

## Full-length article

## Curcumin inhibits cellular cholesterol accumulation by regulating SREBP-1/caveolin-1 signaling pathway in vascular smooth muscle cells<sup>1</sup>

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### Key words

curcumin; cholesterol accumulation; vascular smooth muscle cells; caveolin-1; sterol response element-binding protein-1

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### Abstract

**Aim:** To investigate the protective effect and the possible mechanism of curcumin on anti-atherosclerosis. **Methods:** Morphological changes of atherosclerotic lesions taken from apoE knockout (apoE<sup>-/-</sup>) mice were determined by hematoxylin-eosin staining. Intracellular lipid droplets and lipid levels were assayed by oil red O staining and HPLC. The protein expression of caveolin-1 was quantified by Western blotting. Translocation and the expression of sterol response element-binding protein-1 (SREBP-1) were indirectly detected by an immunofluorescence analysis. **Results:** The administration of 20 mg·kg<sup>-1</sup>·d<sup>-1</sup> curcumin to apoE<sup>-/-</sup> mice for 4 months induced a 50% reduction of atherosclerotic lesions and yielded a 5-fold increase in the caveolin-1 expression level as compared to the model group. Rat vascular smooth muscle cells (VSMC) pretreated with 50 mg·L<sup>-1</sup> ox-lipid density lipoprotein(ox-LDL) for 48 h increased cellular lipid contents, and stimulated SREBP-1 translocation, but decreased the caveolin-1 expression level. Lipid-loaded cells exposed to curcumin at various concentrations (12.5, 25, and 50 μmol·L<sup>-1</sup>) for different durations (0, 6, 12, 24, and 48 h) significantly diminished the number and area of cellular lipid droplets, total cholesterol, cholesterol ester, and free cholesterol accompanying the elevation of the caveolin-1 expression level (approximately 3-fold); the translocation of SREBP-1 from the cytoplasm to the nucleus was inhibited compared with the models. Lipid-loaded VSMC exposed to N-acetyl-Leu-Leu-norleucinal, a SREBP-1 protease inhibitor, showed increased nuclear translocation of SREBP-1, reduced caveolin-1 expression level, and upregulated cellular lipid levels. **Conclusion:** Curcumin inhibits ox-LDL-induced cholesterol accumulation in cultured VSMC through increasing the caveolin-1 expression via the inhibition of nuclear translocation of SREBP-1.

### Introduction

Curcumin is extracted from *Curcuma longae*, and has been demonstrated to have a variety of pharmacological effects, such as antitumor, anti-inflammatory, as well as anti-oxidative effects<sup>[1-3]</sup>. Some studies have revealed that curcumin can decrease lipid peroxidation and cholesterol levels of sera and tissues in mice and human<sup>[4,5]</sup>. It is well known that atherosclerosis largely results from oxidative stress and massive lipid deposition in the aorta. Olszanecki *et al*<sup>[6]</sup> re-

ported a preventive effect of curcumin on atherosclerotic development in apoE/LDL-Receptor double-knockout mice. A recent study showed that some genes, such as LDL-R, sterol response element-binding protein (SREBP), and CD36, involved in cholesterol homeostasis were influenced by curcumin<sup>[7]</sup>. However, the detailed anti-atherosclerotic mechanisms of curcumin are still to be elucidated.

Caveolin-1 is a 22-24 kDa constructive protein in caveolae<sup>[8]</sup>. Evidence demonstrates that the caveolin-1 is able to directly

bind free cholesterol (FC) and form a cholesterol transport complex with other elements<sup>[9-11]</sup>. In addition, the upregulation of the caveolin-1 expression promotes intracellular cholesterol efflux and an improved lipid-loaded state<sup>[12]</sup>. SREBP-1 exists in the cytoplasm and nucleus, and is associated with cholesterol catabolism<sup>[13]</sup>. Its active form can translocate into the nucleus and regulate target gene transcription through binding with the sterol regulatory element (SRE) sequence within the promoter<sup>[14]</sup>. It was found that the SRE sequence also exists in the caveolin-1 promoter that is negatively regulated by SREBP-1<sup>[15]</sup>. A more recent report showed that SREBP expression could be regulated by curcumin and is associated with reverse cholesterol transport<sup>[7]</sup>.

The present paper addresses whether curcumin prevents ox-LDL-induced cholesterol from accumulation in cultured vascular smooth muscle cells (VSMC) through inhibiting the nuclear translocation of SREBP-1 followed by raising the caveolin-1 expression.

## Materials and methods

**Reagents** Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum were purchased from Gibco/BRL (Grand Island, NY, USA). Curcumin ([E,E]-1,7-bis[4-hydroxy-3-methoxyphenyl]-1,6-heptadiene-3,5-dione, purity =99%), N-acetyl-Leu-Leu-norleucinal (ALLN; purity =97%) and oil red O powder were purchased from Sigma-Aldrich (St Louis, MO, USA) and dissolved by DMSO (Sangon, Shanghai, China). All reagents were of analytical grade.

**Animals and diets** The apoE<sup>-/-</sup> mice were obtained from the barrier unit at the Laboratory Animal Center of Chongqing Medical University (Chongqing, China). The apoE<sup>-/-</sup> mice were randomized into 3 groups and had similar body weights ( $n=7$  in the apoE<sup>-/-</sup> mice group;  $n=8$  in the apoE<sup>-/-</sup> mice with curcumin group; and  $n=8$  in the apoE<sup>-/-</sup> mice with lovastatin group). C57BL/6J mice ( $n=7$ ) were used as the control. Three groups of apoE<sup>-/-</sup> mice were given a high-fat diet (21% lard and 0.15% cholesterol). The C57BL/6J mice were fed a normal diet. The animals were housed in single pens under controlled conditions (temperature between 18 °C and 22 °C, relative air humidity between 30%–70%, with 4 air changes per hour) and fed 3 times daily on a restricted schedule. All mice received the same amount of food (4% body weight). The dose of curcumin (purity =94%) administered (mixed with diet) for the curcumin group was 20 mg·kg<sup>-1</sup>·d<sup>-1</sup> per mouse (4 months). The dose of lovastatin administered for the lovastatin group was 40 mg·kg<sup>-1</sup>·d<sup>-1</sup>. The total study period was 5 months. Morphological changes of atherosclerotic lesions in the aorta were measured by histological section and hematoxylin-eosin (HE) staining.

## Morphological examination of atherosclerotic lesions

At the end of the experimental period, the animals were euthanized by phlebotomy under light anesthesia with sodium pentobarbital (30 mg·kg<sup>-1</sup> intravenously; Jilin Northern Medicine, Jilin, China). The atherosclerotic lesions were analyzed as described previously<sup>[16]</sup>. Briefly, the aorta was cut into transversal sections of 4 μm and fixed in 10% formalin for 24 h. The tissue slices were routinely processed and embedded in paraffin. Lipid deposition in the aorta was determined by the morphological assessment of the percentage of lesion-covered aortas as visualized by HE staining under a light microscope at 40× magnification.

**Serum lipid and lipoprotein analysis** Non-fasting mice were anesthetized with sodium pentobarbital, and blood was collected. Serum total cholesterol (TC), triglyceride (TG), High Density Lipoprotein-Cholesterol (HDL-C) and Lower Density Lipoprotein (LDL-C) were determined by commercial enzymatic methods (test kits, Shanghai Rongsheng Biotechnology, Shanghai, China).

**Cell culture** Rat VSMC were maintained in DMEM containing 10% fetal bovine serum in a humidified atmosphere of 5% CO<sub>2</sub> and 95% O<sub>2</sub>. The cells were pre-incubated with 50 mg·L<sup>-1</sup> ox-LDL for 48 h and then treated with various concentrations of curcumin (purity =94%; 12.5, 25, and 50 μmol·L<sup>-1</sup>) or ALLN (25 μmol·L<sup>-1</sup>) for 24 h. Untreated cells were used as controls.

**Oil Red O staining** The culture was washed 3 times by phosphate-buffered saline (PBS) to remove suspended cells. The VSMC were fixed with 10% formalin for 10 min. After washing with PBS, the cells were stained with oil red O solution (solubilized in isopropanol: water, 3:2) for 30 min. Then the cells were washed with 2-propanol for 10 s to remove background staining. The cells were then stained with HE for 5 min to stain the nuclei, and images were taken at 40× magnification.

**Intracellular lipid level analysis by HPLC** Cellular lipid (TC, FC, and cholesterol ester [CE]) contents were analyzed by our method described previously<sup>[17]</sup>. Briefly, the VSMC were scraped from the culture flasks into 0.9% NaCl (1 mL/50 cm<sup>2</sup> flask) and homogenized on ice by sonication for 10 s. After the protein concentration was determined using a BCA kit (Pierce Biotechnology, Rockford, IL, USA), an equal volume of freshly-prepared, cold (-20 °C) potassium hydroxide in ethanol (150 g·L<sup>-1</sup>) was added to the cell lysates, and the mixture was repeatedly vortexed until clear. An equal volume of 3:2 hexane isopropanol (v/v) was then added. The mixture was vortexed for 5 min, followed by centrifugation at 800×g (15 °C) for 5 min. The extraction procedure was repeated twice. The combined organic phase was transferred

to clean tapered glass tubes and thoroughly dried under nitrogen at 40 °C. The tubes were allowed to cool to room temperature. One hundred microliters of isopropanol-acetonitrile 20:80 (v/v) was added. The sample was solubilized in an ultrasound water bath at room temperature for 5 min. After centrifugation at 800×g for 5 min, the samples were introduced into the HPLC device (Agilent 1100, Agilent Technologies, Palo Alto, CA, USA). Cholesterol was eluted with 1 mL·min<sup>-1</sup> of eluent consisting of 20:80 isopropanol-acetonitrile (v/v), and detected by UV absorption at 206 nm.

**Protein isolation and Western blotting** The proteins were isolated from flash-frozen apoE<sup>-/-</sup> mice aortas as previously described<sup>[18]</sup>. The total proteins (10-50 µg/lane) were electrophoresed, separated on 10% SDS-PAGE, and transferred to a polyvinylidene difluoride membrane (Millipore Corporation, Billerica, MA, USA), which was blocked in 5% non-fat dry milk in Tris-buffered saline Tween (TBST; pH 7.6). The membrane was incubated overnight with a rabbit polyclonal antibody to rat caveolin-1 or a monoclonal antibody to mouse caveolin-1 and SREBP-1p68 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1:1000 on a rotating platform at 4 °C. Subsequently, the membrane was rinsed in TBST (pH 7.6) for 40 min and incubated with horseradish peroxidase (HRP)-conjugated antirabbit immunoglobulin G (IgG) antibodies (Boster, Wuhan, China) diluted in TBST (1:4000) for 1 h on a rotating platform at 37 °C. Bands were visualized using a HRP developer, and background-subtracted signals were quantified on a laser densitometer (Bio-Rad, Hercules, California, USA). Blots were probed with a mouse anti- $\beta$ -actin monoclonal antibody (Boster, China) to ensure equal protein loading. All protein levels were assessed by densitometry, with  $\beta$ -actin used as a control.

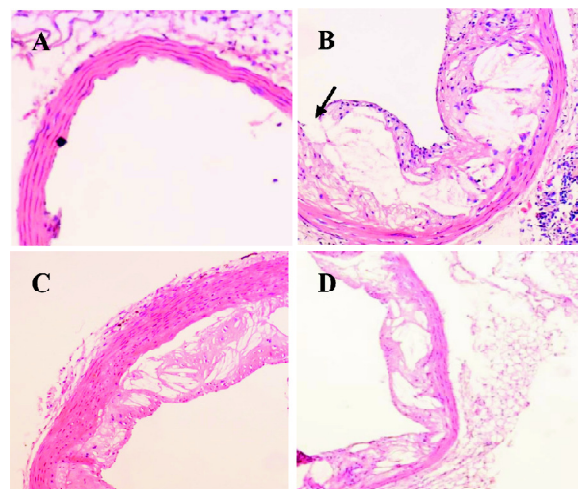
**Indirect immunofluorescence with double staining** For the immunofluorescence analysis, 2×10<sup>5</sup> cells were plated into each well of 6-well plate preplaced coverslips. The cells were pretreated with ox-LDL for 48 h and treated with or without curcumin. Then the cells were fixed with methanol acetic acid (3:1) for 15 min at room temperature. The samples were then permeabilized with 0.25% Triton X-100 in PBS (Amresco, Solon, OH, USA) and 5% DMSO for 20 min at -20 °C, then washed twice with PBS containing 0.25% Triton X-100. The cells were incubated with an anti-SREBP antibody (Santa Cruz Biotechnology, USA) overnight at 4 °C in a humidified chamber. After washing 3 times with PBS, the cells were incubated with Cy3-labeled goat antirabbit IgG (Sigma, USA) for 1 h and then incubated with 4',6-diamidino-2-phenylindole (DAPI)(from 0.1 to 1.0 mg·mL<sup>-1</sup>) for 10 min to display the cell nucleus protected from light at room temperature. The cells were then visualized using a fluorescence microscope (Olympus,

Tokyo, Japan), with red and blue representing the cytoplasm and nucleus, respectively. Data were acquired with a Pixera camera (Los Angeles, CA, USA).

**Statistical analysis** The results were expressed as mean±SD. The two-tailed Student's *t*-test was used for statistical comparisons. *P*<0.05 was considered to be statistically significant. For non-quantitative data, results are representative of at least 3 independent experiments.

## Results

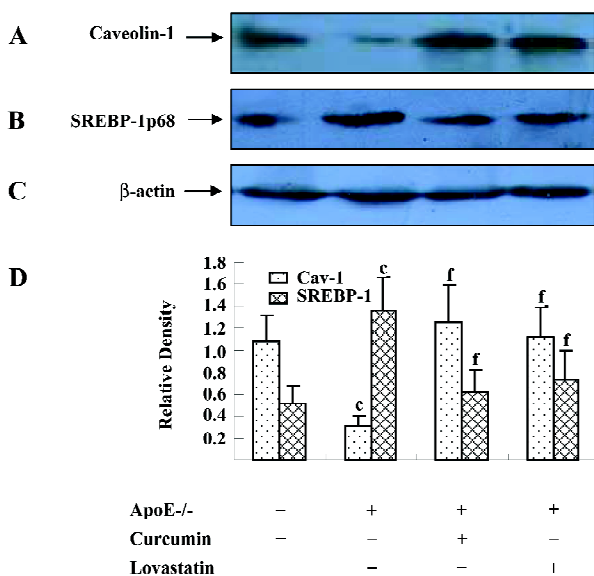
**Curcumin improved atherosclerotic lesions and increased plaque stability in apoE<sup>-/-</sup> mice** To identify the anti-atherosclerotic role of curcumin, we first observed its effects on plaque size and stability in apoE<sup>-/-</sup> mice. HE staining showed that the lesion area in apoE<sup>-/-</sup> mice seemed larger, the aorta lumen became narrower, and the structure fibrous cap in the lesions became brittle (Figure 1B). After administration of curcumin (20 mg·kg<sup>-1</sup>·d<sup>-1</sup>) and lovastatin (40 mg·kg<sup>-1</sup>·d<sup>-1</sup>) for 4 months, the lesion area was reduced by 50%, and the plaque size became more stable (Figure 1C, 1D).



**Figure 1.** Effects of curcumin on atherosclerotic lesions and plaque stability in apoE<sup>-/-</sup> mice. Aorta slices taken from C57BL/6J and apoE<sup>-/-</sup> mice were stained with HE. (A) aorta from C57BL/6J mice; (B) aorta from apoE<sup>-/-</sup> mice fed a high-fat diet for 4 months. Arrow points to the plaque brittleness. (C) aorta from curcumin-treated apoE<sup>-/-</sup> mice fed a high-fat diet for 4 months; (D) aorta from lovastatin-treated apoE<sup>-/-</sup> mice fed a high-fat diet for 4 months. Images were taken at 40× magnification.

**Curcumin increased the caveolin-1 expression and decreased the SREBP-1p68 expression of the aortic wall in apoE<sup>-/-</sup> mice** Since caveolin-1 promotes intracellular cholesterol efflux and SREBP-1 regulates caveolin-1 expression, we were interested in examining whether the effect of curcumin in

reducing atherosclerotic lesions in apoE<sup>-/-</sup> mice was related to the SREBP-1/caveolin-1 pathway. The Western blotting analysis showed an 80% lower caveolin-1 expression in apoE<sup>-/-</sup> mice as compared to C57BL/6J mice. However, the caveolin-1 level in the curcumin group showed a 5-fold elevation as compared with the models. In contrast, apoE<sup>-/-</sup> mice showed a 2-fold higher level of SREBP-1p68, an active form of SREBP-1, compared to C57BL/6J mice. Curcumin significantly inhibited high fat-induced the SREBP-1p68 expression in apoE<sup>-/-</sup> mice. The effects of curcumin were similar to lovastatin (Figure 2).



**Figure 2.** Effect of curcumin on the expression of caveolin-1 and SREBP-1 in aortic walls from C57BL/6J and apoE<sup>-/-</sup> mice. Proteins were isolated from flash-frozen apoE<sup>-/-</sup> mice aortas. Total proteins (10-50 g/lane) were electrophoresed and separated on 10% SDS-PAGE. Protein expression of caveolin-1 and SREBP-1p68 was detected by Western blotting analysis. (A-C) original expression photos of caveolin-1, SREBP-1p68, and  $\beta$ -actin. (D) semiquantitative analysis of caveolin-1 and SREBP-1p68 expression. Relative density means the ratio of caveolin-1 or SREBP-1p68 over  $\beta$ -actin. Data were expressed as mean $\pm$ SD of 3 independent experiments. <sup>c</sup> $P$ <0.01 vs control, <sup>f</sup> $P$ <0.01 vs apoE<sup>-/-</sup>.

**Table 1.** Effects of curcumin on serum lipid levels in apoE<sup>-/-</sup> mice.  $n=6$ . Data expressed as mean $\pm$ SD. <sup>b</sup> $P$ <0.05, <sup>c</sup> $P$ <0.01 vs control; <sup>e</sup> $P$ <0.05, <sup>f</sup> $P$ <0.01 vs ox-LDL.

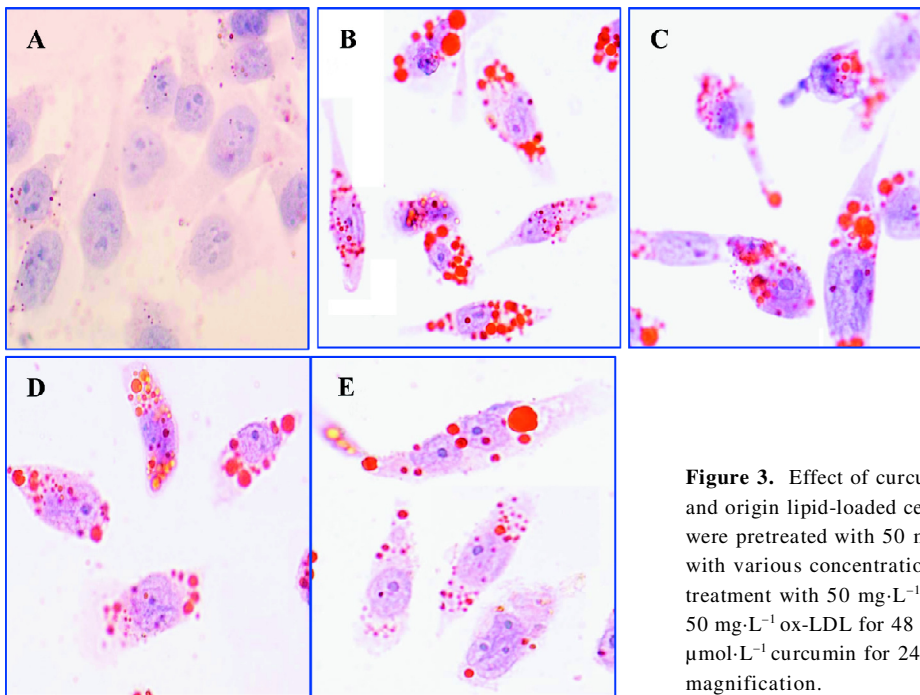
	TG	TC	HDL-C	LDL-C
Control	2.61 $\pm$ 0.31	10.58 $\pm$ 0.83	2.49 $\pm$ 0.28	7.87 $\pm$ 0.92
apoE <sup>-/-</sup>	4.94 $\pm$ 0.35 <sup>c</sup>	19.42 $\pm$ 1.58 <sup>c</sup>	0.65 $\pm$ 0.16 <sup>c</sup>	16.51 $\pm$ 1.01 <sup>c</sup>
Curcumin	3.25 $\pm$ 0.24 <sup>e</sup>	15.35 $\pm$ 1.26 <sup>e</sup>	1.29 $\pm$ 0.23 <sup>f</sup>	10.46 $\pm$ 0.93 <sup>f</sup>
Lovastatin	2.93 $\pm$ 0.30 <sup>f</sup>	13.12 $\pm$ 1.79 <sup>f</sup>	1.76 $\pm$ 0.31 <sup>f</sup>	9.36 $\pm$ 0.74 <sup>f</sup>

**Curcumin improved plasma levels of lipids in apoE<sup>-/-</sup> mice** TC, TG, and LDL-C were significantly increased in the apoE<sup>-/-</sup> mice fed a high-fat diet than those in the control group (C57BL/6J mice). The administration of curcumin markedly decreased plasma TC, TG, and LDL-C levels. Furthermore, a significant increase in HDL cholesterol was observed in curcumin-treated apoE<sup>-/-</sup> mice. The regulating effect of curcumin on blood lipids was similar to that of lovastatin (Table 1).

**Curcumin inhibited cholesterol accumulation in rat VSMC and origin lipid-loaded cells** To further investigate the mechanism of curcumin in inhibiting plaque size and stabilizing plaque, we observed the effects of curcumin on cholesterol accumulation in VSMC and origin lipid-loaded cells induced by ox-LDL. Oil red O staining demonstrated that many lipid droplets were full of lipid-loaded cells. Treatment with curcumin (12.5, 25, and 50  $\mu\text{mol}\cdot\text{L}^{-1}$ ) for 24 h markedly reduced the number of lipid droplets (Figure 3) without significant cellular toxicity (data not shown). The HPLC analysis showed that curcumin decreased intracellular lipid levels with a peak at 25  $\mu\text{mol}\cdot\text{L}^{-1}$  for 24 h; CE was reduced from 141 $\pm$ 15 to 45 $\pm$ 3.7  $\text{mg}\cdot\text{g}^{-1}$  protein (Table 2).

**Curcumin facilitated caveolin-1 expression in rat VSMC and origin lipid-loaded cells** As already stated, caveolin-1 plays a critical role in cholesterol flux<sup>[10,11]</sup>. We tried to determine whether the intracellular caveolin-1 expression could be influenced by curcumin during lipid-loaded state improvement. Lipid-loaded cells were exposed to curcumin at various concentrations (12.5, 25, and 50  $\mu\text{mol}\cdot\text{L}^{-1}$ ) for different durations (0, 6, 12, 24, and 48 h). Curcumin treatment for 24 h significantly increased the caveolin-1 expression in a concentration-dependent manner (Figure 4A) and a peak at 25  $\mu\text{mol}\cdot\text{L}^{-1}$  (Figure 4B).

**Curcumin inhibited the nuclear translocation of SREBP-1 in rat VSMC and origin lipid-loaded cells** It has been reported that SREBP-1 regulates the caveolin-1 expression<sup>[22]</sup>. To examine the role of SREBP-1 under curcumin treatment, we observed SREBP-1 translocation by immunofluorescence with double staining. Compared with the controls, ox-LDL



**Figure 3.** Effect of curcumin on cholesterol accumulation in VSMC and origin lipid-loaded cells measured by oil red O staining. VSMC were pretreated with 50 mg·L<sup>-1</sup> ox-LDL for 48 h and then incubated with various concentrations of curcumin for 24 h. (A) control; (B) treatment with 50 mg·L<sup>-1</sup> ox-LDL for 48 h; (C-E) pretreatment with 50 mg·L<sup>-1</sup> ox-LDL for 48 h and then incubation with 12.5, 25, and 50 μmol·L<sup>-1</sup> curcumin for 24 h, respectively. Images were taken at 40× magnification.

**Table 2.** Effects of curcumin on lipid contents of VSMC-derived lipid-loaded cells (mg/g). *n*=3. Data expressed as mean±SD. <sup>b</sup>*P*<0.05, <sup>c</sup>*P*<0.01 vs control, <sup>e</sup>*P*<0.05, <sup>f</sup>*P*<0.01 vs ox-LDL.

Index	TC	FC	CE
Control	188±21	181±16	77±0.9
Ox-LDL	393±40 <sup>c</sup>	250±23 <sup>b</sup>	143±15 <sup>c</sup>
Cur 12.5 μmol/L	310±32 <sup>e</sup>	220±25	90±7.2 <sup>e</sup>
Cur 25 μmol/L	250±27 <sup>e</sup>	205±26 <sup>e</sup>	45±3.7 <sup>f</sup>
Cur 50 μmol/L	260±30 <sup>e</sup>	210±25 <sup>e</sup>	50±4.0 <sup>f</sup>
Control	185±19	177±15	70±0.9
Ox-LDL	389±39 <sup>c</sup>	248±24 <sup>b</sup>	141±15 <sup>c</sup>
Cur 12 h	330±33	250±25	80±7.2 <sup>e</sup>
Cur 24 h	262±30 <sup>e</sup>	217±26 <sup>e</sup>	45±3.7 <sup>f</sup>
Cur 48 h	265±30 <sup>e</sup>	217±25 <sup>e</sup>	48±4.0 <sup>f</sup>
Cur 72 h	254±27 <sup>e</sup>	204±21 <sup>e</sup>	50±3.8 <sup>f</sup>

VSMC were pretreated with 50 mg·L<sup>-1</sup> ox-LDL for 48 h and then treated with different concentrations of curcumin for different periods of time. Intracellular lipid levels (TC, FC, and CE) were detected by HPLC. Lipid-loaded cells treated with different concentration of curcumin for 24 h. Treatment of lipid-loaded cells with 25 μmol·L<sup>-1</sup> curcumin for different periods of time. Cur: curcumin.

enhanced the SREBP-1 expression and stimulated SREBP-1 translocation from the cytoplasm into the nucleus (Figure 5). Yet there was a redistribution of SREBP-1 after the curcumin treatment. The SREBP-1 expression decreased gradually,

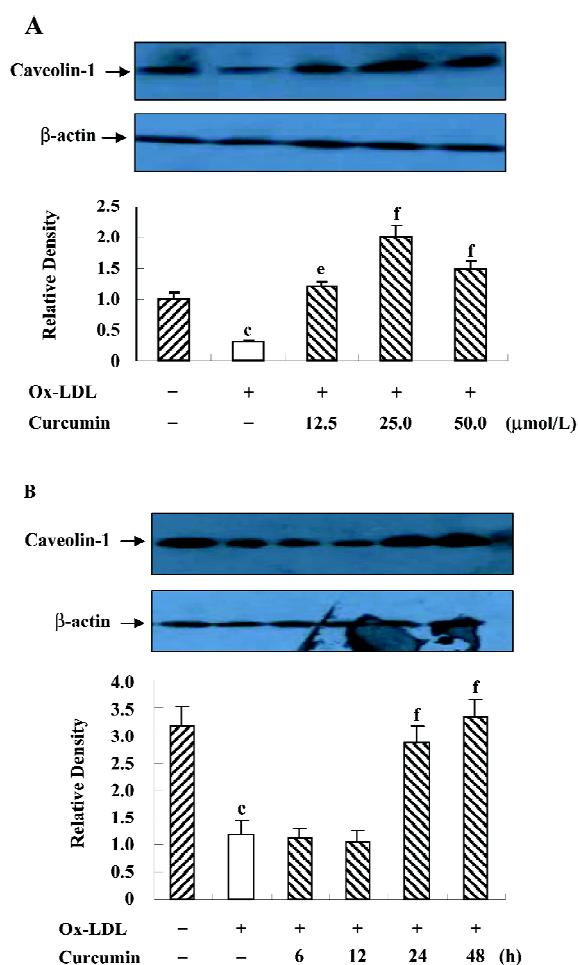
and its substantial portion accumulated in the cytoplasm, and not in the nucleus (Figure 5).

**ALLN abolished the effect of curcumin on SREBP-1 translocation in rat VSMC and origin lipid-loaded cells** To further confirm the relationship between SREBP-1 and curcumin, the lipid-loaded cells were treated with 25 μmol·L<sup>-1</sup> curcumin for 24 h and then with or without ALLN, an inhibitor of SREBP-1 metabolism (or a promoter of SREBP-1). Compared with the controls, curcumin inhibited the SREBP-1 expression and its nuclear translocation. However, ALLN abolished the effect of curcumin and raised the SREBP-1 expression, as shown in Figure 6, with an increased density of red staining in the cell nuclei accompanying its nuclear translocation augmentation.

**ALLN attenuated the effect of curcumin on the caveolin-1 expression in rat VSMC-derived lipid-loaded cells** In an effort to understand the role of the SREBP-1/caveolin-1 pathway in the anti-atherosclerosis of curcumin, we observed the caveolin-1 expression in the presence or absence of ALLN. The Western blotting analysis indicated that ALLN could lower caveolin-1 expression in both conditions with or without ox-LDL (Figure 7A). Further experiments revealed that ALLN attenuated the curcumin-increased caveolin-1 expression in the presence of ox-LDL (Figure 7B), indicating that curcumin enhanced caveolin-1 levels by inhibiting the SREBP-1 expression.

**ALLN attenuated the effect of curcumin inhibiting**





**Figure 4.** Effects of curcumin on the caveolin-1 expression in VSMC and origin lipid-loaded cells. Cells were pretreated with 50  $\mu\text{g}\cdot\text{mL}^{-1}$  ox-LDL for 48 h and then exposed to curcumin. Total proteins were electrophoresed and separated on a 10% SDS-PAGE. (A) lipid-loaded cells were treated with indicated concentrations of curcumin for 24 h. Upper panel is a representative Western blot of caveolin-1. Lower panel shows semiquantitative analysis of the caveolin-1 expression in a dose-dependent manner. (B) lipid-loaded cells were treated with 25  $\mu\text{mol}\cdot\text{L}^{-1}$  curcumin for the indicated times. Upper panel is an original expression photo of caveolin-1. Lower panel shows a time course of the caveolin-1 expression. Relative density means the ratio of caveolin-1 over  $\beta$ -actin. Data were expressed as mean $\pm$ SD of 3 independent experiments. <sup>c</sup> $P<0.01$  vs control, <sup>e</sup> $P<0.05$ , <sup>f</sup> $P<0.01$  vs ox-LDL.

#### cellular cholesterol accumulation in rat VSMC and origin lipid-loaded cells

Because ALLN influenced the effects of curcumin on SREBP-1 translocation and caveolin-1 expression, whether intracellular lipid levels could be eventually affected by ALLN requires further study. We noticed that the pretreatment of ALLN for 24 h counteracted the effects of curcumin on decreasing cellular lipid levels (Table 3),

indicating that curcumin inhibits cellular cholesterol accumulation through the regulation of the SREBP-1/caveolin-1 signaling pathway.

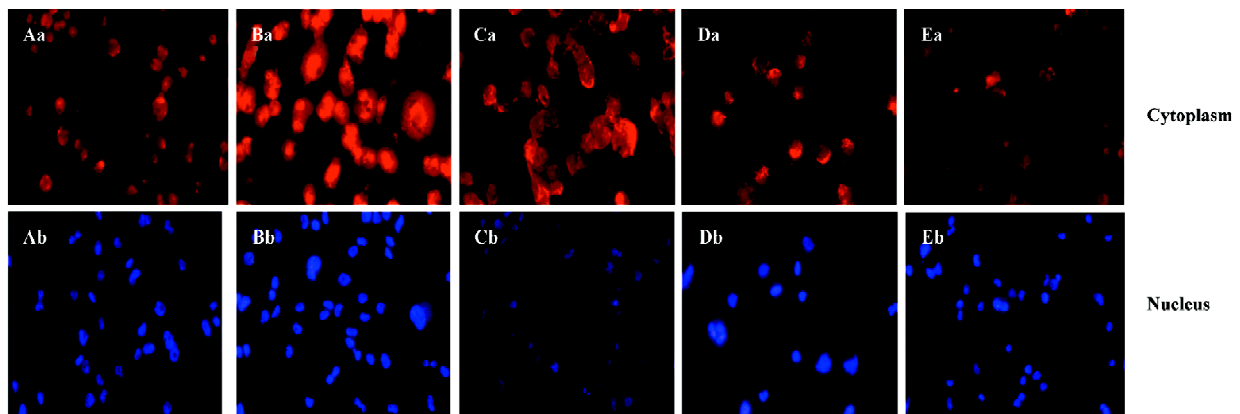
## Discussion

To clarify the pharmacological mechanism of curcumin on anti-atherosclerosis, we observed its effects on apoE<sup>-/-</sup> mice and cultured rat VSMC and origin lipid-loaded cells. In the present study, we reproduced the anti-atherosclerosis of curcumin at animal and cellular levels, and further documented that these effects were related to the inhibition of SREBP-1 nuclear translocation followed by the increment of the caveolin-1 expression.

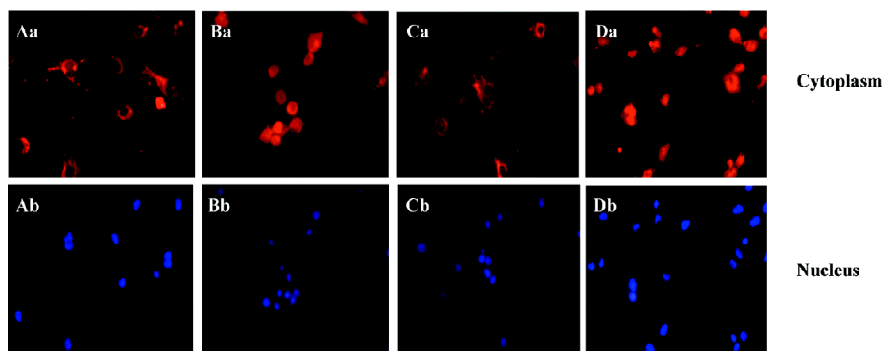
As a constituent of the spice turmeric, the antitumor and anti-inflammatory effects of curcumin have been studied<sup>[1-3]</sup>. Although several lines of evidence strongly suggest that curcumin could prevent atherosclerotic development by regulating some elements in cholesterol homeostasis<sup>[6,7]</sup>, the potential mechanism was unclear. Our animal experiment showed that the administration of curcumin inhibited plaque formation and enhanced plaque stability in apoE<sup>-/-</sup> mice.

Caveolae, discovered approximately 50 years ago, was mainly formed by the caveolin family and is known as an important hub not only in signal transmission, but also in lipid transport across the plasma membrane<sup>[19,20]</sup>. Considering that plaque regression may be linked to intracellular lipid efflux, and caveolin-1 may play a key role, we subsequently studied the relationship between the caveolin-1 expression and anti-atherosclerosis of curcumin in apoE<sup>-/-</sup> mice and cholesterol accumulation in cultured VSMC. Our results demonstrated that curcumin promoted the caveolin-1 expression in apoE<sup>-/-</sup> mice. In rat VSMC and origin lipid-loaded cells induced by ox-LDL, curcumin promoted the caveolin-1 expression and downregulated lipid levels in a dose-dependent manner. The data demonstrated that curcumin anti-atherosclerosis and this effect are perhaps linked to caveolin-1 expression enhancement.

The SREBP-1 precursor is located on membrane of the endoplasmic reticulum. When intracellular FC changes, its active fragment (SREBP-1p68) transfers into the nucleus to regulate caveolin-1 transcription<sup>[13,15]</sup>, which implies that SREBP-1 probably participates in the effects of curcumin on the caveolin-1 expression and lipid levels. To identify this possibility, we observed the expression and translocation of SREBP-1 in cultured VSMC treated with ox-LDL or curcumin. The results showed that ox-LDL induced the high expression of SREBP-1, but curcumin counteracted this effect and inhibited its nuclear translocation. Meanwhile, curcumin upregulated the caveolin-1 expression and reduced the in-



**Figure 5.** Effects of curcumin on nuclear translocation of SREBP-1 in VSMC and origin lipid-loaded cells. SREBP-1 in the cytoplasm (red) or nucleus (blue) was detected by indirect immunofluorescence with double staining. Lane a: cytoplasm; lane b: nucleus. (A) control; (B) treatment with 50 mg·L<sup>-1</sup> ox-LDL for 48 h; (C-E) pretreatment with 50 mg·L<sup>-1</sup> ox-LDL for 48 h and then 12.5, 25, and 50 μmol·L<sup>-1</sup> curcumin for 24 h, respectively. Images were taken with a fluorescence microscope (×400).



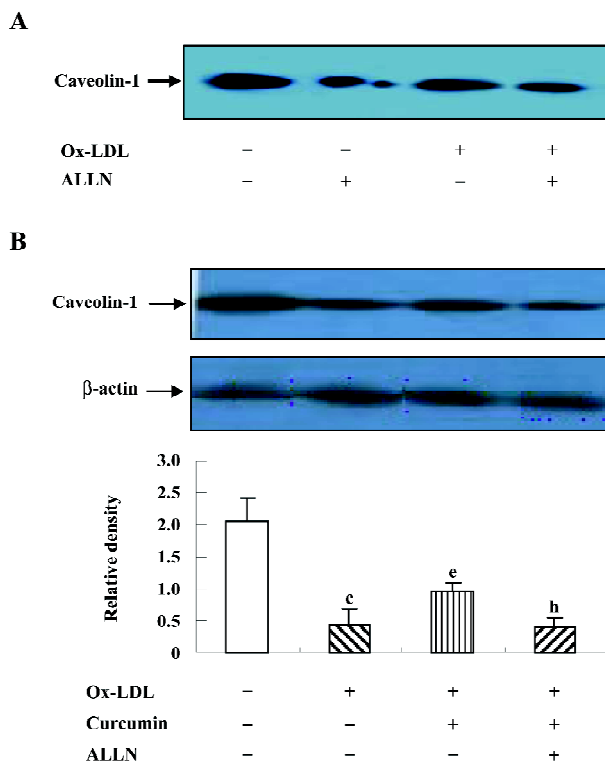
**Figure 6.** ALLN abolished the effect of curcumin on SREBP-1 translocation in rat VSMC and origin lipid-loaded cells. SREBP-1 in the cytoplasm (red) or nucleus (blue) was detected by indirect immunofluorescence with double staining. Lane a: cytoplasm; lane b: nucleus. (A) control; (B) treatment with 50 mg·L<sup>-1</sup> ox-LDL for 48 h; (C) pretreatment with 50 mg·L<sup>-1</sup> ox-LDL for 48 h and then incubation with 25 μmol·L<sup>-1</sup> curcumin for 24 h; (D) exposure to 50 mg·L<sup>-1</sup> ox-LDL for 48 h and then treatment with 25 μmol·L<sup>-1</sup> curcumin and 25 μmol·L<sup>-1</sup> ALLN for 24 h. Images were taken with a fluorescence microscope (×400).

tracellular lipid levels correspondingly. The relationship between SREBP-1 and caveolin-1 in our experiment was consistent with a previous study<sup>[15]</sup>.

It has been reported that ALLN could enhance the SREBP-1 level by inhibiting the catabolism of SREBP-1, and the latter could negatively regulate the caveolin-1 expression<sup>[15,21]</sup>. However, we were interested in whether the effects of curcumin on SREBP-1 translocation and subsequent caveolin-1 expression, as well as cellular lipid levels, could be decreased by ALLN. Interestingly, in the present of ALLN at a concentration of 25 μmol·L<sup>-1</sup>, when the lipid-loaded cells were incubated with curcumin, the curcumin-induced blockage of SREBP-1 translocation was obviously eliminated, curcumin-

induced caveolin-1 expression was reduced, and intracellular lipid levels were upregulated again. These findings strongly suggest the effects of curcumin on increasing the caveolin-1 expression and lowering intracellular lipid levels, which were mediated by the inhibition of the nuclear translocation of SREBP-1.

In conclusion, our data demonstrate that curcumin inhibits ox-LDL-induced cholesterol accumulation in cultured VSMC by regulating the SREBP-1/caveolin-1 pathway. This new mechanism may be beneficial for anti-atherosclerotic research. Together with previous studies, our results indicate the potential of curcumin or its chemically-modified derivatives as ideal anti-atherosclerotic drugs.



**Figure 7.** Effects of ALLN on the caveolin-1 expression in VSMC and origin lipid-loaded cells with or without curcumin. Total proteins were separated by 10% SDS-PAGE, and the expression of caveolin-1 was detected by Western blotting analysis. (A) effect of 25  $\mu\text{mol}\cdot\text{L}^{-1}$  ALLN on the caveolin-1 expression in normal or lipid-loaded VSMC; (B) VSMC were exposed to 50  $\text{mg}\cdot\text{L}^{-1}$  ox-LDL for 48 h and then treated with 25  $\mu\text{mol}\cdot\text{L}^{-1}$  ALLN and/or 25  $\mu\text{mol}\cdot\text{L}^{-1}$  curcumin for 24 h. Upper panel is a representative Western blot of caveolin-1. Lower panel shows a semiquantitative analysis of the caveolin-1 expression. Relative density means the ratio of caveolin-1 over  $\beta$ -actin. Data were expressed as mean $\pm$ SD of 3 independent experiments. <sup>a</sup> $P<0.01$  vs control, <sup>c</sup> $P<0.05$  vs ox-LDL, <sup>h</sup> $P<0.05$  vs curcumin.

**Table 3.** ALLN inhibited the effects of curcumin on lipid levels in VSMC-derived foam cells (mg/g).  $n=3$ . Data expressed as mean $\pm$ SD. <sup>b</sup> $P<0.05$ , <sup>c</sup> $P<0.01$  vs control, <sup>e</sup> $P<0.05$ , <sup>f</sup> $P<0.01$  vs ox-LDL, <sup>h</sup> $P<0.05$ , <sup>i</sup> $P<0.01$  vs curcumin.

Index	TC	FC	CE
Control	188 $\pm$ 21	181 $\pm$ 16	77 $\pm$ 0.9
Ox-LDL	393 $\pm$ 40 <sup>c</sup>	250 $\pm$ 23 <sup>b</sup>	143 $\pm$ 15 <sup>e</sup>
Cur(25 $\mu\text{mol/L}$ )	255 $\pm$ 26 <sup>e</sup>	210 $\pm$ 19 <sup>e</sup>	45 $\pm$ 5.2 <sup>f</sup>
Cur+ALLN	370 $\pm$ 39 <sup>h</sup>	240 $\pm$ 28 <sup>h</sup>	130 $\pm$ 12 <sup>i</sup>

VSMC were pretreated with 50  $\text{mg}\cdot\text{L}^{-1}$  ox-LDL for 48 h and then processed by 25  $\mu\text{mol}\cdot\text{L}^{-1}$  curcumin and/or 25  $\mu\text{mol}\cdot\text{L}^{-1}$  ALLN for 24 h. Intracellular lipid levels (TC, FC, and CE) were detected by HPLC. Cur: curcumin.

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