Original Article

Curcumin enhances vascular contractility via induction of myocardin in mouse smooth muscle cells

Shao-wei SUN¹, Wen-juan TONG², Zi-fen GUO¹, Qin-hui TUO³, Xiao-yong LEI¹, Cai-ping ZHANG¹, Duan-fang LIAO^{3,*}, Jian-xiong CHEN^{3,4}

¹Institute of Pharmacy and Pharmacology, School of Life Science and Technology, University of South China, Hengyang 421001, China; ²Department of Gynecology and Obstetrics, First Affiliated Hospital, University of South China, Hengyang 421001, China; ³Medical School, Hunan University of Chinese Medicine, Changsha 410208, China; ⁴Department of Pharmacology and Toxicology, University of Mississippi Medical Center, School of Medicine, Jackson, MS, USA

Abstract

A variety of cardiovascular diseases is accompanied by the loss of vascular contractility. This study sought to investigate the effects of curcumin, a natural polyphenolic compound present in turmeric, on mouse vascular contractility and the underlying mechanisms. After mice were administered curcumin (100 mg·kg⁻¹·d⁻¹, ig) for 6 weeks, the contractile responses of the thoracic aorta to KCl and phenylephrine were significantly enhanced compared with the control group. Furthermore, the contractility of vascular smooth muscle (SM) was significantly enhanced after incubation in curcumin (25 μmol/L) for 4 d, which was accompanied by upregulated expression of SM marker contractile proteins SM22α and SM α-actin. In cultured vascular smooth muscle cells (VSMCs), curcumin (10, 25, 50 μmol/L) significantly increased the expression of myocardin, a “master regulator” of SM gene expression. Curcumin treatment also significantly increased the levels of caveolin-1 in VSMCs. We found that as a result of the upregulation of caveolin-1, curcumin blocked the activation of Notch1 and thereby abolished Notch1-inhibited myocardin expression. Knockdown of caveolin-1 or activation of Notch1 signaling with Jagged1 (2 μg/mL) diminished these effects of curcumin in VSMCs. These findings suggest that curcumin induces the expression of myocardin in mouse smooth muscle cells via a variety of mechanisms, including caveolin-1-mediated inhibition of Notch1 activation and Notch1-mediated repression of myocardin expression. This may represent a novel pathway, through which curcumin protects blood vessels via the beneficial regulation of SM contractility.

Keywords: curcumin; smooth muscle; vascular contractility; myocardin; caveolin-1; Notch1

Acta Pharmacologica Sinica (2017) 38: 1329–1339; doi: 10.1038/aps.2017.18; published online 1 May 2017

Introduction

Blood vessels, which are part of the circulatory system, are responsible for the transport of blood and delivery of essential nutrients to downstream tissues. The primary structural, muscular, and functional unit of a blood vessel is the medial wall, which is composed primarily of vascular smooth muscle (SM). Vascular SM provides a critical regulatory role in vessel relaxation and contraction to ensure adequate tissue blood flow and to maintain proper localized arterial blood pressures and perfusion of downstream tissues. Vascular dysfunction is the root cause of a variety of important disease processes, including hypertension, myocardial infarction, and atheroscle-

rosis. The underlying pathophysiology of vascular dysfunction occurs in numerous forms and often involves vascular SM losing its contractile characteristics^[1–3]. Recent studies also point to dysfunctional vascular SM as an underlying factor of cardiovascular disease, such as atherosclerosis and vascular aging^[4, 5]. Logically, therapeutically targeting vascular SM for further discovery and potential therapeutic utility against cardiovascular disease is highly significant and essential. Vascular smooth muscle cells (VSMCs) are highly specialized cells that constitute basic structural and functional elements of vascular SM. By contracting and relaxing, they regulate lumen caliber and thus arterial and venous tone and vascular resistance, which enables vascular SM to control the distribution of blood flow throughout the body. However, VSMCs are also thought to play a major role in vascular dysfunction in a variety of disease states^[6, 7]. Mature VSMCs are normally

*To whom correspondence should be addressed.

E-mail dfliao66@aliyun.com

Received 2017-01-14 Accepted 2017-02-20

highly differentiated and characterized by the expression of a range of SM-specific contractile proteins, including SM α -actin, SM22 α , and h1-calponin^[8, 9]. A number of works have shown that the transcription of these muscle genes and almost all SM genes were regulated by myocardin^[10]. Myocardin is a master regulator of VSMC differentiation that is found only in smooth and cardiac muscle and regulates gene expression by forming a higher order complex with serum response factor (SRF)^[11]. Critical roles of myocardin in SM have been revealed in mice with global or conditional knockout of the myocardin gene. Mice with homozygous null mutations for myocardin die during early aortic development, and embryos fail to express SMC markers^[12]. Mice with myocardin conditional mutations in SMCs also died at six months and exhibited deformation of the structure of the great arteries, along with increased dilation of the stomach, small intestine, bladder and ureters^[13]. Forced expression of myocardin increases smooth muscle contractile proteins and contributes to the development of SM contractility^[14]. Thus, myocardin could be a prime target for regulation of vascular contractility.

Curcumin is a natural polyphenolic compound present in turmeric and one of the most extensively studied natural products. Curcumin exerts a wide range of bioactivities implicated in anti-oxidant, anti-inflammatory, anti-carcinogenic and wound-healing effects^[15-19]. It is renowned for its medical properties and as a cure for several types of diseases, including diabetes, cancers and cardiovascular diseases. Mounting evidence suggests that curcumin has vascular protective effects in cardiovascular disease^[20, 21]. Our study found that curcumin increased vascular contractility in C57BL/6 mice. However, it remains unknown whether curcumin enhances contractility of blood vessels via upregulation of myocardin and its target genes. To test the hypothesis that curcumin improves vascular SM function via the induction of myocardin, we examined myocardin expression in vascular tissues from mice treated with curcumin and their contractility *in vitro*. The underlying mechanisms were further investigated using cultured VSMCs.

Materials and methods

Reagents and antibodies

Fetal bovine serum (FBS) and Dulbecco's minimum essential medium (DMEM) were purchased from Gibco (Grand Island, USA). KCl and phenylephrine (PE) were purchased from Sigma Aldrich (Sigma-Aldrich, USA). Jagged1 recombinant human protein was purchased from Thermo Fisher Scientific (Waltham, USA), and Human Caveolin-1 Adenovirus (Ad-Caveolin-1) from Vector BioLabs (Cat. No. 1364). Curcumin (purity >98%) was purchased from Sinopharm Chemical Reagent Co, Ltd (Shanghai, China). For use in cell culture, curcumin was dissolved in dimethyl sulfoxide (DMSO) to give a 50 mmol/L stock solution. The concentration of DMSO in the experimental solutions was less than 0.1%. Antibodies specific for SM α -actin, SM22 α , myocardin, caveolin-1 and cleaved Notch1 (NICD) were purchased from Abcam (Cambridge, USA). β -actin and horseradish peroxidase-conjugated

secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, USA).

Animals

C57BL/6 male mice, 6–8 weeks of age, were purchased from Jingda Laboratory Animal Technology Co Ltd (Changsha, China). Male homozygous caveolin-1 knockout (caveolin-1 KO) mice with a C57BL/6 genetic background were purchased from the Jackson Laboratory (Stock No: 007083). All mice were acclimatized with 12 h dark-light cycles under a constant temperature (22 $\pm$ 2°C) and had free access to water and food. All experiments were carried out in accordance with the internationally accepted guide for the care and use of laboratory animals and were approved by the Animal Ethics Committee of Hunan University of Chinese Medicine. One set of mice was fed with curcumin. The curcumin was dissolved in ethanol and administered to the mice via gavages of 100 mg/kg body weight/day for 6 weeks. An equivalent amount of ethanol was used for the control group.

Cells

Human VSMCs were purchased from Cell Bank of Chinese Academy of Sciences in Shanghai. Cells were grown in high glucose DMEM medium with supplements (10% FBS, 100 U/mL penicillin G, 100 μ g/mL streptomycin, and 2 mmol/L glutamine). According to the IC₅₀ value of VSMCs determined previously by us, curcumin was applied at an end concentration of 10–50 μ mol/L for all assays. For controls, the carrier (DMSO) diluted accordingly to the curcumin working solutions was applied to the cells.

RNA interference studies

RNA interference was based on the pGreenPuro system (Addgene, USA) expressing small-hairpin RNA (shRNA). pGreen-puro-caveolin-1-shRNA and pGreenPuro-vector constructs, encoding shRNA for caveolin-1 or a negative control (vector), were prepared by inserting the target sequence into pGreen-Puro. The shRNA oligonucleotide was designed with 19 bases in the sense strand (5'-ACCAGAAGGGACACACAGT-3') and the antisense strand (5'-ACTGTGTGTCCTTCTGGT-3'), which targeted region nt 424–442 of the caveolin-1 coding sequence, separated by a hairpin loop (TTCAAGAGA). The ligation products were transformed into *E coli*, clones with the shRNA insert were selected and amplified, the plasmid DNA was isolated, and selected reconstructed plasmids for transfection were purified with a plasmid purification mini kit (ComWin Biotech Co, Ltd, China). For transfection, VSMCs were seeded in 6-well plates at 1 $\times$ 10⁶ cells/well and allowed to grow overnight to 80%–90% confluency. They were transfected with a mixture of 2 μ g plasmid DNA and 10 μ L lipofectamine 2000 (Invitrogen, USA) in 2 mL serum-free medium. At 6 h after transfection, 500 μ L FBS/well was added. At 26 h after transfection, the medium was replaced by normal medium containing 10% FBS and antibiotics. VSMCs transfected with caveolin-1 shRNA or the control vector were

selected by 1 $\mu\text{g}/\text{mL}$ of puromycin.

Western blot analysis

VSMCs were washed with ice-cold PBS three times and lysed with RIPA lysis buffer [50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L sodium chloride, 10 mmol/L NP-40, 5 mmol/L deoxycholic acid, 1 mmol/L sodium dodecyl sulfate (SDS), 1 mmol/L EDTA, and 1 mmol/L phenylmethylsulfonyl fluoride] for 10–15 min on ice. Crude lysates were centrifuged at $13000\times g$ for 10 min at 4°C. Protein concentrations in the supernatants were determined by a BCA protein assay kit according to the manufacturer's instructions, and the samples were stored at -80°C. For vascular tissues, three mice from each group were used for the Western blotting. All vascular tissues were collected and stored in liquid nitrogen for protein extraction. Liquid nitrogen-frozen tissues were cut into pieces, homogenized in lysis buffer, and left on ice for 30 min. After centrifugation, protein concentrations were determined with the BCA assay. The Western blot analysis procedures were performed as described elsewhere. Equal amounts of protein from each lysate were mixed with loading buffer at 95°C for 5 min to denature the protein. Proteins were separated by SDS-PAGE and electro-transferred to polyvinylidene difluoride membranes. Nonspecific antigens were blocked by incubation at room temperature for 2 h with 5% nonfat milk in TBST (20 mmol/L Tris-HCl pH 7.6, 136 mmol/L NaCl, and 0.1% Tween-20). The membranes were probed for 1.5 h or overnight with the following primary antibodies: SM α -actin, SM22 α , myocardin, NICD, caveolin-1, and β -actin. After five washes in TBST, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 1.5 h at room temperature. The membranes were washed five times in TBST, and the protein was detected by using the ECL reagent (Beyotime, China). Emitted light was quantified using the AlphaImager gel documentation systems (formerly Alpha Innotech), and signal intensities were normalized to β -actin using Quantity One software (Bio-Rad Laboratories).

Adenovirus infection

For *in vivo* adenovirus infection, mice were injected with 10^9 PFU of Ad-caveolin-1 via the tail vein once every 7 d. For cell adenovirus infection, VSMCs were grown in 6-well plates at a density of 2×10^5 cells/well. After 24 h, cells were incubated in a low FBS medium (2%) in the presence of the Ad-caveolin-1 virus (100 MOI) for 6 h, then returned to a 10% FBS medium. On the fourth day post infection, the caveolin-1 levels were determined by Western blot.

Vascular tissue myography

The mice were sacrificed by cervical dislocation for vascular tissue collection. Thoracic aortas were excised from the mice and transferred into a dish containing cold PBS. Periaortic fibroadipose tissues were carefully removed under surgical microscopy. The aortas were cut into 2 mm length rings, and endothelium was removed by gently rubbing the intimal surface of the vessels. The aortic rings were mounted hori-

zontally in the 5 mL wire myograph chamber of a Multi Wire Myograph System (Danish Myo Technology, Skejbyparken, Denmark), maintained at 37°C in PSS solution (114 mmol/L NaCl, 4.7 mmol/L KCl, 0.8 mmol/L KH_2PO_4 , 1.2 mmol/L MgCl_2 , 11 mmol/L *D*-glucose, 25 mmol/L NaHCO_3 , and 2.5 mmol/L CaCl_2 , pH 7.4), and gassed with a 95% O_2 -5% CO_2 mixture at a resting tension of 1 g (9.8 mN). Two tungsten wires (25 μm in diameter) were guided through the lumen of each ring segment. Vascular tissues were allowed to equilibrate for 60 min before the experiments were carried out. Isometric tension was recorded using MyoDaq/MyoData 2.1 software (Danish Myo Technology). One set of the aortic rings was then cultured in DMEM medium with or without addition of curcumin for 4 d, and KCl (50 mmol/L)- and PE (1 $\mu\text{mol}/\text{L}$)-induced contractile responses were measured by the tissue myograph system after culture.

Statistical analysis

Data are presented as either representative recordings or mean $\pm$ SEM. The number of replicates (*n*) represents the number of independent assays. Differences were evaluated using Student's *t*-test or one-way ANOVA. $P<0.05$ was considered statistically significant.

Results

Curcumin increased vascular contractility

To determine whether curcumin affects vascular contractility, C57BL/6 mice received curcumin (100 mg/kg) daily for 6 weeks by gavage. Thoracic aortas were excised from the mice and cut into rings (approximately 2 mm in length). We examined the contractile responses of the aortic rings to membrane depolarization with KCl (50 mmol/L) and $\alpha 1$ -adrenoceptor activation by PE (1 $\mu\text{mol}/\text{L}$). The results showed that administration of curcumin exhibited a significantly increased ($P<0.05$) vascular contractility (Figure 1).

The contractility of vascular smooth muscle (SM) was enhanced after exposure to curcumin

We then observed the effects of curcumin on the regulation of vascular SM functions. Thoracic aortas were removed from the mice and dissected, as previously described. The endothelium was removed by gently rubbing the intimal surface of the vessels. Contractile responses of the vascular tissues were examined by the tissue myograph system and then cultured in DMEM medium with or without the addition of curcumin (25 $\mu\text{mol}/\text{L}$) in a 5% CO_2 incubator at 37°C for 4 d. After culture, the contractile responses of the vascular tissues to KCl and PE were examined again by the myograph system. We also detected the expression of contractile protein in vascular tissues after exposure to curcumin. Our results showed that the contractile responses of vascular tissues to KCl and PE were conserved after 4 d incubation with vehicle (Figure 2A). However, after treatment with curcumin for 4 d, the vascular tissues exhibited an enhanced contractile response to KCl and PE (Figure 2B), and the expression of contractile proteins SM α -actin and SM22 α were increased (Figure 2E).

Figure 1. Curcumin increased vascular contractility. Thoracic aortas were excised from mice treated with or without curcumin and cut into rings (about 2 mm in length). The contractile responses of the aortic rings to KCl and PE were examined. (A) KCl (50 mmol/L) and PE (1 μ mol/L) responses of control mouse aortic rings. (B) KCl and PE responses of aortic rings from mice received curcumin (100 mg/kg) daily for 6 weeks. (C and D) Cumulative data showing the effects of curcumin on vascular contractile response to KCl and PE. $n=6$. Mean $\pm$ SEM. * P <0.05.

Contractile protein expression was upregulated by curcumin in VSMCs

To study the underlying mechanisms of curcumin-induced increases in vascular SM contractility, we examined the effect of curcumin on the expression of contractile proteins in cultured human vascular smooth muscle cells (VSMCs). VSMCs were incubated with different concentrations of curcumin (10, 25, 50 μ mol/L) for 24 h. Western blotting was used to detect the expression of SM α -actin and SM22 α in VSMCs. The results showed that the expression of SM α -actin and SM22 α in VSMCs increased significantly in a dose-dependent manner when exposed to curcumin (Figure 3).

Curcumin increased the expression of myocardin

Since most SM contractile genes, including SM α -actin and SM22 α , are controlled by myocardin, we incubated VSMCs with varying concentrations of curcumin (10, 25, 50 μ mol/L) for 24 h or incubated VSMCs with 25 μ mol/L curcumin for different amounts of time (12, 24, 48 h). Western blotting was used to evaluate the expression of myocardin in VSMCs after

curcumin treatment. Similar to the effects of curcumin on the expression of contractile proteins in vascular tissues, Western blotting of the VSMCs showed that the expression of myocardin increased significantly in a dose- and time-dependent manner following induction with curcumin (Figure 4).

Curcumin upregulated expression of myocardin by repressing Notch1 activation

It has been demonstrated that notch signaling represses myocardin-induced VSMC differentiation. Binding of ligand Jagged1 to the Notch1 receptor releases NICD (Notch1 intracellular domain) to the nucleus, which inhibits myocardin-dependent transcription of SM-restricted genes^[22]. To identify whether curcumin-induced myocardin expression depends on the inhibition of Notch1 activity, we pretreated VSMCs with 2 μ g/mL of Jagged1 to activate Notch1 signaling, followed by incubation with or without curcumin (25 μ mol/L) for 24 h. We found that curcumin decreased the generation of NICD (Figure 5A) and upregulated the expression of myocardin (Figure 5B). However, curcumin-induced expression of myo-

Figure 2. The contractility of vascular smooth muscle (SM) was enhanced after exposure to curcumin. Thoracic aortas were excised, cut into rings, removed the endothelium, and then incubated with vehicle or curcumin. (A) KCl (50 mmol/L) and PE (1 μ mol/L) responses of vascular tissues before and after 4-d treatment with vehicle (DMSO, 0.1% v/v). (B) KCl and PE responses of vascular tissues before and after 4-d treatment with 25 μ mol/L curcumin. (C and D) cumulative data showing the effects of curcumin and vehicle on vascular contractile response to KCl and PE. $n=4$. Mean $\pm$ SEM. $^*P<0.05$. Mouse thoracic aortas were excised from mice and removed the endothelium, then cultured in DMEM medium with 25 μ mol/L curcumin for 4 d. After culture, the vascular tissues were harvested for measurement of the level of SM α -actin and SM22 α . (E) Western blot detection of the expression of contractile proteins SM α -actin and SM22 α . Left: representative Western blots. Right: cumulative data showing the relative amounts of SM α -actin and SM22 α after curcumin treatment as indicated. $n=4$. Mean $\pm$ SEM. $^*P<0.05$.

cardin was abrogated by Jagged1, and exogenous Jagged1 significantly inhibited the expression of myocardin (Figure 5B). These results suggest that curcumin enhances the expression of myocardin by repressing Notch1 activation.

Repression of Notch1 by curcumin was caveolin-1 dependent

Our previous study demonstrated that curcumin regulated intracellular cholesterol content via induction of caveolin-1 expression^[23]. In this study, we determined if caveolin-1 plays a role in the regulation of vascular contractility. Male homozygous caveolin-1 knockout (caveolin-1 KO) mice with a C57/BL6 genetic background, C57/BL6 mice injected with adenovirus encoding caveolin-1 (Ad-caveolin-1), and age-matched C57/BL6 wild-type (WT) mice were used in this experiment. The contractile responses of aortic rings removed from 6-week-old mice (caveolin-1 KO, Ad-caveolin-1 and WT) were

examined using a tissue myograph system. VSMCs stably expressing caveolin-1-shRNA and VSMCs infected with Ad-caveolin-1 were used in this experiment to observe the effects of caveolin-1 on the expression of myocardin and contractile proteins. Our results showed that the contractility of aortic rings from caveolin-1 KO mice declined by approximately 20% compared to those from wild-type mice but increased in Ad-caveolin-1 mice (Figure 6B, C and D). In cultured VSMCs, infection of adenovirus encoding caveolin-1 markedly increased the expression of myocardin, SM α -actin and SM22 α (Figure 6G). To determine whether caveolin-1 is involved in curcumin-enhanced SM contractility, the contractile responses of caveolin-1 KO mouse aortic rings to KCl and PE were examined, and the activity of Notch1 in wild-type, caveolin-1 over-expressed and knocked-down (caveolin-1-shRNA) VSMCs was analyzed by Western blot after curcumin (25 μ mol/L)

Figure 3. Contractile protein expression was upregulated by curcumin in VSMCs. VSMCs were incubated with different concentrations of curcumin for 24 h. Representative western blot and quantitative analysis of SM α -actin and SM22 α in VSMCs after treatment. $n=3$. Mean $\pm$ SEM. * $P<0.05$.

treatment. As shown in Fig. 6, curcumin increased the contractile responses of WT mice but had little effect on caveolin-1 KO mice (Figure 6H and I). Western blot revealed that curcumin inhibited the expression of NICD in normal VSMCs and the inhibitory effect of curcumin on Notch1 was less or disappeared in caveolin-1-shRNA VSMCs, while in Ad-caveolin-1 VSMCs, overexpression of caveolin-1 enhanced the inhibitory effect on Notch1 (Figure 6L). These results suggest that caveolin-1 plays a role in the regulation of vascular contractility and contributes to curcumin-mediated NICD down-regulation.

Discussion

Turmeric is commonly used as a food additive to enhance the taste and flavor of food. It has medicinal uses because of the discovered therapeutic effects^[24, 25]. Curcumin, a natural polyphenolic compound present in turmeric, is considered one of the active ingredients that contributes to the health function of turmeric and has been extensively investigated for its chemopreventive potential^[26, 27]. The putative mechanisms of action for curcumin are numerous, among which are its anti-inflammatory, antiproliferative, antiangiogenic, proapoptotic and immune-modulatory properties^[28-30]. In addition, mounting evidence suggests that curcumin has vascular protective effects in aging animals and humans. In this study, curcumin was found to be effective in regulating vascular contractility.

Vascular contractility depends on vascular SM function. It

Figure 4. Curcumin increased the expression of myocardin. VSMCs were incubated with varying concentrations of curcumin for 24 h, or incubated with 25 μmol/L curcumin for different times. (A) Representative Western blot and quantitative analysis of myocardin in VSMCs after incubation with different concentrations of curcumin for 24 h. (B) Representative Western blot and quantitative analysis of myocardin in VSMCs after incubation with 25 μmol/L curcumin for different time. $n=3$. Mean $\pm$ SEM. * $P<0.05$.

Figure 5. Curcumin upregulated expression of myocardin by repressing Notch1 activation. VSMCs were treated with different concentrations of curcumin for 24 h. (A) Representative Western blot and quantitative analysis of NICD in VSMCs after treatment. VSMCs were pretreated with 2 $\mu\text{g}/\text{mL}$ of Jagged1 to activate Notch1 signaling, followed by incubation with or without curcumin (25 $\mu\text{mol}/\text{L}$) for 24 h. (B) Representative Western blot and quantitative analysis of NICD and myocardin in VSMCs after incubation. $n=3$. Mean $\pm$ SEM. * $P<0.05$.

is well known that contractility of vascular SM is critical in the regulation of the lumenal diameter of the resistance vessels, thereby contributing significantly to setting the level of blood pressure and blood flow to vascular beds. SM contractility can be regulated by various signaling pathways^[31-34]. We harvested thoracic aortas from C57BL/6 mice, treated them with curcumin to determine the effects of curcumin on SM contractility, and found that the contractility of vascular SM was enhanced after incubation with curcumin and the expression of contractile proteins in vascular tissues was increased. VSMCs are highly specialized cells that constitute basic structural and functional elements in vascular SM. By contracting and relaxing, they alter the tension of vascular smooth muscle, enabling vascular SM to maintain an appropriate blood pressure. Mature VSMCs are characterized by the expression of a range of SM-specific contractile proteins, such as SM α -actin, SM22 α , and h1-calponin. The findings of the present study well demonstrate that the expression of contractile proteins, SM α -actin and SM22 α was dramatically increased by curcumin in VSMCs. It has been known that most SM contractile genes, such as SM α -actin and SM22 α , are controlled by myocardin. Myocardin was reported to be a key regulator of cardiac and SM differentiation, forming a ternary complex with SRF on a sequence known as a CarG box, and specifically induces the expression of smooth and cardiac muscle-specific genes. In VSMCs, the level of myocardin is abundantly expressed along with key SM-restricted genes and decreases in

parallel with the loss or attenuation of SM marker expression. Although the importance of myocardin in SM development is firmly established, the regulatory mechanism of myocardin is not well understood.

Multiple signaling pathways are expected to modulate myocardin, such as Nkx2.5 and Notch1^[22, 35-37]. In the present study, we found that curcumin induced the expression of contractile proteins, as well as myocardin, in a concentration- and time-dependent manner in VSMCs. So far, there is no direct evidence for the role of curcumin in the regulation of Nkx2.5, Notch1 or myocardin. Critically, it was demonstrated that caveolin-1 was involved in the regulation of Notch1 activation^[38], and our previous study showed that curcumin upregulated the expression of caveolin-1 in VSMCs^[23]. It is possible that caveolin-1 mediates the effect of curcumin on myocardin expression by the regulation of notch signaling. The Notch signaling pathway is a highly conserved cell signaling system present in most multicellular organisms. Mammals possess four different notch receptors, referred to as Notch1-4. The constitutive expression of the Notch family has been reported in a variety of tissues, such as vascular endothelial and smooth muscle cells. Notch1 belongs to a family of transmembrane receptors that regulate fate decisions and differentiation during normal development^[39]. Binding of the ligand Jagged1 to the extracellular domain of Notch1 induces proteolytic cleavage and release of the intracellular domain NICD, which translocates to the nucleus and forms complexes with specific

Figure 6A–6F. Repression of Notch1 by curcumin was caveolin-1 dependent. (A) Representative Western blot and quantitative analysis of caveolin-1 in VSMCs after treatment with different concentrations of curcumin for 24 h. $n=3$. Mean $\pm$ SEM. * $P<0.05$. The contractile responses of aortic rings excised from 6 weeks old mice (caveolin-1 KO, Ad-caveolin-1 and WT) were examined by a tissue myograph system. (B) KCl and PE responses of aortic rings from WT mice. (C) KCl and PE responses of aortic rings from caveolin-1 KO mice. (D) KCl and PE responses of aortic rings from mice injected with Ad-caveolin-1 adenovirus. (E and F) Cumulative data showing the effects of caveolin-1 on aortic rings contractile response to KCl and PE. $n=6$. Mean $\pm$ SEM. * $P<0.05$.

DNA-binding proteins and transcriptionally regulates target genes^[40]. It was reported that Notch1 signaling repressed myocardin-induced SM-specific contractile proteins expression^[22]. In our experiments, curcumin-induced expression of myocardin was abrogated by Jagged1, and exogenous Jagged1 significantly inhibited the expression of myocardin. These results suggest that curcumin upregulates the expression of myocardin by repressing Notch1 activation.

Caveolin-1 is an anchoring protein in the plasma membrane of most animal cells that interacts with several signaling mechanisms and participates in many important physiological processes, including the ability to regulate blood vessel function^[41–44]. One of the most important functions of caveolin-1 is to promote the formation of caveolae, a special type of lipid raft, on the surface of VSMCs^[45, 46]. A number of signaling molecules and enzymes are located in the caveolae/

raft including γ -secretase^[47], which is required for the cleavage and activation of Notch1^[48]. γ -Secretase activity has been reported to be enriched in caveolae/raft microdomains^[49], but the proteolytic cleavage of Notch1 occurs primarily in non-raft membranes^[50]. It is highly conceivable that caveolin-1 could intensify the spatial segregation of γ -secretase imposed by caveolae/raft membrane domains and inhibit its switching to non-raft membranes where Notch1 becomes easily accessible to this protease. To determine whether caveolin-1 mediates the effects of curcumin on Notch1 activation, we analyzed the expression of NICD, an activated form of Notch1, in caveolin-1-overexpressed and knocked-down VSMCs after treatment with curcumin. Our experiments showed that curcumin inhibited the expression of NICD in normal VSMCs but had no effect in caveolin-1 knocked-down VSMCs, while overexpression of caveolin-1 enhanced the inhibitory effect of cur-

Figure 6G–6L. (G) Representative Western blot and quantitative analysis of myocardin, SM α -actin and SM22 α in VSMCs (caveolin-1-shRNA, Ad-caveolin-1 and wild-type). (H) KCl and PE responses of aortic rings from caveolin-1 KO mice. (I) KCl and PE responses of aortic rings from caveolin-1 KO mice treated with curcumin (100 mg/kg) daily for 6 weeks. (J and K) Cumulative data showing the effects of curcumin on aortic rings contractile response to KCl and PE in caveolin-1 KO mice. (L) Representative Western blot and quantitative analysis of NICD in wild-type, caveolin-1 overexpressed and knocked-down VSMCs after curcumin (25 μ mol/L) treatment. $n=3$. Mean $\pm$ SEM. * $P<0.05$. WT, wild-type; CAV1-shR, caveolin-1-shRNA; Ad-CAV1, Ad-caveolin-1.

cumin on NICD. We also observed the effect of caveolin-1 on vascular contractility and found that mice with homozygous null mutations for caveolin-1 showed an impaired vascular contractility, and Ad-caveolin-1 mice exhibited an enhanced contractility. These results suggest that caveolin-1 critically contributes to regulate SM contractility by mediating the effect of curcumin on Notch1 activity.

In summary, we identified a previously unknown role for curcumin as a regulator of the vascular contractile response by inducing myocardin, which increases contractile protein expression and vascular contractility. Curcumin upregulated the expression of myocardin via a variety of mechanisms, including caveolin-1-mediated inhibition of Notch1 activation and Notch1-mediated repression of myocardin expression. This study extends our understanding of the role of curcumin

in vascular protection and may represent a new approach for the treatment of vascular diseases.

Acknowledgments

This work was supported by grants from the National Natural Science Foundation of China (No. 31371161, 81670268, 30971170 and 30971267) and Natural Science Foundation of Hunan Province (No. 2016JJ3109).

Author contribution

Duan-fang LIAO designed research; Wen-juan TONG, Zi-fen GUO, Qin-hui TUO and Cai-ping ZHANG performed research; Jian-xiong CHEN contributed new analytical tools and reagents; Xiao-yong LEI analyzed data; Shao-wei SUN wrote the paper.

References

- Brunner F, Wolkart G, Pfeiffer S, Russell JC, Wascher TC. Vascular dysfunction and myocardial contractility in the JCR:LA-corpulent rat. *Cardiovasc Res* 2000; 47: 150–8.
- Kang S, Woo HH, Kim K, Lim KM, Noh JY, Lee MY, *et al*. Dysfunction of vascular smooth muscle and vascular remodeling by simvastatin. *Toxicol Sci* 2014; 138: 446–556.
- Wurster S, Wang P, Dean R, Chaudry I. Vascular smooth muscle contractile function is impaired during early and late stages of sepsis. *J Surg Res* 1994; 56: 556–61.
- El Assar M, Angulo J, Vallejo S, Peiro C, Sanchez-Ferrer C, Rodriguez-Manas L. Mechanisms involved in the aging-induced vascular dysfunction. *Front Physiol* 2012; 3: 132.
- Dupasquier CMC, Weber A-M, Ander BP, Rampersad PP, Steigerwald S, Wigle JT, *et al*. Effects of dietary flaxseed on vascular contractile function and atherosclerosis during prolonged hypercholesterolemia in rabbits. *Am J Physiol Heart Circ Physiol* 2006; 291: H2987–96.
- Bennett MR, Sinha S, Owens GK. Vascular smooth muscle cells in atherosclerosis. *Circ Res* 2016; 118: 692–702.
- Michel J-B, Li Z, Lacolley P. Smooth muscle cells and vascular diseases. *Cardiovasc Res* 2012; 95: 135–7.
- Yoshida T, Owens GK. Molecular determinants of vascular smooth muscle cell diversity. *Circ Res* 2005; 96: 280–91.
- Owens G. Molecular control of vascular smooth muscle cell differentiation. *Acta Physiol Scand* 1998; 164: 623–35.
- Wang Z, Wang D-Z, Pipes GCT, Olson EN. Myocardin is a master regulator of smooth muscle gene expression. *Proc Natl Acad Sci U S A* 2003; 100: 7129–34.
- Zhou N, Lee J-J, Stoll S, Ma B, Wiener R, Wang C, *et al*. Inhibition of SRF/myocardin reduces aortic stiffness by targeting vascular smooth muscle cell stiffening in hypertension. *Cardiovasc Res* 2016; cvw222.
- Li S, Wang D-Z, Wang Z, Richardson JA, Olson EN. The serum response factor coactivator myocardin is required for vascular smooth muscle development. *Proc Natl Acad Sci U S A* 2003; 100: 9366–70.
- Huang J, Wang T, Wright AC, Yang J, Zhou S, Li L, *et al*. Myocardin is required for maintenance of vascular and visceral smooth muscle homeostasis during postnatal development. *Proc Natl Acad Sci U S A* 2015; 112: 4447–52.
- Yoshida T, Kawai-Kowase K, Owens GK. Forced expression of myocardin is not sufficient for induction of smooth muscle differentiation in multipotential embryonic cells. *Arterioscler Thromb Vasc Biol* 2004; 24: 1596–601.
- Chadalapaka G, Jutooru I, Chintharlapalli S, Papineni S, Smith R III, Li X, *et al*. Curcumin decreases specificity protein expression in bladder cancer cells. *Cancer Res* 2008; 68: 5345–54.
- Na HS, Cha MH, Oh D-R, Cho C-W, Rhee JH, Kim YR. Protective mechanism of curcumin against *Vibrio vulnificus* infection. *FEMS Immunol Med Microbiol* 2011; 63: 355–62.
- Scapagnini G, Foresti R, Calabrese V, Stella AMG, Green CJ, Motterlini R. Caffeic acid phenethyl ester and curcumin: a novel class of heme oxygenase-1 inducers. *Mol Pharmacol* 2002; 61: 554–61.
- Shankar S, Srivastava RK. Bax and Bak genes are essential for maximum apoptotic response by curcumin, a polyphenolic compound and cancer chemopreventive agent derived from turmeric, *Curcuma longa*. *Carcinogenesis* 2007; 28: 1277–86.
- Zhang L, Luo J, Zhang M, Yao W, Ma X, Yu SY. Effects of curcumin on chronic, unpredictable, mild, stress-induced depressive-like behaviour and structural plasticity in the lateral amygdala of rats. *Int J Neuropsychopharmacol* 2014; 17: 793–806.
- Fleenor B, Sindler A, Marvi N, Howell K, Zigler M, Yoshizawa M, *et al*. Curcumin ameliorates arterial dysfunction and oxidative stress with aging. *Exp Gerontol* 2013; 48: 269–76.
- Zhao J, Ching L, Huang Y, Chen C, Chiang A, Kou Y, *et al*. Molecular mechanism of curcumin on the suppression of cholesterol accumulation in macrophage foam cells and atherosclerosis. *Mol Nutr Food Res* 2012; 56: 691–701.
- Proweller A, Pear WS, Parmacek MS. Notch signaling represses myocardin-induced smooth muscle cell differentiation. *J Biol Chem* 2005; 280: 8994–9004.
- Yuan HY, Kuang SY, Zheng X, Ling HY, Yang YB, Yan PK, *et al*. Curcumin inhibits cellular cholesterol accumulation by regulating SREBP-1/caveolin-1 signaling pathway in vascular smooth muscle cells. *Acta Pharmacol Sin* 2008; 29: 555–63.
- Park S, Lim H, Hwang S. Evaluation of antioxidant, rheological, physical and sensorial properties of wheat flour dough and cake containing turmeric powder. *Food Sci Technol Int* 2012; 18: 435–43.
- Rao S, Dinkar C, Vaishnav LK, Rao P, Rai MP, Fayad R, *et al*. The Indian spice turmeric delays and mitigates radiation-induced oral mucositis in patients undergoing treatment for head and neck cancer: an investigational study. *Integr Cancer Ther* 2014; 13: 201–10.
- Gowda NKS, Ledoux DR, Rottinghaus GE, Bermudez AJ, Chen YC. Efficacy of turmeric (*Curcuma longa*), containing a known level of curcumin, and a hydrated sodium calcium aluminosilicate to ameliorate the adverse effects of aflatoxin in broiler chicks. *Poult Sci* 2008; 87: 1125–30.
- Keshavarz K. The Influence of turmeric and curcumin on cholesterol concentration of eggs and tissues. *Poult Sci* 1976; 55: 1077–83.
- Bengmark S. Curcumin, an atoxic antioxidant and natural NF[κ]B, cyclooxygenase-2, lipooxygenase, and inducible nitric oxide synthase inhibitor: a shield against acute and chronic diseases. *J Parenter Enteral Nutr* 2006; 30: 45–51.
- Heger M, van Golen RF, Broekgaarden M, Michel MC. The molecular basis for the pharmacokinetics and pharmacodynamics of curcumin and its metabolites in relation to cancer. *Pharmacol Rev* 2013; 66: 222–307.
- Lopresti AL, Hood SD, Drummond PD. Multiple antidepressant potential modes of action of curcumin: a review of its anti-inflammatory, monoaminergic, antioxidant, immune-modulating and neuroprotective effects. *J Psychopharmacol* 2012; 26: 1512–24.
- Ye GJ, Nesmith AP, Parker KK. The role of mechanotransduction on vascular smooth muscle myocytes' [corrected] cytoskeleton and contractile function. *Anat Rec (Hoboken)* 2014; 297: 1758–69.
- Schauwienold D, Plum C, Helbing T, Voigt P, Bobbert T, Hoffmann D, *et al*. ERK1/2-Dependent Contractile Protein Expression in Vascular Smooth Muscle Cells. *Hypertension* 2003; 41: 546–52.
- Dietrich A, Mederos y Schnitzler M, Gollasch M, Gross V, Storch U, Dubrovskaya G, *et al*. Increased vascular smooth muscle contractility in TRPC6^{-/-} mice. *Mol Cell Biol* 2005; 25: 6980–9.
- Stull JT, Gallagher PJ, Herring BP, Kamm KE. Vascular smooth muscle contractile elements. Cellular regulation. *Hypertension* 1991; 17: 723–32.
- Creemers EE, Sutherland LB, McAnally J, Richardson JA, Olson EN. Myocardin is a direct transcriptional target of Mef2, Tead and Foxo proteins during cardiovascular development. *Development* 2006; 133: 4245–56.
- Ueyama T, Kasahara H, Ishiwata T, Nie Q, Izumo S. Myocardin expression is regulated by Nkx2.5, and its function is required for cardiomyogenesis. *Mol Cell Biol* 2003; 23: 9222–32.
- Xie WB, Li Z, Miano JM, Long X, Chen SY. Smad3-mediated myocardin silencing: a novel mechanism governing the initiation of smooth muscle differentiation. *J Biol Chem* 2011; 286: 15050–7.
- Kapoor A, Hsu W, Wang B, Wu G, Lin T, Lee S, *et al*. Caveolin-1

- regulates γ -secretase-mediated A β PP processing by modulating spatial distribution of γ -secretase in membrane. *J Alzheimers Dis* 2010; 22: 423–42.
- 39 Fiuza UM, Arias AM. Cell and molecular biology of Notch. *J Endocrinol* 2007; 194: 459–74.
- 40 Bray SJ. Notch signalling in context. *Nat Rev Mol Cell Biol* 2016; 17: 722–35.
- 41 Parat MO, Riggins GJ. Caveolin-1, caveolae, and glioblastoma. *Neuro Oncol* 2012; 14: 679–88.
- 42 Frank P, Lisanti M. Role of caveolin-1 in the regulation of the vascular shear stress response. *J Clin Invest* 2006; 116: 1222–5.
- 43 Jia G, Sowers JR. Caveolin-1 in cardiovascular disease: a double-edged sword. *Diabetes* 2015; 64: 3645–7.
- 44 Hardin CD, Vallejo J. Caveolins in vascular smooth muscle: Form organizing function. *Cardiovasc Res* 2006; 69: 808–15.
- 45 Cohen AW, Hnasko R, Schubert W, Lisanti MP. Role of caveolae and caveolins in health and disease. *Physiol Rev* 2004; 84: 1341–79.
- 46 Gratton JP, Bernatchez P, Sessa WC. Caveolae and caveolins in the cardiovascular system. *Circ Res* 2004; 94: 1408–17.
- 47 Matsumura N, Takami M, Okochi M, Wada-Kakuda S, Fujiwara H, Tagami S, *et al*. γ -Secretase associated with lipid rafts: multiple interactive pathways in the stepwise processing of β -carboxyl-terminal fragment. *J Biol Chem* 2014; 289: 5109–21.
- 48 Olsauskas-Kuprys R, Zlobin A, Osipo C. Gamma secretase inhibitors of Notch signaling. *Onco Targets Ther* 2013; 6: 943–55.
- 49 Urano Y, Hayashi I, Isoo N, Reid PC, Shibasaki Y, Noguchi N, *et al*. Association of active γ -secretase complex with lipid rafts. *J Lipid Res* 2005; 46: 904–12.
- 50 Vetrivel KS, Cheng H, Kim SH, Chen Y, Barnes NY, Parent AT, *et al*. Spatial segregation of γ -secretase and substrates in distinct membrane domains. *J Biol Chem* 2005; 280: 25892–900.