Ezetimibe suppresses cholesterol accumulation in lipid-loaded vascular smooth muscle cells in vitro via MAPK signaling

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Aim: To investigate the mechanisms of anti-atherosclerotic action of ezetimibe in rat vascular smooth muscle cells (VSMCs) in vitro.

Methods: VSMCs of SD rats were cultured in the presence of Chol:MβCD (10 μg/mL) for 72 h, and intracellular lipid droplets and cholesterol levels were evaluated using Oil Red O staining, HPLC and Enzymatic Fluorescence Assay, respectively. The expression of caveolin-1, sterol response element-binding protein-1 (SREBP-1) and ERK1/2 were analyzed using Western blot assays. Translocation of SREBP-1 and ERK1/2 was detected with immunofluorescence.

Results: Treatment with Chol:MβCD dramatically increased the cellular levels of total cholesterol (TC), cholesterol ester (CE) and free cholesterol (FC) in VSMCs, which led to the formation of foam cells. Furthermore, Chol:MβCD treatment significantly decreased the expression of caveolin-1, and stimulated the expression and nuclear translocation of SREBP-1 in VSMCs. Co-treatment with ezetimibe (3 μmol/L) significantly decreased the cellular levels of TC, CE and FC, which was accompanied by elevation of caveolin-1 expression, and by a reduction of SREBP-1 expression and nuclear translocation. Co-treatment with ezetimibe dose-dependently decreased the expression of phosphor-ERK1/2 (p-ERK1/2) in VSMCs. The ERK1/2 inhibitor PD98059 (50 μmol/L) altered the cholesterol level and the expression of p-ERK1/2, SREBP-1 and caveolin-1 in the same manner as ezetimibe did.

Conclusion: Ezetimibe suppresses cholesterol accumulation in rat VSMCs in vitro by regulating SREBP-1 and caveolin-1 expression, possibly via the MAPK signaling pathway.

Keywords: atherosclerosis; vascular smooth muscle cells; cholesterol; ezetimibe; caveolin-1; SREBP-1; MAPK

Introduction

Together, cardiovascular diseases (CVDs) such as atherosclerosis and stroke remain the leading cause of death worldwide. Numerous studies have indicated that excessive accumulation of vascular smooth muscle cells (VSMCs) has a preferential role in the development and progression of atherosclerosis. Cholesterol accumulation in VSMCs and monocytes/macrophages leads to the formation of lipid-loaded cells or foam cells, which is a pathological feature of atherosclerosis.[1]

Ezetimibe is a potent inhibitor of the intestinal absorption of cholesterol and is widely used for the treatment of hypercholesterolemia in humans.[2, 3] More recently, ezetimibe has been reported to possess pleiotropic effects beyond cholesterol lowering both in vitro and in animal models.[4] The beneficial effect of ezetimibe on the development of atherosclerosis was first shown in apolipoprotein E-deficient mice fed with a high-fat diet.[5] Similarly, ezetimibe reduced atherosclerotic lesion formation in a diet-induced atherosclerosis model in hypercholesterolemic rabbits.[6] However, the exact anti-atherosclerotic mechanism of ezetimibe remains to be elucidated.

Caveolin-1 (Cav-1) is a 22-kDa protein that forms the main structure of caveolae[7] and is believed to play a crucial role in the regulation of cellular signaling and cholesterol transport[8].
The established Cav-1 knockout mouse line has confirmed the importance of Cav-1 for the formation of morphologically distinct caveolae\cite{9,10}. In addition, Cav-1 is also involved in the maintenance of cellular cholesterol homeostasis and lipid transport. An increasing number of studies have demonstrated that caveolae and Cav-1 expression are associated with enhancement of cholesterol efflux\cite{11,12}, which contributes to the inhibition of cholesterol accumulation. Smart et al found that the Cav-1 and Annexin-2 complex is a target of ezetimibe in the regulation of intestinal cholesterol transport\cite{13,14}, and ezetimibe therapy has been shown to downregulate SREBP-1 and SREBP-2 expression\cite{15,16}.

It is evident that SREBP-1 exists in the cytoplasm and nucleus and is associated with cholesterol metabolism\cite{17}. Its active form can translocate into the nucleus and regulate target gene transcription through binding with the sterol regulatory element (SRE) sequence within target gene promoters\cite{18,19}. Interestingly, an SRE sequence exists in the Cav-1 promoter, and Cav-1 is negatively regulated by SREBP-1\cite{19,20}. A previous study showed that SREBP expression can be regulated by ezetimibe and is associated with cholesterol transport and metabolism\cite{21}. Recently, accumulating evidence has shown that SREBPs are not only participants in cholesterol-regulated events but are also gene regulatory targets of intracellular signaling pathways, such as MAP kinase cascades that are associated with cholesterol transport\cite{22}. More importantly, MAP kinase-mediated effects on SREBP-1-regulated target genes are linked to the phosphorylation site of MAP kinases-phosphorylation site\cite{23}.

The purpose of this study was to address the underlying mechanism of ezetimibe-dependent effects on cholesterol accumulation in cultured VSMCs. We hypothesized that ezetimibe exerted beneficial effects on atherosclerosis partially through regulating the expression of Cav-1 and SREBP-1 via the MAPK pathway, subsequently inhibiting cellular cholesterol accumulation.

**Materials and methods**

**Reagents**

Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco/BRL (Grand Island, NY, USA). Ezetimibe was purchased from Merck/Schering-Plough Pharmaceuticals (0.021%, w/w) and Oil Red O powder was obtained from Sigma-Aldrich (St Louis, MO, USA) and dissolved with DMSO (Sangon, Shanghai, China). All reagents were of analytical grade.

**Isolation and culture of VSMCs**

Primary VSMCs were derived from the thoracic aorta of adult male Sprague-Dawley (SD) rats as described previously\cite{24,25}. Rat aortic VSMCs were isolated from the thoracic aortas of six-week-old male SD rats using the explant technique\cite{26}. Briefly, after the removal of the endothelium and adventitia, the aortic explants were cultured in DMEM containing 20% FBS. Within 9–10 d, VSMCs migrating from explanted vessels were evident. Cells growing in DMEM containing 10% FBS were used at low passage numbers (≤ten passages).

**Cellular cholesterol assay (Oil Red O staining)**

Foam cell formation was verified by fixation with 4% paraformaldehyde and subsequent staining with 0.5% Oil Red O. Hematoxylin was used as a counterstain, and the cells were photographed at 400× magnification.

**Quantitation of intracellular cholesterol (HPLC and Enzymatic Fluorescence Assay)**

Cellular free cholesterol (FC) and total cholesterol (TC) were analyzed by HPLC\cite{27}. Briefly, cells were placed in 0.9% NaCl solution and homogenized on ice by sonication for 10 s. After the protein concentration was measured with the bicinechonic acid (BCA) assay (Pierce Biotechnology Company, Rockford, IL, USA), an equal volume of freshly prepared cold (-20 °C) potassium hydroxide in ethanol (150 g/L) was added to the cell lysate and vortexed until clear. An equal volume of hexane isopropanol (3:2, v/v) was added and vortexed for 5 min followed by centrifugation at 800×g for 5 min. The organic phases were collected and dried in a SpeedVac. The samples were dissolved in 100 μL isopropanol-acetonitrile (20:80, v/v) and placed in an ultrasonic water bath at room temperature for 5 min. Finally, the samples were subjected to HPLC analysis (Agilent 1100, Agilent Technology, USA). HPLC was performed on a Hypersil-C18 column, and elution was performed with an isopropanol:acetonitrile (20:80, v/v) solvent system at a flow rate of 1 mL/min. Cholesterol ester (CE) was determined by subtracting the FC values from the TC values\cite{19}.

Another method was to use Cholesterol Quantitation Kit (Catalogue No. K603-100, Biovision, Inc, Mountain View, CA, USA). Cells (1×10⁶) were extracted with 200 μL of chloroform: isopropanol: NP-40 (7:11:0.1) in 1.5 mL EP, and then cell membrane was destructed by ultrasonic, the extract was spun at 15000×g for 10 min. The liquid was transferred to a new tub, air dried at 50 °C for 4 h to remove chloroform. The samples were put under vacuum at 50 °C for 30 min to remove trace organic solvent. The dried lipids were dissolved with 200 μL Cholesterol Assay buffer, 20 μL for Cholesterol Quantitation following the protocols provided by the manufacturer. Contents of cholesterol was measured by fluorescence at Ex/Em=535/590. Protein concentrations were determined by the BCA™ Protein Assay Kit according to the protocol provided by the manufacturer (Catalogue No P0012, Beyotime, Inc, Haimen, China). The values of intracellular cholesterol were calculated as μg cholesterol/μg cellular protein.

**Immunofluorescence staining**

For immunofluorescence analysis\cite{28} 2×10⁶ cells were plated into each well of a 6-well plate with coverslips. The cells were incubated with 10 μg/mL Chol:MβCD in the presence or absence of 3 μmol/L ezetimibe for 72 h. After the treatment, the cells were washed with PBS. Then, the cells were fixed with 4% paraformaldehyde for 10 min. All fixed cells were permeabilized in 0.1% Triton X-100 for 10 min. The cells were incubated with SREBP-1 antibody for 60 min at room tempera-
ture. Next, the cells were incubated with rhodamine-conjugated anti-rabbit IgG for 30 min and subsequently incubated with DAPI (10 μg/mL) (Sigma, USA) for 10 min to display the cell nuclei. Coverslips were mounted and analyzed by fluorescence microscopy.

Western blot analyses

Western blot analysis was performed as previously described\textsuperscript{[24, 29, 30]}. Briefly, cells were lysed in a buffer containing 10 mmol/L HEPES (pH 7.9), 1.5 mmol/L MgCl\textsubscript{2}, 10 mmol/L KCl and 0.5% NP-40, and the cell lysates were centrifuged at 13 000 r/min for 15 min at 4°C. The protein concentrations of the cell lysates were determined using a BCA protein assay kit according to the manufacturer’s instructions. The proteins were separated on 6%-10% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore Corporation, Billerica, MA, USA). Nitrocellulose membranes were incubated in blocking solution [PBS with 0.05% (v/v) Tween 20 (PBST) containing 5% (w/v) skim milk or 1% (w/v) BSA (Sigma-Aldrich)] for 1 h at room temperature to block nonspecific binding. The blot was incubated overnight with anti-apoE (Santa Cruz Biotechnology, USA), anti-SR-B1 (Abcam, USA), anti-Cav-1, anti-CD36 (Santa Cruz Biotechnology, USA), anti-p-ERK1/2 and anti-T-ERK1/2 (Cell Signaling Technology, Danvers, MA, USA) or anti-α-tubulin (Santa Cruz Biotechnology, USA) primary antibodies in blocking solution. The blot was washed 3 times for 10 min each in PBST at room temperature, incubated for 45 min in species-appropriate horseradish peroxidase-conjugated secondary antibody (Sigma, St Louis, MO, USA) in blocking solution, and subsequently washed 3 times in PBST. Specific proteins were visualized using an Enhanced Chemiluminescence Kit (New England Biolabs, Beverly, MA, USA).

mRNA extraction and real-time quantitative PCR (RT-qPCR)

Total RNA was prepared using an RNeasy Mini Kit, and 2 μg was reverse transcribed using a QuantiTect Reverse Transcription Kit with Oligo dT as a primer. RT-qPCR was carried out using target gene primers with the SYBR Green PCR master mix (Applied Biosystems, Warrington, UK). The following primers were used: β-actin forward primer: 5’-CCCTGTAC-GCCAACACAGTG-3’; β-actin reverse primer: 5’-ATACTCT-GCTGTGCTGATCC-3’; Cav-1 forward primer: 5’-ATGCTCT-GGCGCAAATACGTG-3’, Cav-1 reverse primer: 5’-CGCGT-CATACACTTGTCTTCT-3’. For SREBP-1, 5’-TGCCCTAAAGGG-TCAAACCACA-3’ and 5’-TGCCGGGCACACTTATTAGAA-3’ served as the forward and reverse primers, respectively. Quantitative measurements were determined using the ΔΔCt method, and average mRNA expression was normalized to β-actin expression.

Statistical analysis

The data are expressed as the mean±SEM. The statistical significance of differences was assessed by ANOVA; a value of \( P<0.05 \) was considered statistically significant.

Results

Ezetimibe suppressed cholesterol accumulation in lipid-loaded VSMCs stimulated by Chol:MβCD

It is well known that cholesterol is involved in the pathogenesis of atherosclerosis. Excessive cholesterol accumulation in cells leads to the formation of foam cells, which is one of the hallmarks of atherosclerotic plaques within the arterial wall. In a previous study\textsuperscript{[31]}, we successfully established a foam cell model by using 10 μg/mL Chol:MβCD, which is “water-soluble cholesterol” containing approximately 50 mg of cholesterol per g of solid (molar ratio, 1:6 cholesterol:MβCD) that delivers cholesterol rapidly and directly to the plasma membrane\textsuperscript{[32]}. Using Oil Red O staining, we determined the morphological changes in cells after incubation of VSMC with Chol:MβCD in the absence or presence of 3 μmol/L ezetimibe for 72 h. Our results showed that ezetimibe treatment dramatically reduced cellular lipid accumulation in VSMCs treated with Chol:MβCD (Figure 1). HPLC was then conducted to determine cellular cholesterol content. The concentrations of TC, FC and CE in ezetimibe-treated cells stimulated by Chol:MβCD were significantly lower than those in the Chol:MβCD group without ezetimibe treatment (Table 1). Thus, ezetimibe suppressed intracellular cholesterol accumulation effectively.

![Figure 1](image-url)  

*Figure 1.* Effects of ezetimibe on cholesterol accumulation in lipid-loaded VSMCs induced by Chol:MβCD. VSMCs were co-incubated with 10 μg/mL Chol:MβCD in the absence or presence of 3 μmol/L ezetimibe for 72 h. After treatment, the cells were stained with Oil Red O. Cellular nuclei were stained with hematoxylin. The magnification of each panel is ×400. All the results were obtained from 3 independent experiments, and each experiment was performed in triplicate. Arrows indicate the foam cells.
Ezetimibe downregulated SREBP-1 and upregulated Cav-1 protein expression in lipid-loaded VSMCs induced by Chol:MβCD

Caveolins, especially Cav-1, have been implicated in the regulation of cellular cholesterol metabolism and lipid uptake as well as cholesterol and lipid efflux \[33\]. It has been reported that Cav-1 is regulated by SREBP-1 due to SREBP-1 binding to the Cav-1 promoter. Therefore, we measured protein levels to determine whether Cav-1 and SREBP-1 are affected by ezetimibe. As shown in Figure 2A and 2B, ezetimibe upregulated the expression of Cav-1 and downregulated the expression of SREBP-1 in VSMCs in the presence of Chol:MβCD at both the protein and mRNA levels. These data indicate that the inhibitory effect of ezetimibe on cholesterol accumulation is most likely linked to the enhancement of Cav-1 expression and reduction of SREBP-1 expression.

Ezetimibe inhibited the nuclear translocation of SREBP-1 in lipid-loaded VSMCs induced by Chol:MβCD

The SREBP-1 precursor is located on the membrane of the endoplasmic reticulum. When the intracellular cholesterol level changes, active SREBP-1 is transferred into the nucleus to regulate Cav-1 transcription \[34\]. To examine the activity of SREBP-1 under ezetimibe treatment, we observed SREBP-1 translocation by double immunofluorescence staining. Compared with the control group, Chol:MβCD stimulated SREBP-1 translocation from the cytoplasm into the nucleus (Figure 3). However, there was a redistribution of SREBP-1 after ezetimibe treatment. A substantial portion of SREBP-1 accumulated in the cytoplasm after ezetimibe treatment, suggesting that ezetimibe reversed the process of SREBP-1 nuclear translocation (Figure 3).

Table 1. The effect of ezetimibe on cellular cholesterol content (μg/mg protein). Intracellular lipid content in VSMCs. VSMCs were incubated with 10 μg/mL Chol:MβCD in the absence or presence of 3 μmol/L ezetimibe for 72 h. Cholesterol ester (CE) and the ratio of CE/TC was calculated. The results are expressed as the means±SD from 3 independent experiments, each performed in triplicate. *P<0.01 vs control. **P<0.05 vs Chol:MβCD.

| Group                  | FC       | TC       | CE       | CE/TC (%) |
|-----------------------|----------|----------|----------|-----------|
| Control               | 42.8±2.3 | 47.5±3.1 | 5.2±2.5  | 11.2±2.1  |
| Chol:MβCD             | 95.2±5.1 | 212.7±5.9| 112.8±9.2| 56.4±3.3  |
| Chol:MβCD+ezetimibe   | 80.6±4.2 | 150.3±7.5| 62.7±8.1 | 39.5±5.7  |

Figure 2. Effects of ezetimibe on the expression of Cav-1 and SREBP-1 in VSMCs in the presence of Chol:MβCD. (A) VSMCs were co-incubated with 10 μg/mL Chol:MβCD in the absence or presence of 3 μmol/L ezetimibe for 72 h. Protein expression was detected by Western blot analysis (30 μg protein/lane). α-Tubulin was used as a loading control. The data were obtained from three independent experiments. (B) VSMCs were co-incubated with 10 μg/mL Chol:MβCD in the absence or presence of 3 μmol/L ezetimibe for 72 h. mRNA levels were evaluated by real-time quantitative polymerase chain reaction (PCR). The data were obtained from three independent experiments. *P<0.01 vs control. **P<0.01 vs Chol:MβCD.
cells stimulated by Chol:MβCD compared with the group treated with Chol:MβCD alone. More importantly, the inhibitory effects of ezetimibe on the expression of p-ERK1/2 and SREBP-1 were enhanced after addition of PD98059 (Figure 4D). Collectively, these results indicate that the ERK1/2 pathway is involved in the regulation of Cav-1 and SREBP-1 by ezetimibe in VSMCs.

Discussion
The formation of VSMC- or macrophage-derived foam cells is a key pathologic process in atherosclerosis. It is believed that controlling the balance of cholesterol transport is strongly associated with regulation of atherosclerosis. In foam cells, the ratio of cholesterol ester (CE) to total cholesterol (TC) is more than 50%, while VSMCs are referred to as lipid-loaded cells if the ratio is less than 50%\(^\text{[12]}\). In our study, the intracellular CE/TC ratio exceeded 50% when VSMCs were treated with 10 μg/mL Chol:MβCD for 72 h, while ezetimibe decreased the intracellular CE/TC ratio dramatically. Our findings clearly demonstrated that ezetimibe suppressed intracellular cholesterol accumulation effectively. Clinically, ezetimibe has been used in hypercholesterolemia for cholesterol reduction\(^\text{[38, 39]}\). Therefore, our findings are consistent with the observations in clinical practice.

To date, stimulating cholesterol removal from lipid-loaded cells is believed to be a promising strategy to delay atherosclerosis. The accumulation of intracellular cholesterol is associated with an imbalance in cholesterol influx and efflux. Cav-1 plays a key role in cholesterol efflux in the form of an intracellular trafficking complex containing Cav-1, HSP56, cyclophilin A and cyclophilin 40\(^\text{[40]}\). In the present study, we found that ezetimibe could increase the level of the efflux-related protein Cav-1, suggesting that the suppressive effect of ezetimibe in cytoplasmic cholesterol accumulation might be due to the promotion of cholesterol efflux. Meanwhile, we also found that SREBP-1, a transcriptional regulator of Cav-1, was activated by Chol:MβCD. Active SREBP-1 translocates into the nucleus to regulate Cav-1 transcription via binding to two SREs in the promoter of Cav-1\(^\text{[41]}\).

It has been shown that ezetimibe can effectively alter the transcription of some surface receptors (eg, NPC1L1) by downregulating the gene expression of the nuclear receptors SREBP-1 and SREBP-2 in Caco-2 cells\(^\text{[42]}\). Our results showed that Chol:MβCD-induced elevation of SREBP-1 and translocation of SREBP-1 from the cytoplasm into the nucleus could be inhibited by ezetimibe. ERK1/2 and p44/42 mitogen-activated protein kinases (p44/42 MAPK) belong to a highly conserved family of Ser-Thr protein kinases. The role of ERK1/2 in the development of atherosclerosis was described in a previous study\(^\text{[43]}\). Inhibition of ERK1/2 significantly increases

![Figure 3. Effects of ezetimibe on nuclear translocation of SREBP-1 in lipid-loaded VSMCs induced by Chol:MβCD. VSMCs were co-incubated with 10 μg/mL Chol:MβCD in the absence or presence of 3 μmol/L ezetimibe for 72 h. SREBP-1 in the cytoplasm (red) or nucleus (blue) was detected by indirect immunofluorescence with double staining. Images were obtained with a fluorescence microscope (×400).](image-url)
cellular cholesterol efflux, which suggests that ERK1/2 activity is closely linked to cholesterol trafficking.[43]. Once phosphorylated, ERK becomes an active kinase that translocates to the nucleus, where it has been shown to regulate the transcription of target genes. Moreover, the MAP kinase pathway appears to be critical for SREBP-1 stimulation in skeletal muscle.[44]. Evidence from several studies has shown that SREBPs are substrates for ERK1 as well as ERK2 in vitro.[45, 46]. Furthermore, SREBP-1 is phosphorylated by the ERK subfamily of MAP kinases at serine 117.

As mentioned above, Muller-Wieland and co-workers[45] demonstrated a strong linkage between the MAP kinase-dependent pathway and expression of SREBP-1. In the present study, ezetimibe downregulated p-ERK1/2 expression in a dose-dependent manner, and a similar suppressive effect of ezetimibe on SREBP-1 expression was noted. More importantly, ezetimibe provoked a redistribution of SREBP-1 and ERK1/2, and the nuclear accumulation of ERK1/2 and SREBP-1 stimulated by Chol:MβCD was reversed by ezetimibe in VSMCs. Furthermore, the inhibitory effects of ezetimibe on the expression of p-ERK1/2 and SREBP-1 were enhanced after addition of PD98059. These findings indicate that the effects of

Figure 4. Ezetimibe regulates MAPK signaling in lipid-loaded VSMCs induced by Chol:MβCD. (A) The expression of p-ERK1/2, Cav-1, SREBP-1, and α-tubulin was detected by Western blot analysis in VSMCs treated with ezetimibe at the indicated concentration in the presence of 10 μg/mL Chol:MβCD for 72 h. (B) VSMCs were co-incubated with 10 μg/mL Chol:MβCD in the absence or presence of 3 μmol/L ezetimibe for 72 h. The localization of p-ERK1/2 (red) was determined by immunofluorescence. Nuclei were stained with DAPI (blue). (C) VSMCs were incubated with or without 3 μmol/L ezetimibe for 72 h in the presence of 10 μg/mL Chol:MβCD followed by treatment with the ERK1/2 inhibitor PD98059 (50 μmol/L) for 1 h. n=3. Mean±SEM. *p<0.05 vs 10 μg/mL Chol:MβCD group. (D) VSMCs were incubated with or without 3 μmol/L ezetimibe for 72 h in the presence of 10 μg/mL Chol:MβCD followed by treatment with the ERK1/2 inhibitor PD98059 (50 μmol/L) for 1 h. The expression levels of p-ERK1/2, total ERK1/2 (T-ERK1/2), Cav-1, SREBP-1, and α-tubulin were measured by Western blot analysis. α-Tubulin served as a control. The data were obtained from three independent experiments.
ezetimibe on the regulation of Cav-1 and SREBP-1 expression involve MAPK signaling.

In summary, ezetimibe strongly suppresses the intracellular accumulation of cholesterol by regulating the expression of SREBP-1 and Cav-1 via the MAPK pathway. Our study has revealed a novel mechanism underlying the anti-atherosclerosis properties of ezetimibe, which may aid the discovery of novel targets for anti-atherosclerosis therapy.

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Author contribution
All authors have made substantial contributions to this paper. Dr. Duan-fang LIAO conceived and designed the current study and approved the final submitted version of the manuscript. Dr. Li QIN and Yun-bo YANG performed a majority of the experiments and drafted the article. Dr. Yi-xin YANG performed cell culture. Neng ZHU performed the HPLC assays. Ms. Yong-zhen GONG and Dr. Shun-xiang LI performed the immunofluorescence staining experiments. Dr. Cai-ping ZHANG performed the matic fluorescence staining Assays.

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