

Full-length article

PKC promotes proliferation of airway smooth muscle cells by regulating cyclinD1 expression in asthmatic rats¹Li-fen QIAO², Yong-jian XU^{1,2}, Xian-sheng LIU², Jun-gang XIE², Jin WANG³, Chun-ling DU², Jian ZHANG², Wang NI², Shi-xin CHEN²*Departments of ²Respiratory Medicine and ³Emergency Medicine, Huazhong University of Science and Technology, Wuhan 430030, China***Key words**

asthma; muscle; smooth; protein kinase C; cyclinD1; cell proliferation

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Abstract

Aim: To determine whether protein kinase C (PKC) has any effect on the expression of cyclinD1, a key regulator of growth control and G₁/S transition, and to investigate the underlying molecular mechanisms of PKC involving the remodeling of the asthmatic airway smooth muscle (ASM). **Methods:** The treatment of synchronized ASM cells from asthmatic rats with PKC-specific agonist phorbol 12-myristate 13-acetate (PMA) and antagonist 2-[1-[3-(amidinothio) propyl]-1H-indol-3-yl]-3-(1-methylindol-3-yl) maleimide methanesulfonate salt (Ro31-8220) was followed by the proliferation assay. PKC α and cyclinD1 expressions in ASM cells (ASMC) were detected by RT-PCR and Western blotting. The relation between PKC α and cyclinD1 was assessed by linear regression analysis. The effect of the construct recombinant plasmid pcDNA3.1-antisense cyclinD1 (pcDNA3.1-ascyclinD1) on the proliferation of ASMC was found to be induced by PMA. **Results:** The data showed phorbol ester-dependent PKC α promoted the proliferation of ASMC. The closely-positive correlation existed between the expression of PKC α and cyclinD1 at the transcriptional ($r=0.821$, $P<0.01$) and translational ($r=0.940$, $P<0.01$) levels. pcDNA3.1-ascyclinD1 could inhibit the proliferation of ASMC. pcDNA3.1-ascyclinD1 almost completely attenuated the PMA-induced proliferation effect as Ro31-8220+pcDNA3.1. **Conclusion:** The proliferation of ASMC by PKC might be regulated by the cyclinD1 expression in asthmatic rats.

Introduction

Asthma is characterized by airway chronic inflammation, which is the pathophysiological base of airway hyperresponsiveness and remodeling^[1,2]. The latter is a complex process that involves all of the component tissues of the airway from the epithelium to the adventitia, which includes epithelial cell damage and mucus gland hypertrophy, reticular basement membrane thickening, alterations in connective tissue composition, and an increase in the content of smooth muscle in the airway wall as a result of hyperplasia and hypertrophy^[3]. Increasing evidence indicates that the asthmatic airway smooth muscle (ASM) plays an important role in the remodeling of the airway in asthma^[4-6]. The remodeling of the asthmatic rat airway is extensively characterized *in vivo*; smooth muscle mass is considered to

be the main determinant in the airway response to allergen stimulus. McVicker and colleagues concluded that repeat allergen inhalation induced cytoskeletal remodeling in the smooth muscle of rat bronchioles. Increased F-actin content resulted in alterations in the composition and/or arrangement of the contractile apparatus after albumin exposure; such a mechanism may have relevance for airway hyperresponsiveness (AHR) found in allergic asthma^[1]. Janssen found that some therapeutic drugs, such as corticosteroids and β_2 -agonists, were potent inhibitors of ASM cell (ASMC) proliferation, which suggested ASMC was used as a target of asthma therapy^[4]. Although the structural changes in morphology concerning ASM in asthma is clear, the mechanism responsible for cellular hyperplasia has not been determined.

Protein kinase C (PKC), a cellular mediator of signal

transduction, has also been implicated in controlling a number of mitotic events in mammalian cells^[7]. The PKC family consists of at least 11 structurally-related serine/threonine protein kinases and can be divided into 3 subgroups based on differences in their structures and biochemical properties. PKC α , β I, β II, and γ are calcium-dependent and phorbol ester/diacylglycerol-dependent. PKC θ , δ , ϵ , η , and μ only respond to phorbol ester/diacylglycerol. PKC ζ , ι , and λ belong to an atypical PKC subclass that is insensitive to calcium and phorbol ester/diacylglycerol^[8]. They are not merely isozymes of identical function, but have specialized roles in cell signaling and specific cellular responses. In recent years we have reported that PKC α contributes to the pathogenesis of asthma by promoting the proliferation of passively-sensitized human ASMC^[9]. ASMC are normally quiescent, proliferate at low indices, and remain at the G₀/G₁ phase of the cell cycle. Studies of bronchial biopsies in mild asthmatics reveal that extracellular stimuli transducer proliferative responses move ASMC through the cell cycle, which comprises of distinct phases G₁, S (DNA synthesis), G₂, and M (mitosis). D-type cyclins (cyclins D1, D2, and D3), in conjunction with cyclin-dependent kinases (CDK)4 and CDK6, are thought to be key regulators of growth control and G₁/S transition^[10-12]. The expression of cyclinD1 is upregulated primarily by specific mitogens and is needed for the subsequent expression of cyclins E and A. Mammary gland tissues overexpressing cyclinD1 result in tumor development, which further supports the crucial role of cyclinD1 in regulating cell growth and proliferation^[13-15].

However, the results that PKC is involved in the process of ASM remodeling by regulating the expression of cyclinD1 in asthmatic rats have not been reported before. Elucidation of the PKC pathway and its downstream will no doubt lead to novel therapies designed to prevent or reverse these changes, so in this present study, we determined whether PKC α could promote the proliferation of ASMC by regulating the expression of cyclinD1.

Materials and methods

Animal sensitization and allergen exposure Twelve pathogen-free male Brown Norway rats weighing 250-350 g (Experimental Animal Center, Huazhong University of Science and Technology, Wuhan, China) were actively sensitized by an intraperitoneal injection of 1 mg of ovalbumin (OA; Sigma, St Louis, MO, USA) together with 200 μ g Al(OH)₃ in 0.5 mL of 0.9% NaCl. An 1 mL Bordetella pertussis vaccine containing 6 \times 10⁹ heat-killed organisms was given intraperitoneally as an adjuvant on the first day of the

study (d 0). An intraperitoneal injection of 1 mg OA and 200 μ g Al(OH)₃ in 0.5 mL of 0.9% NaCl was administered on d 7. On d 14-28, the rats were exposed to aerosolized OA 3 times a week^[16]. Challenges took place for 30 min in a 0.8-m³ chamber, with free-breathing animals being exposed to 2% (w/v) OA, which was generated by a 402T ultrasonic nebulizer (Siling Instrument, Shanghai, China) with 3 mL/min output. The Animal Care Committee of our institute approved the experimental protocol, and the care and handling of the animals were in accordance with the National Institute of Health guidelines.

Cell culture and groups The ASMC were obtained from asthmatic rats, as previously described^[17]. The ASMC were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 1 \times 10⁵ U/L penicillin, and 100 mg/L streptomycin. The cultures were maintained in a humidified atmosphere of 95% air/5% CO₂. Third to sixth generation cells were applied in the experiment. The ASMC were cultured in 0.5% serum for 24 h to synchronize at the G₀/G₁ phase. Subsequently, 10% FBS and DMEM were added to the cultures with or without various treatments for 24 h. The ASMC were divided into 4 groups: (1) Control group, where the cells were cultured in growth medium containing DMSO vehicle after serum starvation; (2) phorbol 12-myristate 13-acetate (PMA; Sigma, USA) group, where the cells were cultured in growth medium containing 10 nmol/L PMA; (3) PMA+2-[1-[3-(amidinothio) propyl]-1H-indol-3-yl]-3-(1-methylindol-3-yl) (Ro31-8220; Sigma, USA) group, where the cells were incubated with 5 μ mol/L Ro31-8220 for 1 h before adding 10 nmol/L PMA; and (4) Ro31-8220 group, where the cells were incubated with 5 μ mol/L Ro31-8220 only.

Transfection with cyclinD1 recombinant plasmid The constructed expression plasmids of the antisense cyclinD1 (ascyclinD1) gene with the pcDNA3.1 plasmid preserved in our laboratory were digested by *Bam*H1, *Xho*1, as previous described^[18]. Plasmid DNA was extracted using the Endo-free plasmid mini kit (Omega Bio-Tek, Lutterworth, Leicestershire UK). Cells were seeded onto a 24-well plate followed by the transient transfection of the cyclinD1 recombinant plasmid. The transfection was performed with Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Transfected ASMC were randomly divided into 5 groups: (1) pcDNA3.1 group; (2) PMA+pcDNA3.1 group; (3) PMA+pcDNA3.1-ascyclinD1 group; (4) pcDNA3.1-ascyclinD1 group; and (5) Ro31-8220+pcDNA3.1 group.

DNA profile analysis Cells were harvested, washed twice with cold 1 \times phosphate-buffered saline (PBS), and fixed with

75% ethanol overnight at 4 °C. The samples were subsequently stained with 10 g/L RNase and 50 mg/L propidium iodide and incubated in the dark for 30 min at room temperature. A FACScan machine (Becton Dickinson, Franklin Lakes, NJ, USA) was used to analyze the samples.

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay Approximately 5×10³ cells were grown in 200 μL 10% FBS-supplemented DMEM medium in 96-well, flat-bottomed plates overnight. After being released from the starvation medium, every group of cells was cultured in the different media 4 times for 20 h according to the group type. At the end-point, the cells were treated with 5 g/L 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, USA) for 3–4 h. Colorimetric quantification was determined at a 490 nm wavelength by an EL808 ultra micro-plate reader (Bio-Tek Instrument, Winooski, VT, USA) after the addition of DMSO.

Immunocytochemistry staining for proliferating cell nuclear antigen The ASMC were seeded onto 6-well plates at a density of 1×10⁵ cells/mL with covering slides, cultivated, and divided into groups as mentioned before. Immunocytochemistry staining was performed for the expression of proliferating cell nuclear antigen (PCNA; Table 1). The expression of PCNA was performed using a streptavidin/peroxidase kit (Zhongshan Biotek, Beijing, China). The sections were examined light microscopically; the cells positive

for PCNA staining were brown in the nuclei. On each slide, 100 cells were randomly counted and then the positive percentage was calculated.

RT-PCR analysis Total RNA was isolated from the cells using TRIzol reagent (Invitrogen, USA) according to the manufacturer’s instructions. RT was performed with 2 μg RNA using the Moloney murine leukemia virus reverse transcriptase (Promega, Madison, WI, USA) according to the manufacturer’s protocol. The PCR amplifications were carried out in the presence of 1.5 mmol/L MgCl₂ by using standard PCR buffer, 0.2 mmol/L dNTP, 0.4 mmol/L of each forward and reverse primer, 1 μL RT products, and 0.1 unit *Taq* DNA polymerase in a total volume of 25 μL. We designed the PCR primers based on GeneBank (Table 2). β -Actin was used as an internal control in the semiquantitative RT-PCR experiments. The PCR conditions were as follows: initial denaturation for 5 min at 94 °C, followed by 30 cycles (for PKC α) or 35 cycles (for cyclinD1) at 94 °C for 30 s, annealing at specific temperature (Table 2) of 72 °C for 1 min and 30 s, and a final extension of 10 min at 72 °C. The relative expression level of each target gene was calculated by normalization against the β -actin level. The PCR products were electrophoresed through 1.0% agarose gel stained with ethidium bromide. The strap of amplified products was analyzed by the Gel Doc XR gel analysis system (Bio-Rad, Hercules, CA, USA), and the densities of the products were

Table 1. Primary antibody information.

Primary antibodies	Source	Catalog No.	Class
PKC α	Santa Cruz	Sc-208	Rabbit polyclonal
CyclinD1	Santa Cruz	Sc-8396	Mouse monoclonal
PCNA	Santa Cruz		
β -Actin	Santa Cruz	Sc-25280	Mouse monoclonal
Smooth muscle actin (4) (B4) α -actin	Santa Cruz	Sc-53142	Mouse monoclonal IgG1

Table 2. Primers and conditions used for RT-PCR.

Primers	ID	Primer sequence	Annealing temperature
PKC	X52479 (gi35482)	Forward 5'-CGA CTG TCT GTA GAA ATC TGG-3'	55 °C
		Reverse 5'-CAC CAT GGT GCA CTC CAC GTC-3'	
CyclinD1	NM_171992 (gi148356228)	Forward 5'-TCAGATGTCCACATCTCGGACGTCG-3'	65 °C
		Reverse 5'-ATGGAACACCAGCTCCTGTGCTGCG-3'	
β -Actin	EF156276.1 (gi119959829)	Forward 5'-ACCCAGATCATGTTTGAGACCT-3'	55 °C
		Reverse 5'-TCAGGAGGAGCAATGATCTTG-3'	
Antisense CyclinD1 (restriction site)	NM_171992 (gi148356228)	Forward 5'-AAGGATCCTCAGATGTCCACATCTCGGACGTCG-3'	65 °C
		Reverse 5'-GACTCGAGATGGAACACCAGCTCCTGTGCTGCG-3'	

quantified with Quantity One 4.6 (Bio-Rad, Hercules, CA, USA). All assays were conducted in triplicate.

Western blot analysis Cells were washed twice with ice-cold PBS, and the whole-cell lysates were obtained using lysis buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 0.02% NaN_3 , 0.1% SDS, 0.5% sodium deoxycholate, 1% NP-40, 100 mg/L phenylmethylsulfonyl fluoride, and 1 mg/L aprotinin). The protein content was quantified using Bradford reagent (Bio-Rad, Hercules, CA, USA), and then 100 μg of total protein was subjected to SDS-PAGE on 10% acrylamide gels, followed by transfer to cellulose nitrate membranes (Amersham, Little Chalfont, Buckinghamshire, UK) using a wet transfer unit (Bio-Rad, USA) for 1 h at 200 mA. The membranes were blocked for 1 h with TBS-T (TBS [150 mmol/L NaCl and 25 mmol/L Tris-HCl, pH 7.4] with 0.05% Tween-20) containing 5% (*w/v*) non-fat dry milk, washed 3 times with TBS-T for 10 min each, and then incubated with the primary antibody (Table 1) at a dilution of 1:500 in TBS-T containing 2% non-fat dry milk overnight at 4 °C, finally with a horseradish peroxidase-conjugated secondary antirabbit antibody (Pierce, Rockford, IL, USA) at a dilution of 1:10000 for 1 h. The bands were developed using an enhanced chemiluminescence detection system (Pierce, USA).

Statistical analysis All data were expressed as mean \pm SD. *F*-test was used for the comparison of variance among several groups. The significance of difference between two groups was tested by *q*-test. The correlations were evaluated using linear regression assay. For all tests, $P < 0.05$ was considered statistically significant.

Results

Identification of ASMC The cells were observed by an inverted phase-contrast microscope. They appeared spindle-shaped, having central oval nuclei with prominent nucleoli, as well as the characteristic “hill and valley” appearance (Figure 1A). Confluent monolayers of cells were identified with anti-smooth muscle-actin antibody using an immunofluorescent method to show that the cells were of ASM origin (Figure 1B).

Proliferation levels regulated by PKC in ASMC The activity of PKC has also been indicated to be important in the regulation of cell growth in various types of cells. Furthermore, the asthmatic ASMC were in an active proliferation state. These findings led us to focus on the effect of PKC in the pathophysiology of asthma.

We first investigated the effect of PMA on ASMC proliferation. The cultures were maintained in DMEM containing 10% FBS with or without treatments. They were subjected to MTT assay, a DNA profile analysis, and immuno-

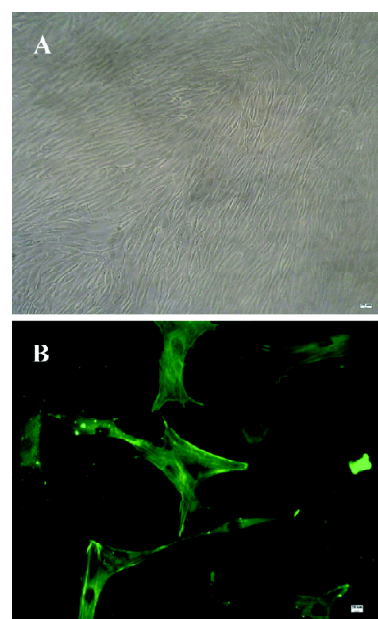


Figure 1. Identification of ASMCs. (A) Peak and valley phenomenon of airway smooth muscle cells of rats observed by inverted phase contrast microscope ($\times 100$). (B) Identification of ASMCs by immunofluorescent assay for smooth muscle actin by fluorescence microscope ($\times 200$). Scale bar = 20 μm .

cytochemistry staining for PCNA.

The MTT assay detects mitochondrial dehydrogenase activity and produces the resulting formazan in living cells. Therefore, optical density (*OD*) represents the living cell numbers. As shown in Figure 2A, PMA increased the *OD* level markedly, indicating that ASMC proliferation was prompted. The Ro31-8220 treatment reduced the *OD* value simultaneously.

To further examine the growth pattern of ASMC in response to PKC, flow cytometry analysis was conducted. After addition of PMA, the majority of the cells were present at the S+G₂M phases in comparison with the untreated control cells, in which a proportion of the cells is still presented at the G₀/G₁ phase. The opposite effect occurred in the cells treated with Ro31-8220. However, the combination of PMA and Ro31-8220 did not affect the cell cycle progression (Figure 2B), suggesting that the observed growth effects of PMA and Ro31-8220 in ASMC were due to cell cycle progression.

Next, we examined the effect of PMA on the expression of PCNA. The cells were cultured in medium containing PMA or Ro31-8220, alone or in combination for 24 h, and immunocytochemistry staining was performed for the expression of PCNA (Figure 2C). The values of the positive expression rate were 56% \pm 3.6%, 96.9% \pm 7.3%, 52.7% \pm 4.5%, and

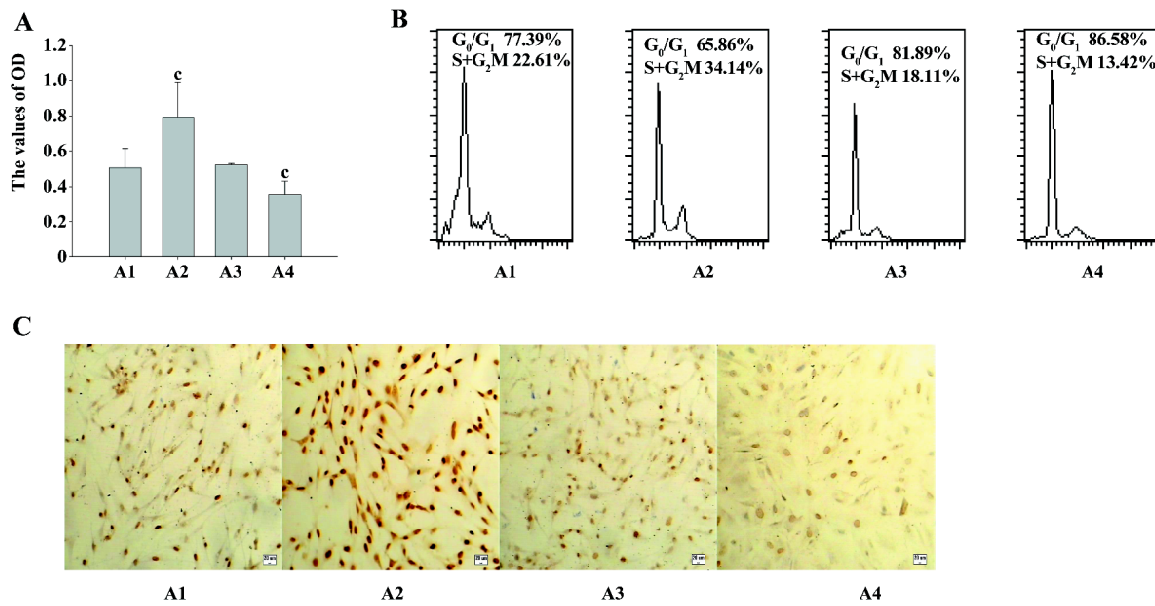


Figure 2. The proliferation levels of ASMCs stimulated by PMA, Ro31-8220 and PMA+Ro31-8220. A1: Control; A2: PMA; A3: PMA+Ro31-8220; A4: Ro31-8220. (A) MTT assay of ASMCs. Subconfluent exponentially growing ASMCs in 96-well plates were incubated in different mediums (A1) Control group, the growth medium containing dimethyl sulphoxide (DMSO) vehicle after serum starvation. (A2) PMA group, the growth medium containing 10 nmol/L PMA. (A3) PMA+Ro31-8220 group, the cells were incubated with 5 μmol/L Ro31-8220 for an hour, and then 10 nmol/L PMA was added. (A4) Ro31-8220 group, the cells were incubated with 5 μmol/L Ro31-8220 only for 24 h. Cell viability was determined by MTT assay. Results were expressed as mean±SD. n=6 rats for each group. *P<0.01 vs control group. (B) DNA profile analysis by FACS. ASMCs were incubated in serum free medium for 24 h to induce quiescence, followed by addition of PMA or Ro31-8220, and cultured for 24 h, and cell DNA content were analyzed. (C) Immunocytochemistry staining for PCNA. 5×10⁴ ASMCs were seeded on the slides, after serum starvation for 24 h, and then cultured in various mediums for 24 h. Immunocytochemistry staining was performed with antibodies specific for PCNA (×100). Scale bars=20 μm.

42.3%±5.8%, respectively. Collectively, these data indicated that PKC induced the proliferation of asthmatic ASMC.

PKCα and cyclinD1 levels stimulated by PMA in ASMC

Although the PKC family consists of more than 11 members that participate in diverse cellular responses, it is known that PKCα is the most common isozyme expressed at the protein and mRNA levels in the human lung and airway smooth muscle. To determine whether PMA treatment can induce the activation of PKCα, we examined the mRNA and protein levels. Compared with the control group, the mRNA expression and protein levels of PKCα increased significantly by PMA stimulation and was decreased by Ro31-8220. Treatment with a combination of PMA and Ro31-8220 had no effect on the expression levels of PKCα (Figure 3A). The cyclinD1 mRNA expression level was similar to PKCα (Figure 3B). There was a positive correlation between PKCα and cyclinD1 in the mRNA (r=0.821, P<0.01) and protein (r=0.940, P<0.01) levels (Figure 4). These results suggested that the activation of a conventional PKC, PKCα, probably played an important role in regulating cyclinD1 in asthmatic ASMC.

Proliferation levels regulated by PKC in transfected

ASMC It was confirmed that the expression plasmid of ascyclinD1 was successfully recombinated according to the DNA sequence analysis conducted by Invitrogen (Carlsbad, CA, USA).

The MTT results showed that compared with the pcDNA3.1 group, the OD values of cell vitality increased in the PMA+pcDNA3.1 group and decreased in the pcDNA3.1-ascyclinD1 group. Compared with the PMA+pcDNA3.1 group, there was a significant decrease in the cell vitality OD values after PMA+pcDNA3.1-ascyclinD1, pcDNA3.1-ascyclinD1, and Ro31-8220+pcDNA3.1 treatment. There was no difference between the PMA+pcDNA3.1-ascyclinD1, pcDNA3.1-ascyclinD1, and Ro31-8220+pcDNA3.1 groups (Figure 5A).

The flow cytometry results showed that the DNA content was consistent with the MTT assay. The percentage of the S+G₂M phases, an index reflecting the proliferation characteristic of cells, were 19.61%, 44.32%, 10.22%, 8.25%, and 6.75%, respectively. PMA+pcDNA3.1 shortened the G₁ phase of the cell cycle (55.68%), with an accelerating transition from the G₁ phase to the S phase. pcDNA3.1-ascyclinD1

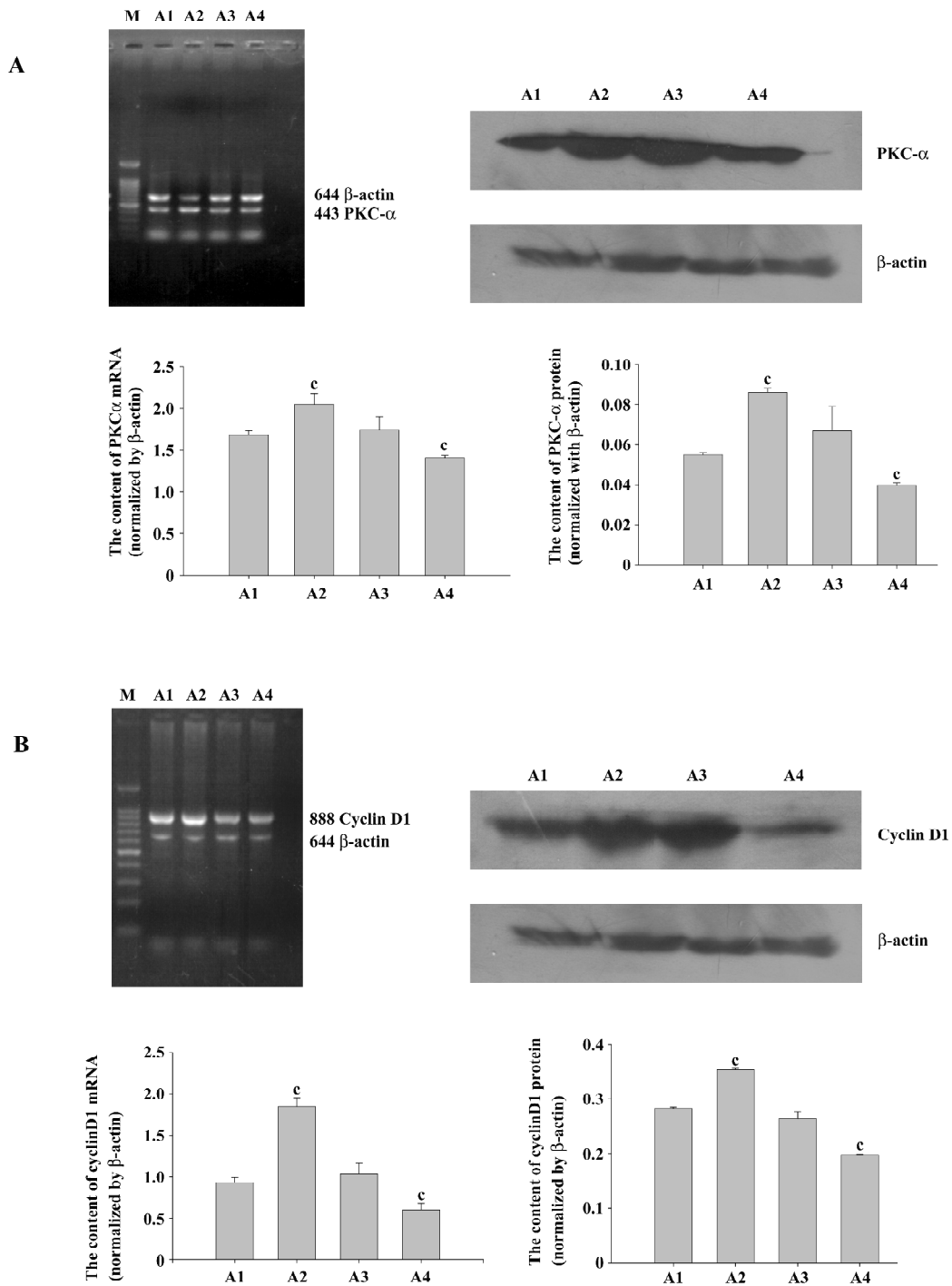


Figure 3. PMA increased the expression of PKC α and cyclinD1. The expression of PKC α (A) and cyclinD1 (B) were detected by RT-PCR and Western blot and summarized. PMA increased the expression of PKC α and cyclinD1, but Ro31-8220 inhibited both PKC α and cyclinD1 expression. A1: Control; A2: PMA; A3: PMA+Ro31-8220; A4: Ro31-8220. ^c*P*<0.01 vs control group.

arrested the cell cycle progression at the G₀/G₁ phase (91.75%) and attenuated the accelerating transition effects induced by PMA (89.78%; Figure 5B), which may explain, at least in part, why the cells being treated with PMA+pcDNA3.1–

ascyclinD1 had a lower OD value than the cells transfected with PMA+pcDNA3.1.

Quantifying PCNA may also indicate the activity of cell proliferation in transfected ASMC. Similar to MTT, the re-

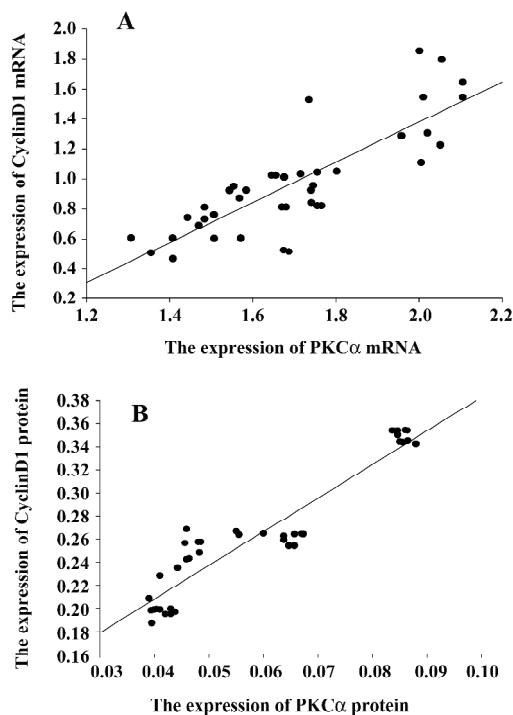


Figure 4. Correlation between PKC α and cyclinD1 expressed by ASMCs. (A) All the data from 6 rats were presented as means \pm SD, and then subjected to correlation analysis. There was positive correlation between PKC α and cyclinD1 at mRNA level ($r=0.821$, $P<0.01$). (B) There was positive correlation between PKC α and cyclinD1 at protein level ($r=0.940$, $P<0.01$).

sults of the immunocytochemistry staining showed an increase in the positive expression rate of PCNA in the PMA+pcDNA3.1 group and a reduction in the pcDNA3.1–ascyclinD1 group. Compared with the PMA+pcDNA3.1 group, pcDNA3.1–ascyclinD1 also attenuated the effects induced by PMA in quantifying PCNA (Figure 5C).

CyclinD1 expression levels in pcDNA3.1–ascyclinD1-transfected ASMC regulated by PKC α To investigate whether cyclinD1 was involved in the PKC pathway, we constructed an expression plasmid of the ascyclinD1 gene and transfected it to asthmatic ASMC to study the effects of cyclinD1 on the proliferation of cells in asthmatic rats. It was confirmed by restriction endonuclease digestion and the DNA sequence analysis (Invitrogen, Shanghai, China) that the ascyclinD1 plasmid was successfully recombinated.

The cyclinD1 expression levels in PMA+pcDNA3.1-transfected cells were higher than that of the pcDNA3.1 group. The expression levels of cyclinD1 in the pcDNA3.1–ascyclinD1-transfected cells were lower than that of the pcDNA3.1 group. Furthermore, pcDNA3.1–ascyclinD1 attenuated the increased cyclinD1 levels induced by PMA as the Ro31-8220+pcDNA3.1 (Figure 6).

Discussion

The specific aim of our study was to observe the effect of PKC rather than the single PKC isoform on the expression of cyclinD1, so PMA, the non-specific agonist for PKC, was chosen as the tool drug. The PKC α isoform was merely selected to support the expression alternation induced by PMA. Our findings showed that PKC α was involved the process of cell proliferation. The increased expression of the cyclinD1 response to activated PKC α may be associated with mechanisms by which PMA promotes the proliferation of ASMC. In addition, pcDNA3.1–ascyclinD1 almost completely attenuated the PMA-induced growth acceleration of ASMC as the Ro31-8220+pcDNA3.1 group, which indicates that the PMA-mediated upregulation of cyclinD1 involves the remodeling of the ASM via the PKC signaling pathway in asthmatic rats.

Although PKC is important for cell proliferation in a number of cell types, little is known about the signaling pathways involved in PKC in ASMC. In the present study, we focused on the involvement of the PKC α isoform in regulating cell growth and revealed whether or not cyclinD1 was at the downstream of PKC pathway in the ASMC of asthmatic rats.

Published observations on the mechanisms of asthma have suggested that there may be a link between the incidence of asthma and various inflammatory mediators, cytokines, and growth factors^[19-22]. PKC signaling pathways have been considered as a signaling pathway involved in proliferation, cell cycle progression, differentiation, apoptosis, cytoskeletal remodeling, modulation of ion channels, and secretion initiated by the mitogenic stimulators mentioned earlier^[23]. Experiments *in vivo* and *in vitro* have suggested their potent activity in the respiratory tracts. In the present study, PMA, a phorbol ester, which is a known substitute for diacylglycerol as a high-affinity ligand for conventional PKC and novel PKC isoforms^[24], stimulated PKC α gene expression and protein production in ASMC, and accelerated cell proliferation. On the contrary, Ro31-8220, the selective inhibitor of PKC, repressed the expression of PKC α compared with the control and arrested the cell cycle at the G₀/G₁ phase, suggesting that increasing PKC α induced by PMA might have contributed to the proliferation of asthmatic ASM. The results were consistent with the article reported earlier, but contrasted with other studies concerning the role of PKC. These divergent observations could at least be partly explained by differences in the subtypes of PKC, the kinds of tissues, and the various concentrations of PMA.

Cyclins have been shown to play an important role in the growth progression of various cells. For example, G₁-phase cell cycle proteins stimulate human intestinal smooth muscle

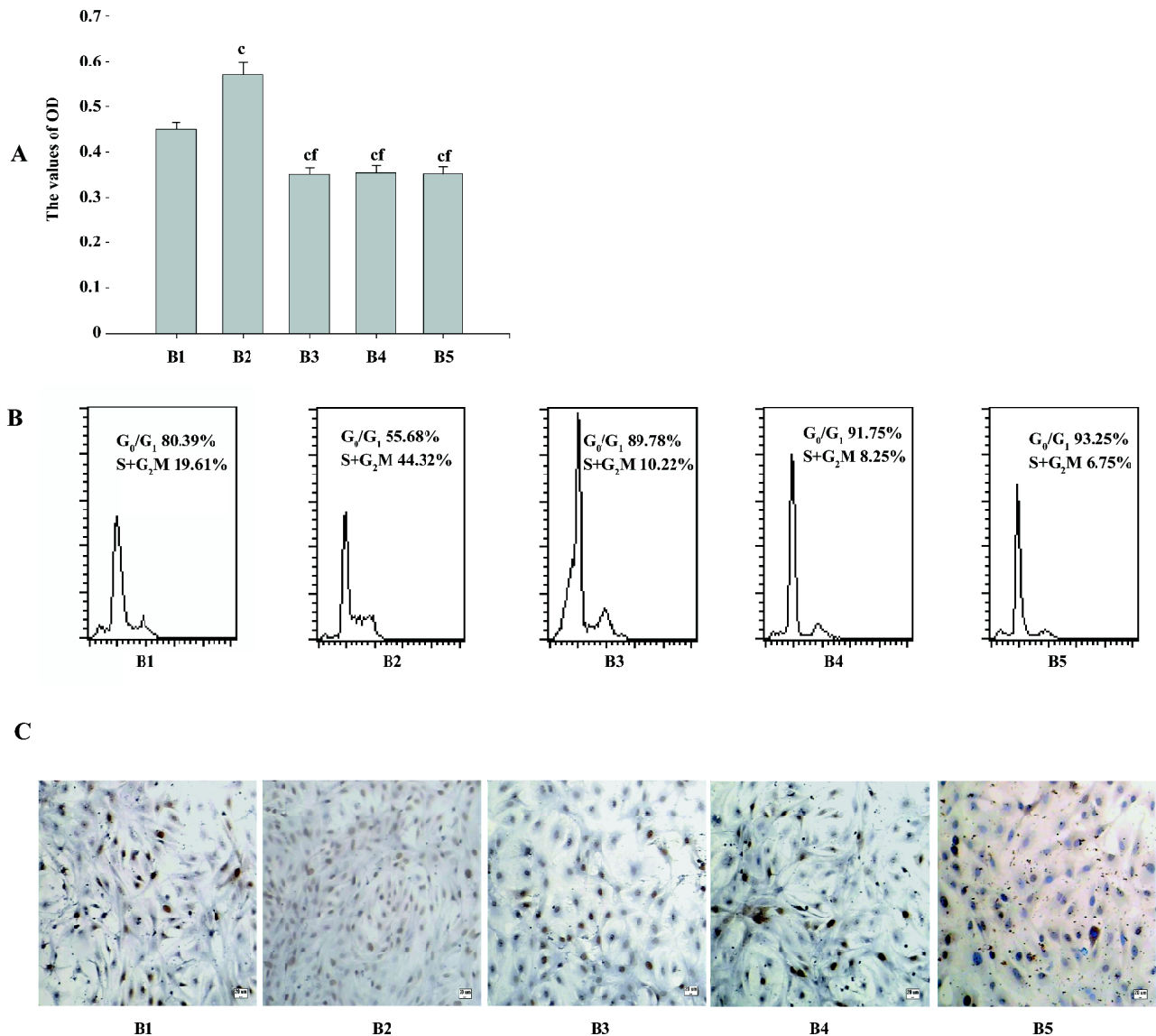


Figure 5. Effects of pcDNA3.1-ascyclinD1 on the proliferation of ASMCs stimulated by PMA. B1: pcDNA3.1; B2: PMA+pcDNA3.1; B3: PMA+pcDNA3.1-ascyclinD1; B4: pcDNA3.1-ascyclinD1; B5: Ro31-8220+pcDNA3.1. $n=6$ rats for each group. ^c $P<0.01$ vs pcDNA3.1 group, ^f $P<0.01$ vs PMA+pcDNA3.1 group. (A) MTT assay of ASMCs. Subconfluent exponentially growing ASMCs in 96-well plates were incubated in mediums transfected recombinants for 24 h. Cell viability was determined by MTT assay. (B) DNA profile analysis by FACS. ASMCs were incubated in serum free medium for 24 h to induce quiescence, followed by addition of PMA or pcDNA3.1-ascyclinD1, and cultured for 24 h, and FACS analyses were conducted. (C) Immunocytochemistry staining for PCNA (×100). 5×10^4 ASMCs were seeded on the slides, after serum starvation for 24 h and then cultured in various mediums for 24 h, immunocytochemistry staining were performed with antibodies specific for PCNA. Scale bars=20 μm.

cell growth induced by IGF-1^[25]. Cyclin-dependent kinase (CDK) inhibited coronary artery smooth muscle cell proliferation and migration^[26]. Hammerhead ribozymes targeting against cyclin and E2F1 cooperated to downregulate coronary smooth muscle proliferation^[27]. G₁ phase cyclins played a role in the cell cycle progression. The 3 D-type cyclins display significant homology; this has been interpreted as

indicating that they may have overlapping functions^[28]. However, their expression patterns vary widely among different cell types, suggesting the possibility of distinct functions. CyclinD1 has been the most studied cyclin in ASM biology and has been qualified as a proto-oncogene based on its overexpression in human and animal malignancies as well as its tumorigenic effects in gene transfer experi-

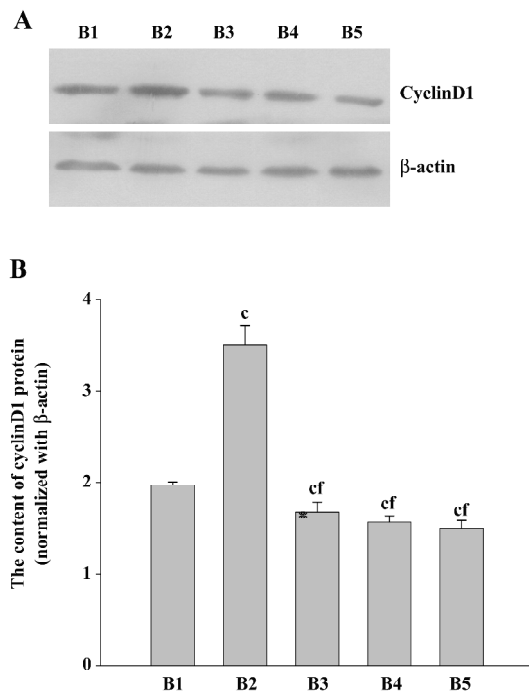


Figure 6. (A) Effects of cyclinD1-*asc*pcDNA3.1 on cyclinD1 level induced by PMA. B1: pcDNA3.1; B2: PMA+pcDNA3.1; B3: PMA+pcDNA3.1-*asc*cyclinD1; B4: pcDNA3.1-*asc*cyclinD1; B5 Ro31-8220+pcDNA3.1. *n*=6 rats for each group. (B) Data are summarized for cyclinD1 and expressed as mean±SD. **P*<0.01 vs pcDNA3.1 group, [†]*P*<0.01 vs PMA+pcDNA3.1 group.

ments with cultured cells and transgenic mice. In bovine ASM, mitogenic stimulation with platelet-derived growth factor (PDGF) induced cyclinD1 transcriptional activation and protein synthesis, consequently resulting in the hyperphosphorylation of phospho-retinoblastoma protein (pRb), which then released the elongation factor E2F that activates DNA polymerase^[29]. Consistent with a previous study, the conclusion from our experiment illustrates that cyclinD1 is related to the proliferation of asthmatic ASMC *in vitro*. The expression of cyclinD1 increased in response to PMA as well as the proliferative activity, but the effect of Ro31-8220 was the opposite, indicating that the PMA-mediated upregulation of cyclinD1 is involved in ASMC proliferation. After being transfected with the pcDNA3.1-*asc*cyclinD1 plasmid containing the Brown Norway rat *asc*cyclinD1 CDS fragment, our data of cell proliferation assessed by MTT, flow cytometry, and the expression rate of PCNA, showed that the ASMC were in a growth suppression state, which further suggested that cyclinD1 could stimulate the proliferation of asthmatic ASMC *in vitro*.

However, the mechanism of the increased proliferative activity of ASMC induced by PMA is unclear. It should be

noted that the upstream of the signaling pathway ultimately regulates the cell cycle through the key proteins of the cell cycle. Another study has also demonstrated that vascular smooth muscle cells exhibit acceleration from the G₀ phase to the S phase in the cell cycle progression in response to the activation of PKC^[30]. Skaletz-Rorowsk found that the PKC inhibitor can inhibit human and bovine aortic smooth muscle DNA synthesis through the downregulation of basic fibroblast growth factor (bFGF) induced by cyclinD1, yet PMA increased the synthesis of DNA^[31]. Accumulating studies have shown that PKC regulates the cell cycle procession by G₁-phase cyclins. It is known that phorbol ester-sensitive PKC isoforms, as the cellular receptor for phorbol esters and other tumor promoters, play a significant role in transmitting mitogenic signaling and promoting cell proliferation^[32,33]. Thus, the PKC activation-induced cyclinD1 expression further emphasizes the connection between cell cycle regulation and signaling transduction. To the best of our knowledge, the present study is the observation on the expression of cyclinD1 in response to PKC in ASMC. Our results clearly demonstrate that the expression of cyclinD1 is positively relative to the expression of PKCα as expected. ASMC transfected with the pcDNA3.1-*asc*cyclinD1 plasmid simultaneously inhibited PMA-induced S-phase traversal as Ro31-8220+pcDNA3.1, suggesting that PKC signaling pathways lead to the production of cyclinD1 in asthmatic ASMC, and a PKC-cyclinD1 cascade might be responsible for the proliferation in response to PMA. CyclinD1 could be a key downstream target of PKCα; that downstream transcription factor target of PKCα could regulate cyclinD1 transcriptional activity and cell cycle progression, which elucidates the mechanism that PKCα is involved in the remodeling of airway through regulating the cyclinD1 expression.

In conclusion, PKCα stimulates the proliferation of ASMC via the PMA-mediated upregulation of cyclinD1 in asthmatic rats. These findings may provide new insight into the signaling transduction system and the cell cycle regulation in the human respiratory tract, especially in cases of asthma. Further investigations should focus on the different roles of PKC isoforms stimulated by PMA, the cyclins-CDK complexes and CDK inhibitors, and the transduction pathway to determine the pathogenesis of asthma.

Author contributions

Yong-jian XU, Xian-sheng LIU, Jun-gang XIE designed research; Li-fen QIAO, Jin WANG performed research; Chunling DU, Jian ZHANG, Wang NI, Shi-xin CHEN contributed new reagents or analytic tools; Li-fen QIAO, Jin WANG analyzed data; Li-fen QIAO wrote the paper.

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