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Blocking of Akt/NF-κB Signaling by Pentoxifylline Inhibits Platelet-Derived Growth Factor–Stimulated Proliferation in Brown Norway Rat Airway Smooth Muscle Cells

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ABSTRACT: The proliferation of airway smooth muscle cells (ASMC) can cause airway hyperresponsiveness (AHR). It has been reported that platelet-derived growth factor (PDGF) can stimulate the proliferation of ASMC through phosphatidylinositol 3-kinase (PI3 K) signaling pathway, which can activate Akt protein. Activated-Akt can activate downstream signal protein [$p70^{S6\ K}$, nuclear factor (NF)-κB, and extracellular signal regulated kinase (ERK)], increasing the cyclin D1 level and suppressing the transcription of $p27^{Kip1}$ to enable cell cycle entry. This investigation demonstrated that pentoxifylline (PTX) inhibited the PDGF-stimulated proliferation of ASMC by suppressing activation of the Akt/NF-κB pathway. ASMC were treated with PTX for 48 h, which attenuated the PDGF-stimulated proliferation of ASMC. PTX and wortmannin, a PI3 K inhibitor, not only inhibited the PDGF-activated phosphorylation of Akt but also suppressed $p70^{S6\ K}$ expression and IκBα degradation, inhibiting nuclear translocation and the DNA binding activity of NF-κB. However, PTX did not influence the phosphorylation of ERK1/2. The suppression of $p70^{S6\ K}$ by rapamycin did not influence cyclin D1 expression in PDGF-stimulated cells. These data reveal that the down-regulation of the Akt/NF-κB signaling pathway by PTX inhibited the proliferation of ASMC. PTX may provide information on the pathogenesis of asthma. (***Pediatr Res* 60: 657–662, 2006**)

In asthma pathogenesis, an injured epithelium releases several mediators, including endothelin-1 (ET-1), epidermal growth factor (EGF), IGF, and PDGF, to induce the proliferation of ASMC (1–4). The proliferation of ASMC is believed to be important in causing AHR, a prominent feature of airway remodeling (5). Two major pathways—ERK 1/2 and PI3 K—are involved in the proliferation of ASMC. PI3 K pathway is involved in a very important way (6,7). PDGF stimulates ASMC to enter the cell cycle (8). PDGF activates downstream effectors, including Raf-1, which activates Akt, a major downstream substrate of PI3 K pathway. Activated-Akt transmits signals that then increase the cyclin D1 level (9,10), repressing the transcription of CDK inhibitors, including $p27^{Kip1}$, permitting entry into the cell cycle (11). Cyclin D1 is an important key regulator of the G1/S transition and blocks the expression of cyclin D1, inhibiting the proliferation of ASMC (12). The cyclin D1 promoter contains nuclear factor (NF)-κB regulatory elements (13), and NF-κB also appears to be a target of activated Akt (14). However, the role of Akt/ NF-κB in the proliferation of AMSC is unclear.

PTX, a phosphodiesterase inhibitor, effectively inhibits the development of autoimmune diabetes mellitus (15), experimental allergic encephalomyelitis (16), and asthma in animal models (17). Furthermore, PTX effectively reduces the proliferation of vascular smooth muscle (18) and renal mesangial cells (19). However, whether PTX suppresses the proliferation of ASMC is unknown. PTX can reduce NF-κB activity to inhibit the proliferation of cells (20). The relationship between the Akt and the NF-κB activity, and whether PTX can inhibit the proliferation of ASMC by inhibiting Akt/NF-κB pathway, are unclear.

In this study, the mechanism by which PTX inhibits the proliferation of ASMC was investigated. PTX and wortmannin, a PI3 K inhibitor, inhibited the PDGF-activated phosphorylation of Akt, IκBα degradation and nuclear translocation, and the DNA binding activity of NF-κB. PTX did not affect the phosphorylation of ERK1/2 but did inhibit the phosphorylation of $p70^{S6\ K}$. However, the blocking of the $p70^{S6\ K}$ pathway by rapamycin did not influence the expression of cyclin D1 induced by PDGF. These results indicated that PTX did not suppress expression of cyclin D1 *via* the Akt/$p70^{s6k}$ signaling pathway. Instead, PTX inhibited the expression of cyclin D1 and cell cycle entry by suppressing the Akt/NF-κB pathway.

MATERIALS

Culture of ASMC. Adult, 6–8 wk, male Brown Norway rats were obtained from the National Laboratory Animal Center (Taipei, Taiwan). These animals were raised in normal conditions in the animal center of Changhua Christian Hospital. The study was approved by the Institutional Animal Care and Use Committee of Changhua Christian Hospital. Tracheas and bronchi were taken from Brown Norway rats. Tissues were incubated in Hanks' balanced salt solution (HBSS) with 0.1% collagenase solution (Sigma Chemical Co., St.

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Abbreviations: AHR, airway hyperresponsiveness; **ASMC,** airway smooth muscle cells; **EMSA,** electrophoretic mobility shift assay; **ERK,** extracellular signal regulated kinase; **PI3 K,** phosphoinositide-3 kinase; **PTX,** pentoxifylline

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Louis, MO) at 37°C for 20 min. Loosened connective tissues were scraped. The tissues were cut into 0.5 mm^3 fragments, which were placed in a culture dish with complement Dulbecco's modified Eagle's medium (DMEM)/F12, 10% fetal bovine serum (FBS), 2 mM glutamine, and 100U/mL penicillin/streptomycin (all from Invitrogen, Carlsbad, CA). They were then incubated at 37°C. Passages six to eight were used in the experiments. Cultured ASMC expressed α-smooth muscle actins, detected immunocytochemically.

Evaluation of cell proliferation and cell cycle progression. XTT labeling mixture reagents were used (cell proliferation Kit II, Roche Molecular Biochemicals, Indianapolis, IN). Growth-arrested cells were stimulated with recombinant rat PDGF-BB (40 ng/mL; R & D Systems, Minneapolis, MN) in the presence or absence of PTX (1–10 mM) (Sigma Chemical Co.) or PTX alone for 48 h. XTT mixture reagent was added to each well and incubated for 4 h; the absorbance at 490 nm was measured.

Growth-arrested cells were stimulated with PDGF-BB in the presence or absence of PTX for 18 h to elucidate cell cycle progression. The harvested cell pellet was added to 3 mL of cold 70% ethanol and maintained at –20°C for 30 min. The cell pellet was resuspended with 1% Triton X-100, 0.1 mg/mL RNase A and 4 μg/mL propidium iodide after centrifuging. The flow cytometry (FC 500, Beckman Coulter, Inc., Fullerton, CA) was used to elucidate cell cycle progression.

Apoptosis assay and necrosis assay. Terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) assay (Mebstain apoptosis kit II, Immunotech, Marseille, France) was used to determine the percentage of cells that exhibited apoptosis. Briefly, cells were fixed with 4% paraformaldehyde, permeated with 70% ethanol, and labeled with TdT reagent before 30 μL of avidin-FITC solution was added. The cells were washed twice with PBS and the pellet was resuspended in the dark. The cells were then ready for analysis using flow cytometry.

Annexin V-FITC Apoptosis Detection kit (Strong Biotech Corporation, Taiwan) was used to determine the percentage of necrotic cells. Briefly, the treatment of cells was washed with PBS and centrifuged cells at 200 g for 5 min. The cell pellet was resuspended in staining buffer for 15 min at 25°C. The cells were ready for analysis using flow cytometry. The cells were in the range of annexin V positive and PI positive, indicating necrosis cells.

RNA extraction and reverse transcriptase PCR. Total RNA was isolated using TRI reagent (Molecular Research Center, Cincinnati, OH). The cDNA was primed with oligo$(dT)_{12-18}$ and extended with reverse transcriptase (CLONTECH, Palo Alto, CA). cDNA was amplified by PCR using following primer pairs: cyclin D1, forward, 5′-CTGACACCAATCTCCTCAAC-3′; reverse, 5′-GTAGATGCACAACTT CTGG-3. GAPDH, forward, 5′-TGAAGGTCGGAGTCAACGGATTTGG-3′; reverse, 5′-CATGTGGGC-CATGAGGTCCACCAC-3′. The PCR reaction production was run on 2% agarose gel in a TAE buffer and identified after staining with ethidium bromide. The intensity of the signal was quantified (Image Station 2000R, Eastman Kodak, Rochester, NY) and normalized against GAPDH messages.

Extraction of protein and Western blot analysis. Total cellular proteins were extracted using a lysis buffer, and cytoplasm and nuclear proteins were obtained using NE-PER reagents (Pierce Biotechnology, Rockford, IL). The protein concentration was determined using Bio-Rad protein assay (Bio-Rad, Hercules, CA). Antibodies against cyclin D1, Akt, Ser473-phosphorylated Akt, phosphorylated glycogen synthase kinase 3α/β (GSK3α/β), phosphorylated $p70^{S6\ K}$ (Thr389, Thr421/Ser424), $p70^{S6\ K}$, phosphorylated ERK1/2, ERK1/2, IκBα and β-actin were purchased from Cell Signaling Technology (Beverly, MA). Antibodies against NF-κB and $p27^{kip1}$ were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The secondary antibodies were purchased from Pierce Biotechnology. Cell proteins were fractionated by electrophoresis on a 10% SDS-polyacrylamide gel and were transferred onto nitrocellulose membrane, blocked, and probed with various primary antibodies. Following incubation with primary antibodies (1:1000) overnight, the membrane was washed and incubated with horseradish peroxidase–conjugated secondary antibody (1:10000) for 1 h. The blot was washed and visualized by enhanced chemiluminescence (Pierce Biotechnology). The bands were quantified and normalized against β-actin messages.

Akt kinase assays. Akt activity was assayed using an Akt kinase assay kit (Cell Signaling Technology). Briefly, 100 μg of total cellular extracts were immunoprecipitated with anti-Akt antibody and incubated with 200 μM ATP and 1 μg of GSK3 fusion protein for 30 min. The reactions were separated by SDS-PAGE and analyzed by Western blot with antibody against p-GSK3α/β and Akt.

EMSA. An EMSA gel-shift kit was used (Pierce Biotechnology). In a binding reaction, 6 μg nuclear protein was incubated with 2 μL of 3′ end-labeled probe and 1 μg poly (dI-dC) for 20 min. Electrophoresis was performed on 4.8% polyacrylamide gel at 4°C. Samples were transferred to (+) nylon membranes, blocked in blocking buffer, and then incubated with anti-biotin fragments (1:10,000) conjugated with horseradish peroxidase. Finally, bands were visualized by enhanced chemiluminescence.

Immunocytochemistry. Treated cells that grew on coverslips were washed with PBS, fixed for 20 min with 3.7% paraformaldehyde, permeabilized with 0.2% Triton/PBS for 10 min, blocked with 1% BSA in PBS overnight, and incubated with anti-NF-κB (p65) antibodies (2 μg/mL) for 1 h. Following washing, rhodamine-conjugated secondary antibodies (1:400) were incubated for 1.5 h. The cells on coverslips were mounted in DAPI reagent (Molecular Probes, Eugene, OR) and were analyzed using a fluorescence microscope (IX-50, Olympus Optical, Hamburg, Germany).

Statistical analysis. Statistical analyses were carried out using SPSS/Windows (SPSS Science, Chicago, IL) software. The statistical significance was evaluated by one-way ANOVA.

RESULTS

PTX inhibited PDGF-stimulated proliferation of ASMC. The role of PTX in the PDGF-stimulated proliferation of ASMC was investigated. After serum deprivation for 48 h, growth-arrested ASMC were treated with 10, 20, 40, 60, 80, and 160 ng/mL rrPDGF-BB. After incubation for 48 h, the rate of proliferation of cells was assayed by XTT. The net proliferation rate (ΔOD) was determined ($OD_{48\ h} - OD_{0\ h}$) (Fig. 1*A*). PDGF 40 ng/mL stimulated the greatest proliferation rate: PDGF 40 ng/mL, 0.53 ± 0.07; PDGF 10 ng/mL, 0.43 ± 0.01; PDGF 20 ng/mL, 0.45 ± 0.05; PDGF 60 ng/mL, 0.52 ± 0.05; PDGF 80 ng/mL, 0.50 ± 0.02; PDGF 160 ng/mL, 0.44 ± 0.02; medium only, 0.27 ± 0.02; $p < 0.05$. Growth-arrested cells were treated with PDGF 40 ng/mL and PTX (0.1, 0.5, 1, 5, and 10 mM) for 48 h. The proliferation of

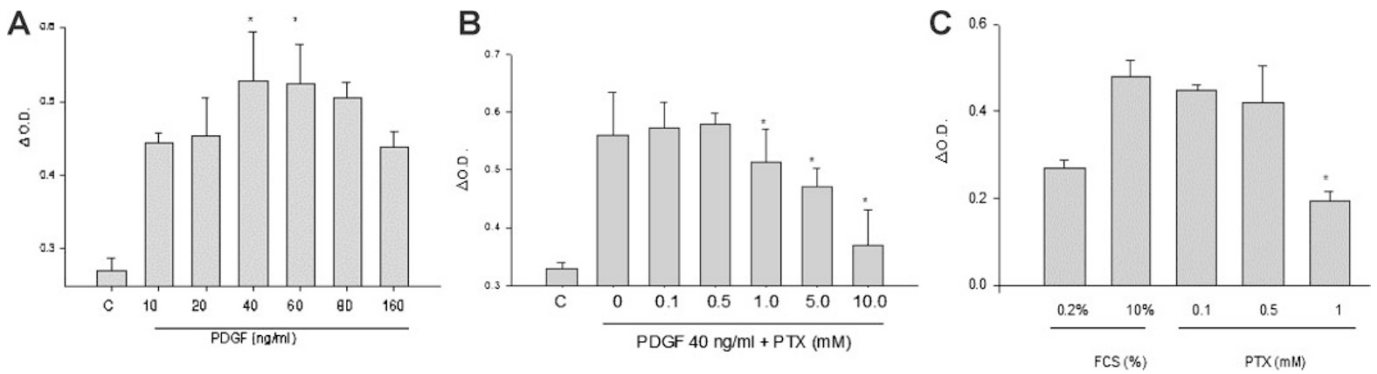

Figure 1. (*A*) Proliferation of cells in the presence of rrPDGF-BB for 48 h. ASMC were starved for 48 h and treated with rrPDGF-BB (10–160 ng/mL). (*B*) PTX inhibited the PDGF-stimulated proliferation of cells. Starved ASMC were treated with rrPDGF-BB (40 ng/mL) and PTX (1–10 mM). (*C*) ASMC were cultured in the complement of medium and treated with PTX (1 mM). After 48 h of incubation, OD values were obtained by XTT assay. ΔOD values were calculated ($\Delta OD = OD_{48\ h} - OD_{0\ h}$). Results are mean ± SD from three individual experiments. *$p < 0.05$.

cells was reduced: PDGF 40 ng/mL, 0.56 ± 0.07; PDGF 40 ng/mL + PTX 1 mM, 0.51 ± 0.05; $p < 0.05$ (Fig. 1*B*). The treatment of PTX alone affected growth of cells cultured in 10% FBS-containing medium (Fig. 1C). These results indicated that PTX inhibited the proliferation of PDGF-stimulated ASMC.

The effects of PTX on the cell cycle progression were elucidated. Growth-arrested cells were stimulated with PDGF in the presence or absence of PTX (1 mM) for 18 h, and cell cycle profiles were obtained by flow cytometric analysis. When growth-arrested ASMC were stimulated with PDGF for 18h, only 44.74% of the cells remained in G_1/G_0 phase. PTX significantly reduced the PDGF-stimulated G_1-to-S progression, and 64.82% of cells had remained in G_1/G_0 phase (Fig. 2*A*).

The percentage of apoptotic cells was determined by TUNEL assays to confirm that the antiproliferative effect of PTX could not be attributed to its cytotoxicity. The results indicated that PTX (1 mM) did not induce an apoptosis response (PDGF, 9.1 ± 1.9%; PTX, 3.8 0.2%) (Fig. 2*B*). The percentage of necrotic cells was determined by annexin V-FITC apoptosis detection kit (Strong Biotech Corporation), and these results also indicated that PTX (1 mM) did not induce a necrosis response (PDGF, 4.3 ± 0.3%; PTX, 3.87 ± 0.5%) (Fig. 2*C*). PTX inhibited the proliferation of cells by arresting the cell cycle, not by apoptosis or necrosis.

PTX regulates cyclin D1 and p27^{kip1} expression in ASMC. Cyclin D1 is an important regulator protein in the early G_1 phase of the cell cycle (21). In this investigation, PTX arrested PDGF-stimulated cells in G_1/G_0 phase and influenced the level of cyclin D1.

Growth-arrested ASMC were stimulated with PDGF (40 ng/mL) in the presence or absence of PTX (1 mM). The level of cyclin D1 mRNA increased 2–24 h after PDGF stimulation (arrest group: 0.95 ± 0.01; PDGF 40 ng/mL, 6 h: 2.75 ± 0.18) (Fig. 3*A*). The levels of cyclin D1 mRNA after stimulation of PDGF and PTX were lower than those of PDGF-treated cells at all times (PDGF 40 ng/mL, 6 h: 2.75 ± 0.18; PDGF 40 ng/mL + PTX 1 mM, 6 h: 0.75 ± 0.04) (Fig. 3*A*). PTX reduced expression of cyclin D1 protein, as did wortmannin (Fig. 3*B*). Furthermore, PTX increased expression of p27^{kip1} protein in PDGF-stimulated cells (Fig. 3*B*). Taken together,

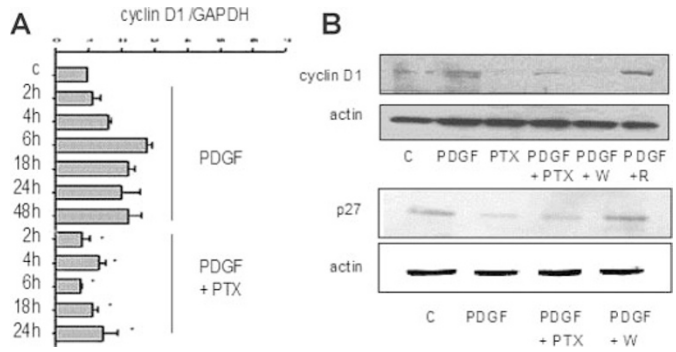

Figure 3. (*A*) Effect of PDGF on cyclin D1 mRNA expression. The growth-arrested cells were stimulated by adding PDGF 40 ng/mL for 2, 4, 6, 18, 24, and 48 h. PTX inhibited the expression of cyclin D1 mRNA. The growth-arrested cells were stimulated with PDGF 40 ng/mL in the presence or absence PTX for 2, 4, 6, 18, 24, and 48 h. (*B*) PTX and wortmannin inhibited PDGF-stimulated cyclin D1 protein expression and the up-regulation of p27 expression. The growth-arrested cells were stimulated with PDGF 40 ng/mL alone, PDGF 40 ng/mL + PTX 1 mM, PDGF 40 ng/mL + wortmannin 10 nM, or PDGF 40 ng/mL + rapamycin 20 nM for 2 h. Results are mean ± SD from three experiments. *Significantly different from the value obtained with medium only. **Significantly different from the value obtained with PDGF, $p < 0.05$.

these results indicated that PTX, like wortmannin, not only reduced the expression of cyclin D1 but also increased expression of p27 ^{kip1}, preventing entry to the cell cycle.

PTX reduces expression of cyclin D1 by PI3 K/Akt pathway. The mechanisms by which PTX suppressed cyclin D1 expression were investigated. In ASMC, PDGF can activate PI3 K pathway, increasing the expression of cyclin D1 (22). PDGF induces the phosphorylation of Akt (ser473), ERK1/2, and p70$^{S6\ K}$ (23). In this study, PTX and wortmannin reduced the phosphorylation of Akt (PDGF group, 4.4 ± 0.6; PDGF + PTX group, 0.4 ± 0.05; PDGF + wortmannin group, 0) (Fig. 4*A*) and phosphorylation of p70$^{S6\ K}$ (Fig. 4*B*), but did not influence the phosphorylation of ERK1/2 (Fig. 4*C*). However, the inhibition of p70$^{S6\ K}$ by rapamycin, a p70$^{S6\ K}$ inhibitor, did not influence the expression of cyclin D1 (Fig. 3*B*). The down-regulation of Akt phosphorylation by PTX reduced the activity of Akt using Akt kinase assay *in vitro* (Fig. 4*D*).

Akt translocates to the plasma membrane and is phosphorylated by phosphoinositide-dependent kinase, a product of PI3

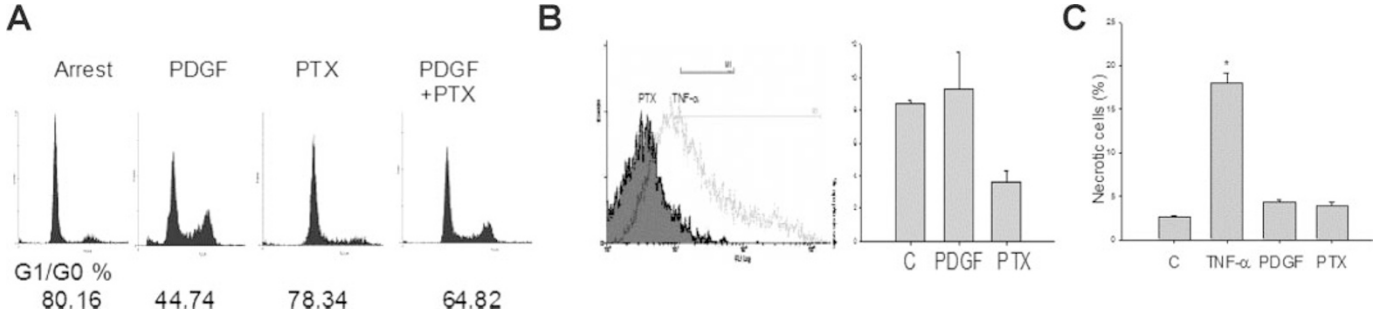

Figure 2. (*A*) PTX inhibited the G1 phase of cell cycle progression in PGDF-stimulated ASMC. Starved cells were treated with rrPDGF-BB (40 ng/mL) in the presence or absence PTX (1 mM) for 18 h. Cell cycle profiles were determined by flow cytometric analysis. (*B,C*) PTX did not induce cytotoxicity. Starved cells were treated with rrPDGF-BB (40 ng/mL) or PTX (1 mM) for 18 h. TUNEL assay and necrotic cells assay were determined by flow cytometric analysis. Results are mean ± SD from three experiments. *Significantly different from the value obtained with medium only. **Significantly different from the value obtained with PDGF, $p < 0.05$.

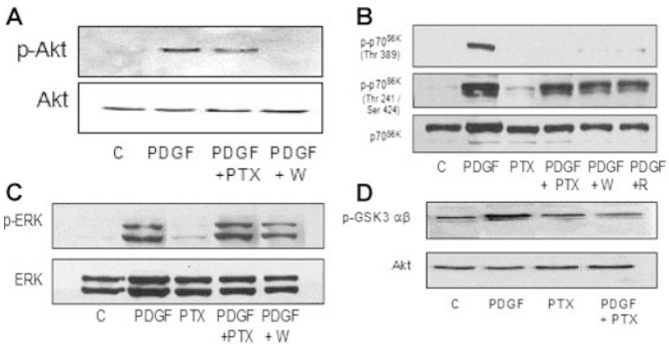

Figure 4. (*A,B,C*) PTX, wortmannin, and rapamycin effectively blocked the PDGF-induced phosphorylation of Akt and $p70^{S6\ K}$ in ASMC. PTX did not inhibit PDGF-induced phosphorylation of ERK1/2. Growth-arrested cells were treated with or without PDGF (40 ng/mL), PTX (1 Mm), and wortmannin (10 nM) for 15 min. Western blot was performed with various antibodies. Normalized signals from three independent experiments are presented in the bar charts as mean ± SD. (*D*) PTX inhibits PDGF-induced Akt activity. Growth-arrested cells were treated with or without PDGF and PTX for 15 min. Akt was immunoprecipitated by anti-Akt antibody and underwent an *in vitro* kinase assay using GSK3α/β as a substrate. Phosphorylation of GSK3α/β in kinase reactions was detected by Western blot using an antibody to p-GSK3α/β. *Significantly different from the value obtained with medium only. **Significantly different from the value obtained with PDGF, $p < 0.05$.

K (24). The effects of PTX on translocation of Akt were investigated. PDGF stimulation markedly increased the level of phosphorylated Akt in the cell membrane (PDGF group, 1.97 ± 0.02. control group, 0) (Fig. 5). However, PTX treatment reduced the phosphorylation of Akt by a factor of three in the cell membrane fraction (PDGF group, 1.97 ± 0.02; PTX group, 0.67 ± 0.08). These results indicated that PTX inhibited phosphorylation of Akt in the cell membrane and PTX did not affect translocation of Akt in this experiment.

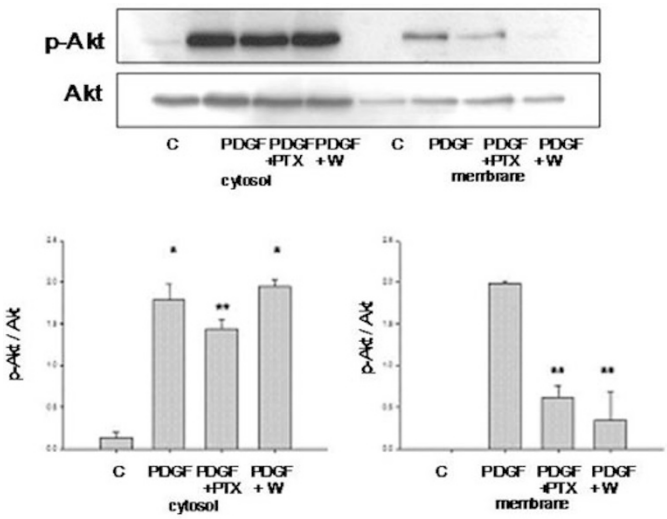

Figure 5. PTX inhibited phosphorylation of Akt on membrane fraction. Growth-arrested cells were stimulated with PDGF for 15 min in the presence or absence of PTX. Cytosol and membrane fractions were prepared and Western blot analyses performed using Akt and p-Akt antibodies. Normalized signals from three independent experiments are presented in the bar charts as mean ± SD. *Significantly different from the value obtained with medium only. **Significantly different from the value obtained with PDGF, $p < 0.05$.

PTX inhibits the degradation of IκBα protein and the translocation of NF-κB in PDGF-stimulated ASMC. NF-κB is a target protein of Akt, which is activated in PDGF-stimulated proliferation of cells (14). Whether changes in NF-κB activation caused by treatment with PTX and wortmannin, inhibiting the activation of Akt, was investigated. Growth-arrested cells were stimulated with PDGF in the presence or absence of PTX for 30 min. The translocation level of NF-κB was determined in cytoplasm and nuclear protein fractions. PDGF increased the translocation of NF-κB into nuclei after treatment for 30 min. However, wortmannin and PTX inhibited the translocation of NF-κB. PTX and wortmannin also could suppress IκBα degradation in PDGF-stimulated cells (Fig. 6*A*). PTX and wortmannin had a significantly lower level of NF-κB in fraction of nuclei than did the PDGF-treatment group (Fig. 6*A*). The densities of NF-κB in cytosol protein fractions in different groups were compared with the level of phosphorylated NF-κB. PTX and wortmannin reduced a markedly level of phosphorylated-NF-κB (Fig. 6*B*). NF-κB activity was also determined using EMSA. PDGF promoted the activity of NF-κB, which was markedly inhibited by PTX and wortmannin (Fig. 6*C*).

The translocation of NF-κB (p65) at the single cell level was also investigated by immunofluorescence microscopy. The stimulation of ASMC with PDGF for 30 min was clearly associated with the nuclear translocation of NF-κB (p65) in 90 ± 10% of cells. The nuclear localization of NF-κB (p65 subunit) was observed in 4 ± 5% of a group of unstimulated cells. The stimulation of ASMC with PDGF plus PTX for 30 min reduced the nuclear translocation of NF-κB (p65) to a level close to that of the control group (Fig. 6*D*). These data indicated that PTX and wortmannin blocked PDGF-induced NF-κB (P65) nuclear translocation and activity, and that PTX suppressed the PDGF-stimulated down-regulation of Akt/NF-κB to inhibit the expression of cyclin D1.

DISCUSSION

In the pathogenesis of asthma, the proliferation of ASMC is a key factor in airway remodeling. ASMC are also a source of chemokines and cytokines as they increase the recruitment and activation of inflammatory cells involved in airway inflammation (25). Most studies on airway smooth muscle have focused on reducing the contraction in response to stimuli. Studies of the inhibition of the proliferation of ASMC are few. This study demonstrated that PTX inhibited the hyperplasia of ASMC as a potential therapeutic drug for asthma.

According previous studies, PTX significantly attenuates AHR *in vivo* and inhibits vascular and mesangial cell proliferation *in vitro*. Therefore, this study proposed that PTX inhibited the proliferation of ASMC and achieved the goal of attenuating AHR. PDGF was demonstrated to induce the proliferation of ASMC in a dose-dependent manner and PTX inhibited PDGF-stimulated cell proliferation. PTX prevented activation of Akt in cell membrane to inhibit the expression of cyclin D1 and promote $p27^{kip1}$ activation. PTX also reduced inflammatory cytokine expression and extracellular matrix accumulation (16,26,27). Treatment with PTX also inhibits

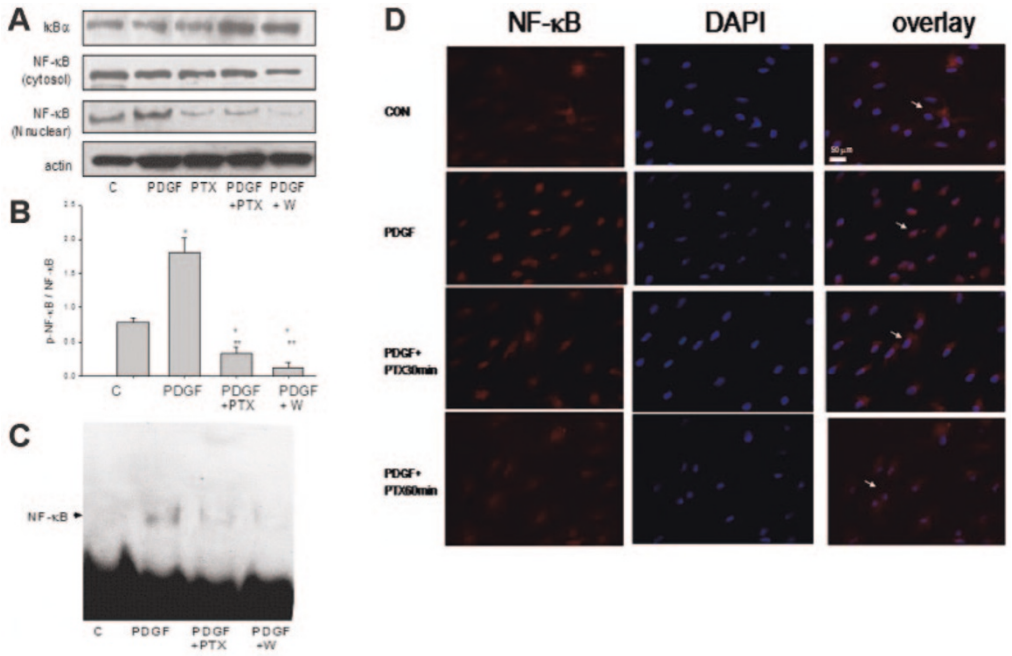

Figure 6. (*A,B*) PTX and wortmannin affected the translocation of NF-κB/P65, degradation of IκBα, and phosphorylation of NF-κB/P65. PTX and wortmannin blocked the translocation and phosphorylation of NF-κB/P65 (p-NF-κB) that would have otherwise been induced by PDGF for 30 min. Results are mean ± SD. *Significantly different from the value obtained with medium only. **Significantly different from the value obtained with PDGF, $p < 0.05$. (*C*) PTX and wortmannin inhibited PDGF-stimulated NF-κB/P65 activation in an EMSA. Activity of NF-κB/P65 in response to PDGF stimulated at 30 min. PTX and wortmannin suppressed NF-κB/P65 activation. Results are mean ± SD. *Significantly different from the value obtained with control group, $p < 0.05$. (*D*) Effect of PTX on the immunostaining of nuclear translocated NF-κB/P65 induced by PDGF. (*a*) Control, (*b*) stimulation of ASMC with PDGF (40 ng/mL) for 30 min induced nuclear translocation of NF-κB/P65. (*c,d*) PTX blocked the translocation of NF-κB/P65 that would otherwise be induced by PDGF for 30 min and 60 min. *Arrows* indicate cytosolic fraction. All panels are representative microphotographs (magnification: ×400) of three independent sets of experiments.

Th1 cytokine production. These effects may contribute to the effectiveness of PTX in reducing AHR. These results demonstrate that PTX serves as a potential drug for treating asthma, not only improving of airway remodeling but also reducing the inflammatory response.

PTX cannot change either PDGF binding to the receptor or phosphorylation of the receptor (28). A possible mechanism of the inhibition of PDGF postreceptor signaling by treatment of PTX, particularly the PI3 K signaling pathway, has been investigated (19). A possible mechanism by which PTX prevented Akt phosphorylation was also demonstrated. PTX inhibits phosphodiesterase activity, resulting in an increase in cAMP (18). An increase in cAMP can inhibit proliferation (29). cAMP inhibited the activation of Rap1 and reduced Akt phosphorylation (30). Furthermore, PTX reduced Akt phosphorylation through cAMP-dependent protein kinase, protein kinase A (PKA), by increasing of cAMP (19). The mechanism by which PTX reduces Akt phosphorylation in the cell membrane fraction may involve PKA, affected by an increase in cAMP.

PTX blocks proliferation of cell by inhibiting activation of NF-κB by an unclear pathway (31). The one possible mechanism is that PTX blocks the PI3 K/Akt signaling pathway and then suppresses activation of NF-κB. Akt enhances the degradation of IκBs and cooperates with other factors to activate NF-κB (32). Akt has also been demonstrated to be both necessary and sufficient for PDGF to induce NF-κB activation (14). This study demonstrated that PTX inhibited the proliferation of ASMC by blocking the Akt/NF-κB pathway. PTX and wortmannin have been established to inhibit the activation of Akt, the degradation of IκBα, and the activity of NF-κB. It also inhibited the expression of cyclin D1, reducing the proliferation of ASMC. These results might indicate that PTX attenuates the PDGF-induced Akt/NF-κB pathway, inhibiting cyclin D1 expression and cell proliferation.

The activation of Akt can activate $p70^{S6\ K}$. In this study, administration of PTX reduced the phosphorylation of $p70^{S6\ K}$. The blocking of the $p70^{S6\ K}$ pathway by rapamycin did not influence the expression of cyclin D1 induction by PDGF. Other factors might be involved in inducing the expression of cyclin D1 in the mTOR/$p70^{S6\ K}$ pathway.

In conclusion, this study demonstrated the mechanism of PTX in asthma *in vitro*. These results establish that PTX inhibited the proliferation of PDGF-stimulated ASMC by inhibiting the Akt/NF-κB pathway, thereby reducing the expression of cyclin D1. These data might provide useful information for treating asthma and show that PTX has potential for reducing remodeling and the inflammatory responses.

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