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Original Article

Protocatechuic acid inhibits TGF-β1-induced proliferation and migration of human airway smooth muscle cells

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Abstract: Protocatechuic acid (3,4-dihydroxybenzoic acid, PCA) is a major metabolite of anthocyanins and was reported to possess anti-allergic response. However, the effects of PCA on airway smooth muscle cells (ASMCs) proliferation and migration remain unclear. Therefore, this study aims to investigate the effects of PCA on proliferation and migration of ASMCs. ASMCs were pre-incubated with various concentrations of PCA for 30 min before stimulation with TGF-β1 for different times. Cell proliferation was determined using the colony formation assay. Cell migration was detected using the Transwell chamber assay. The levels of type I collagen, fibronectin, phosphorylated Smad2, Smad2, phosphorylated Smad3 and Smad3 were detected by western blot analysis. Our results demonstrated that PCA inhibited the proliferation and migration of ASMCs, as well as suppressed the expression of type I collagen and fibronectin in ASMCs induced by transforming growth factor-beta1 (TGF-β1). Furthermore, PCA obviously down-regulated the phosphorylation levels of Smad2/3 in ASMCs exposed to TGF-β1. Taken together, the present results have revealed that PCA inhibits asthma airway remodeling by suppressing proliferation and extracellular matrix (ECM) protein deposition in TGF-β1-mediated ASMCs via the inactivation of Smad2/3 signaling pathway. Therefore, PCA may be useful for the prevention or treatment of asthma airway remodeling.

Keywords: Asthma, protocatechuic acid (PCA), airway smooth muscle cells (ASMCs), transforming growth factor-beta1 (TGF-β1)

Introduction

Asthma is a chronic airway inflammatory disorder in childhood. It is characterized by airway hyperresponsiveness (AHR), inflammation and remodeling [1]. Although multiple pharmacological agents have been introduced to reduce the growing morbidity associated with asthma, asthma is a major health concern for children worldwide [2-4]. Airway smooth muscle cell (ASMC) was known to involve in the pathophysiology of asthma. Abnormal proliferation of ASMC directly contributes to the airway remodeling during development of asthma [5]. Moreover, previous studies showed that ASMC migration toward the airway epithelium in response to inflammatory mediators such as transforming growth factor-beta1 (TGF-β1) contributes to pathology in airway remodeling [6]. Thus, inhibition of ASMC proliferation and migration may be a good way to prevent the development and progression of childhood asthma.

Protocatechuic acid (3,4-dihydroxybenzoic acid, PCA) is a major metabolite of anthocyanins. It is found in a large variety of edible plants. It possesses numerous pharmacologic properties, including antioxidant, anti-inflammatory, anti-apoptosis, anti-hyperglycemia and anti-tumor activities [7-9]. For example, Chan *et al*. reported that PCA obviously suppressed the migration of vascular smooth muscle cells in a dose- and time-dependent manner [10]. In addition, PCA treatment significantly decreased ovalbumin-induced airway hyperresponsiveness (AHR) to inhaled methacholine, as well as reduced the number of inflammatory cells in bronchoalveolar lavage (BAL) fluid [11]. However, the effects of PCA on AS-

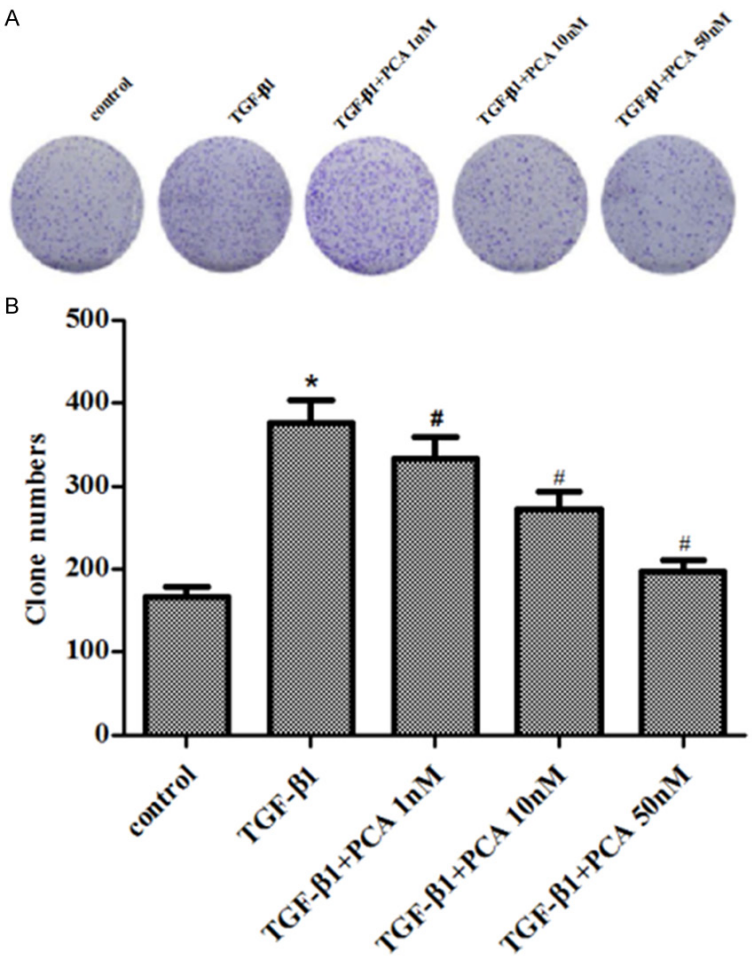

Figure 1. PCA inhibits TGF-β1-induced ASMC proliferation. ASMCs at a density of 1×10^3 cells/well were pre-incubated with PCA (1, 10 and 50 nM) for 30 min before stimulation with TGF-β1 (10 ng/ml), and then cultured for 14 days. Cell proliferation was determined using the colony formation assay. A, Representative photographs of colonies formed in ASMCs. B, Data were presented as means ± SD, n=3, *P < 0.05 versus control, #P < 0.05 versus TGF-β1.

MC proliferation and migration remain unclear. Therefore, this study aims to investigate the effects of PCA on proliferation and migration of ASMCs. Our results demonstrated that pretreatment with PCA inhibited TGF-β1-induced ASMC proliferation and migration.

Materials and methods

Cell culture and treatment

Human bronchial ASM cell line was purchased from American Type Culture Collection (ATCC, Manassas, VA) and cultured in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Gibco, Rockville, MD) and 100 μg/ml penicillin, and 100 μg/ml streptomycin (Sigma, St. Louis, MO, USA). Cells were incubated in an atmosphere of 37°C with 5% CO_2. Passages between 3 and 8 were used in all experiments. ASMCs were plated in a 24-well tissue culture plate and then pre-incubated with PCA (1, 10 and 50 nM) for 30 min before stimulation with TGF-β1 (10 ng/ml) for different times.

Cell proliferation assay

Cell proliferation was evaluated using the colony formation assay. In brief, after treatment, ASMCs at a density of 1×10^3 cells/well were plated in 96-well plates and cultured for 14 days. The resulting colonies were fixed with 4% paraformaldehyde and stained with 5% Giemsa (Sigma, St. Louis, MO, USA). The total number of colonies was counted. Stained single clones were observed under a microscope (Olympus, Tokyo, Japan).

Transwell migration assay

Cell migration was measured by the Transwell® cell culture chambers (Abcam PLC, Cambridge, UK). In brief, the treated ASMCs at a density of 1×10^5 cells/well were added to upper chamber, the lower chamber of the transwell plates were filled with 500 μl of DMEM containing 10% FBS. After 24 h, cells remaining on the upper surface of the membrane were wiped off using a cotton swab. The migrated cells on the lower surface of the filter were fixed in 4% paraformaldehyde in PBS at 4°C, stained with Giemsa and counted under the microscope (Olympus, Tokyo, Japan) by counting five independent visual fields.

Western blot

ASMCs were lysed with RIPA buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Nonidet

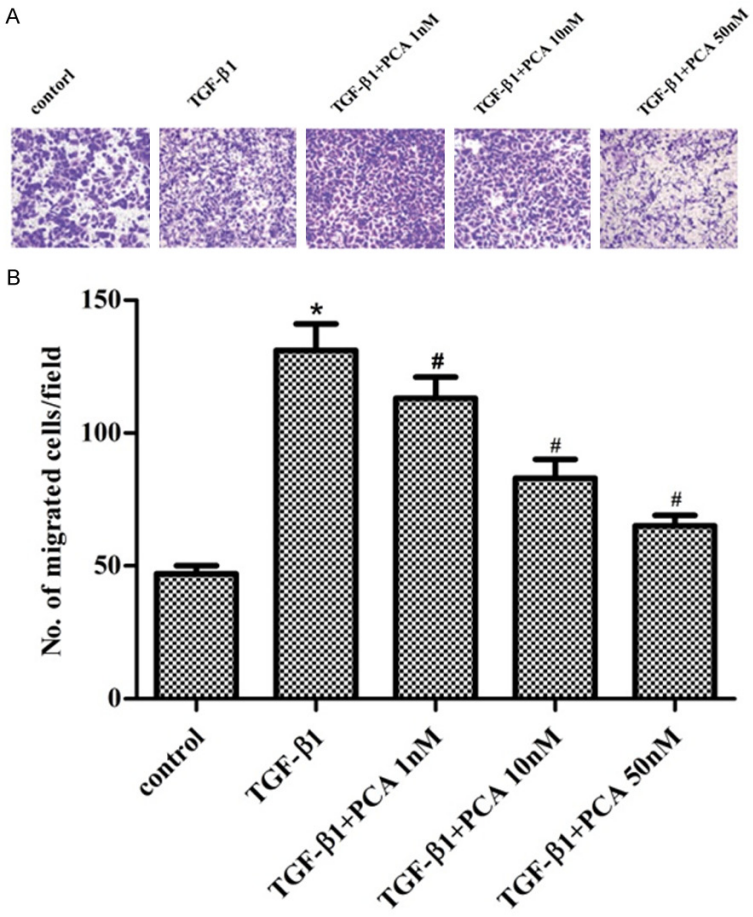

Figure 2. PCA inhibits TGF-β1-induced ASMC migration. ASMCs at a density of 1×10^4 cells/well were pre-incubated with PCA (1, 10 and 50 nM) for 30 min before stimulation with TGF-β1 (10 ng/ml) for 24 h. Cell migration was detected using the Transwell chamber assay. A. Representative photographs of cell migration. B. Data were presented as means ± SD, n=3, *P < 0.05 versus control, #P < 0.05 versus TGF-β1.

P-40, 0.5% deoxycholate, 0.1% SDS) containing protease inhibitor cocktail (Roche, Basel, Switzerland). The protein concentration in the lysates was determined by BCA protein assay kit (Beyotime, Nantong, China). The proteins (30 mg/lane) were separated by 10% by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to Immobilon-P Transfer Membranes (Millipore). Non-specific binding was blocked by incubating with 5% non-fat milk in TBS buffer (50 mmol/L NaCl, 10 mmol/L Tris, pH 7.4) for 1 h at room temperature. Then, the blots was incubated with primary antibodies (dilution, 1: 1,000) targeting type I collagen, fibronectin, p-Smad2, Smad2, p-Smad3, Smad3 and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 4°C, followed by incubation with the corresponding horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Following washing, the sites of antibody binding were visualized by enhanced chemiluminescence (ECL) detection system (Amersham, Little Chalfont, UK). The grey intensity analysis was performed using Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

Statistical analysis

All experiments were carried out in at least triplicates and are expressed as mean ± SD. Statistical significance was analyzed with one-way factorial ANOVA or Student's two-tailed *t*-test. A value of $P < 0.05$ was considered statistically significant.

Results

PCA inhibits TGF-β1-induced ASMC proliferation

We first examined the effect of PCA on ASMC proliferation using the colony formation assay. Representative photographs of colonies formed in ASMCs were shown in **Figure 1A**. The results showed that TGF-β1 treatment significantly promoted the proliferation of ASMCs compared with the control. At the same time, we observed that PCA pre-treatment markedly suppressed TGF-β1-induced ASMC proliferation, exhibiting a concentration-dependent manner (**Figure 1B**).

PCA inhibits TGF-β1-induced ASMC migration

Then, we examined the effect of PCA on ASMC migration using the Transwell chamber assay. Representative photographs of cell migration were shown in **Figure 2A**. ASMCs exposed to TGF-β1 for 24 h exhibited significantly increased cell migration compared with the control. However, PCA pre-treatment substan-

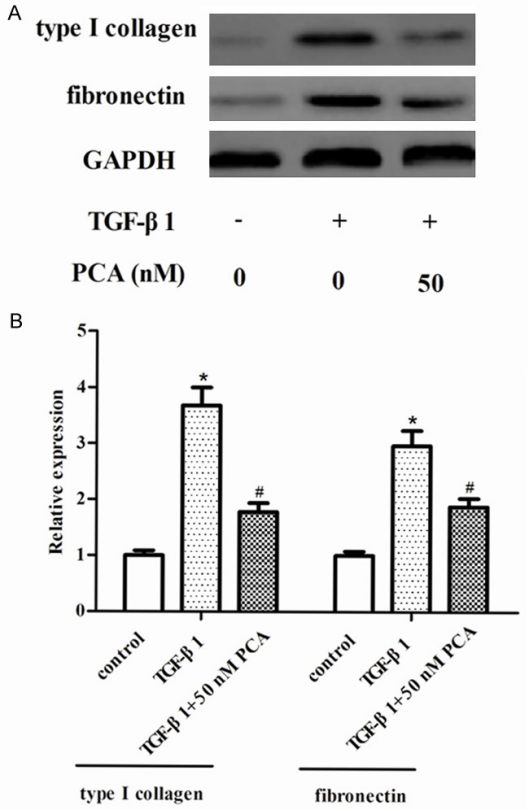

Figure 3. PCA inhibits the expression of ECM in TGF-β1-stimulated ASMCs. ASMCs at a density of 1×10^4 cells/well were pre-incubated with 50 nM PCA for 30 min before stimulation with TGF-β1 (10 ng/ml) for 24 h. A. The protein expression levels of type I collagen and fibronectin were examined using western blot assay. B. Quantification analysis was performed using Image-Pro Plus 6.0 software. Data were presented as means ± SD, n=3, *P < 0.05 versus control, #P < 0.05 versus TGF-β1.

tially suppressed TGF-β1-induced ASMC migration, compared with the TGF-β1 group (**Figure 2B**).

PCA inhibits the expression of extracellular matrix (ECM) in TGF-β1-stimulated ASMCs

ECM accumulation has been recognized as a major pathogenic event in the progression of asthma. Therefore, we examined the effect of PCA on ECM expression in ASMCs exposed to TGF-β1. The results of western blot analysis demonstrated that the expression of type I collagen and fibronectin was significantly increased in ASMCs cultured by TGF-β1. However, PCA pre-treatment obviously attenuated ECM protein deposition reflected as a marked decrease in the expression of type I collagen and fibronectin in ASMCs induced by TGF-β1 (**Figure 3A**). Quantification analysis of type I collagen and fibronectin was performed using Image-Pro Plus 6.0 software (**Figure 3B**).

PCA inhibits the activation of Smad2/3 pathway in AMSCs

TGF-β1/Smad signaling pathway plays an important role in regulating the proliferation, migration and ECM expression of ASMCs. To further explore the molecular mechanisms by which PCA inhibited TGF-β1-induced ASMCs proliferation and migration, we examined the effect of PCA on the activation of TGF-β1/Smad pathway using western blot. As shown in **Figure 4A**, TGF-β1 treatment significantly increased the phosphorylation of Smad2/3 in ASMCs. However, pre-treatment of ASMCs with PCA markedly inhibited TGF-β1-induced the phosphorylation of Smad2/3. Quantification analysis of p-Smad2 and p-Smad3 was performed using Image-Pro Plus 6.0 software (**Figure 4B**).

Discussion

In this study, we showed for the first time that PCA inhibited the proliferation and migration of ASMCs exposed to TGF-β1. In addition, PCA suppressed the expression of type I collagen and fibronectin in ASMCs induced by TGF-β1. Furthermore, PCA obviously down-regulated the phosphorylation levels of Smad2/3 in ASMCs exposed to TGF-β1.

Increasing evidences have reported that TGF-β1 plays a critical role in the pathophysiology of asthma [12-14]. It was reported that the expression of TGF-β1 was increased in the airway sub-mucous eosinophils, as compared with the control subjects [15]. Increased levels of TGF-β1 have been reported to induce the proliferation and migration in ASMCs [16-18]. In agreement with the previous studies, herein, we observed that ASMCs exposed to TGF-β1 for 24 h exhibited significantly increased cell proliferation and migration. Furthermore, PCA has been shown to possess anti-proliferative and anti-migratory activities. Tsao *et al*. confirmed that PCA treatments dose-dependently inhibited the proliferation of non-small cell lung cancer (NSCLC) cells [9]. Lin *et al*. reported that PCA inhibited oleic acid-induced VSMC pr-

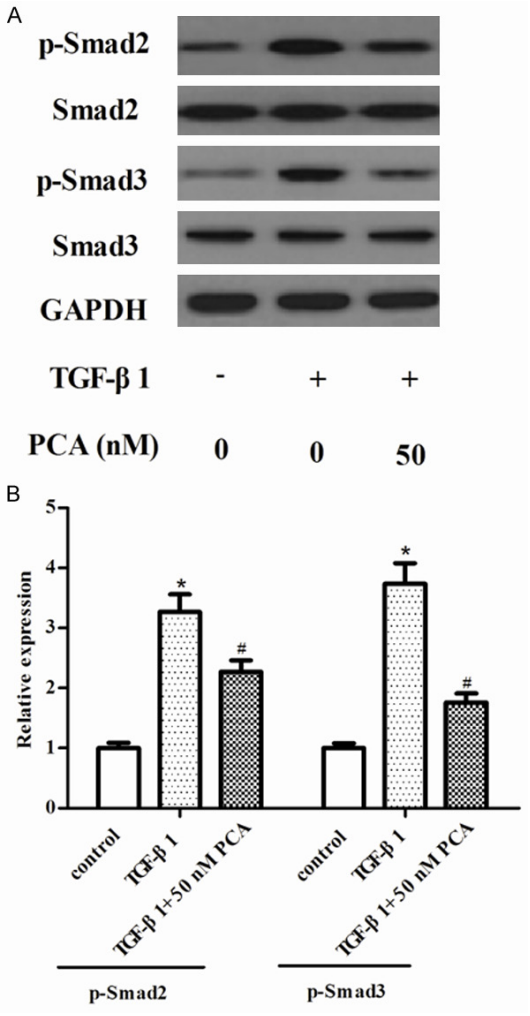

Figure 4. PCA inhibits the activation of Smad2/3 pathway in AMSCs. ASMCs at a density of 1×10^4 cells/well were pre-incubated with 50 nM PCA for 30 min before stimulation with TGF-β1 (10 ng/ml) for 24 h. A. The levels of phosphorylated Smad2, Smad2, phosphorylated Smad3 and Smad3 were detected by western blot analysis. B. Quantification analysis was performed using Image-Pro Plus 6.0 software. Data were presented as means ± SD, n=3, *P < 0.05 versus control, #P < 0.05 versus TGF-β1.

oliferation through blocking G0/G1 phase cell cycle progression [19]. Similarly, in the current study, we observed that PCA significantly inhibited the proliferation and migration of ASMCs exposed to TGF-β1.

ECM accumulation has been suggested to contribute to pathology in airway remodeling in asthma [20-22]. Several studies demonstrated that TGF-β1 is a potent factor responsible for the synthesis of ECM in a wide variety of cell types, including ASMCs, which lead to the development of airway remodeling [23-25]. Consistent with the results of previous studies, herein, we observed that the expression of type I collagen and fibronectin was significantly increased in ASMCs cultured by TGF-β1. Conversely, PCA pre-treatment markedly inhibited TGF-β1-induced ECM expression in ASMCs. These data suggest that PCA suppressed TGF-β1-induced ASMC proliferation and migration may be mediated partly via inhibition of the expression of ECM.

The TGF-β1/Smad signaling pathway is one of the important mechanisms involved in the development of airway remodeling in asthma [26-28]. TGF-β1 exerts its biological functions via a heteromeric receptor complex of type II and type I receptor serine-threonine kinases. Activated TβRI kinase phosphorylates Smad2 and -3, which then form a complex with Smad4 and translocate to the nucleus where they regulate the expression of target genes, including ECM proteins, and finally results in cell proliferation, migration, cell cycle arrest and apoptosis [29]. It has been reported that the expression levels of phosphorylated Smad2 in bronchial biopsy specimens from asthmatic subjects were higher than those in specimens from normal subjects [30]. Thus, targeting the TGF-β1/Smad signaling pathway may provide a novel therapeutic method for asthma airway remodeling. In this study, we found that TGF-β1 treatment significantly increased the phosphorylation of Smad2/3 in ASMCs. At the same time, pre-treatment with PCA obviously down-regulated the phosphorylation levels of Smad2/3 in ASMCs exposed to TGF-β1. All these data suggest that PCA inhibits asthma airway remodeling by suppressing proliferation and ECM protein deposition in TGF-β1-mediated ASMCs at least partly via the inactivation of Smad2/3 signaling pathway.

In conclusion, the present results have revealed that PCA inhibits asthma airway remodeling by suppressing proliferation and ECM protein deposition in TGF-β1-mediated ASMCs via the inactivation of Smad2/3 signaling pathway. Therefore, PCA may be useful for the prevention or treatment of asthma airway remodeling.

Disclosure of conflict of interest

None.

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