

Up-regulation of cyclin D1 expression in asthma serum-sensitized human airway smooth muscle promotes proliferation via protein kinase C α

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ABSTRACT

Abnormal hypertrophy and hyperplasia of airway smooth muscle cells play an important role in airway remodeling in chronic asthma. The authors' previous studies have indicated that protein kinase C α (PKC α) is involved in the proliferation of passively sensitized human airway smooth muscle cells (HASMCs). However, the underlying mechanisms remain unknown. Here, the authors examined the possible role of the α isoform of PKC in the control of cyclin D1 expression, using HASMCs passively sensitized on human atopic asthmatic serum as a model system. Cultured HASMCs were passively sensitized with serum from atopic asthmatic patients. Cell proliferation was measured by [³H]thymidine incorporation and an MTT assay. Cell cycle status was analyzed by flow cytometry. The mRNA and protein expression profiles of cyclin D1 were measured by reverse transcriptase–polymerase chain reaction (RT-PCR) and Western blotting, respectively. Furthermore, the authors assessed the role of cyclin D1 in PKC α -induced HASMC proliferation by transfection with a recombinant cyclin D1 antisense construct. The activation of PKC α with phorbol myristate acetate (PMA), a PKC activator, up-regulated cyclin D1 expression and increased the proliferation of passively sensitized HASMCs. This effect was significantly decreased by specific inhibition of PKC α with Go6976. In addition, the authors showed that transfection with antisense cyclin D1 abolished PMA-induced G1/S progression and HASMC proliferation. These results demonstrate that PKC α promotes the proliferation of HASMCs sensitized with atopic asthmatic serum via up-regulation of cyclin D1 expression.

KEYWORDS cyclin D1, human airway smooth muscle cell (HASMCs), passively sensitized, proliferation, protein kinase C α (PKC α)

Asthma is highly prevalent worldwide as a chronic disease characterized by airway hyperreactivity, inflammation, and remodeling [1], and it is now recognized as a major cause of disability, medical expense, and preventable death [2]. Despite great advances in medication to suppress airway inflammation and relax the airway, some patients with chronic and severe asthma still do not receive effective treatment, due to airway remodeling and subsequent poorly reversible airway obstruction. Many studies have demonstrated that abnormal proliferation of

airway smooth muscle cells (ASMCs) plays an important role in asthmatic airway remodeling [3–6]. However, the underlying mechanism remains unclear.

Protein kinase C (PKC) is a family of serine/threonine kinases that includes 3 types of isoenzymes. The conventional isoforms (α , β_1 , β_2 , and γ) are activated by calcium, phorbol esters, and phosphatidylserine, whereas the novel isoforms (δ , ϵ , η , θ , and μ) are calcium-insensitive but also activated by phorbol esters and phosphatidylserine. The atypical isoforms (ζ and τ/λ) are calcium- and phorbol ester-insensitive but activated by phosphatidylserine. PKC isoforms α , β_1 , β_2 , δ , ϵ , and ζ are expressed in human tracheal myocytes [7]. Differences between isoforms have been reported in terms of tissue distribution and subcellular localization as well as kinase

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activity characteristics *in vitro*. These isoforms are involved in the signaling pathways that regulate cell proliferation, differentiation, apoptosis, motility, and adhesion [8–10]. Many reports have demonstrated that the activation of PKC is a necessary event for the proliferation of ASMCs [11–15], and that different PKC isoforms may have distinct roles in the regulation of cell proliferation. In NIH3T3 cells, PKC ϵ is a powerful growth stimulus, whereas PKC α and PKC δ inhibit growth [16]. PKC δ also inhibits cell cycle progression in A7r5 vascular smooth muscle cells [17], capillary endothelial cells [18], and rat colonic epithelial cells [19]. Fukumoto *et al.* demonstrated that PKC δ inhibits the proliferation of vascular smooth muscle cells by arresting the cells in G₁, mainly via inhibition of the expression of cyclin D1 and cyclin E [17]. In human, bovine, and rat tracheal smooth muscle cells, PKC δ negatively regulates cyclin D1 transcription [20].

In smooth muscle cells, the relationship between PKC δ and cyclin D1 is well known, but the relationships between other PKC isoforms and cyclin D1 are unclear. Our laboratory has reported that PKC α contributes to the proliferation of human airway smooth muscle cells (HASMCs) passively sensitized with asthmatic serum [21]. However, the downstream mechanism of PKC α regulation is unknown. Control of the cell cycle is a fundamental part of cell proliferation. Recent studies have examined the signal transduction pathways that regulate the expression of specific cell cycle proteins in ASMCs [22]. D-type cyclins (cyclins D1, D2, and D3) have been found to be key regulators of G1 progression in mammalian cells. Progression through the G1 phase initially depends on holoenzymes composed of one or more of the D-type cyclins (*i.e.*, D1, D2, and/or D3) in association with a cyclin-dependent kinase (CDK), CDK4 or CDK6. This is followed by the activation of a cyclin E–CDK2 complex as the cells approach the G1/S transition.

Cyclin D1 is one of the major targets for several growth stimulatory signaling pathways [23, 24]. In ASMCs, cyclin D1 may be the predominant cyclin. In agreement with previous reports [25, 26], we have recently confirmed that cyclin D1 up-regulation is a key event associated with the proliferation of passively sensitized HASMCs [27].

However, whether PKC α regulates the proliferation of ASMCs via cyclin D1 expression remains unknown. Therefore, in the present study, we examined the possible role of α isoforms of PKC in the control of cyclin D1 expression, using HASMCs passively sensitized with human atopic asthmatic serum as a model system.

MATERIALS AND METHODS

Patients

The study was approved by the ethics committee of the Tongji Hospital, Tongji Medical College. Human atopic asthmatic serum containing more than 1000 IU/mL immunoglobulin E (IgE) was obtained from 16 allergic patients (7 males and 9 females, ages 22 to 40 years) with moderate to severe asthma who exhibited positive skin test responses to one or more common allergens. The sera were obtained when the subjects were in clinically stable phases and had not taken oral corticosteroids for at least 4 weeks before the study. Control serum, with a total IgE concentration of less than 20 IU/mL, was derived from 13 nonatopic, nonasthmatic individuals (5 males and 8 females, ages 25 to 35 years) who were negative for skin allergen tests [28].

Human airway smooth muscle (HASM) was obtained from the lobar or main bronchus of 7 males (average age, 45 ± 9 years; nonsmokers) undergoing lung resection for carcinoma of the bronchus, as previously described in detail by Johnson *et al.* [29]. Large bronchi (3 to 6 mm internal diameter) not invaded by the carcinoma were selected for HASM culture.

Materials

Cell culture medium, fetal bovine serum (FBS), cell culture reagents, TRIZOL reagent, and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium-bromide (MTT) were purchased from Gibco-BRL, USA. The PKC activator phorbol 12-myristate 13-acetate (PMA), presidium iodide, and ribonuclease A were purchased from Sigma-Aldrich, USA. The PKC α inhibitor Go6976 [12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo[2,3-*a*]pyrrolo[3,4-*c*]carbazole] was from Calbiochem (San Diego, CA, USA). Primary antibodies (rabbit polyclonal antibodies against β -actin, cyclin D1, CDK4, and PKC α , and a mouse monoclonal antibody against α -smooth muscle actin) were purchased from Santa Cruz Biotech (Santa Cruz, CA, USA). Antibody against phosphorylated PKC α (p-PKC α) was from BD Biosciences Pharmingen (USA), and antibody against phosphorylated PKC β 1 (p-PKC β 1) was from Millipore Corporation (USA). SP Histostain™-Plus Kits were purchased from Beijing Zhongshan Biotechnology (Beijing, China). Taq DNA polymerase and restriction enzymes were purchased from Fermentas (California, USA). Moloney murine leukemia virus (M-MLV) reverse

transcriptase was purchased from Promega (Madison, WI, USA). Lipofectamine 2000 was purchased from Invitrogen (USA). T4 DNA ligase was purchased from Takara Biotechnology (Dalian, China). Goat anti-rabbit horseradish peroxidase-conjugated secondary antibody, rabbit anti-mouse horseradish peroxidase-conjugated secondary antibody, and SuperSignal West Pico chemiluminescent substrate were purchased from Pierce Biotechnology, USA. Other reagents were commercial products with analytic purity.

Plasmid construction

Total cellular RNA was extracted from cells with TRIZOL reagent. A 1122-bp cDNA fragment encoding the full-length coding sequence of human cyclin D1 (PubMed BC023620 [gi:33991562]) was amplified from cDNA of normal HASMCs by reverse transcriptase-polymerase chain reaction (RT-PCR) and cloned in an antisense orientation into the EcoRI/XbaI-digested eukaryotic expression plasmid PCDNA3.1(+), using standard procedures. The recombinant plasmid, PCDNA3.1(+)/as-cyclinD1, was confirmed by restriction digest mapping and sequencing. Then, the antisense cyclin D1 construct was transiently transfected into HASMCs in order to reduce the level of cyclin D1 expression.

HASM cell culture and preparation

Isolated large bronchi were dissected free of connective and parenchymal tissue under sterile conditions in ice-cold Hanks' balanced salt solution with antibiotics (100 U/mL penicillin G, 100 U/mL streptomycin), and fibrous tissue and epithelium were also removed under a dissecting microscope. The tissue samples were finely chopped (into pieces approximately 1 mm³), seeded into 25-cm² vented tissue culture flasks in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% FBS, 100 U/mL penicillin, and 100 U/mL streptomycin, and placed in a 5% CO₂ humidified incubator at 37°C. After 14 to 18 days, human airway smooth muscle cells (HASMCs) migrated from the tissue explants and approached confluence around the explants. The explanted tissue was removed, and the HASMCs remaining in the culture were passaged with 0.25% trypsin. Under phase-contrast microscopy, the cells possessed a spindle shape, presented central oval nuclei with prominent nucleoli, and displayed the typical "hill and valley" morphology at confluence. Smooth muscle cell identity was verified by immunocytochemical staining for α -smooth muscle actin.

HASMCs (passages 3 to 7) from 7 patients were seeded at a density of 1×10^4 cells/cm² in DMEM supplemented with 10% FBS for all experiments. At subconfluence (80%), HASMCs were synchronized by FBS deprivation for 24 hours. The cells were then treated for 24 hours with either DMEM supplemented with 10% human normal serum or DMEM supplemented with 10% atopic asthmatic serum. Cells cultured in DMEM without serum were used as a control. In additional experiments, after synchronization, the HASMCs were treated with atopic asthmatic serum (DMEM supplemented with 10% atopic asthmatic serum) in the absence or presence of PMA (10 nM), or PMA (10 nM) following pretreatment with Go6976 (2.3 nM) for 30 minutes. In additional experiments, HASMCs were transiently transfected with PCDNA3.1(+)/as-cyclinD1 plasmid or the PCDNA3.1(+) empty vector, after a 4-hour pretreatment with PMA (10 nM), and passively sensitized with atopic asthmatic serum. Forty-eight hours later, the treated cells were harvested for further assays. Every group had 3 replicate wells for each patient.

MTT assay for cell proliferation

Cells grown in 96-well plates were treated as indicated above. MTT (5 mg/mL) was added to the wells (20 μ L/well). After a 4-hour incubation at 37°C, medium was removed from the wells, and dimethylsulfoxide was added (150 μ L/well). The plates were agitated gently at room temperature for 30 minutes. The plate cover was removed, and the absorbance (optical density) was measured for each well, including the blanks, using a spectrophotometer set to 490 nm.

Tritiated thymidine assay

[³H]thymidine incorporation was used as an indicator of cell proliferation [30]. ASMCs were seeded in triplicate in 96-well plates. [³H]thymidine was added to each well (1 μ Ci/well final concentration [3.7×10^7 kBq/mL]). After 6 hours of exposure, [³H]thymidine incorporation was assessed. Cells were washed with Dulbecco's phosphate-buffered saline (PBS), fixed with 10% trichloroacetic acid, washed again with Dulbecco's PBS, and then lysed with 1 M NaOH. The cell lysate was added to 0.5 mL scintillant and mixed. Counts per minute (cpm) were determined on a Beckman scintillation counter. Results are expressed as a percentage of the cpm in the presence of 10% atopic asthmatic serum.

Flow cytometric analysis of DNA content

HASMCs were seeded in 6-well plates. After the same treatment described above, the cells were harvested from the wells using 0.25% trypsin. Cells were washed twice, resuspended in PBS, and then fixed with cold 75% ethanol at 4°C overnight. After resuspension in PBS containing 1% bovine serum albumin (BSA), cells were treated with propidium iodide (50 µg/mL) and RNase A (1 mg/mL). Data from the stained cell suspensions were acquired on a FACSort Flow Cytometer (BD Bioscience, USA) using Cell Quest software. The resulting DNA profiles were analyzed to determine the percentage of cells in each phase of the cell cycle.

Reverse transcriptase–polymerase chain reaction

Semiquantitative RT-PCR was used to measure mRNA expression. Total cellular RNA was extracted from cells with TRIZOL reagent, and the gene of interest was amplified by RT-PCR. Reverse transcription reactions were performed using 1 µg of mRNA extract and the M-MLV reverse transcriptase kit (Promega), and PCR was performed with a Taq polymerase kit (Fermentas, USA). β -Actin mRNA was used as an internal housekeeping gene control. PCR primer sequences, PCR conditions used, and the expected sizes of the respective amplicons are shown in Table 1. The PCR mixture consisted of final concentrations of 1.5 mM MgCl₂, 1× PCR buffer, 0.2 mM dNTP mixture, 2.5 units Taq polymerase, 20 pmol of the upstream and downstream primers, as well as the synthesized cDNA strand. Each of the 35 PCR cycles was programmed to carry out denaturation at 94°C for 60 seconds, primer annealing for 50 seconds, extension at 72°C for 60 seconds, all followed by a final extension at 72°C for 10 minutes. PCR products were visualized by ethidium bromide staining after 2% agarose gel electrophoresis, and the sizes of the bands were determined by comparison with DNA molecular weight markers. The calculation of the size of the amplicons and the semiquantitative PCR analysis of the agarose gels was performed using the Gel Doc XR system and Quantity One analysis software

(version 4.6) (Bio-Rad Laboratories, USA). Densitometry data were normalized against β -actin values.

Western blotting

HASMCs were washed with cold PBS and detached with trypsin. Whole-cell protein was extracted on ice for 30 minutes with lysis buffer (50 mM Tris-HCl [pH 8.0], 0.15 M NaCl, 0.1% sodium dodecyl sulfate [SDS], 100 µg/mL phenylmethylsulfonylfluoride, 1 µg/mL aprotinin, 0.02% sodium azide, 0.5% sodium deoxycholate, and 1% Nonidet P-40). Samples were centrifuged at 12,000 rpm for 10 minutes at 4°C. The supernatants were collected as whole-cell fractions. Protein concentration was measured using the Bradford method. Equal amounts of protein extracts (40 µg) were combined with a 5× gel loading buffer (10% SDS [*w/v*], 50% glycerol [*v/v*], 0.25 M Tris-HCl [pH 6.8], 0.025% bromophenol blue [*w/v*], and 0.5 M dithiothreitol) and boiled for 10 minutes before cooling to room temperature. Proteins were subjected to SDS–polyacrylamide gel electrophoresis (12% *w/v*) at 80 V for 2 hours in a buffer consisting of 0.1% SDS, 25 mM Tris, and 250 mM glycine (pH 8.4) and then transferred onto a nitrocellulose membrane. The membrane was blocked with Tris-buffered saline (TBS: 10 mM Tris and 150 mM NaCl, pH 7.6) containing 0.05% Tween 20 (TBS-T) and 5% nonfat milk at room temperature for 2 hours. After a brief rinse with TBS-T, the membrane was incubated overnight at 4°C with a monoclonal or polyclonal antibody at a 1:500 dilution (except the polyclonal antibody against β -actin, which was used at a 1:1000 dilution) in TBS-T containing 5% dried skim milk. The membrane was washed three times with TBS-T before being incubated with horseradish peroxidase–conjugated anti-mouse or anti-rabbit IgG antibody (1:2000 to 1:3000 in TBS-T containing 5% dried nonfat milk) for 1 hour. The membrane was then washed again and visualized using the SuperSignal West Pico Chemiluminescent Substrate kit before exposure to Kodak film. To control for equal loading of protein, the membrane was probed with anti- β -actin antibody. Relative absorbances of the resultant bands were determined using the Quantity One imaging system (Bio-Rad). Densitometry data were normalized to β -actin values.

TABLE 1 Summary of PCR Primer Sequences, PCR Conditions Used, and the Expected Size of the Respective Amplicons

Gene	Upstream primer	Downstream primer	Annealing (°C)	Cycle number	Amplicon (bp)
cyclin D1	5'-GCGAGGAACAGAAGTGC-3'	5'-GGCGGATTGGAAATGA-3'	50	35	397
CDK4	5'-CATCCCGAACTGACCG-3'	5'-GCTGGCAAGCCTGGTG-3'	50	35	103
β -Actin	5'-TCGACAACGGCTCCGGCAT-3'	5'-AAGGTGTGGTGCCAGATTTTC-3'	50	35	241

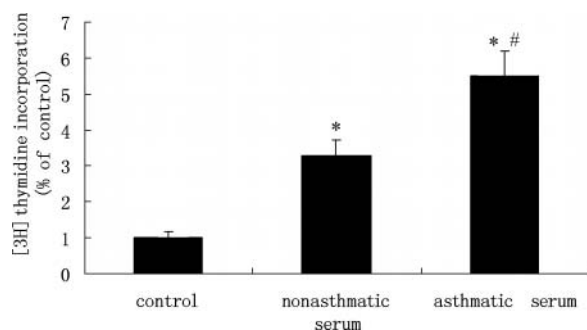


FIGURE 1 Effect of human atopic asthmatic serum on the proliferation of HASMCs. [^{3} H]thymidine incorporation was used as an indicator of cell proliferation. Cells were derived from 4 donors. Values (mean $\pm$ SEM) are expressed as percentages of the control. * P < .01 compared with the control; # P < .01 compared with HASMCs treated with nonasthmatic serum.

Data analysis

Data are expressed as mean $\pm$ standard error of the mean (SEM). Each result for HASMCs was from triplicate wells for each patient. The significance of differences between experimental groups was analyzed by ANOVA (Bonferroni-Dunn test) or a t test, with values of P < .05 considered statistically significant.

RESULTS

Human atopic asthmatic serum induced proliferation of HASMCs

In this study, HASMCs were incubated with atopic asthmatic serum. [^{3} H]thymidine incorporation data indicated that there was a significant difference (P < .01, n = 4) between the proliferation of HASMCs treated with atopic asthmatic serum and those treated with nonatopic nonasthmatic serum (Figure 1). These results suggest that HASMCs were passively sensitized.

PMA promoted PKC α activation in passively sensitized HASMCs, whereas Go6976 specifically inhibited PKC α

To determine the effect of α isozymes of PKC on the proliferation of passively sensitized HASMCs, we stimulated the cells with the PMA to activate PKC α and used Go6976 to inhibit PKC α . Go6976 is an isotype-selective PKC inhibitor that is selective for α (IC_{50} = 2.3 nM) and β 1 (IC_{50} = 6.2 nM) isotypes. Sirosh M. Bokhari and colleagues have shown that Go6976 (2.3 nM) specifically inhibits PKC α in

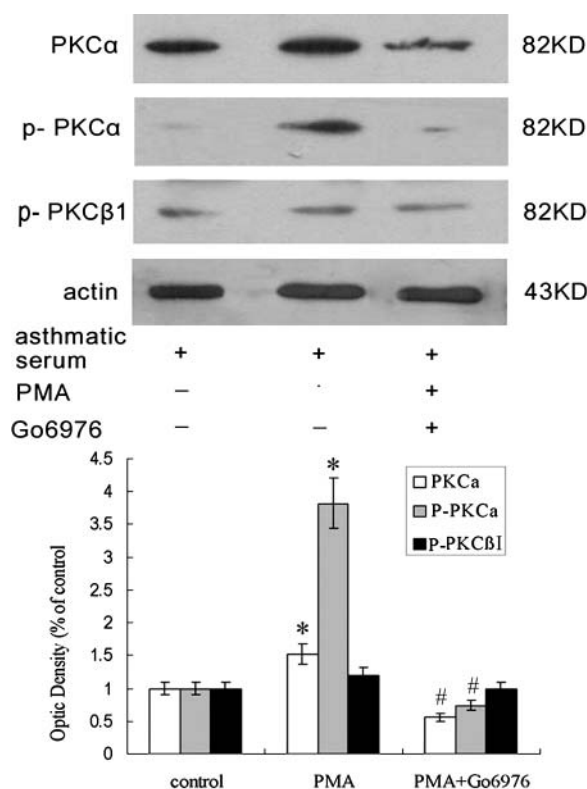


FIGURE 2 Effects of PMA and Go6976 on PKC α activation and expression. Densitometric evaluation of immunoblots obtained from 4 independent experiments using cells derived from different donors. Values (mean $\pm$ SEM) are expressed as percentages of the control. * P < .01 compared with the control (HASMCs treated with human asthmatic serum alone); # P < .01 compared with the HASMCs treated with human asthmatic serum in the presence of PMA.

human dermal microvascular endothelial cells [31]. Our preliminary experiments have determined that Go6976 (2.3 nM) also specifically inhibits PKC α in human airway smooth muscle cells. Immunoblotting was performed on cell lysates from the HASMCs to detect PKC α phosphorylation at threonine 638, an indicator of PKC α activity [32–34], and the overall expression of the PKC α protein. p-PKC β 1 levels in these cells were also detected (Figure 2). In HASMCs that were treated with a combination of PMA and atopic asthmatic serum, the protein expression levels of PKC α , p-PKC α , and p-PKC β 1 were all significantly elevated, compared with those of the HASMCs treated with atopic asthmatic serum alone. The specificity of Go6976 for PKC α is demonstrated in Figure 2. When HASMCs were pretreated with Go6976, followed by activation with PMA, there was a marked inhibition of p-PKC α as compared to levels in HASMCs treated with PMA alone, whereas p-PKC β 1 was not affected significantly (P > .05, n = 4).

PMA induced proliferation of passively sensitized HASMCs, and this effect was attenuated by pretreatment with Go6976

The number of viable, adherent cells remaining was assessed by an MTT assay (Figure 3A). The number of adherent, viable HASMCs markedly increased upon PMA stimulation ($P < .01$, $n = 4$). After pretreatment with Go6976, the stimulatory effect of PMA was significantly reduced ($P < .01$, $n = 4$).

[^3H]thymidine incorporation assays to detect DNA synthesis and cell cycle activation were also performed after stimulation with PMA and/or Go6976. As shown in Figure 3B, PMA induced a 2.5-fold increase in HASMC proliferation relative to untreated controls. However, when Go6976 was added 30 minutes before PMA administration, the proliferative effect of PMA was markedly inhibited.

Cell cycle distribution was analyzed by flow cytometry (Figure 3C). After PMA treatment, the percentage of cells in S phase significantly increased, whereas Go6976 pretreatment markedly inhibited the transition of the cells into S phase.

These results implicate PKC α in the positive regulation of the proliferation of passively sensitized HASMCs. In addition, we also found that the specific inhibition of PKC α did not completely abrogate the PMA-induced proliferation.

PMA increased both mRNA and protein expression of cyclin D1 in HASMCs treated with atopic asthmatic serum, and this effect was reduced by Go6976

To examine the effect of PMA and Go6976 on cyclin D1 and CDK4 expression, we detected both the mRNA and protein expression of the genes of interest by semiquantitative RT-PCR and Western blotting, respectively. There was a significant difference ($P < .01$, $n = 4$) between cyclin D1 mRNA levels in the presence and absence of PMA in passively sensitized HASMCs. Pretreatment with Go6976 significantly diminished cyclin D1 up-regulation in response to PMA ($P < .01$, $n = 4$; Figure 4A). Consistent with the changes in cyclin D1 mRNA levels, there was a significant increase in the protein expression of cyclin D1 in the presence of PMA as compared to that in the absence of PMA, as determined by densitometry of Western blots. Pretreatment with Go6976 significantly attenuated the effect of PMA ($P < .05$, $n = 4$; Figure 4B). In contrast, there was no significant change in CDK4 mRNA or protein expression in either the group treated with PMA alone or the group treated with PMA and Go6976 ($P > .05$, $n = 4$). From the data presented above, we found that the

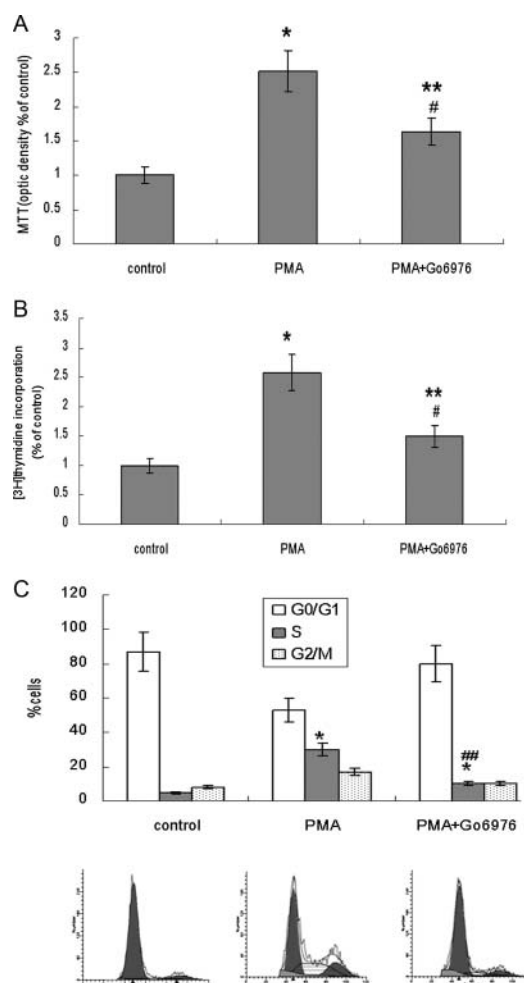


FIGURE 3 Effects of PMA and Go6976 on the proliferation of passively sensitized HASMCs. The number of viable, adherent cells remaining was assessed by MTT assays, cell proliferation was examined by [^3H]thymidine incorporation assays, and cell cycle distribution was determined by flow cytometric analysis. **(A)** MTT assay on HASMCs 24 hours after treatment with human atopic asthmatic serum in the absence (control) or presence of PMA (10 nM) or the presence of PMA (10 nM) after 30-minute pretreatment with Go6976 (2.3 nM). Optical density values were obtained from 4 independent experiments using cells derived from different donors. Values (mean $\pm$ SEM) are expressed as percentages of the control. * $P < .01$ compared with the control; ** $P < .05$ compared with the control, # $P < .01$ compared with PMA-treated cells. **(B)** DNA synthesis was evaluated with a 6-hour pulse of [^3H]thymidine after treatment. Results are expressed as percentages of the control (cells treated with asthmatic serum alone). Values (mean $\pm$ SEM) were obtained from cells derived from four donors. Criteria for significance were as above. **(C)** Cells were stained with propidium iodide and analyzed by flow cytometry. Representative panels depicting cell cycle distribution are shown. Similar results were observed in four independent experiments. * $P < .01$ compared with the control; ## $P < .05$ compared with PMA-treated cells.

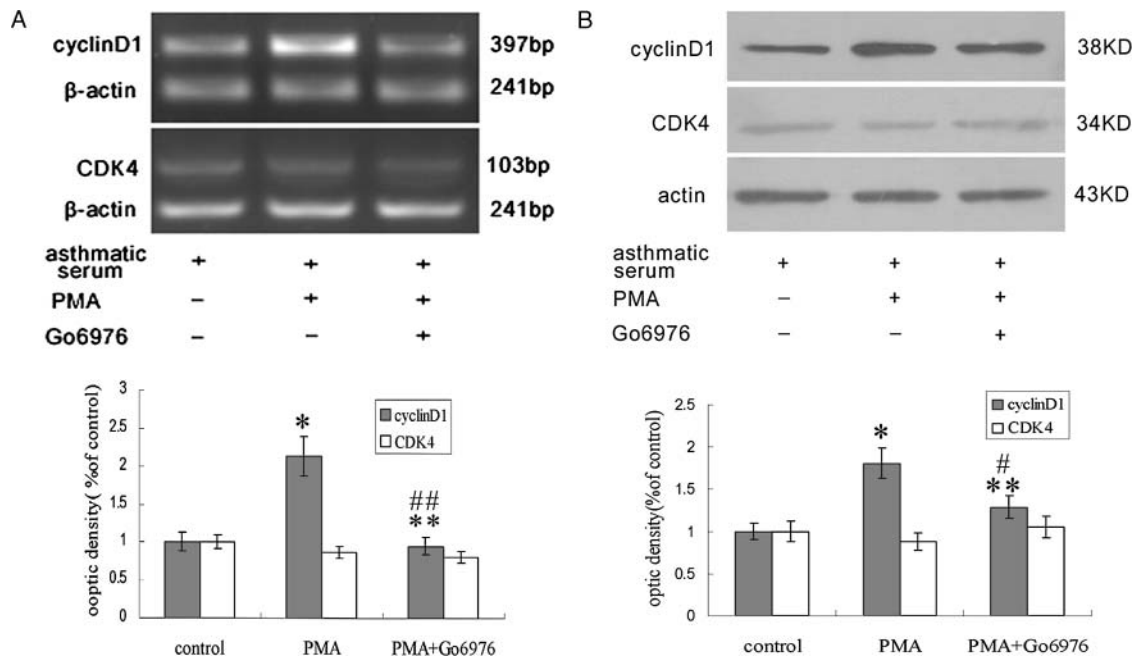


FIGURE 4 Effects of PMA and Go6976 on the expression of cyclin D1 in passively sensitized HASMCs. **(A)** mRNA levels were normalized against β -actin mRNA levels and listed as percentages of the β -actin control. Values (mean $\pm$ SEM) were from 4 independent experiments performed from cells from different donors. * $P < .01$ compared with the control; ** $P < .05$ compared with the control; ## $P < .05$ compared with PMA-treated cells. **(B)** Densitometric evaluation of immunoblots obtained from 4 independent experiments. Values (mean $\pm$ SEM) are expressed as percentages of the response in control cells. * $P < .01$ compared with the control; ** $P < .05$ compared with the control, # $P < .01$ compared with PMA-treated cells.

trend of changes in cyclin D1 expression was the same as that of PKC α in response to PMA and Go6976. In addition, we also found that the specific inhibition of PKC α could not abrogate the PMA-induced up-regulation of cyclin D1 expression.

Transfection with PCDNA3.1(+)/as-cyclinD1 abolished PMA-stimulated HASMC proliferation

Based on the above results, we hypothesized that cyclin D1 may play a key role in the proliferation of passively sensitized HASMCs in response to PKC α . To confirm this hypothesis, HASMCs were transiently transfected with PCDNA3.1(+)/as-cyclinD1 after being synchronized and treated with PMA (10 nM) for 4 hours. After 48 hours, the number of HASMCs was quantified by MTT and [^{3} H]thymidine incorporation assays and flow-cytometric analysis. Indeed, upon transfection with a cyclin D1 antisense construct, cyclin D1 protein expression was significantly decreased (Figure 5A), and the G1/S progression and proliferation induced by PMA were abolished (Figure 5B, C, D). These data confirm that cyclin D1 is required for PKC α to induce the proliferation of passively sensitized HASMCs.

DISCUSSION

Our previous studies have indicated that PKC α is involved in the proliferation of passively sensitized HASMCs [21], and a recent study suggested that cyclin D1 up-regulation was a key event associated with the proliferation of passively sensitized HASMCs [27]. Therefore, we hypothesized the existence of a causal relationship between PKC α and cyclin D1 in the proliferation of HASMCs.

Because airway smooth muscle cells from asthmatic patients are difficult to acquire, many research groups have adopted the technique of passive sensitization, an induced model of hyperresponsiveness in vitro. The use of serum from allergic asthmatic individuals induces nonasthmatic airway smooth muscle to demonstrate changes in contractile responses, cytokine production, and proliferation that may reflect characteristics of asthmatic smooth muscle [28, 29]. In this study, we incubated HASMCs with atopic asthmatic serum to passively sensitize the cells. [^{3} H]thymidine incorporation data indicated that there was a significant difference in the proliferation of HASMCs in the atopic asthmatic serum group and nonatopic nonasthmatic serum group. This result was consistent with previous studies

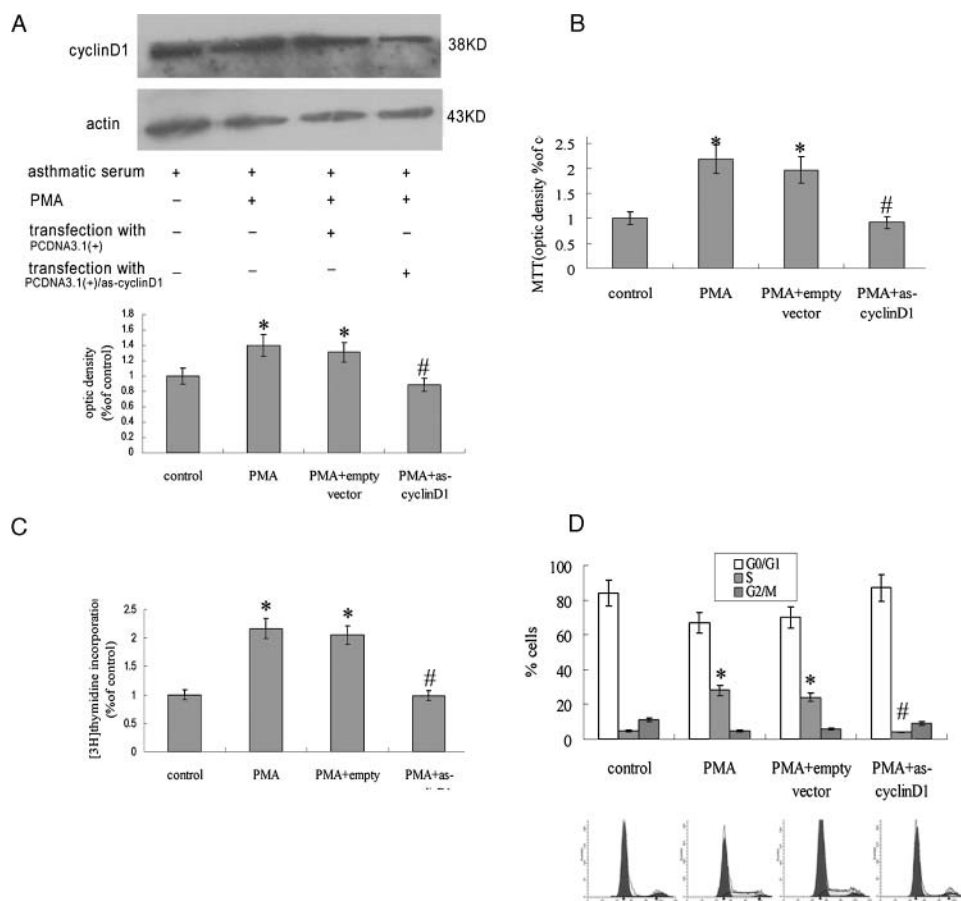


FIGURE 5 The effect of transfection with PCDNA3.1(+)/as-cyclinD1 on the proliferation of sensitized HASMCs. **(A)** Blots from 4 independent experiments using cells from different donors were probed for cyclin D1. Densitometry values (mean $\pm$ SEM) are expressed as percentages of the control. * $P < .01$ compared with the control; # $P < .01$ compared with PMA-treated cells. **(B)** Optical density values from the MTT assay were obtained from 4 independent experiments performed from cells from different patients. Values (mean $\pm$ SEM) are expressed as percentages of the control. * $P < .01$ compared with the control; # $P < .01$ compared with PMA-treated cells. **(C)** DNA synthesis was evaluated with a 6-hour pulse of [^{3} H]thymidine after treatment. Results (mean $\pm$ SEM) are expressed as percentages of the control. * $P < .01$ compared with the control; # $P < .01$ compared with PMA-treated cells. **(D)** After transfection, cells were stained with propidium iodide and analyzed by flow cytometry. Representative panels depicting cell cycle distribution are shown. Similar results were observed in 4 independent experiments. * $P < .01$ compared with the control; # $P < .01$ compared with PMA-treated cells.

[28] and confirmed that passive sensitization was successful.

In the current study, the proliferation of HASMCs increased dramatically with increased expression of PKC α (specifically, PKC α phosphorylated at T638). Conversely, when PKC α and p-PKC α levels decreased, the proliferation of HASMCs also significantly decreased. These results show that in response to PMA, both the increase and activation of PKC α play a predominant role in cell proliferation, rather than being a consequence of proliferation.

In our study, PMA up-regulated cyclin D1 protein levels and induced cell proliferation, whereas Go6976

decreased cyclin D1 protein levels and attenuated the PMA-induced proliferation. Neither the pharmacological activator nor the inhibitor showed any effect on CDK4 expression. The change in cyclin D1 protein levels was consistent with PKC α and p-PKC α protein levels and with our hypothesis that cyclin D1 is required for the proliferation of passively sensitized HASMCs in response to PKC α . Therefore, to test this hypothesis, we transiently transfected HASMCs with PCDNA3.1(+)/as-cyclinD1 to inhibit cyclin D1 expression. The results further increased the strength of the evidence for a causal relationship between PKC α and cyclin D1 in HASMCs. The fact that

PMA-induced proliferation was abolished by cyclin D1 antisense indicates that cyclin D1 lies downstream of PKC- α and is required for PKC α -induced proliferation. These results are consistent with the report that activation of PKC α in mouse fibroblasts plays an important role in enhancing cell cycle progression and cell proliferation [35].

Understanding the signaling pathways that regulate cyclin D1 expression in airway smooth muscle may provide insight into the pathogenesis of asthma, which has been associated with an increase in airway smooth muscle cell number. Previous work in this area has focused on positive regulatory pathways of cyclin D1 expression, particularly the extracellular signal-regulated kinase (ERK) [36, 37] and phosphoinositol 3-kinase (PI3K) signaling pathways [38, 39]. In the present study, we have demonstrated that PKC α positively regulates cyclin D1 expression, but the precise mechanism by which PKC α regulates cyclin D1 has not been elucidated. Indeed, the increase in proliferative capacity observed in HASMCs overexpressing PKC α is perhaps not surprising when the downstream targets of PKC α are considered. PKC α has been shown to phosphorylate Raf-1 kinase, which leads to the activation of the ERK-mitogen-activated protein kinase (MAPK) cascade, thus promoting cellular proliferation in a number of different cell types [40–43]. PKC α can also promote transcriptional activity at the cyclin D1 promoter, mediated mainly through the activator protein (AP)-1 enhancer element [35]. Additionally, previous reports suggest that PKC α acts upstream of PKC θ in the T-cell receptor-initiated pathway that leads to nuclear factor (NF)- κ B activation [44]. The events presented above represent a possible mechanism for PKC α -induced cyclin D1 expression in passively sensitized HASMCs. However, these signals and other unknown signaling pathways involved in the proliferative effect of PKC await confirmation through further research.

In the present study, although Go6976 significantly inhibited PMA-mediated responses, PMA-induced up-regulation of cyclin D1 and cell proliferation were not completely abolished by the inhibition of PKC α . It is likely that even though PKC α plays a predominant role in cell proliferation in response to PMA, it is not the only pathway that participates in this process. As an activator of conventional and novel PKCs, PMA may induce the up-regulation of cyclin D1 and proliferation of passively sensitized HASMCs by signal transduction through other PKC isoforms.

Because cyclin D1 expression is required for airway smooth muscle DNA synthesis [45], further studies into the suppression of cyclin D1 expression may be helpful for the development of therapeutic interven-

tions for asthma and other diseases in which abnormal cellular proliferation is an important feature.

CONCLUSIONS

In conclusion, our results provide evidence that the activation of PKC α in passively sensitized HASMCs induces cyclin D1 expression, thereby promoting cell cycle progression and cell proliferation. Thus, PKC α promotes the proliferation of HASMCs sensitized with atopic asthmatic serum by up-regulating cyclin D1 expression.

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