Curcuminol inhibits PDGF-BB-induced proliferation and migration of airway smooth muscle cells by suppressing ERK/CREB pathway

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Received 9 September 2021; Accepted 29 September 2021
Available online 1 January 2022

Abstract

Background: Curcuminol, possessing antiviral, antifungal, antimicrobial, anticancer, and anti-inflammatory properties, has been widely used in treating cancers and liver fibrosis. The aim of this study was to determine the effect of curcuminol on the progression of asthma.

Materials and methods: Curcuminol was administrated to platelet-derived growth factor (PDGF)-BB-stimulated airway smooth muscle cells (ASMCs). The proliferation of ASMCs was assessed by MTT and EdU incorporation assays. The apoptosis of ASMCs was measured by flow cytometry and Western blotting. The migration of ASMCs was evaluated by Transwell migration assay and Western blotting. The regulatory effects of curcuminol on extracellular signal-regulated protein kinase (ERK)/cAMP response element-binding protein (CREB) pathway was evaluated by Western blotting.

Results: The proliferation and migration of ASMCs induced by PDGF-BB was suppressed, and the apoptosis of ASMCs was elevated by curcuminol in a dose-dependent manner. The activation of ERK/CREB pathway induced by PDGF-BB was suppressed by curcuminol.

Conclusion: Curcuminol could suppress ERK/CREB pathway to inhibit proliferation and migration and promote apoptosis of PDGF-BB-stimulated ASMCs. These findings suggest that curcuminol may act as a potential drug for asthma treatment.

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KEYWORDS

Curcuminol; asthma; PDGF-BB, ERK/CREB pathway; airway smooth muscle cells

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https://doi.org/10.15586/aei.v50i1.501
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Introduction

Asthma is a common respiratory disorder affecting people of all age groups.\(^1\) It can be triggered by various factors such as respiratory infections, allergies, and airway irritants.\(^1,2\) The major symptoms of asthma are cough, chest tightness, wheezing, and shortness of breath, which arise from inflammation, hyperresponsiveness, and airway remodeling.\(^3,4\) The airway smooth muscle cells (ASMCs) are involved in the inflammatory process, and secretory, proliferative, and contractile functioning of asthmatic airways, which play an important role in asthma pathophysiology.\(^5\) The increase of proliferation and migration of ASMCs can promote airway remodeling and cause irreversible obstruction, thereby aggravating asthma.\(^7,8\) The augment of ASMCs in asthma is induced by contractile agonists, proinflammatory cytokines as well as growth factors such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF)-BB, and insulin-like growth factor-1 (IGF-1).\(^10,11\) PDGF and EGF have been reported to activate mitogen-activated protein kinase (MAPK)/extracellular signal-regulated protein kinase (ERK) and phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) signaling pathways to induce ASMCs proliferation.\(^12,13\) Thus, specific targeting of ASMCs for treating asthma has attracted a lot of attention.\(^16\)

Current asthma treatments focus on reducing the symptoms through ameliorating inflammation, and the drugs used primarily contain beta-adrenoceptor-2 (β2) agonists, leukotriene antagonists, and corticosteroids.\(^15\) However, these drugs produce various side effects such as osteoporosis, cardiovascular effects, headaches, and vomiting.\(^16\) Traditional herbal medicines have been widely applied for the treatment, cure, and prevention of many diseases.\(^17\) Accumulating literature suggests that traditional herbal medicines may be recognized as a potential alternative for treating asthma, because the extracts of some medical plants (e.g., Herissantia tiubae, Aster yomena) can effectively attenuate asthma symptoms and have comparable efficacies with the commonly used asthma drugs in the modern system of medicine.\(^18,19\) Nevertheless, there is still a lack of information on the study of the toxicological profiles and clinical tests of plant extracts.\(^16\) Curcumol, as a bioactive sesquiterpenoid, is extracted from Rhizoma Curcumae.\(^6\) It has various pharmacological properties (e.g., antiviral, antifungal, antimicrobial, anticancer, and anti-inflammatory) with low cytotoxicity.\(^17\) For instance, curcumol can inhibit cellular proliferation in different cancers, including hepatocarcinoma, colorectal cancer, and lung carcinoma.\(^21-23\) Curcumol suppresses the migration and adhesion of hepatic stellate cells to decrease liver fibrosis.\(^7\) Moreover, curcumol has been proved to ameliorate airway remodeling and lung inflammation in a mouse model of chronic asthma.\(^8\) However, the molecular mechanisms of curcumol in asthma are rarely reported.

It is accepted that curcumol affects different diseases by regulating many signaling pathways such as Nuclear factor kappa B (NF-κB), PI3K/AKT, and MAPK/ERK pathways.\(^9\) Lee et al. have demonstrated that ERK activation promotes the proliferation of ASMCs\(^10\). Deng et al. have indicated that inhibiting the activation of ERK and AKT caused by FSTL1 knockdown suppresses the proliferation and migration of ASMCs\(^11\). Besides, a key downstream target of ERK, cAMP Response Element-Binding (CREB) protein, is associated with inflammation, deposition of extracellular matrix (ECM), and proliferation of different cells.\(^29,30\) However, whether curcumol regulates ERK/CREB pathway to affect the growth of ASMCs has not been reported.

Hence, in the present study, we have evaluated the effect of curcumol on the proliferation, apoptosis, and migration of ASMCs. In addition, we explored the interactive relationship between curcumol and ERK/CREB pathway in ASMCs.

Materials and methods

Cell culture

Human ASMCs isolated from primary and lobar bronchi were purchased from Bena Culture Collection (Beijing, China). ASMCs were cultured in Dulbecco’s Modified Eagle’s medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C in a humidified incubator containing 5% CO₂.

Groups

Curcumol (MedChemExpress [MCE], Monmouth Junction, NJ, USA) was dissolved in absolute ethyl alcohol to a concentration of 10 mg/mL and diluted with serum-free DMEM to the required concentration before use. PDGF-BB (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in DMEM with 0.1% bovine serum albumin (BSA). ASMCs were incubated for 24 h with DMEM containing 10% FBS and starved in serum-free DMEM for 24 h to synchronize cell cycle. After cell synchronization, cells were divided into the following five groups: control, PDGF-BB, PDGF-BB + curcumol 10 μmol/L, PDGF-BB + curcumol 20 μmol/L, and PDGF-BB + curcumol 40 μmol/L. Control group: both PDGF-BB and curcumol were not added into the cells, and cells were incubated only with serum-free DMEM. PDGF-BB group: serum-free DMEM containing 0.1% ethyl alcohol was added into cells for 1 h for pre-incubation and PDGF-BB (20 ng/mL) was added and incubated for another 48 h. PDGF-BB + curcumol 10 μmol/L group: cells were pre-incubated with curcumol (10 μmol/L) for 1 h to promote drug absorption; PDGF-BB (20 ng/mL) was then added into the cells for 48 h to stimulate re-entry into the cell cycle and induce proliferation. PDGF-BB + curcumol 20 μmol/L group: the same treatment as performed in PDGF-BB + curcumol 10 μmol/L group, except the dose of curcumol was 20 μmol/L. PDGF-BB + curcumol 40 μmol/L group: the same treatment as performed in PDGF-BB + curcumol 10 μmol/L group, except that the dose of curcumol was 40 μmol/L.

Cell viability assay

The effect of curcumol on the viability of ASMCs or PDGF-BB-induced ASMCs was measured by MTT assay. ASMCs were cultured with DMEM containing 10% FBS in 96-well
plates (200 μL per well, 2.5 × 10^4 cells/mL) for 24 h. These were starved in serum-free DMEM for 24 h to synchronize cell cycle. Next, for the effect of curcumin on ASMC viability, the cells were treated with curcumin (0, 10, 20, 40, 60, 80, or 100 μmol/L) for 48 h. For the effect of curcumin on the viability of PDGF-BB-induced ASMC, the cell groups were designed and treated as described above.

After 48 h of incubation, MTT (20 μL, 5 mg/mL; Sigma-Aldrich) was added to each well in 96-well plates and incubated for 4 h to form formazan crystals. After discarding the medium, dimethyl sulfoxide (DMSO; Sigma-Aldrich) was added to solubilize the crystals. The absorbance of the samples was evaluated by a microplate (Tecan’s Infinite 200 PRO, Myron, Switzerland) at 570 nm (detection wavelength) and 630 nm (reference wavelength; background). The data were calculated as follows:

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\text{Cell viability (%) = } \frac{\text{Experimental group OD value} - \text{Blank group OD value}}{\text{Control group OD value} - \text{Blank group OD value}} \times 100.
\]

**EdU incorporation assay**

For EdU incorporation assay, ASMCs were incubated with DMEM containing 10% fetal bovine serum (FBS) for 24 h in 24-well plates (Density: 1 × 10^4 cells per well) and synchronized by 24-h serum starvation. ASMCs were treated with curcumin (0, 10, 20, or 40 μmol/L) for 1 h and stimulated with or without PDGF-BB (20 ng/mL) for 48 h. Next, EdU Staining Proliferation Kit (ABCAM), Abcam, Cambridge, UK, was used following manufacturer’s protocol, and ASMCs were visualized by Leica DM2500 fluorescence microscope (Wetzlar, Germany).

**Cell apoptosis assay**

ASMCs were digested with trypsin for 3 min, added with complete medium, and centrifuged for 5 min at 1000 rpm. After removing the supernatant and washing twice with PBS, ASMCs were added with 1 × binding buffer. Each group was divided into four equal subgroups, including unstained control, only Annexin-V stain, only PI stain, and Annexin-V + PI stain subgroups. The cell suspension (1 × 10^6 cells/mL, 100 μL) was added into a test tube with Annexin-V (5 μL) and/or PI (5 μL) gently mixed and incubated in the dark for 30 min, followed by adding 400 μL of 1 × binding buffer. The apoptosis was detected by flow cytometry (BD Bioscience, San Diego, CA, USA) and the apoptotic ratio was analyzed as described in literature.(12)

**Cell migration assay**

The migration of ASMCs was measured by Transwell migration assay (Corning Incorporated, Corning, NY, USA). After cell synchronization, ASMCs were harvested, resuspended in serum-free DMEM, and added in the upper chamber. The same medium was added to the lower chamber as well. After allowing to adhere for 1 h, cells were pretreated with curcumin for 1 h in the upper chamber, and PDGF-BB was added to the lower chamber. ASMCs were allowed to migrate for 24 h at 37°C. Next, the cells remaining on the upper surface of the chamber were wiped off with cotton swab, and the migrated cells were fixed with 4% formaldehyde and stained with 5% crystal violet. The cells were imaged and counted using a microscope (Nikon Corporation, Tokyo, Japan). The cell number was counted by the ImageJ software.

**Western blot test**

ASMCs were lysed by RIPA lysis buffer, centrifuged at 4°C for 15 min at 12,000 rpm and the supernatant was procured. The concentration of proteins was assessed by the BCA method. Proteins (20 μg per hole) were added in the loading holes of 10% SDS-PAGE, followed by transferring to PVDF membranes. After being blocked with 5% non-fat milk for 2 h, the membranes were rinsed with Tris-buffered saline plus Tween 20 (TBST) for five times and incubated with primary antibodies at 4°C overnight. Primary antibodies, purchased from Cell Signaling Technology (Boston, MA, USA) (dilution 1/1000), included Bax (#14796), Bcl-2 (#3498), cleaved Caspase-3 (#9654), MMP9 (#13667), α-SMA (#19245), p-ERK1/2 (#8544), ERK1/2 (#9102), p-CREB (#9198), CREB (#9197), and β-actin (#4970). Subsequently, the membranes were incubated with secondary antibodies (anti-rabbit IgG, HRP-linked antibody, #7074, dilution 1/2000; Cell Signaling Technology) at 37°C for 1 h and washed with TBST for four times. Proteins in the membranes were visualized by an enhanced chemiluminescence reagents (Sangon Biotech, Shanghai, China). The quantification of the proteins was analyzed by the ImageJ software as described in literature.(12)

**Statistical analysis**

Each experiment was independently repeated for three times. Data were expressed as mean ± standard error (SE) and analyzed by the GraphPad Prism 7.0 software. Statistically significant difference was evaluated by Student’s t-test for comparisons between two groups and ANOVA with Bonferroni multiple comparisons test for comparisons between more than two groups. Statistical significance was designated as P < 0.05.

**Results**

**Curcumol inhibits the proliferation of ASMCs induced by PDGF-BB**

The effect of curcumol on the proliferation of ASMCs was assessed by MTT and EdU incorporation assays. MTT assay indicated that curcumin at the concentration of 10, 20, 40 μmol/L had no significant effect on the viability of ASMCs, but the higher concentration of curcumin (60, 80, 100 μmol/L) demonstrated an obvious cytotoxicity to ASMCs, evidenced by decreased cell viability (Figure 1A). PDGF-BB stimulation significantly enhanced the viability of ASMCs compared with the control group (Figure 1B). Nevertheless,
Curcumol suppresses the migration of ASMCs induced by PDGF-BB

Western blotting was used to explore whether the molecular mechanism of curcumol in the progression of asthma was related to ERK/CREB pathway. Compared with the control group (Figures 3C and 3D). However, these enhancements were reversed by curcumol in a dose-dependent manner (Figures 3C and 3D). Therefore, curcumol promoted the apoptosis of ASMCs induced by PDGF-BB.

Curcumol suppresses the migration of ASMCs induced by PDGF-BB

The effect of curcumol on the migration of ASMCs was detected by transwell migration assay and Western blot test. In comparison with the control, PDGF-BB greatly elevated the migration number of ASMCs (Figures 3A and 3B). Western blot test indicated that PDGF-BB obviously increased the protein expressions of MMP9 and α-SMA as compared with the control group (Figures 3C and 3D). However, these enhancements were reversed by curcumol in a dose-dependent manner (Figures 3C and 3D). Therefore, curcumol suppressed the migration of ASMCs induced by PDGF-BB.

Curcumol suppresses the activation of ERK/CREB pathway induced by PDGF-BB

Western blotting was used to explore whether the molecular mechanism of curcumol in the progression of asthma was related to ERK/CREB pathway. Compared with the
Curcumol inhibits ASMC growth 21 Discussion

Since the aberrant increase in proliferation and migration of ASMC is intensely associated with the formation of airway remodeling, 7 asthma treatment that targets ASMCs helps to attenuate structural changes in the airway wall(13). PDGF-BB is critical for promoting the proliferation control, PDGF-BB significantly increased the expressions of p-ERK1/2 and p-CREB, indicating that PDGF-BB activated ERK/CREB pathway (Figure 4). Nevertheless, curcumol treatment decreased the expressions of p-ERK1/2 and p-CREB in a dose-dependent manner (Figure 4). Hence, curcumol suppressed the activation of ERK/CREB pathway induced by PDGF-BB.

Discussion

Since the aberrant increase in proliferation and migration of ASMC is intensely associated with the formation of airway remodeling, PDGF-BB is critical for promoting the proliferation

Figure 2 Curcumol promotes the apoptosis of ASMCs induced by PDGF-BB. (A) and (B) The apoptosis of ASMCs was detected by flow cytometry. (C) The protein expressions of Bax, Bcl-2, and cleaved Caspase-3 were determined by Western blotting. "P < 0.01 (PDGF-BB versus control); "P < 0.05, ""P < 0.01 (PDGF-BB + curcumol [10, 20, or 40 μmol/L] versus PDGF-BB).

Figure 3 Curcumol suppresses the migration of ASMCs induced by PDGF-BB. (A) and (B) The migration of ASMCs was determined by transwell migration. (C) and (D) The protein expressions of MMP9 and α-SMA were examined by Western blotting. "P < 0.01 (PDGF-BB versus control); "P < 0.05, ""P < 0.01 (PDGF-BB + curcumol [10, 20, or 40 μmol/L] versus PDGF-BB).
Nevertheless, deactivation of ERK/CREB signaling inhibits proliferation and migration of ASMC. This work demonstrated that the activation of ERK/CREB pathway was induced by PDGF-BB in ASMCs; however, its activation was suppressed by curcumol. Taken together, it is speculated that curcumol could suppress ERK/CREB pathway to inhibit proliferation and migration and promote apoptosis in PDGF-BB-stimulated ASMCs.

Conclusion

Results of the present study indicated that curcumol had inhibitory effect on the proliferation and migration of PDGF-BB-induced ASMCs by suppressing the activity of ERK/CREB pathway. These findings contribute to understanding the molecular mechanism of curcumol in the progression of asthma and suggest that curcumol could act as a potential drug for treating asthma. Nevertheless, the present study used ASMCs from a subset of asthmatic patients, and therefore the findings of this study have its limitations. Hence, these observations require to be further validated by extensive biological experiments and clinical trials.

Funding

This study was supported by the following grants to Qian Zhang: Jiangsu Province Social Development Project (BE2020651), in part Jiangsu Province “333 Talents” Project (BRA2020015), and the Changzhou Sci & Tech Program (CE20205023).

Competing interests

The authors state that there are no conflicts of interest to disclose.
Author Contributions

Shihui Ling and Liya Zhang designed the study, supervised data collection; Yan Qian and Zhiguang Liu analyzed and interpreted the data; Zhengdao Mao and Qian Zhang prepared the manuscript for publication and reviewed its draft. All authors read and approved the final manuscript.

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