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Effects and underlying mechanisms of curcumin on the proliferation of vascular smooth muscle cells induced by Chol:MBCD

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Abstract

Proliferation of vascular smooth muscle cells (VSMCs) contributes to the development of various cardiovascular diseases. Curcumin, extracted from *curcumae longae*, has been shown a variety of beneficial effects on human health, including anti-atherosclerosis by mechanisms poorly understood. In the present study, we attempted to investigate whether curcumin has any effect on VSMCs proliferation and the potential mechanisms involved. Our data showed curcumin concentration-dependently abrogated the proliferation of primary rat VSMCs induced by Chol:MBCD. To explore the underlying cellular and molecular mechanisms, we found that curcumin was capable of restoring caveolin-1 expression which was reduced by Chol:MBCD treatment. Moreover, curcumin abrogated the increment of phospho-ERK1/2 and nuclear accumulation of ERK1/2 in primary rat VSMCs induced by Chol:MBCD, which led to a suppression of AP1 promoter activity stimulated by Chol:MBCD. In addition, curcumin was able to reverse cell cycle progression induced by Chol:MBCD, which was further supported by its down-regulation of cyclinD1 and E2F promoter activities in the presence of Chol:MBCD. Taking together, our data suggest curcumin inhibits Chol:MBCD-induced VSMCs proliferation via restoring caveolin-1 expression that leads to the suppression of over-activated ERK signaling and causes cell cycle arrest at G1/S phase. These novel findings support the beneficial potential of curcumin in cardiovascular disease.

Keywords

curcumin; proliferation; ERK1/2; VSMCs; caveolin-1

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Introduction

Curcuma longa is the source of the spice Turmeric, which is not only used in preparing Asian curries dishes but was also a component of some ancient herbal remedies for various diseases. Recently, curcumin, an extract from *curcuma longa*, has been demonstrated to have a variety of health beneficial effects, including anti-oxidative, anti-inflammatory, anti-atherosclerosis, and anticancer effects [1,2,3,4], and thus has been recently used as a daily health supplement. However, the cellular and molecular mechanisms of curcumin's beneficial effects on human health are not well characterized. We are interested in the anti-atherosclerosis property of curcumin. Abnormal vascular smooth muscle cells (VSMCs) proliferation within the arterial intima contributes to the progression of atherosclerotic lesions [5], where high level of cholesterol promotes VSMCs proliferation [6]. To mimic this pathophysiological process, Chol:M β CD complex, one of "water-soluble cholesterol" has been widely used as an experimental replacement for cholesterol [7]. By depleting cellular cholesterol, Chol:M β CD destroys the construct of caveolae [8], a center for normal cell signal transduction. The destruction of caveolae results in aberrant signaling that leads to VSMCs proliferation via mechanisms yet to be elucidated [9,10]. However, it is known that the formation of caveolae depends on caveolins especially caveolin-1[11]. Evidence showed that caveolin-1 plays an important role in cardiovascular diseases mostly due to its regulation of cell proliferation through MAPK [12]. Therefore, in this study, we investigated the effect of curcumin on Chol:M β CD-induced VSMCs proliferation and the underlying cellular and molecular mechanisms, particularly on caveolin-1/MAPK signaling.

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). Curcumin ([E,E]-1,7-bis[4-hydroxy-3-methoxyphenyl]-1,6-heptadiene-3,5-dione, purity =99%), MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenylterazoliumbromide) and dimethylsulfoxide (DMSO) were products of AMERSCO (Solon, Ohio, USA). The Chol:M β CD complex was purchased from Sigma (St Louis, MO, USA). The primary antibodies of ERK1/2, phospho-ERK1/2 (p-ERK1/2), caveolin-1 were purchased from Santa Cruz Biotechnology (California, USA). The secondary antibody was purchased from DAKO (Produktionsvej, Glostrup, Denmark).

Cell culture

Primary VSMCs were derived from the thoracic aorta of adult male Sprague–Dawley rats as described previously [13]. First, the aorta was isolated and the adventitia were stripped off. Then, the aorta was minced and cultured in a DMEM media containing 20% FBS until VSMCs were isolated. Cells grown in DMEM containing 10% FBS were used at low passages (<= the tenth passage).

Cell counting and MTT assay

Cell proliferation was measured using MTT assay and cell counting [14]. In brief, 5,000 cells/well were seeded into flat-bottomed 96-well plates. After 24 h, the medium was removed and replaced with serum-free medium for 24 h to achieve synchronous growth arrest. Forty-eight hours after the experimental stimulation, MTT was added (final concentration 0.5 μ g/mL) for 4 h, followed by the addition of 150 μ L DMSO to dissolve the formed formazan crystals. The absorbance was recorded at 570 nm with a microplate reader (Bio-Rad, Hercules, CA, USA). Results were reported as relative optical densities from at least 3 independent experiments. In parallel experimental plates, VSMCs were lifted after trypsin incubation and then counted microscopically using a hemocytometer after trypan blue staining.

Flow cytometry analysis

VSMCs grown to 80% confluence were treated with Chol:M β CD combined with or without curcumin (30 μ M) for 48 h, cells were washed twice with PBS, and fixed overnight with cold ethanol (70%) at 4 °C, and resuspended in PBS. The cells were incubated with RNaseA for 45 min and then stained for DNA content (PBS, 0.1% Triton X-100, 0.1 mmol/L EDTA, 0.05 μ g/mL RNaseA and 50 μ g/mL propidium iodide). Fluorescence stained nuclei were measured with a FACScan flow cytometer (Becton-Dickinson, Bedford, MA). The proportions of cells at the G0/G1, S, and G2/M phases were analyzed by FACScan software programs as previously described [15]

Western blotting

Western blot analysis was performed as previously described [16]. Briefly, VSMCs were lysed in a buffer containing 10 mmol/L HEPES (pH 7.9), 1.5 mmol/L MgCl₂, 10 mmol/L KCl, and 0.5% NP-40, and cell lysates were centrifuged at 13,000 \times g for 15 min at 4°C. The protein concentrations of cell lysates were determined by a BCA protein assay kit (Thermo scientific, Rockford, USA) according to the manufacturer's instructions. The proteins (total 30 μ g /lane) were separated on 10% SDS-PAGE, and transferred to a polyvinylidene difluoride membrane (Millipore Corporation, Billerica, MA, USA). After incubated with appropriate antibodies, specific proteins were visualized using Enhanced Chemiluminescence kit (New England Biolabs, Beverly, MA, USA) (16).

Immunofluorescence analysis

For immunofluorescence analysis, 2 \times 10⁵ cells were plated into each well of a 6-well plate with cover-slips. The cells were incubated with Chol:M β CD in the presence or absence of curcumin (30 μ M) for 48 h. Then the cells were fixed as described previously [17] and incubated with p-ERK1/2 antibody overnight at 4 °C in a humidified chamber. The following day, the cells were incubated with Cy3-labeled goat anti-mouse IgG (Sigma, USA) for 1 h and then incubated with DAPI (10 μ g/ml) for 10 min to display the cell nuclei. Then the cover-slips were analyzed by confocal laser scanning microscopy (LSM410; Zeiss, Jena, Germany). The images at randomly-selected microscopic fields were acquired with a Pixera camera (Pixera Corporation, Los Gatos, USA). The experiments were repeated 3 times.

Promoter Luciferase Reporter Assay

The plasmids of activator protein 1 (AP1) promoter luciferase reporter construct were kindly provided by Dr. JJ Li (National Research Center and Beckman Research Institute, USA), The plasmids of cyclinD1 promoter luciferase reporter construct were kindly provided by Dr. Strauss[18], and the plasmids of E2F promoter luciferase reporter construct were gifts from Dr GY Li (Central South University Cancer Research Institute, China). The promoter luciferase reporter assay was performed as previously described [16]. In brief, 5 \times 10⁴ cells were seeded into each well of a 12-well plate and incubated with Chol:M β CD firstly and followed by a 48h incubation of 30 μ M curcumin or vehicle only. Reporter plasmids transfections were performed using LipofectAMINE according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Luciferase activities were determined using the dual luciferase assay kit from Promega in the luminometer. All activities were normalized to the activity of the co-transfected β -galactosidase reporter plasmid. All experiments were repeated 3 times.

Statistical analysis

A two-tailed Student's *t*-test was used for statistical comparisons between any two specific experimental groups as indicated in text and figure legends. *P* < 0.05 was considered statistically significant. The results were expressed as Mean \pm SEM

Results

Curcumin inhibits the proliferation of VSMCs induced by Chol:M β CD

To examine the effect of curcumin on the proliferation of VSMCs, cell counting and MTT assay were performed. As expected, VSMC number was increased significantly by the treatment of 10 μ g/ml Chol:M β CD (Fig. 1). Curcumin attenuated the VSMCs proliferation effect of Chol:M β CD in a concentration-dependent manner (1 μ M 100 μ M) (Fig. 1A), which is further confirmed by MTT assay (Fig. 1B). Based on the concentration curve study, we used 30 μ M of curcumin for the subsequent experiments.

Curcumin regulates caveolin-MAPK signaling in VSMCs induced by Chol:M β CD

Since caveolin-MAPK signaling pathway plays a critical role in the proliferation of VSMCs, we investigated whether curcumin has any effects on the expression of caveolin-1 and the phosphorylation of ERK1/2 (p-ERK1/2). Caveolin-1 was down-regulated in VSMCs stimulated with Chol:M β CD, which was rescued by curcumin (Fig. 2A), confirming the notion that caveolin-1 plays an important role in cardiovascular diseases [19]. The phosphorylation level of ERK1/2 was strongly increased in VSMCs treated with Chol:M β CD, which was suppressed by curcumin (Fig. 2A). Immunofluorescence analysis showed that the proportion of VSMCs with ERK1/2 in nuclei was significantly increased upon treatment with Chol:M β CD, indicating ERK1/2 was translocated to nuclei under this condition. The nuclear translocation of ERK1/2 stimulated by Chol:M β CD was abolished when cells were co-incubated with curcumin (Fig. 2B). Furthermore, the Chol:M β CD-induced AP-1 promoter activity, a downstream of ERK1/2 signaling, was dramatically reduced by curcumin treatment (Fig. 3).

Curcumin inhibits cell cycle progression and down-regulates the activities of cyclinD1 and E2F in VSMCs induced by Chol:M β CD

To further investigate the cellular and molecular mechanisms of curcumin on VSMCs proliferation induced by Chol:M β CD, the cell cycle profile was monitored by FACS. As shown in Table 1, the proportion of VSMCs in G0/G1 phase was decreased when 10 μ g/ml Chol:M β CD was incubated [38.5 \pm 3.6 % vs 67.9 \pm 5.8 % (in control group), $P < 0.01$], meanwhile the percentage of cells at S phase was increased (46.7 \pm 5.4 % vs 28.5 \pm 3.2 % in control group, $P < 0.01$). Consistent with results shown in Fig. 1, curcumin significantly reversed the alterations of VSMC's cell cycle induced by Chol:M β CD: 38.5 \pm 3.6 % (in Chol:M β CD only group) vs 54.7 \pm 4.9 % (in Chol:M β CD plus curcumin group) for G0/G1 phase, $P < 0.01$; 46.7 \pm 5.4 % (in Chol:M β CD only group) vs 30.4 \pm 2.6 % (in Chol:M β CD plus curcumin group) for S phase, $P < 0.01$ (Table 1). We observed identical cell numbers at G2/M phase under the treatment of Chol:M β CD with or without curcumin (Table 1). These data indicated curcumin inhibited cell proliferation by causing a cell cycle arrest at G1/S phase. As both cyclinD1 and E2F regulate cell cycle progression from G1 to S transition [20], luciferase reporter analysis for both cyclinD1 and E2F promoters were performed. As showed in Fig. 3, curcumin indeed decreased the both promoter activities of cyclinD1 and E2F in VSMCs treated with Chol:M β CD, which strengthens the notion that curcumin caused cell cycle arrest at G1/S phase in primary rat VSMCs.

Discussion

Atherosclerosis arises from a multifaceted pathophysiology characterized by chronic inflammation, ubiquitous accumulation of lipids, and vascular cells modifications in the arterial wall [21]. VSMCs are the major cellular component of the vascular media, and their migration and proliferation lead to the formation of the neointima which renders the vessels particularly sensitive to atherosclerosis [22,23]. Therefore, inhibition of VSMCs proliferation is an

important component of strategies to prevent or halt the development of atherosclerosis. Curcumin, a health supplement, has recently been shown of such potential [24]. However, the detailed mechanism remains unclear. In this study, we demonstrated that curcumin significantly inhibits the proliferation of VSMCs induced by Chol:MBCD via modulating caveolin-MAPK signaling pathway which leads to cell cycle arrest.

Firstly we have successfully established the cellular model of primary VSMCs proliferation induced by Chol:MBCD, which can be inhibited by curcumin concentration-dependently (Fig. 1). The data showed that curcumin may target a pathophysiological process in atherosclerosis, namely VSMCs proliferation caused by high level of cholesterol. Then we focused our attention to caveolin-1. Caveolins (1, 2, and 3) are the major scaffolding proteins in caveolae, which are 50- to 100-nm vesicles within the plasma membrane of most cell types including VSMC [25]. Caveolin-1 is most abundantly expressed in terminally differentiated cells such as epithelial and endothelial cells, adipocytes, fibroblasts, and smooth muscle cells [26]. Signaling molecules have been shown to associate with caveolae, including receptor tyrosine kinases and their downstream targets (e.g., epidermal growth factor receptor, c-Neu, platelet-derived growth factor receptor, insulin receptor, nerve growth factor receptor, neurotrophin receptor, H-Ras, Raf-1, and ERK [27]). Thus caveolae and caveolins are implicated in various cellular processes, including transcytosis, cholesterol transport, migration, proliferation and programmed cell death (apoptosis), which are also important in the pathophysiology of vascular proliferative diseases [28]. In addition, caveolin-1 also acts as a membrane adaptor that links the integrin α subunit to the tyrosine kinase Fyn, and results in activation of the Ras-Erk pathway and promotion of cell cycle progression [29]. In our experimental model, Caveolin-1 is reduced by Chol:MBCD, and this down-regulation could be recovered by curcumin (Fig. 2A). This also coincides with our previous report, showing that curcumin inhibited ox-LDL-induced cholesterol accumulation in cultured VSMCs, by increasing the caveolin-1 expression [1]. Together these studies highlight an important molecular mechanism of curcumin actions, which is to recover caveolin-1 expression and thus restore related signals to normal levels. One of such targeted signals may be MAPK pathway, as we will discuss below.

Mitogen-activated protein kinases (MAPKs) are evolutionarily conserved enzymes that play key roles in cellular responses to inflammatory stimuli and environmental stresses. There are three independent pathways in MAPKs family—extracellular signal-regulated kinases (ERK1/2), C-Jun N-terminal kinase(JNK), and p38 MAPK. Phosphorylation and then nuclear translocation of ERK1/2 are critical steps in the regulation of cell proliferation, migration, and programmed death [30]. In this study, we revealed that curcumin was capable to inhibit the rising-levels of phosphorylated ERK1/2 in VSMCs stimulated by Chol:MBCD (Fig. 2A). The nuclear accumulation of ERK1/2 stimulated by Chol:MBCD was also reversed by curcumin in VSMCs (Fig. 2B), further confirming that ERK1/2 are targets of curcumin. As AP-1 is a transcription factor located downstream of ERK1/2 pathway that is closely linked with cell proliferation and transformation, we further investigated the effects of curcumin on AP-1 promoter activity. As shown in Fig. 3, AP1 promoter activity stimulated by Chol:MBCD was significantly reduced by curcumin in primary rat VSMCs, which further supports the suppression effect of curcumin on ERK signaling. In agreement with our data, Lev-Ari et al [31] reported that curcumin inhibited pancreatic and lung adenocarcinoma cell survival via blocking ERK1/2 activity.

We next asked whether the suppression of ERK signaling by curcumin would translate into a cell cycle arrest. There are two major “check points” in the cell cycle, the G1→S transition and the G2→M transition, both transitions are tightly controlled by cyclins /Cdks and their inhibitors. Our data showed that curcumin indeed induced cell cycle arrest at G1/S phase in primary rat VSMCs treated by Chol:MBCD (Table 1). Furthermore, we demonstrated that

cyclin D1, one of cyclins regulating G1 to S, is targeted by curcumin to modulate the VSMCs cell cycle progression induced by Chol:M β CD, as cyclin D1 promoter activity in the cells treated with Chol:M β CD was reduced significantly by curcumin (Fig. 3). In addition, E2F, another key regulator for G1 to S phase, is also targeted by curcumin, since the E2F promoter activity in VSMCs induced by Chol:M β CD was also abrogated by curcumin (Fig. 3). These results are consistent with our flowcytometry data shown in Table 1, and also in a good agreement with literature showing that curcumin induced cell cycle arrest at G1/S phase via inhibiting the expression of cyclin D1 and cyclin E in some cancer cells [32]. However, there was a report indicating that curcumin induced G2/M cell cycle arrest in a p53-dependent manner in human glioma cells [33]. In addition, an accumulation of the cells in the G2/M phase was also observed in Lovo cells and HCT-116 cells under curcumin treatment [34,35]. These conflicting reports may be due to different cells used.

In summary, our present study showed that curcumin inhibits primary rat VSMCs proliferation induced by Chol:M β CD via restoring caveolin-1 expression which leads to the suppression of ERK signaling and cell cycle arrest at G1/S phase. These data shed new insights in understanding the beneficial effects of curcumin in cardiovascular diseases.

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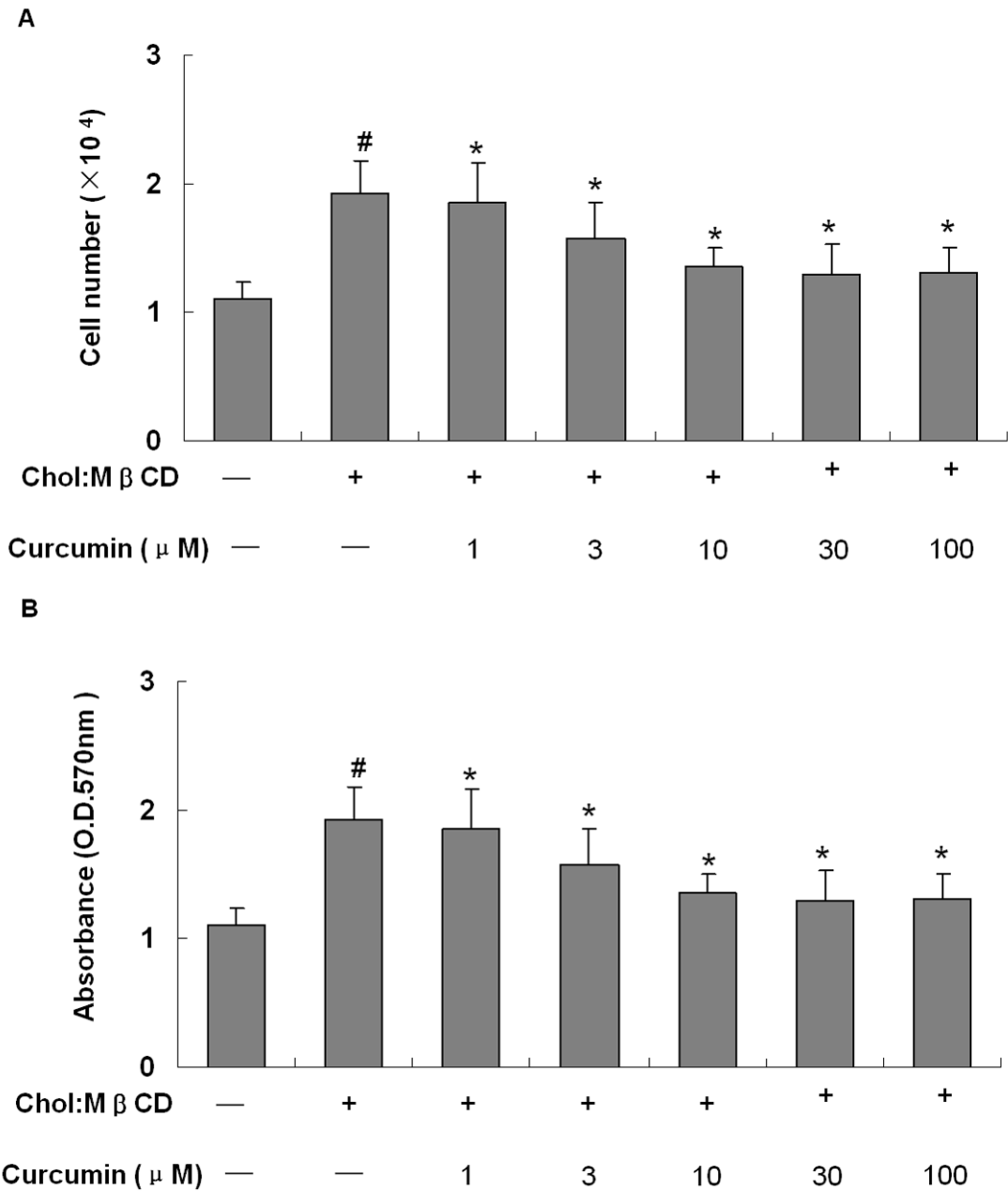


Figure 1. Curcumin inhibits the proliferation of VSMCs induced by Chol:MBCD

VSMCs were treated as indicated for 48 h, and cell proliferation was evaluated by cell counting (A) and MTT assay (B). n=4, [#]*P* < 0.01 vs control group (without both Chol:MBCD and curcumin), ^{*}*P* < 0.01 vs Chol:MBCD treatment alone.

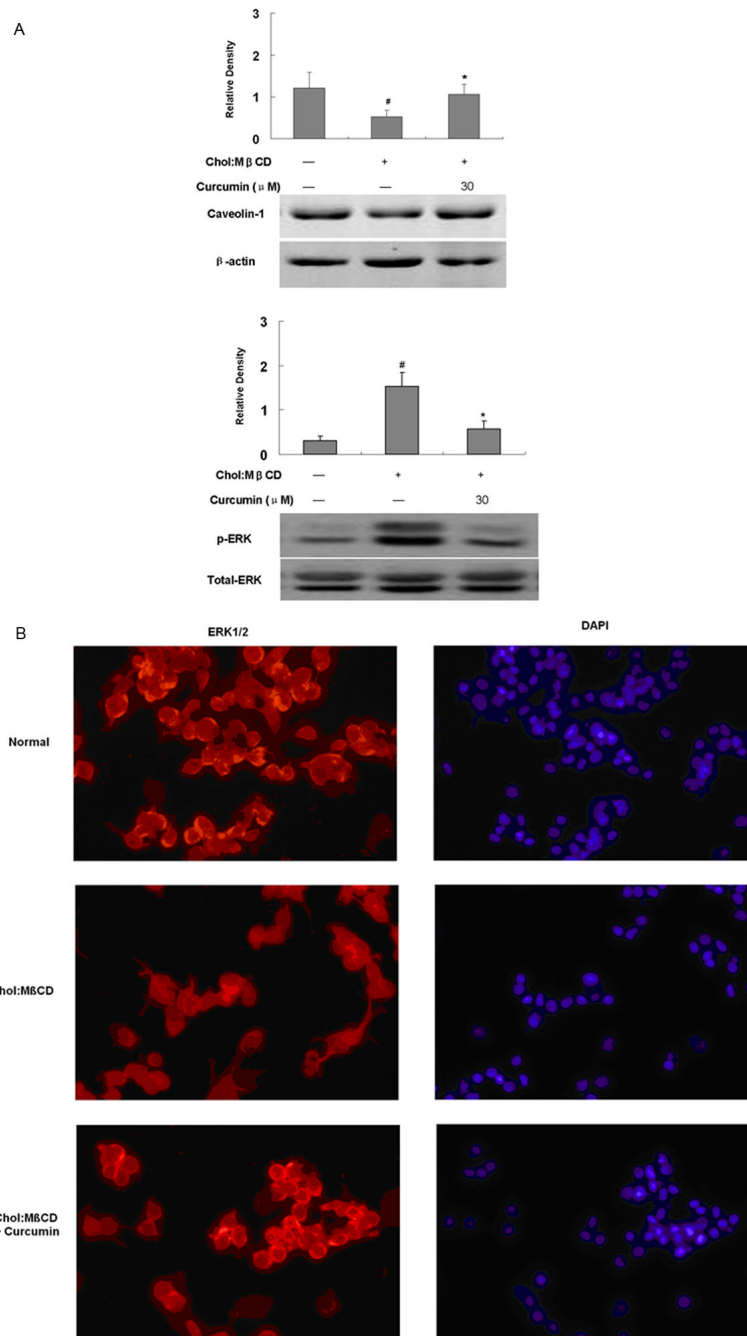


Figure 2. Curcumin regulates caveolin-MAPK signaling in VSMCs treated by Chol:MβCD
VSMCs were treated as indicated for 48 h. The expression of phospho-ERK1/2, total ERK1/2, β-actin and caveolin were detected by western blot analysis (30μg protein /lane), and then the densities were quantitated and shown in the bar graph (A). Values are expressed as Mean ± SEM (n=3). #*p* < 0.01 vs Control; **p* < 0.01 vs Chol:MβCD. The localization of phospho-ERK1/2 (red) was determined by immunofluorescence, and nuclear staining by DAPI is blue (B).

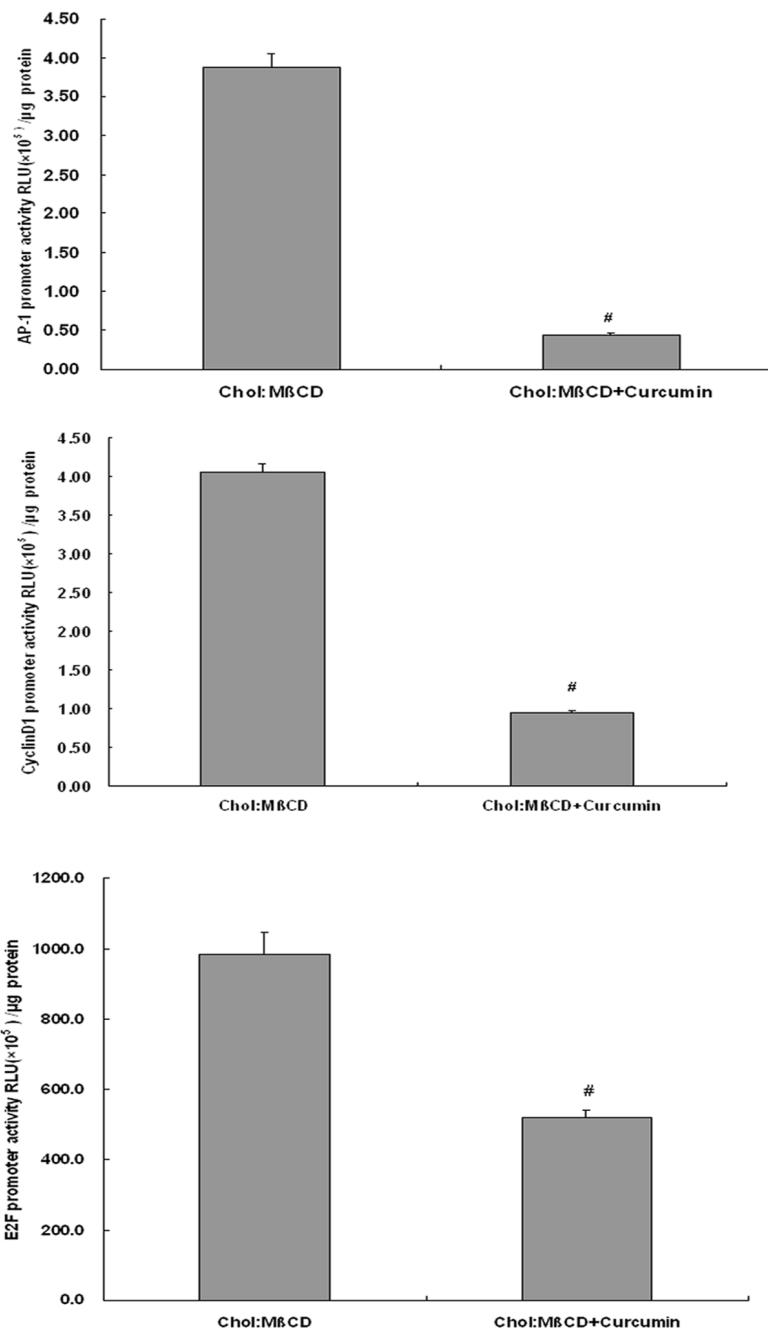


Fig 3. Curcumin down-regulates the promoter activities of AP1, cyclinD1 and E2F in VSMCs induced by Chol:MβCD

The VSMCs were treated as shown in Fig. 2, and then the promoter activities of AP-1, cyclinD1 and E2F were detected by luciferase assays. Values are expressed as Mean ± SEM (n=3). #*p* < 0.01 vs Chol:MβCD.

Table 1**Curcumin inhibits cell cycle progression in VSMCs induced by Chol:MBCD**

VSMCs were treated as indicated for 48 h, and the proportion of cells in different phases are determined by flow cytometry analysis as described in text. The experiments were carried out in triplicate. Values are expressed as Mean \pm SEM (n=3).

Group	G0/G1 (%)	S (%)	G2/M (%)
Normal	67.9 \pm 5.8	28.5 \pm 3.2	3.6 \pm 1.2
Chol:MBCD	38.5 \pm 3.6 [#]	46.7 \pm 5.4 [#]	14.8 \pm 2.1
Chol:MBCD+curcumin	54.7 \pm 4.9 [*]	30.4 \pm 2.6 [*]	14.9 \pm 1.9

[#] $p < 0.01$ vs control group;

^{*} $p < 0.01$ vs Chol:MBCD group.