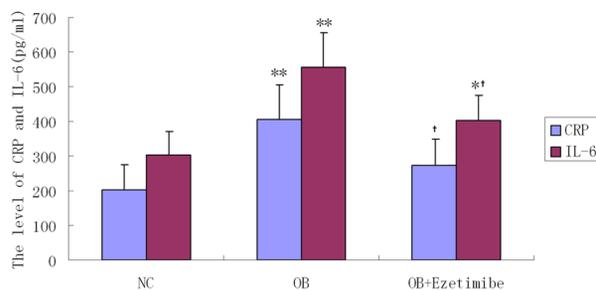


Figure 3. Comparison of serum CRP and IL-6 levels.

Data were shown as the means \pm S.E.M., ** $P < 0.01$ vs. NC group, † $P < 0.01$ vs. OB group. NC group (n=10); OB group (n=10); OB+Ezetimibe group (n=10).

concentric hypertrophy ($P < 0.05$). FS, representing LV systolic function, did not differ between the HF-fed and NC rats ($P > 0.05$). However, E/A was decreased significantly in the OB rats compared to the NC rats ($P < 0.05$) (Table 2). At the end of the study period, there were more marked changes in IVS, LVPW, and E/A in the OB rats (Table 2). Additionally, when compared to the OB rats, treatment with ezetimibe slowed the progression of cardiac hypertrophy and protected the diastolic function (Table 2).

Ezetimibe improved the ultrastructure of cardiomyocytes in obese rats

TEM analysis showed increased collagen fiber and mitochondria swelling in the cardiomyocytes of OB rats compared to the NC rats (Fig. 1). Moreover, after

ezetimibe treatment, the cardiac ultrastructure was obviously improved (Fig. 1).

Ezetimibe reduced the collagen content in obese rats

Masson staining revealed an increase of interstitial fibrosis in the myocardium of OB rats compared to the NC group. However, interstitial fibrosis was reduced after ezetimibe treatment (Fig. 2).

Ezetimibe decreased serum CRP and IL-6 levels in obese rats

Serum CRP and IL-6 levels of the OB rats were significantly higher than that of the NC rats. However, serum levels of CRP and IL-6 decreased significantly in the ezetimibe-treated group compared to the OB group (Fig. 3).

Ezetimibe decreased P-p38MAPK and IL-6 levels in the hearts of obese rats

The levels of P-p38MAPK and IL-6 in the rat hearts were evaluated by Western blot. In the OB rats, the levels of P-p38MAPK and IL-6 were significantly increased in the myocardial tissues compared to the NC group. However, both P-p38MAPK and IL-6 levels decreased significantly in ezetimibe-treated rats when compared to the OB rats (Fig. 4).

Ezetimibe decreased ICAM-1 and VCAM-1 levels in the hearts of obese rats

In the OB rats, ICAM-1 and VCAM-1 levels in the hearts were significantly higher compared to the NC group. However, the levels of ICAM-1 and VCAM-1

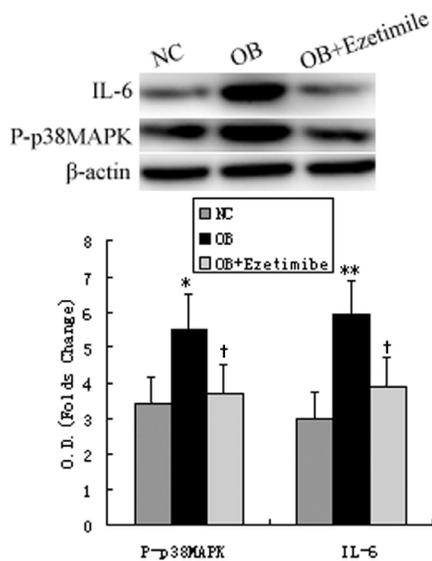


Figure 4. Western blot analysis of P-p38MAPK and IL-6 levels in the myocardial tissues of rats.

Representative blots were shown. The bar graph displays the relative protein levels. Each bar represents the mean \pm SEM. ** $P < 0.01$; * $P < 0.05$ vs. NC group; † $P < 0.05$ vs. OB group. NC group (n=10); OB group (n=10); OB+Ezetimibe group (n=10).

were significantly lower in the OB + ezetimibe group compared to the OB group (Fig. 5).

DISCUSSION

In this study, a rat model of obesity was used to demonstrate that obesity-enhanced cardiac inflammation

and remodeling could be inhibited by treatment with ezetimibe. Mechanistically, we found that obesity led to the activation of p38MAPK and enhanced the expression of inflammatory factors, such as IL-6, ICAM-1, and VCAM-1. Treatment with ezetimibe inhibited such obesity-induced activation of p38MAPK and the upregulation of several inflammatory factors.

Obesity is one known risk factor for CVD, for which the underlying mechanism is thought to be related to lipotoxicity and insulin resistance (IR) (Avalos-Soriano *et al.*, 2016). In the present study, HF-fed rats developed obesity with low levels of HDL-C, as well as elevated cholesterol, TG, and BW. Moreover, most obese patients already exhibit vascular abnormalities by the time they are diagnosed with a metabolic disorder (Ruderman & Schneider, 1992; Ridker *et al.*, 2003). In our study, the obese rat model showed enhanced myocardial remodeling and inflammation, all of which may contribute to the high prevalence of cardiovascular complications associated with obesity. Moreover, the risk factors associated with CVD, including dyslipidemia, hypertension, and hyperglycemia, are considered to be initiation and progression factors of myocardial remodeling. In addition, inflammation is a hallmark sign throughout the distinct stages of myocardial remodeling induced by a HF-fed diet (Klingenberg & Luscher, 2012; Haffner, 2006). Furthermore, a systemic chronic inflammatory response in obesity, characterized by altered cytokine production and the activation of inflammatory signaling pathways, is another important mechanism associated with the initiation and progression of myocardial remodeling (Martínez-Martínez *et al.*, 2016; Mayerl *et al.*, 2006).

IL-6 and CRP are the major mediators of inflammation (Young *et al.*, 2014; Libinaki *et al.*, 2010; Hattori *et al.*, 2003). In addition, the p38MAPK pathway is a key

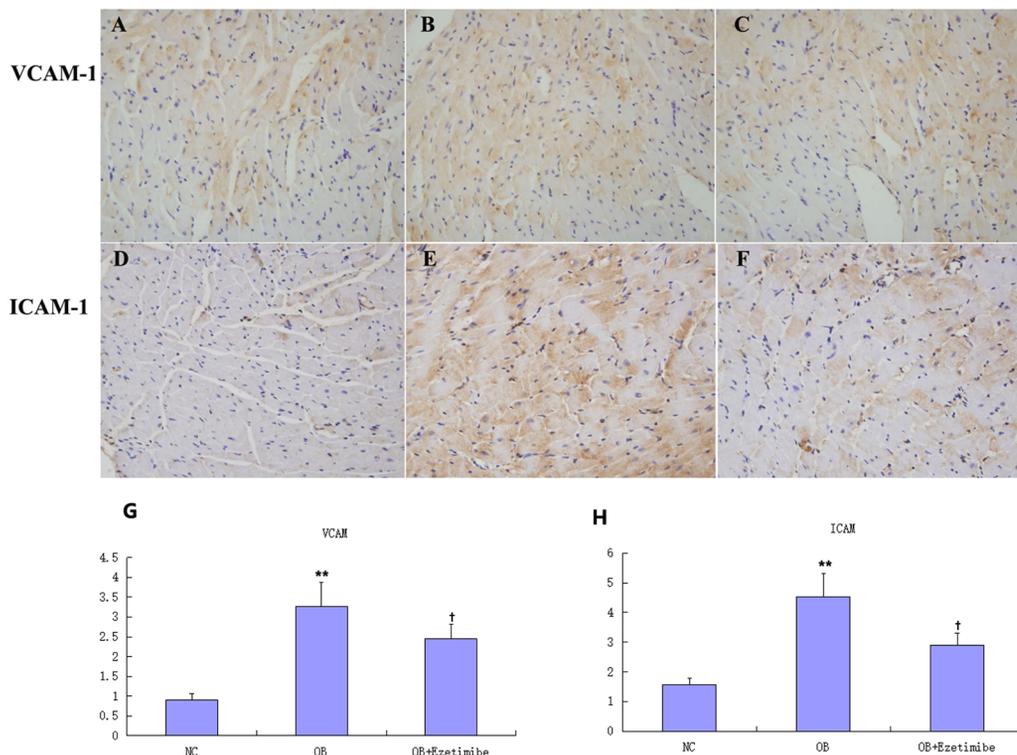


Figure 5. Immunohistochemistry staining of VCAM-1 and ICAM-1 in the myocardial tissues of the rats.

(A and E) NC group (n=10); (B and F) OB group (n=10); (C and G) OB+Ezetimibe group (n=10). Representative micrographs of immunohistochemistry staining were shown. Original magnification: $\times 400$. (D and H) Quantitative analysis of VCAM-1 and ICAM-1 staining. Data are presented as the mean \pm S.E.M. ** $P < 0.01$ vs. the NC group; † $P < 0.01$ vs. the OB group.

regulator of pro-inflammatory cytokine biosynthesis, and its components are potential targets for the treatment of inflammatory diseases associated with obesity (Cuenda & Rousseau, 2007). It is interesting to note that p38MAPK activation is essential for VCAM-1 and ICAM-1 expression in cardiac cells (Kacimi *et al.*, 1998). VCAM-1 and ICAM-1 are important factors that promote inflammation. In this study, the cardiac tissue of obese rats exhibited: cardiomyocytes with an abnormal ultrastructure; increased interstitial fibrosis; thicker walls; reduced diastolic function; p38MAPK activation; and increased expression of CRP, IL-6, VCAM-1, and ICAM-1. Previous studies have indicated that dyslipidemia induces the formation of atherosclerotic lesions by increasing the release of inflammatory molecules, such as TNF- α and IL-6 (Derosa *et al.*, 2011; Li *et al.*, 2015). These results suggest that the obesity-induced activation of p38MAPK may promote cardiac inflammation and plays an important role in the pathogenesis of cardiac remodeling.

Recent studies suggested that ezetimibe may inhibit inflammation and improve metabolic functionality (Shapiro & Fazio, 2016; *et al.*, 2015). Ezetimibe displays cardioprotective activity by inhibiting the expression of vascular CD14, a marker of the infiltration of mononuclear cells (Kuhlencordt *et al.*, 2009). Ezetimibe also interferes with the activity of nuclear factor- κ B in leukocytes and reduces the amount of monocyte and macrophage chemoattractant protein 1 (MCP-1) (Gómez-Garre *et al.*, 2009). In addition, the anti-inflammatory effects of ezetimibe are thought to have a favorable impact on lipid metabolism (Krysiak *et al.*, 2014); however, no studies have assessed the effect of ezetimibe on myocardial remodeling after a high fat diet. Therefore, we investigated the effects of ezetimibe on inflammation and myocardial remodeling in rats with obesity induced by a HF diet. We found that ezetimibe could effectively alleviate myocardial remodeling by inhibiting the level of inflammation. In addition to lowering lipid metabolism, ezetimibe prevented cardiac inflammation and collagen deposition induced by a HF diet.

Taken together, our findings suggest that the upregulation of inflammatory factors promotes myocardial remodeling in an obese rat model, which may be due to the activation of p38MAPK in the heart tissue. Treatment with ezetimibe may have the potential to inhibit p38MAPK activation, reduce the expression of inflammatory factors, and alleviate myocardial remodeling associated with obesity.

Conflict of interests

The authors confirm that there are no conflicts of interest.

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