Original Article
Triptolide suppresses secretion of inflammatory factors and promotes expression of ABCA1

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Abstract: Purpose: The aim of the present study was to explore the roles and molecular mechanisms of triptolide in the progression of atherosclerosis. Method: Genotypes of APOE knock out genes and corresponding wild type genes were tested via PCR assay. Levels of total cholesterol and total triglycerides were detected according to kit protocol. Fat tissues in livers were stained and observed by oil O staining. APOE-/- mice were selected by PCR assay, oil O staining, and H&E staining. ApoE mice were randomly divided into three groups: ApoE-/- mice, wild type mice, and ApoE-/- mice treated with 5 μg/kg TPL and 50 μg/kg of triptolide twice every day. Concentrations of inflammatory cytokines were determined by ELISA assay. Expression of ABCA1 was detected by Western blotting. Results: Body weight showed no statistical differences between the triptolide treated group and ApoE-/- group. Triptolide decreased levels of total cholesterol and total triglycerides in ApoE-/- mice. Secretion of inflammatory cytokines in macrophages was significantly decreased in 50 μg/mL of triptolide treated ApoE-/- mice. Triptolide increased expression of ABCA1 in macrophages. Conclusion: Triptolide treatment decreased secretion of inflammatory cytokines TNF-α, IL-1β, IL-6, and IL-8 and promoted expression of ABCA1 in macrophages. Thus, triptolide may be a new agent for clinical therapy of atherosclerosis.

Keywords: Triptolide, inflammatory cytokines, ApoE, ABCA1

Introduction
Atherosclerosis is an important risk factor of many cardiovascular diseases, such as coronary heart disease, cerebral infarction, and myocardial infarction [1]. The main feature of cardiovascular and cerebrovascular diseases is atherosclerosis, characterized by lipid deposit and inflammatory infiltration [2]. In China, dietary habits of residents have changed from carbohydrate-based to high-fat and high-protein, leading to high incidence of atherosclerosis [3]. Until now, cardiovascular and cerebrovascular diseases have occupied an important position in China’s lethal diseases [4]. Relevant pathological basis and treatments of atherosclerosis have become urgent issues to be clarified. Clarification would be beneficial in the field of medicine at home and abroad for the prevention and treatment of related cardiovascular and cerebrovascular diseases.

Occurrence of atherosclerosis is not only a simple dyslipidemia, but it is caused by a combination of various factors. Known risk factors are hypertension, hyperlipidemia, eating habits, age, stress, diabetes, infections, and smoking. It has been reported that occurrence and progression of atherosclerosis is accompanied by occurrence of inflammatory reactions and disorders of lipid metabolism [5]. Chronic inflammation normally breaks the balance of related lipid metabolism, reduces the biological activity of the blood vessel wall, and finally causes arterial atherosclerosis [6]. Specifically, inflammatory cells often secrete inflammation-related chemokines and various cytokines (such as TNF-α, IL-1β and IL-6 etc.), which promote peripheral blood mononuclear cells differentiating into macrophages and migrating to the arterial intima, inducing progression of atherosclerosis [7]. Lipids are swallowed by the macrophages to form foam cells. Accumulation of foam cells forms the lipid core, which constitutes an atherosclerotic plaque, together with the fibrous cap and inflammatory factors [8].
In mammals, all nucleated cells can synthesize cholesterol, but they do not have the ability to degrade cholesterol. Excess cholesterol will be stored in or released from the cytoplasm. Generally, to maintain the balance of cholesterol, excessive cholesterol in peripheral cells can be eliminated by reverse cholesterol transport (RCT) pathways by transferring protein to the liver, eventually excreting it in the liver with bile. ATP-binding cassette subfamily A, member 1 (ABCA1), is involved in reverse cholesterol transport (RCT) and required for apoA-I-mediated cholesterol efflux. ABCA1 is predominantly expressed at the plasma membrane and widely expressed in almost all tissues, with higher expression in the liver, intestine, adrenal glands, lungs, brain, and macro-saline cells. ABCA1 is normally combined with apolipoprotein A-I and other apolipoproteins, such as ApoE, in the initial stages of RCT. Thus, the present study aimed to detect whether treatment with anti-inflammatory agents would promote RCT in macrophages of ApoE-/- mice.

Triptolide is an epoxy diterpene lactone compound, extracted from roots, leaves, flowers, and fruits of the genus Tripterygium. It has been reported that triptolide possesses potent anti-inflammatory effects [9, 10]. The Zhou group found that inflammatory response was attenuated by triptolide, partly by inhibiting NF-kappaB signaling pathways [11]. Inhibition of inflammatory reactions of triptolide by inhibiting NF-kappaB signaling pathways was also found in LPS-induced acute lung injuries in a murine model [12, 13]. Moreover, in an adjuvant-induced arthritis (AA) murine model of rheumatoid arthritis (RA), triptolide effectively alleviated RA by inhibiting neutrophil inflammatory function [14]. Results suggest that triptolide might work as a potential therapeutic agent for anti-inflammatory response. However, the roles and molecular mechanisms of triptolide in the progression of atherosclerosis have not been clearly clarified. The present study used an ApoE-/- murine model of atherosclerosis, exploring the roles and molecular mechanisms of triptolide in vivo in the progression of atherosclerosis.

Materials and methods

Animals and mice grouping

In this study, 6-8-week-old apolipoprotein E knock-out mice (n=6) and corresponding back-ground mice (n=6) were housed in specific pathogen-free conditions. The 8-week-old male wild type C57BL/6 mice with the same genetic background as ApoE-/- mice were used as negative controls. The mice were divided into four groups, including ApoE-/- mice treated with 5 μg/kg of triptolide and 50 μg/kg of triptolide, ApoE-/- mice, and negative control mice. ApoE-/- mice were fed with 5 μg/kg triptolide or 50 μg/kg of triptolide twice every day, respectively. Each group contained 6 mice. All mice were free to eat and drink. The ambient temperature was kept at (22±2)°C, with a humidity of about 50% to 60%.

PCR assay

The mice were numbered when they were caged, including four female mice and three male mice. DNA samples were extracted with a DNA extraction kit (Bieteke, Beijing, China) in ApoE-/- mice with the genetic background of C57BL/6 and the same background with C57BL/6 mice. DNA was taken from blood in the tail tip of each mouse. The mouse genotype was identified by PCR assay. Primer sequences used were as follows: Primer 1: 5’-GCCTAGCCG-AGGGAGAGCCG-3’, Primer 2: 5’-TGTGACTTGGG-AGCTCTGCAGC-3’, Primer 3: 5’-GCCGCCCGAC-TGCATCT-3’. Thermal cycling conditions were 95°C for 10 minutes, followed by 30 cycles of 30 seconds at 95°C, 30 seconds at 65°C, and 30 seconds at 72°C, then 10 minutes at 72°C for elongation. The length of DNA fragments was 155 bp/155 bp for wild type mice, 245 bp/245 bp for ApoeE-/- mice, and 155 bp/245 bp for heterozygous type mice.

ELISA assay

Kits for total cholesterol (Cat. No. YG-elisa-01-138) and total triglycerides (Cat. No. YG-elisa-01273) were both obtained from Yinggongbiotec Corporation (Shanghai, China). Total cholesterol and total triglycerides in plasma were determined according to kit protocol. Briefly, peritoneal macrophages were obtained in ApoE-/- mice treated with 5 μg/kg triptolide, 50 μg/kg of triptolide, ApoE-/- mice, and negative control mice. The single cell suspension was prepared and cell density was adjusted into 5×10⁴ cells/mL and treated with 12.5 μg/mL of ox-LDL for 48 hours. Next, cell supernatant was harvested and concentrations of TNF-α, IL-1β, IL-6, and IL-8 were determined with ELISA kits (Neobio-
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science, Beijing, China, according to manufacturer instructions, using a Benchmark Microplate Reader (Bio-Rad, Hercules, CA). The experiment was performed and repeated twice in triplicate. Total cholesterol and total triglycerides in plasma were detected using the corresponding ELISA kit. Specifically, the mice were divided into 4 groups, including ApoE-/- mice, 5 μg/kg triptolide-treated ApoE-/- mice, 50 μg/kg of triptolide-treated ApoE-/- mice, and C57BL/6 mice. At the 20th week, blood samples were collected after 12-16 hours of fasting by cutting a piece of the tail of each mouse. Total cholesterol and total triglycerides in plasma were determined by specific kits according to kit protocol.

**Western blot**

The 5 μg/kg of TPL and 50 μg/kg of TPL-treated ApoE-/- mice, as well as the control group, including ApoE-/- mice and control mice with the same background, were sacrificed at the 20th week. Peritoneal macrophage cells were collected and cultured at a density of 3×10^6 cells/mL. Eight hours later, the cells were treated with 12.5 μg/ul oxLDL for 24 hours. Levels of ABCA1 were detected by Western blotting, as previously described [18-20]. Antibodies used in the experiment were as follows: Anti-ABCA1 (NB400-105) antibody was purchased from Novus Biologicals. Anti-β-Actin antibody (cat. no. sc-47778) is a mouse monoclonal antibody obtained from Santa Cruz Biotechnology, Inc.

**Statistical analysis**

Data were analyzed with SPSS software. Data are shown as the mean ± standard error of the mean. Data between the two groups were compared with t-test and multiple comparisons were analyzed using one-way ANOVA and Tukey’s test. Expression levels of ABCA1 in macrophage-derived foam cells were repeated three times. *P<0.05, **P<0.01, compared with control group.

**Results**

Apoprotein E (ApoE) gene-deficient mice are currently the most commonly used animal models in atherosclerosis research. The current study chose ApoE-/- mice as animal models to test the roles of triptolide in the progression of atherosclerosis. First, this study tested the genotype of each mouse by PCR assay, as described in Materials and methods. As shown in Figure 1, ApoE-/- mice had the allele of 245 bp and wild type mice had the allele of 155 bp. The ApoE gene in female mouse No. 1 was wild type and the ApoE gene in female mouse No. 2 and 3 was ApoE knockout type. No. 4 of the female mice had a mixture allele of ApoE knockout and wild type. It was the mixture of 155 bp and 245 bp.

![Figure 1. APOE-/- mice are used as animal model and the genotype is identified by PCR assay.](image)

Liver tissues were obtained from ApoE-/- mice and control mice. They were fixed in 4% paraformaldehyde for 2 hours, then transferred to 20% sucrose for dehydration. Paraffin-embedding was then carried out for HE staining and Oil O red staining, as previously described [15-17].

**HE staining and Oil O red staining**

Liver tissues were obtained from ApoE-/- mice and control mice. They were fixed in 4% paraformaldehyde for 2 hours, then transferred to 20% sucrose for dehydration. Paraffin-embedding was then carried out for HE staining and Oil O red staining, as previously described [15-17].

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No. 3 female mice. Additionally, corresponding wild type mice were collected with the same age. They were used as negative controls, as they had the same genetic background with ApoE-/- mice.

Body weight shows no statistical differences between ApoE-/- mice and corresponding mice

Body weight is an important indicator in the animal experiment. Here, ApoE-/- mice and corresponding mice were free to eat and drink in the cages. They were fed for 20 weeks. The body weight of each mouse was measured and recorded every two days. Body weight was compared between ApoE-/- mice and wild type mice at the 10th week, 15th week, and 20th week. As shown in Figure 2, there were no statistical differences between the ApoE-/- group and corresponding group.

Secretion of inflammatory cytokines in macrophages determined by ELISA assay

Chronic inflammation promotes the progression of atherosclerosis. Thus, the current study detected inflammatory cytokine production in ApoE-/- mice and wild type mice. Macrophages were collected from ApoE-/- mice and wild type mice, then treated with 20 ug/ul of oxLDL for 24 hours. Levels of inflammatory cytokines were tested by ELISA assay. As shown in Figure 2B, levels of TNF-α, IL-1β, and IL-6 were significantly increased in macrophages of ApoE-/- mice, compared with those of wild type mice (**P<0.01). As inflammatory cytokines are important inducers in initiating atherosclerosis, ApoE-/- mice produced more inflammatory cytokines and were more prone to producing atherosclerosis.

Lipid droplets are more and larger in livers of ApoE-/- mice than that of wild type mice

To observe whether the morphology of liver cells was intact, H&E staining of livers in ApoE-/- mice and mice with the same corresponding background was conducted. As shown in Figure 3, H&E staining results showed that the morphology of liver cells was intact, the nucleus was not obviously various, and the hepatic lobule was intact. Moreover, oil red O staining was performed to detect fat in liver tissues. Oil red O staining results showed that fat lipid droplets were more and larger in livers of ApoE-/- mice than in wild type mice, suggesting that fat spontaneously accumulated in ApoE-/- mice (Figure 3).

Tripotolide does not affect the body weight of ApoE-/- mice and decreases total cholesterol and total triglycerides in ApoE-/- mice

Body weight is an important indicator reflecting whether triptolide is toxic or not. Thus, this study measured the body weights of ApoE-/- mice and control mice every two days. As shown in Figure 4A, body weight was gradually and...
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Abnormal lipid metabolism is accompanied with progression of atherosclerosis. Plasma cholesterol and triglycerides are risk factors inducing and promoting the progression of atherosclerosis. Here, total cholesterol and total triglycerides were determined, as described in Materials and methods. Results showed that

Stably increased in the three groups of mice. There were no statistical differences between ApoE-/- mice and 50 μg/kg of triptolide-treated ApoE-/- mice (P>0.05). Data revealed that 50 μg/kg of triptolide caused no toxicity to the mice. Thus, it was an appropriate drug dose in the experiment.

Triptolide treatment decreases levels of total cholesterol and total triglycerides in ApoE-/- mice

Abnormal lipid metabolism is accompanied with progression of atherosclerosis. Plasma cholesterol and triglycerides are risk factors inducing and promoting the progression of atherosclerosis. Here, total cholesterol and total triglycerides were determined, as described in Materials and methods. Results showed that

Figure 3. Lipid droplets are more and larger in livers of ApoE-/- mice than in wild type mice. Oil red O staining and H&E staining of livers in ApoE-/- mice and mice with the same corresponding background.

Triptolide contributes to expression of ABCA1 in macrophages

Macrophage-derived foam cells have an important role in the progression of atherosclerosis. According to the in vitro experiment, macrophage cells were obtained from each group of triptolide treated ApoE-/- mice and control group. Macrophage-derived foam cells were
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Triptolide inhibits progression of atherosclerosis induced by stimulation with 12.5 μg/mL of oxLDL for 24 hours. Expression levels of ABCA1 were determined by Western blot. As shown in Figure 5, levels of ABCA1 were greatly increased in macrophage cells from 50 μg/kg of triptolide-treated ApoE−/− mice. However, levels of ABCA1 in 5 μg/kg of triptolide-treated ApoE−/− mice were not obviously varied, compared to ApoE−/− mice and negative control mice.

**Secretion of inflammatory cytokines obviously inhibited in TPL-treated mice**

Chronic inflammatory reactions promote the progression of atherosclerosis. This study detected the secretion of inflammatory cytokines, including TNF-α, IL-1β, IL-6, and IL-8, by ELISA assay. As shown in Figure 6, secretion of TNF-α, IL-1β, IL-6, and IL-8 was gradually decreased,

**Figure 4. Physiological indicators of atherosclerotic model animals.** Triptolide does not affect the body weight of ApoE−/− mice and decreases the total cholesterol and total triglycerides in ApoE−/− mice. The variation of body weight (A), the total cholesterol (B), and total triglycerides (C) in ApoE−/− mice, 50 μg/kg of triptolide-treated ApoE−/− mice, and the C57/BL6 mice. n=6 in each group. **P<0.01, compared with ApoE−/− mice.

**Figure 5. Triptolide contributes to expression of ABCA1 in macrophages.** The ApoE−/− mice were treated with 5 μg/kg of TPL and 50 μg/kg of TPL every twice day. At the 20th week, peritoneal macrophage cells were obtained and treated with 12.5 μg/ul ox-LDL for 24 hours. A. Expression of ABCA1 was detected by Western blotting. TPL: triptolide. B. Expression levels of ABCA1 were shown in histogram. Levels of ABCA1 in ApoE−/− mice were used as negative control. **P<0.01, compared with the level of ABCA1 in ApoE−/− mice.
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in a dose-dependent manner. Specifically, macrophage cells obtained from 50 μg/kg triptolide-treated ApoE mice produced less TNF-α, IL-1β, IL-6, and IL-8 than ApoE-/- mice (Figure 6). Present results demonstrate that triptolide treatment decreased the secretion of inflammatory cytokines and inhibited the formation of foam cells.

Discussion

Cardiovascular and cerebrovascular diseases are commonly seen with high incidence, characterized by chronic inflammation and abnormal lipid metabolism [21, 22]. The present study used an ApoE-/- mice model, exploring the anti-inflammatory roles of triptolide in the progression of atherosclerosis of mice. It was found that secretion of TNF-α, IL-1β, IL-6, and IL-8 was decreased in mice treated with 50 μg/kg of triptolide, while expression of ABCA1 was obviously increased in macrophages.

The current study found that expression of ABCA1 in macrophages from triptolide-treated ApoE-/- mice was significantly higher than that from negative control ApoE-/- mice, suggesting that triptolide treatment increased levels of

Figure 6. Secretion of inflammatory cytokines is obviously inhibited in TPL-treated mice. The mice were divided into four groups and fed for 20 weeks. Peritoneal macrophage cells were treated with 12.5 μg/ul oxLDL. Secretion of inflammatory cytokines, including TNF-α, IL-1β, IL-6, and IL-8, was determined by ELISA assay, as described in Materials and methods. *P<0.05, **P<0.01, compared with ApoE-/- mice.
ABCA1 and promoted reverse cholesterol transport. This was consistent with the results of Luo et al., in which they found that expression of anti-ATP-binding cassette transporter A1 in the macrophages of ApoE(-/-) mice was upregulated when treated by triptolide. However, expression of LXRalpha was not obviously changed [20]. Thus, triptolide treatment inhibited the formation of foam cells and suppressed the progression of atherosclerosis.

Triptolide has been reported to attenuate inflammatory response in various disease models [12, 13, 23, 24]. As the progression of atherosclerosis is accompanied by chronic inflammatory response, this study tested the roles of triptolide in the formation of atherosclerosis. The ApoE-/- mice model is usually used to study the development and treatment of atherosclerosis [25, 26]. This study also chose ApoE-/- mice as an animal model to test the roles of triptolide in progression of atherosclerosis. This was examined by testing levels of inflammatory cytokines. Data suggests that treatment with triptolide obviously decreased the production of inflammatory cytokines, including TNF-α, IL-1β, IL-6, and IL-8. Ingesting modified low-density lipoprotein and cholesterol efflux affect the formation of macrophage-derived foam cells [27-29]. In the present study, 12.5 μg/mL of ox-LDL was used to treat macrophages in triptolide-treated ApoE-/- mice and negative control mice, aiming to induce the formation of macrophage-derived foam cells. Next, cytokines production was detected by ELISA assay, finding that foam cells in ApoE-/- treated with triptolide produced less inflammatory cytokines than control mice. Results suggest that triptolide showed potent effects, inhibiting inflammatory response in the progression of atherosclerosis.

In conclusion, triptolide showed obvious effects concerning the suppression of chronic inflammatory response. It also increased expression of ABCA1, promoting reverse cholesterol transport in foam cells. Therefore, triptolide might inhibit the formation of atherosclerosis in a mice model. Results suggest the promotion of triptolide as a new agent for clinical therapy of atherosclerosis.

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Disclosure of conflict of interest

None.

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