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Cofilin Phosphorylation Mediates Proliferation in Response to Platelet-Derived Growth Factor-BB in Rat Aortic Smooth Muscle Cells

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Abstract. Cofilin, an actin-binding protein, is essential for a variety of cell responses. In this study, we investigated the correlation between proliferation and cofilin phosphorylation in response to platelet-derived growth factor (PDGF) in rat aortic smooth muscle cells (RASMCs). The phosphorylation of cofilin and activity of mitogen-activated protein kinase (MAPK) were measured by Western analyses and proliferation in RASMCs was measured by BrdU incorporation assays. The phosphorylation of cofilin in RASMCs was decreased by PDGF-BB treatment at 10 min, but recovered to the level of the quiescent state at 60 min. PDGF-BB–induced dephosphorylation of cofilin was inhibited by pretreatment with piceatannol (a spleen tyrosine kinase [Syk] inhibitor), PP2 (a Src inhibitor), or SP600125 (a c-*Jun* N-terminal kinase [JNK] inhibitor), but not by PD98059, an inhibitor of extracellular signal-regulated kinase 1/2. PDGF-BB increased JNK activity and proliferation, and these responses were suppressed by kinase inhibitors and small interference RNA-cofilin. The results suggest that PDGF-BB–induced dephosphorylation of cofilin can be promoted via the JNK pathway, which is regulated by both Syk and Src kinases and that cofilin dephosphorylation may be involved in PDGF-BB–induced RASMC proliferation.

Keywords: cofilin, proliferation, rat aortic smooth muscle cell (RASMC), platelet-derived growth factor-BB (PDGF-BB), c-*Jun* N-terminal kinase (JNK)

Introduction

Vascular smooth muscle cell (VSMC) proliferation is a central event in the pathogenesis of vascular lesions, including plaque development and neointima formation. Platelet-derived growth factor (PDGF) is well known to induce proliferation in variety of cells (1 – 4). PDGF binds its cell surface receptor, a receptor tyrosine kinase, resulting in receptor autophosphorylation and linkage to Src homology 2 (SH2)-domain–containing signaling molecules, such as phospholipase Cγ, phosphatidyl inositol 3-kinase (PI3K), and ras/raf-1 (3, 5). These signaling molecules mediate cellular activities, including actin reorganization, proliferation, migration, and differentiation in response to PDGF in VSMCs (6 – 8).

It is well known that PDGF transits its signaling into the intracellular space through activation of mitogen-activated protein kinases (MAPKs) such as extracellular signal-regulated kinase (ERK) 1/2, p38 MAPK, and c-*Jun* N-terminal kinase (JNK) (6). Three MAPKs contribute to proliferation in rat aortic vascular smooth muscle cells (RASMCs) (9). Spleen tyrosine kinase (Syk), a nonreceptor protein tyrosine kinase, was shown to be expressed in a variety of cells and to be involved in vascular cell functions, including morphogenesis, growth, and survival (10, 11). Recently, Syk was shown to be important in the regulation of proliferation and

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migration in VSMCs (12, 13). Cell responses through Syk kinase are primarily mediated by MAPK (12, 14, 15) and Src, a protein tyrosine kinase, is linked to the regulation of cell functions (16, 17). Src is known to play a major role in VSMC proliferation by regulating the ERK1/2 pathway (18). Src and c-*Jun* regulate PDGF-stimulated VSMC proliferation (19, 20), and Src activity was functionally associated with Syk and involved in RASMC proliferation stimulated by PDGF (13).

The actin cytoskeleton is an essential framework for the control of a variety of cellular functions including growth, movement, and signal transduction. In addition, actin filament dynamics are correlated directly with cell proliferation (21). It is clear that signal transduction processes require the actin cytoskeleton (22). Cofilin, a ubiquitously expressed actin-binding molecule, plays a crucial role in the formation of actin filaments by regulating polymerization and depolymerization (23, 24). The activity of cofilin induced by various extracellular signals is regulated by the phosphorylation of the serine-3 residue of cofilin, which is induced by LIM kinase (LIMK) 1 and 2 or by related testicular protein kinase (TESK) 1 and 2 (23). The activity of cofilin is also regulated by several phosphatases, such as slingshot and chronophin, which dephosphorylate cofilin (25 – 27). Moreover, it has been reported that PI3K regulates cofilin phosphorylation (28). Cofilin phosphorylation decreases the binding of cofilin to actin filaments, and consequently results in actin polymerization, which promotes the accumulation of actin filaments (26, 29). Non-activated cells contain a high proportion of the inactive, phosphorylated form of cofilin, whereas cell stimulation through receptors rapidly dephosphorylate and activate cofilin (26). Moreover, cofilin shifted to its basic site on a 2-dimensional gel as a result of reactive oxygen species–stimulation in VSMCs (30). Recently, investigators have proposed that cofilin dephosphorylation is involved in cell proliferation (31). LIMK1 overexpression induces colon cancer cell proliferation (32). These results support the possible contribution of cofilin to the elevation of cell proliferation. While it has been reported that cofilin and LIMK2 are up-regulated in VSMCs stimulated with PDGF and interleukin-1β (33), a correlation between cofilin and cellular functions, especially proliferation, of RASMCs has not yet been investigated. In this study, we demonstrated the possible contribution of cofilin phosphorylation to RASMC proliferation in response to PDGF-BB.

Materials and Methods

Materials

Piceatannol, PD98059, and SP600125 were purchased from Tocris Bioscience (Bristol, UK). PP2 was obtained from Calbiochem (San Diego, CA, USA). PDGF-BB was from R&D Systems (Minneapolis, MN, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from Hyclone (Logan, UT, USA). Penicillin, Na_3VO_4, calyculin A, and streptomycin were from Sigma (St. Louis, MO, USA). The following antibodies were used: anti-JNK, -β-actin, -phospho JNK, -ERK, -phospho ERK, -phospho^{ser3} cofilin, and anti-cofilin antibodies (Cell Signaling, Beverly, MA, USA).

Cell culture

The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised in 1996) and approved by the Animal Subjects Committee of Konkuk University School of Medicine. All experiments were performed in accordance with the institutional guidelines of Konkuk University. RASMCs (used at passages 5 – 8) were enzymatically isolated from male Sprague Dawley rats (six-week-old, 190 g, n = 4) and cultured in DMEM containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 200 mM glutamine. RASMCs were grown to 70% – 80% confluence and starved in FBS-free DMEM for 24 h.

Immunoblotting

After treatment with stimulants, the cells were lysed with cold buffer (20 mM HEPES [pH 7.5], 1% Nonidet P-40, 150 mM NaCl, 10% glycerol, 10 mM NaF, 1 mM Na_3VO_4, 2.5 mM 4-nitrophenylphosphate, 0.5 mM PMSF, and 1 complete proteinase inhibitor cocktail tablet [Roche, Indianapolis, IN, USA]). The cell lysates were centrifuged (13,000 × *g*, 15 min, 4°C), and the supernatants were collected as protein samples. Protein concentrations were determined using the protein assay reagents (Bio-Rad, Hercules, CA, USA). The protein homogenates were diluted 1:1 (v/v) with SDS buffer containing 40 mM Tris-HCl (pH 6.8), 8 mM EGTA, 4% 2-mercaptoethanol, 40% glycerol, 0.01% bromophenol blue, and 4% SDS, and then boiled for 5 min. Proteins (30 – 50 μg/lane) were separated on 12% – 14% polyacrylamide SDS gels and then transferred electrophoretically to a polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA). The membrane was then blocked for 1 h at room temperature with phosphate-buffered saline (PBS) containing 0.05% Tween 20 and 5% fat-free dried milk. The membranes were incubated overnight at 4°C with antibodies diluted 1:1000 – 2000. Immune complexes were detected with horseradish-peroxidase–conjugated antibodies (Amersham Pharmacia,

Piscataway, NJ, USA) diluted 1:1000 and incubated for 1 h at room temperature. After the application of the secondary antibody, the blots were incubated with enhanced chemiluminescence reagents (Amersham Pharmacia) and exposed to photographic film. Band intensity was measured by computer analysis using Quantitation software (Bio-Rad).

Transfection of siRNA-cofilin

The medium of RASMCs (1×10^5) was replaced with FBS-free DMEM, and then the cells were transfected with the small interference RNA (siRNA) or nonsilencing control siRNA to a final concentration of 300 pM siRNA using a transfection reagent (Welfect-Q™ Gold; Welgene, Korea). The relative expression levels of cofilin were examined using immunoblotting analysis with antibody. Two siRNAs were designed to target the rat cofilin sequence 5'-CUGUUCUUCUGUAGCU CUU-3' (siRNA 1; accession number: NM_017147; Bioneer, Korea) and rat cofilin sequence 5'-CUGAUUC CUCUUGGGUUGA-3' (siRNA 2). The nonsilencing control siRNA was purchased from Bioneer.

Proliferation assay

RASMC proliferation was determined with a 5-bromo-2'-deoxyuridine (BrdU) incorporation assay (Roche). Cells were seeded in 96-well microtiter plates at a density of 2×10^3 cells/well. After 12 h, the cells were incubated in FBS-free DMEM for 6 h and then treated with inhibitors and PDGF-BB for 24 h. BrdU-labeling solution (10 μM) was added to the cells, which were then incubated for 12 h. After the denaturation of the DNA, peroxidase–labeled anti-BrdU monoclonal antibody was added and the samples were incubated at room temperature for 90 min. The BrdU–antibody complexes were detected with a Victor 3 luminometer (PerkinElmer, Boston, MA, USA).

To test proliferation in transfected cell with siRNA-cofilin, the XTT assay was performed using a WelCount™ cell viability assay kit (Welgene). RASMCs were plated in 96-well plates at a density of 1.5×10^3 cells/0.1 ml/well and incubated with siRNA-cofilin for 48 h at 37°C, and then the cells in each well were treated with XTT (a tetrazolium salt). Plates containing XTT dye were incubated for 4 h at 37°C, resulting in the formation of orange formazan crystal by metabolically active cells. Formation of formazan crystal was quantitated with an enzyme-linked immunosorbent assay reader at 450 nm.

Statistical analysis

Data are presented as means ± S.E.M. The statistical evaluation of data was performed using Student's *t*-tests for comparisons between pairs of groups and using ANOVA for multiple comparisons. A value of $P<0.05$ was considered to be a statistically significant difference.

Results

PDGF-BB stimulation of cofilin phosphorylation in RASMCs

To examine if the level of cofilin phosphorylation is altered by PDGF-BB stimulation in RASMCs, PDGF-BB stimulation was carried out in a time- or dose-dependent manner. As shown in Fig. 1A, the level of cofilin phosphorylation in RASMCs was strongly inhibited at 10 min after PDGF-BB (10 ng/ml) stimulation (17.3 ± 9.9% of the control, n = 4). The diminution of cofilin phosphorylation resulted in gradual recovery in a time-dependent manner, which was a level similar to

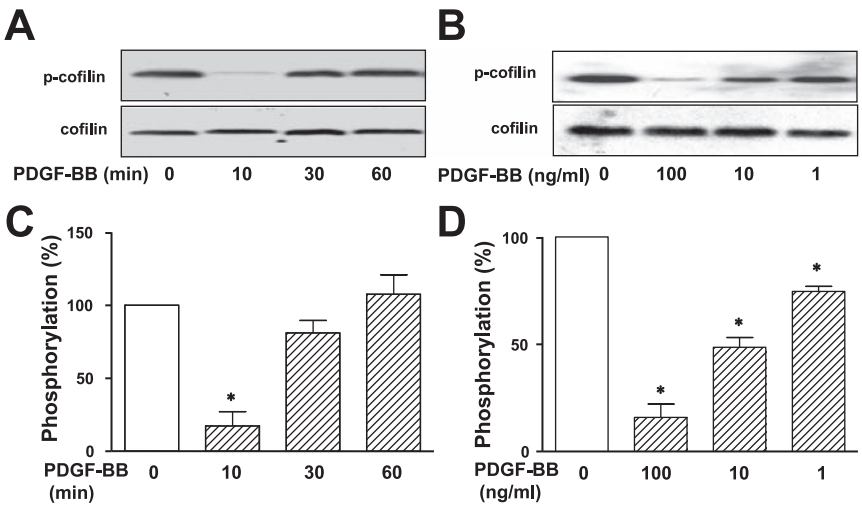

Fig. 1. Time- and concentration-dependent changes of cofilin phosphorylation in response to PDGF-BB in RASMCs. A: Time-dependent change of cofilin phosphorylation induced by PDGF-BB stimulation. RASMCs were treated with PDGF-BB (10 ng/ml) for the indicated times (10, 30, and 60 min). The cell lysates were immunoblotted with anti-phospho-cofilin antibody. The total expression of cofilin was measured by immunoblotting with anti-nonphospho cofilin antibody. B: Concentration-dependent changes of cofilin dephosphorylation stimulated by PDGF-BB. RASMCs were treated with PDGF-BB (1, 10, and 100 ng/ml; n = 4). C and D: The statistical results were obtained from the upper panel A and B, respectively. The basal levels of cofilin phosphorylation were expressed as 100% (n = 4). *Significantly different from the basal levels of cofilin phosphorylation ($P<0.05$). p-cofilin, phosphorylated cofilin.

the quiescent state at 60 min of PDGF-BB treatment (107.7 ± 13.2% of the control, n = 4). Phosphorylation of cofilin in RASMCs was dose-dependently decreased by stimulations of PDGF-BB (1, 10, and 100 ng/ml), which showed a minimal response at 1 ng/ml and a maximal at 100 ng/ml of PDGF-BB (Fig. 1B). However, the treatment with PDGF-BB did not influence total levels of cofilin in RASMCs.

Inhibitors of Src and Syk kinases in PDGF-BB–stimulated dephosphorylation of cofilin

To determine the intracellular signaling pathways that mediate PDGF-BB–induced cofilin dephosphorylation in RASMCs, the effects of inhibitors of Src and Syk kinases on cofilin dephosphorylation stimulated by PDGF-BB were tested. PDGF-BB (10 ng/ml, 10 min) decreased cofilin phosphorylation (24.6 ± 3.8% of the control, n = 4; Fig. 2). This response was attenuated by pretreatment with 30 μM piceatannol, a Syk inhibitor, for 30 min (82.1 ± 12.7% of the control, n = 4) and 10 μM PP2, a Src inhibitor, for 30 min (66.1 ± 12.2% of the control, n = 4). The total expression of cofilin was not affected by PDGF-BB simulation of RASMCs after pretreatment with Src and Syk inhibitors.

MAPK inhibitors and PDGF-BB–stimulated dephosphorylation of cofilin

The effects of inhibitors of MAPKs, PD98059 (an ERK1/2 inhibitor), and SP600125 (a JNK inhibitor), on the inhibition of cofilin phosphorylation was examined in RASMCs. PDGF-BB (10 ng/ml)–induced dephosphorylation of cofilin was significantly inhibited by pretreatment with 30 μM SP600125 for 30 min (52.6 ± 11.1% *vs* 13.1 ± 1.9%, $P<0.05$ compared to PDGF-BB–stimulated cells, n = 4; Fig. 3: A and C). On the other hand, 30 μM PD98059 did not significantly inhibit PDGF-BB–induced dephosphorylation of cofilin (14.2 ± 2.7% of the control, n = 4). In these experiments, PDGF-BB treatment after pretreatments of 30 μM PD98505 and 30 μM SP600125 did not result in alteration of cofilin total expression. We also tested the dose-dependent effect of SP600125 on PDGF-BB–induced dephosphorylation of cofilin. SP600125 (1 – 30 μM) dose-dependently inhibited PDGF-BB (10 ng/ml)-induced dephosphorylation of cofilin (n = 4, Fig. 3: B and D).

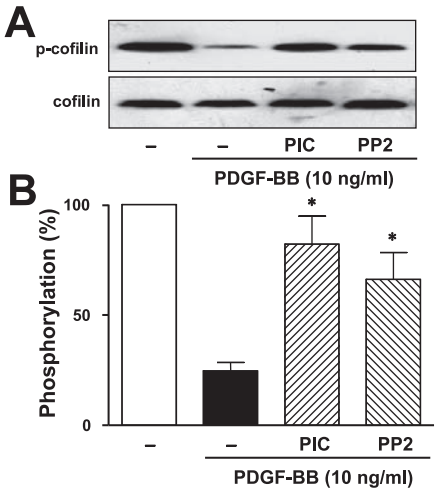

Fig. 2. Effects of tyrosine kinase inhibitors on cofilin phosphorylation in response to PDGF-BB in RASMCs. A: Cells were preincubated with or without PP2 (10 μM) or piceatannol (PIC, 30 μM) for 30 min and then treated with PDGF-BB (10 ng/ml) for 10 min. The cell lysates were subjected to immunoblotting with anti-phospho and anti-nonphospho cofilin antibodies. B: The statistical results obtained from panel A. The basal levels of cofilin phosphorylation were considered to be 100% (n = 4). *Significantly different from PDGF-BB–stimulated states ($P<0.05$). p-cofilin, phosphorylated cofilin.

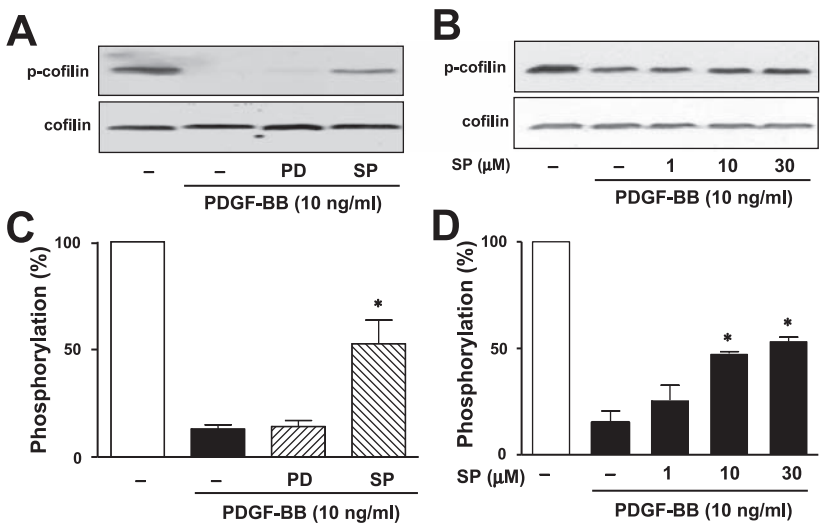

Fig. 3. Effects of MAPK inhibitors on PDGF-BB–induced cofilin phosphorylation in RASMCs. A: Effects of PD98059 and SP600125 on PDGF-BB–stimulated cofilin dephosphorylation in RASMCs. RASMCs were treated with or without PD98059 (PD, 30 μM) or SP600125 (SP, 30 μM) for 30 min and then stimulated with PDGF-BB (10 ng/ml) for 10 min. The cell lysates were immunoblotted with anti-phospho cofilin antibody. B: Dose-dependent effect of SP600125 on PDGF-BB–induced dephosphorylation of cofilin. RASMCs were pretreated with SP600125 (1, 10, and 30 μM). Total expression of cofilin in panel A and B was determined using anti-nonphospho cofilin antibody. C and D: The statistical data obtained from panel A and B. The basal levels of cofilin phosphorylation are expressed as 100% (n = 4 in each experiment). *Significantly different from PDGF-BB–stimulated states ($P<0.05$). p-cofilin, phosphorylated cofilin.

Inhibitors of Src and Syk kinases in PDGF-BB–stimulated MAPK activity

To furthermore evaluate the roles of Syk and Src kinases on the MAPK activation in response to PDGF-BB, the effect of piceatannol and PP2 on the phosphorylation of JNK and ERK1/2 by PDGF-BB was tested. PDGF-BB (10 ng/ml) elevated the phosphorylation of JNK. Piceatannol (30 μM) and PP2 (10 μM) attenuated the phosphorylation of JNK produced by 10 ng/ml of PDGF-BB in RASMCs (n = 4, Fig. 4A). Similarly to our previous study (12, 13), PDGF-BB increased the phosphorylation of ERK1/2, which was inhibited by piceatannol (30 μM) and PP2 (10 μM) (n = 4, Fig. 4B). Pretreatment of cells with piceatannol, PP2, and SP600125 did not alter MAPK expression.

Effects of protein phosphatase inhibitors on PDGF-BB–induced cofilin dephosphorylation and proliferation in RASMCs

It is known that cofilin phosphorylation is regulated by several phosphatases (25, 26). Therefore, the effects of protein phosphatase inhibitors were examined in PDGF-BB–induced dephosphorylation of cofilin in RASMCs. PDGF-BB (10 ng/ml)-induced dephosphorylation of cofilin was inhibited by the pretreatment with 200 μM Na_3VO_4, an inhibitor of protein tyrosine phosphatase, for 30 min (Fig. 5A). The dephosphorylation of cofilin in response to PFGF-BB was diminished by pretreatment with 1 nM calyculin A, an inhibitor of protein phosphatase 1 and 2A, for 30 min (Fig. 5A). We further tested the effects of phosphatase inhibitors, Na_3VO_4 and calyculin A, on PDGF-BB–induced cell

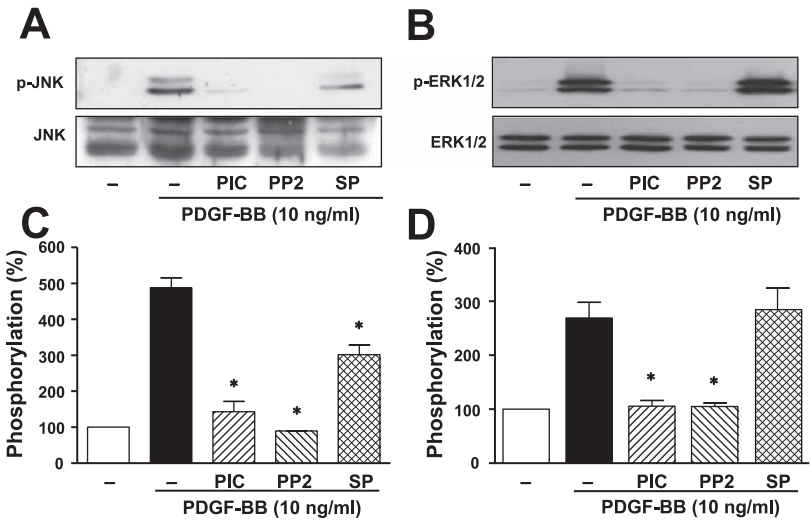

Fig. 4. Effects of kinase inhibitors on PDGF-BB–induced MAPK phosphorylation in RASMCs. Cells were treated with PDGF-BB (10 ng/ml) for 10 min after pretreatment of piceatannol (PIC, 30 μM), PP2 (10 μM), and SP600125 (SP, 30 μM) for 30 min. The RASMC lysates were immunoblotted with anti-MAPK antibodies. The phosphorylations of JNK (A) and ERK1/2 (B) were expressed using anti-phospho antibodies. The total expression was measured using anti-nonphospho MAPK antibodies. C and D: The statistical data obtained from panel A and B. The basal levels of JNK (C) and ERK1/2 (D) phosphorylation are expressed as 100% (n = 4, respectively). *Significantly different from PDGF-BB–stimulated states (P<0.05). p-JNK, phosphorylated JNK; p-ERK1/2, phosphorylated ERK1/2.

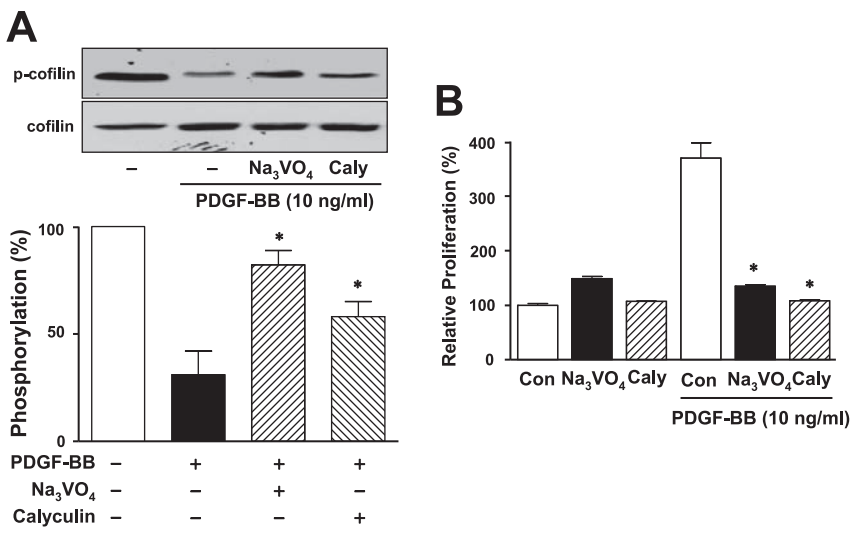

Fig. 5. Effects of phosphatase inhibitors on PDGF-BB–induced cofilin phosphorylation and proliferation in RASMCs. A: Effects of phosphatase inhibitors on PDGF-BB–induced cofilin phosphorylation in RASMCs. RASMCs were left untreated or treated with Na_3VO_4 (200 μM) or calyculin A (1 nM) for 30 min and then stimulated with PDGF-BB (10 ng/ml) for 10 min. The cell lysates were immunoblotted with anti-phospho cofilin antibody. Total expression of cofilin was determined using anti-nonphospho cofilin. The statistical data was obtained from the upper panel. The basal levels of cofilin phosphorylation are expressed as 100% (n = 4). B: Effects of phosphatase inhibitors on PDGF-BB–induced proliferation of RASMCs. RASMCs were incubated with Na_3VO_4 (20 μM) or calyculin A (1 nM) in the absence or the presence of PDGF-BB (10 ng/ml) for 24 h. The basal level in RASMC proliferation is considered as 100% (n = 5). *Significantly different from PDGF-BB–stimulated states (P<0.05). Caly, calyculin A; p-cofilin, phosphorylated cofilin.

proliferation. As shown in Fig. 5B, the treatment with Na_3VO_4 (20 μM) or calyculin A (1 nM) to RASMCs for 24 h inhibited RASMC proliferation in response to PDGF-BB (10 ng/ml).

Effects of kinase inhibitors and siRNA-cofilin transfection on PDGF-BB–induced proliferation

To determine the functional roles of cofilin phosphorylation on PDGF-BB–induced RASMC proliferation, cells were pretreated with kinase inhibitors or transfected with siRNA-cofilin, and then the proliferation in response to PDGF-BB was analyzed. First, PDGF-BB–stimulated proliferation was assessed after pretreatment with kinase inhibitors on the basis of results from Figs. 2 and 3. As shown in Fig. 6A, PDGF-BB increased RASMC proliferation (586.4 ± 49.9%). Piceatannol (30 μM) and PP2 (10 μM) inhibited PDGF-BB (10 ng/ml)-induced cell proliferation to 37.6 ± 2.1% (n = 5) and 186.2 ± 13.0% (n = 5) of the controls, respectively. SP600125 (30 μM) also reduced the PDGF-BB (10 ng/ml)-increased cell proliferation to 377.6 ± 19.9% of the controls (n = 5). PDGF-BB (10 ng/ml)-induced cell proliferation was inhibited by pretreatment with 30 μM PD98059 (Fig. 6A). We further confirmed the dose-dependent effect of SP600125 on PDGF-BB–induced RASMC proliferation. SP600125 (1 – 30 μM) dose-dependently inhibited RASMC proliferation in response to 10 ng/ml of PDGF-BB in a dose-dependent manner (n = 5, Fig. 6B).

Next, we also confirmed the effect of knock down of cofilin on cell proliferation using a siRNA technique in RASMCs. Treatment of cells with siRNA-cofilin decreased the expressions of both non-phosphorylated and phosphorylated cofilin in the absence and the presence of PDGF-BB (10 ng/ml) (Fig. 6C). As shown in Fig 6D, proliferation stimulated by 10 ng/ml of PDGF-BB (399.9 ± 28.4% of non-transfected control) was attenuated in cells transfected with siRNA 1- and 2-cofilin to 327.0 ± 13.4% and 243.2 ± 11.4% of non-transfected control, respectively (n = 5). Cells transfected with nonsilencing siRNA did not exert any inhibitory effect on proliferation in RASMCs.

Discussion

In the present study, we demonstrated, for the first time, that the PDGF induced dephosphorylation of cofilin and increased proliferation in vascular smooth muscle cells, and this proliferation response was inhibited in cofilin knockdown cells. It has been shown that active cofilin inhibits actin polymerization and consequently decreases the formation of actin filaments in a variety of cells (26). Moreover, when cofilin is phosphorylated by kinases, for example, LIMK, it loses

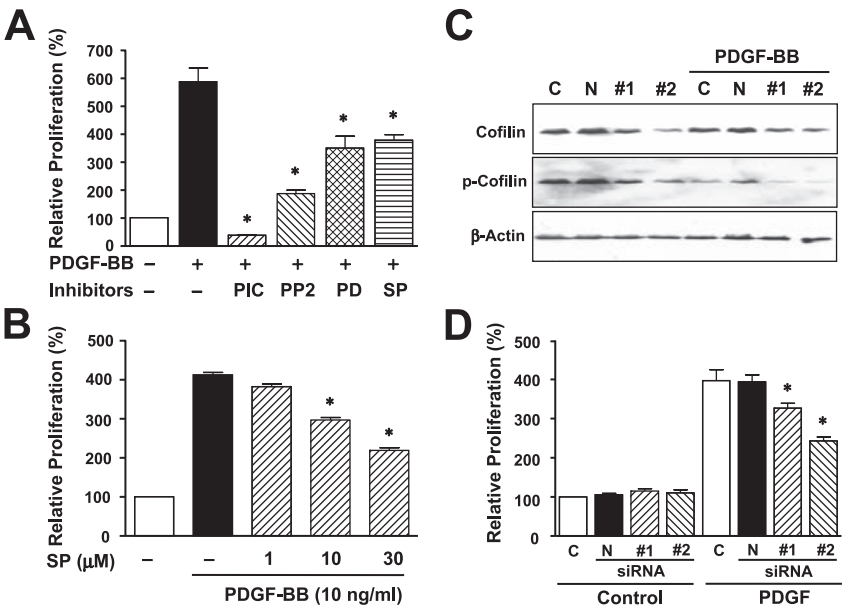

Fig. 6. Effects of kinase inhibitors and siRNA-cofilin transfection on PDGF-BB–induced proliferation of RASMCs. A: Effects of kinase inhibitors on PDGF-BB–stimulated proliferation. RASMCs were left untreated or treated with PDGF-BB (10 ng/ml), piceatannol (PIC, 30 μM), PP2 (10 μM), PD98059 (PD, 30 μM), or SP600125 (SP, 30 μM) for 24 h. B: Dose-dependent effect of SP600125 on PDGF-BB–increased RASMC proliferation. The basal level in RASMC proliferation is considered to be 100% (n = 5 in each experiment). **P*<0.05, compared with PDGF-BB–stimulated states. C: Non-phosphorylated and phosphorylated cofilin expression in RASMCs transfected with siRNA-cofilin in the absence or the presence of PDGF-BB. Cells were transfected with siRNA-cofilin as described in the Methods section and then incubated with or without PDGF-BB (10 ng/ml) for 10 min (n = 3). D: Effect of siRNA-cofilin on PDGF-BB–stimulated proliferation. Cells transfected with siRNA-cofilin were treated with PDGF-BB (10 ng/ml) for 24 h and their proliferation was examined. The basal activity in RASMC proliferation is considered to be 100% (n = 5). **P*<0.05, compared with PDGF-BB–stimulated states. C, nontransfected control; N, nonsilencing siRNA; #1, siRNA 1-cofilin; #2; siRNA 2-cofilin; p-cofilin, phosphorylated cofilin.

its ability to inhibit the formation of actin filaments (23). These results indicate that dephosphorylation of cofilin in response to PDGF can lead to cytoskeletal actin reorganization and may induce actin–dependent cell responses. Moreover, it has been reported that actin filament dynamics correlate directly with cell proliferation (21) and that proliferation is initiated by the actin reorganization (31). Our result also showed that knockdown of cofilin using the siRNA technique in RASMCs attenuated the PDGF-induced cell proliferation. Therefore, PDGF-stimulated cofilin dephosphorylation may mediate RASMC proliferation. In this study, cofilin phosphorylation in response to PDGF resulted in transient decrement and a return to the initial phosphorylation level. Similar results were observed previously (34). It was shown that these transient events trigger stimulation of intercellular molecules and induce long-term activation of intracellular signals (35, 36). From these results, the transient decrease of cofilin phosphorylation may trigger intracellular machinery that increases the proliferation of RASMCs.

In this study, the dephosphorylation of RASMC cofilin in response to PDGF was attenuated by JNK inhibition and proliferation was inhibited by a JNK inhibitor. MAPK and PI3K inhibitors completely blocked cofilin phosphorylation (33), implying that protein kinases may contribute to the phosphorylation of cofilin. Recently, we reported that phorbol ester, a potent PKC activator, induced dephosphorylation of cofilin, implying that cofilin phosphorylation is also regulated by the kinase (30). In the present study, inhibitors of Src and Syk abolished the PDGF responses to cofilin phosphorylation and proliferation. Src and Syk are upper stream signals of MAPK (12, 13), and Src is an important participant in the regulation of cell functions, including proliferation and migration (16, 19). Previous results showed that a Src inhibitor attenuated JNK activity (37). Moreover, both Src and Syk kinases are functionally interacted, and this contributes to cell migration and proliferation in response to PDGF (13). The present results showed that PDGF increased the activity of JNK and this was decreased by the Src and Syk inhibitors. These results imply that PDGF-stimulated JNK activity is mediated by Src and Syk kinases, which are involved in JNK-induced cofilin regulation. Moreover, the dephosphorylation of cofilin in response to PDGF was significantly recovered by the treatment with the phosphatase inhibitors Na_3VO_4 and calyculin A, and these inhibitors attenuated PDGF-induced cell proliferation. It was reported that calyculin A affected cofilin phosphorylation in unstimulated neutrophils (34) and cofilin phosphatases regulate phosphorylation of cofilin (26). These results imply that JNK indirectly regulates cofilin phosphorylation and it may be assumed that cofilin phosphatase participates in this pathway in RASMCs. Additionally, ERK1/2, as well as JNK, contributes to cell proliferation (18). In the present study, ERK1/2 inhibition attenuated the proliferation of RASMC but did not affect the phosphorylation of cofilin. These results indicate that ERK1/2 is involved in PDGF-induced proliferation, which is mediated by cofilin–independent pathways.

In summary, the phosphorylation of cofilin in RASMCs was decreased by PDGF-BB, which was recovered by inhibitors of Syk, Src, and JNK. PDGF-BB increased JNK activity, and this was suppressed by such inhibitors. PDGF-BB–induced proliferation of RASMCs was inhibited by Syk, Src, JNK inhibitor, and cofilin knockdown, as well as ERK1/2 inhibitor that did not affect PDGF-induced cofilin dephosphorylation. Therefore, we suggest that PDGF-BB–induced cofilin dephosphorylation can be promoted via the JNK pathway and that this pathway may be involved in PDGF-BB–induced RASMC proliferation.

Acknowledgment

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References

1 Ross R. Atherosclerosis – an inflammatory disease. N Engl J Med. 1999;340:115–126.
2 Ioroi T, Yamamori M, Yagi K, Hirai M, Zhan Y, Kim S, et al. Dominant negative c-*Jun* inhibits platelet-derived growth factor-directed migration by vascular smooth muscle cells. J Pharmacol Sci. 2003;91:145–148.
3 Raines EW. PDGF-BB and cardiovascular disease. Cytokine Growth Factor Rev. 2004;15:237–254.
4 Millette E, Rauch BH, Kenargy RD, Daum G, Clowes AW. Platelet-derived growth factor-BB transactivates the fibroblast growth factor receptor to induce proliferation in human smooth muscle cells. Trends Cardiovasc Med. 2006;16:25–28.
5 Perona R. Cell signalling: growth factors and tyrosine kinase receptors. Clin Transl Oncol. 2006;8:77–82.
6 Pearson R, Robinson F, Beers Gibson T, Xu BE, Karandikar M, Berman K, et al. Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. Endocr Rev. 2001;22:153–183.
7 Turner CE. Paxillin and focal adhesion signaling. Nat Cell Biol.

2000;2:E231–E236.
8 Newby AC. Matrix metalloproteinases regulate migration, proliferation, and death of vascular smooth muscle cells by degrading matrix and non-matrix substrates. Cardiovasc Res. 2006;69:614–624.
9 Zhan Y, Kim S, Izumi Y, Izumiya Y, Nakao T, Miyazaki H, et al. Role of JNK, p38, and ERK in platelet-derived growth factor-induced vascular proliferation, migration, and gene expression. Arterioscler Thromb Vasc Biol. 2003;23:795–801.
10 Kurosaki T. Functional dissection of BCR signaling pathways. Curr Opin Immunol. 2000;12:276–281.
11 Inatome R, Yanagi S, Takano T, Yamamura H. A critical role for Syk in endothelial cell proliferation and migration. Biochem Biophys Res Commun. 2001;286:195–199.
12 Lee CK, Lee HM, Kim HJ, Park HJ, Won KJ, Roh HY, et al. Syk contributes to PDGF-BB–mediated migration of rat aortic smooth muscle cells via MAPK pathways. Cardiovas Res. 2007;74:159–168.
13 Lee HM, Kim HJ, Park HJ, Won KJ, Kim J, Shin HS, et al. Spleen tyrosine kinase participates in Src-mediated migration and proliferation by PDGF-BB in rat aortic smooth muscle cells. Arch Pharm Res. 2007;30:761–769.
14 Lee HM, Won KJ, Kim J, Park HJ, Kim HJ, Roh HY, et al. Endothelin-1 induces contraction via a Syk-mediated p38 mitogen-activated protein kinase pathway in rat aortic smooth muscle. J Pharmacol Sci. 2007;103:427–433.
15 Takada Y, Aggarwal BB. TNF activates Syk protein tyrosine kinase leading to TNF-induced MAPK activation, NF-κB activation, and apoptosis. J Immunol. 2004;173:1066–1077.
16 Thomas SM, Brugge JS. Cellular functions regulated by Src family kinase. Annu Rev Cell Dev Biol. 1997;13:513–609.
17 Brown MT, Cooper JA. Regulation, substrates and functions of Src. Biochim Biophys Acta. 1996;1287:121–149.
18 Sayeski PP, Ali MS. The critical role of c-Src and the Shc/Grb2/ERK2 signaling pathway in angiotensin II–dependent VSMC proliferation. Exp Cell Res. 2003;287:339–349.
19 Barone MV, Courtneidge SA. Myc but not Fos rescue of PDGF-BB signalling block caused by kinase-inactive Src. Nature. 1995;378:509–512.
20 Zhan Y, Kim S, Yasumoto H, Namba M, Miyazaki H, Iwao H. Effects of dominant-negative c-*Jun* on platelet-derived growth factor–induced vascular smooth muscle cell proliferation. Arterioscler Thromb Vasc Biol. 2002;22:82–88.
21 dos Remedios CG, Chhabra D, Kekic M, Dedova IV, Tsubakihara M, Berry DA, et al. Actin binding proteins: regulation of cytoskeletal microfilaments. Physiol Rev. 2003;83:433–473.
22 Sotiropoulos A, Gineitis D, Copeland J, Treisman R. Signal-regulated activation of serum response factor is mediated by changes in actin dynamics. Cell. 1999;98:159–169.
23 Bamburg JR. Proteins of the ADF/cofilin family: essential regulators of actin dynamics. Annu Rev Cell Dev Biol. 1999;15:185–230.
24 Rosenblatt J, Mitchison TJ. Actin, cofilin and cognition. Nature. 1998;393:739–740.
25 Wang Y, Shibasaki F, Mizuno K. Calcium signal-induced cofilin dephosphorylation is mediated by Slingshot via calcineurin. J Biol Chem. 2005;280:12683–12689.
26 Huang TY, DerMardirossian C, Bokoch GM. Cofilin phosphatases and regulation of actin dynamics. Curr Opin Cell Biol. 2006;18:26–31.
27 Gohla A, Birkenfeld J, Bokoch GM. Chronophin, a novel HAD-type serine protein phosphatase, regulates cofilin-dependent actin dynamics. Nat Cell Biol. 2005;7:21–29.
28 Nebl G, Fischer S, Penzel R, Samstag Y. Dephosphorylation of cofilin is regulated through Ras and requires the combined activities of the Ras-effectors MEK and PI3K. Cell Signal. 2004;16:235–243.
29 Yang N, Higuchi O, Ohashi K, Nagata K, Wada A, Kangawa K, et al. Cofilin phosphorylation by LIM kinase 1 and its role in Rac-mediated actin reorganization. Nature. 1998;393:809–812.
30 Lee CK, Park HJ, So HH, Kim HJ, Lee KS, Choi WS, et al. Proteomic profiling and identification of cofilin responding to oxidative stress in vascular smooth muscle. Proteomics. 2006;6:6455–6475.
31 Subramaniam V, Vincent IR, Jothy S. Upregulation and dephosphorylation of cofilin: modulation by CD44 variant isoform in human colon cancer cells. Exp Mol Pathol. 2005;79:187–193.
32 Bagheri-Yarmand R, Mazumdar A, Sahin AA, Kumar R. LIM kinase 1 increases tumor metastasis of human breast cancer cells via regulation of the urokinase-type plasminogen activator system. Int J Cancer. 2006;118:2703–2710.
33 Bongalon S, Dai YP, Singer CA, Yamboliev IA. PDGF-BB and IL-1 upregulate cofilin and LIMK2 in canine cultured pulmonary artery smooth muscle cells. J Vasc Res. 2004;41:412–421.
34 Zhan Q, Bamburg JR, Badwey JA. Products of phosphoinositide specific phospholipase C can trigger dephosphorylation of cofilin in chemoattractant stimulated neutrophils. Cell Motil Cytoskeleton. 2003;54:1–15.
35 Choi DY, Toledo-Aral JJ, Segal R, Halegoua S. Sustained signaling by phospholipase C mediates nerve growth factor–triggered gene expression. Mol Cell Biol. 2001;21:2695–2705.
36 Lapidot SA, Phair RD. Platelet-derived growth factor causes sustained depletion of both inositol trisphosphate-sensitive and caffeine-sensitive intracellular Ca^{2+} stores in vascular smooth muscle cells. Arterioscler Thromb Vasc Biol. 1995;15:44–51.
37 Kyaw M, Yoshizumi M, Tsuchiya K, Kagami S, Izawa Y, Fujita Y, et al. Src and Cas are essentially but differentially involved in angiotensin II–stimulated migration of vascular smooth muscle cells via extracellular signal-regulated kinase 1/2 and c-*Jun* NH2-terminal kinase activation. Mol Pharmacol. 2004;65:832–841.