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Platelet-derived growth factor stimulated vascular smooth muscle cell proliferation and its molecular mechanism

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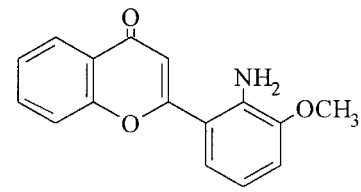
KEY WORDS Ca^{2+}-calmodulin dependent protein kinase；platelet-derived growth factor；vascular smooth muscle；cultured cells；Western blotting；*in situ* hybridization

ABSTRACT

AIM：To study the molecular mechanism of platelet-derived growth factor (PDGF)-BB-stimulated vascular smooth muscle cell (VSMC) proliferation. **METHODS**：DNA synthesis was measured by [^{3}H] thymidine incorporation. Phosphorylation of the 42- and 44-kDa Ca^{2+}-calmodulin dependent protein kinase (CCDPK) was measured by Western blotting method. The expression of c-*myc* specific mRNA was detected by *in situ* hybridization. **RESULTS**：PDGF-BB (2 $\mu g \cdot L^{-1}$) induced DNA synthesis and activated CCDPK in a concentration-dependent manner and a induced a marked c-*myc* mRNA expression. Egtazic acid (EGTA，5 $mmol \cdot L^{-1}$)，genistein (400 $\mu mol \cdot L^{-1}$) or PD 98059 (50 $\mu mol \cdot L^{-1}$) reduced PDGF-BB (2 $\mu g \cdot L^{-1}$)-induced CCDPK activities and inhibited VSMC [^{3}H] thymidine incorporation ($P < 0.05$). PD 98059 (50 $\mu mol \cdot L^{-1}$) also inhibited PDGF-BB (2 $\mu g \cdot L^{-1}$)-induced c-*myc* mRNA expression. **CONCLUSION**：PDGF stimulated VSMC proliferation by activation of p44/p42 CCDPK，which is mediated by Ca^{2+} and protein tyrosine kinase (PTK)，and up-regulation of c-*myc* mRNA expression.

INTRODUCTION

Platelet-derived growth factor (PDGF) is one of the major mitogens in serum and is responsible for proliferation of certain cell types，including vascular smooth muscle cells (VSMC)[1,2]. Excessive VSMC growth has been highlighted in the pathophysiology of hypertension and atherosclerosis[3,4]. Smooth muscle cell proliferation is a central feature of intimal hyperplasia and can be initiated by any one of a number of growth factors，including PDGF，epidermal growth factor (EGF)，and basic fibroblast growth factor (bFGF)，all of which are released at the time of arterial injury[5]. It has been reported that PDGF-BB isoform is a potent inducer of VSMC proliferation. Thus，elucidation of the intracellular signaling pathway of PDGF-BB in VSMC that mediates the growth response is crucial for understanding the vascular biology of cardiovascular diseases. In recent years，many studies have demonstrated that the p44/p42 Ca^{2+}-calmodulin dependent protein kinase (CCDPK) cascades are critical to cellular differentiation，and，in some cells，to the induction of hypertrophy[6]. In this study we aimed to examine PDGF-BB-induced VSMC proliferation and its molecular mechanism.



PD 98059

MATERIALS AND METHODS

Chemicals PDGF (recombinant BB)，egtazic acid，genistein，PD 98059，leuptinin，HEPES，Triton X-100，DMEM，bovine serum albumin (BSA) were purchased from Sigma. Western blot chemiluminescence reagent plus was purchased from NEN™ Life Science Products. DIG Random Labeling and Detection kit was purchased from Boster Bioengineering Ltd Co，Wuhan. All other chemicals were from commercial sources.

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Cell culture[7] VSMC were isolated from aorta of 200 – 250 g Sprague-Dawley rats (supplied by the Experimental Animal Center of Hunan Medical University, grade Ⅱ, Certificate № 20-009) and maintained in DMEM supplemented with 10 % fetal calf serum. Cells used were from 4 to 8 passages.

Experimental protocol VSMC were placed into culture dishes at 1×10^5 cells per well and cultivated in culture medium until 80 % confluency. After 24 h of cultivation in serum-free medium, cells were pretreated either with egtazic acid 5 mmol·L^{-1}, genistein 400 μmol·L^{-1} or PD 98059 50 μmol·L^{-1}, and then stimulated with PDGF-BB 2 μg·L^{-1} for 16 h.

[^{3}H] Thymidine incorporation Quiescent cells grown in 24-well culture dishes were pulsed for 8 h with [^{3}H] thymidine 37 MBq·L^{-1}, washed with cold PBS, trypsinized with trypsine 0.2 %, resuspended in 20 % trichloroacetic acid (TCA), and vortexed vigorously. The cell lysate was vacuum-filtered through a glass-fiber filter. After washing with cold 5 % TCA followed by 70 % ethanol, the filter was dried. The radioactivity of incorporated [^{3}H] thymidine was measured in a liquid scintillation counter. Experiments were performed 6 times in duplicate.

Western blot Cell lysates were prepared as previously described[8]. Protein concentration was estimated by Bradford method[9]. A 1/4 volume of SDS sample buffer [Tris-HCl 0.33 mol/L, SDS 10 % (wt/vol), glycerol 40 % (vol/vol), and dithiothreitol 20 % (vol/vol) containing bromophenol blue 0.4 % (wt/vol)] was added to cell lysates. The extracted protein 20 μg was subjected to SDS-PAGE in a 10 % SDS gel, and the protein was then transferred to nitrocellulose membrane, which was then blocked for 1 h with 5 % BSA in TBST (Tris-HCl 20 mmol·L^{-1}, NaCl 137 mmol·L^{-1}, containing 0.1 % Tween-20). The blots were incubated for 1 h at room temperature with the primary monoclonal antibodies of phosphorated-p44 and p42 CCDPK at a 1∶10 000 dilution, followed by incubation for 1 h with secondary antibody (horseradish peroxidase conjugated) at a 1∶800 dilution. Immunoreactive bands of p42 and p44 CCDPK were visualized by using enhanced chemiluminescence reagents. Quantification of p42 and p44 CCDPK activity was detected by a scanning densitometry of autoradiographs.

***In situ* hybridization (ISH)** VSMC were detected on slides by *in situ* hybridization with the corresponding digoxigenin-labeled DNA probes. Slides were washed in 1 × standard sodium citrate (SSC)/0.05 % Triton X-100 and prehybridization buffer consisting of 6 × SSC, 5 × Denhard's reagent, 40 % formamide, and salmon sperm DNA 200 mg·L^{-1} at 37 ℃ for 30 min. Prehybridization buffer was replaced with hybridization buffer (50 μL hybridization buffer containing c-*myc* probe 10 mg·L^{-1}, 6 × SSC, 5 × Denhard's reagent, 45 % formamide, 10 % dextran sulfate, and salmon sperm DNA 0.1 g·L^{-1}) at 37 ℃ for 12 h. After that, the cells were washed as follows: 2 × SSC containing 50 % formamide (3 × 10 min), 1 × SSC (3 × 10 min) and 0.5 × SSC (2 × 20 min) at 42 ℃. Cells were rinsed with buffer Ⅰ (Tris-HCl 0.1 mol·L^{-1}, NaCl 0.15 mol·L^{-1}, pH 7.5), then incubated with buffer Ⅰ containing 2 % normal sheep serum and 0.3 % Triton X-100 at 37 ℃ for 30 min. Buffer Ⅱ containing anti-digoxigenin at 1∶3000 was applied to the cells, followed by one washing with buffer Ⅰ and buffer Ⅲ (Tris-HCl 100 mmol·L^{-1}, NaCl 100 mmol·L^{-1}, $MgCl_2$ 50 mmol·L^{-1}, pH 9.5). The reaction was visualized using a standard alkaline phosphatase technique with NBT and BCIP as substrate. Finally, the reaction was stopped with buffer Ⅳ (Tris-HCl 10 mmol·L^{-1} and edetic acid 1 mmol·L^{-1}, pH 8.0). Sections were not counterstained.

Statistical analysis Values were expressed as $\bar{x} \pm s$ and assessed by ANOVA and *t*-test.

RESULTS

Effect of PDGF-BB on VSMC DNA synthesis PDGF-BB (0.02 – 20 μg·L^{-1}) induced an increase in [^{3}H] thymidine incorporation into cells in a concentration-dependent manner (Tab 1).

Tab 1. Effect of PDGF-BB on [^{3}H] thymidine incorporation into rat aortic smooth muscle cells. $\bar{x} \pm s$ of 6 experiments in cultured rat VSMC. Average of duplicate constitutes one determination. $^cP < 0.01$ *vs* control.

PDGF/μg·L^{-1}	[^{3}H] Thymidine incorporation/ Bq per well
0	301 ± 61
0.02	582 ± 134^c
0.20	864 ± 196^c
2.00	1601 ± 629^c
20.0	2216 ± 385^c

Effect of PDGF-BB on CCDPK activation Concentration-dependent increases in the phosphorylation of p44/p42 CCDPK were observed from PDGF 0.02 to

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20 $\mu g \cdot L^{-1}$ (Fig 1, Tab 2).

Fig 1. Effect of PDGF-BB on phosphored p42/p44 CCDPK by Western blot. 1) Control; 2) 0.02; 3) 0.2; 4) 2; 5) 20 $\mu g \cdot L^{-1}$.

Tab 2. Densimetry quantification of the effect of PDGF-BB on phosphored p42/p44 CCDP kinase by Western blot. $\bar{x} \pm s$ of 6 independent experiments in cultured rat VSMC. Average of duplicate constitutes one determination. $^bP < 0.05$, $^cP < 0.01$ *vs* control.

PDGF/$\mu g \cdot L^{-1}$	$10^{-3} \times$ Peak area/mm^2
0	44 ± 8
0.02	86 ± 12[b]
0.20	124 ± 14[c]
2.00	164 ± 11[c]
20.0	192 ± 18[c]

Effect of extracelluar Ca^{2+}, PTK, and PD 98059 on CCDPK activation Incubation of VSMC with egtazic acid, genistein, and PD 98059 markedly inhibited CCDPK activation stimulated by PDGF-BB by 48 %, 52 %, and 43 %, respectively, $P < 0.01$ *vs* PDGF-BB alone (Fig 2, Tab 3).

Fig 2. Effect of egtazic acid, genistein, and PD 98059 on expression of rat aortic smooth muscle cell phosphored p42/p44 CCDPK by Western blot. 1) Control; 2) PDGF-BB 2 $\mu g \cdot L^{-1}$; 3) Egtazic acid 5 $mmol \cdot L^{-1}$; 4) Egtazic acid + PDGF-BB; 5) Genistein 400 $\mu mol \cdot L^{-1}$; 6) Genistein + PDGF-BB; 7) PD 98059 50 $\mu mol \cdot L^{-1}$; 8) PD 98059 + PDGF-BB.

Effect of egtazic acid, genistein, and PD 98059 on PDGF-BB-induced DNA synthesis Treatment of cells with egtazic acid, genistein, or PD 98059 resulted in a reduction in PDGF-BB-induced [^{3}H] thymidine incorporation (48 %, 74 %, and 60 % inhibition, respectively; $n = 6$, $P < 0.01$ *vs* PDGF alone) (Tab 4).

Tab 3. Densimetry quantification of the effect of egtazic acid 5 $mmol \cdot L^{-1}$, genistein 400 $\mu mol \cdot L^{-1}$, and PD 98059 50 $\mu mol \cdot L^{-1}$ on expression of phosphored p42/p44 CCDPK by Western blot. $\bar{x} \pm s$ of 6 independent experiments in cultured rat VSMC. Average of duplicate constitutes one determination. $^cP < 0.01$ *vs* PDGF-BB group.

Treatment	$10^{-3} \times$ Peak area/mm^2
Control	44 ± 8
PDGF	156 ± 18
Egtazic acid	43 ± 9
Egtazic acid + PDGF	78 ± 11[c]
Genistein	35 ± 8
Genistein + PDGF	84 ± 12[c]
PD 98059	38 ± 8
PD 98059 + PDGF	89 ± 10[c]

Tab 4. Effect of the treatment of egtazic acid 5 $mmol \cdot L^{-1}$, genistein 400 $\mu mol \cdot L^{-1}$, and PD 98059 50 $\mu mol \cdot L^{-1}$ on [^{3}H] thymidine incorporation. $\bar{x} \pm s$ of 6 independent experiments in cultured rat VSMC. Average of duplicate constitutes one determination. $^cP < 0.01$ *vs* PDGF group.

Treatment	[^{3}H] Thymidine incorporation/ Bq per well
Control	286 ± 54
PDGF	2108 ± 468
PDGF + EGTA	1164 ± 84[c]
PDGF + Genistein	598 ± 109[c]
PDGF + PD 98059	842 ± 180[c]

Effect of PD 98059 on PDGF-BB-induced c-*myc* mRNA expression PDGF-BB induced a marked c-*myc* mRNA expression. On treatment of VSMC with PD 98059 (50 $\mu mol \cdot L^{-1}$), c-*myc* mRNA positive expression could not be detected (Fig 3).

DISCUSSION

The present investigation provides evidence for the involvement of the CCDPK signaling pathway in PDGF-BB-mediated proliferation of VSMC. We used PD 98059, a synthetic inhibitor of the Ca^{2+}-calmodulin dependent protein kinase kinase (CCDPKK, formerly called MEK), to inhibit activation of p42/p44 CCDPK, which could also result in an obvious reduction of the DNA synthesis. Our previous work showed that the p42/p44 CCDPK antisense ODN inhibited VSMC DNA synthesis induced by EGF[10]. These suggest that p42/p44 CCDPK is a requisite for the growth process.

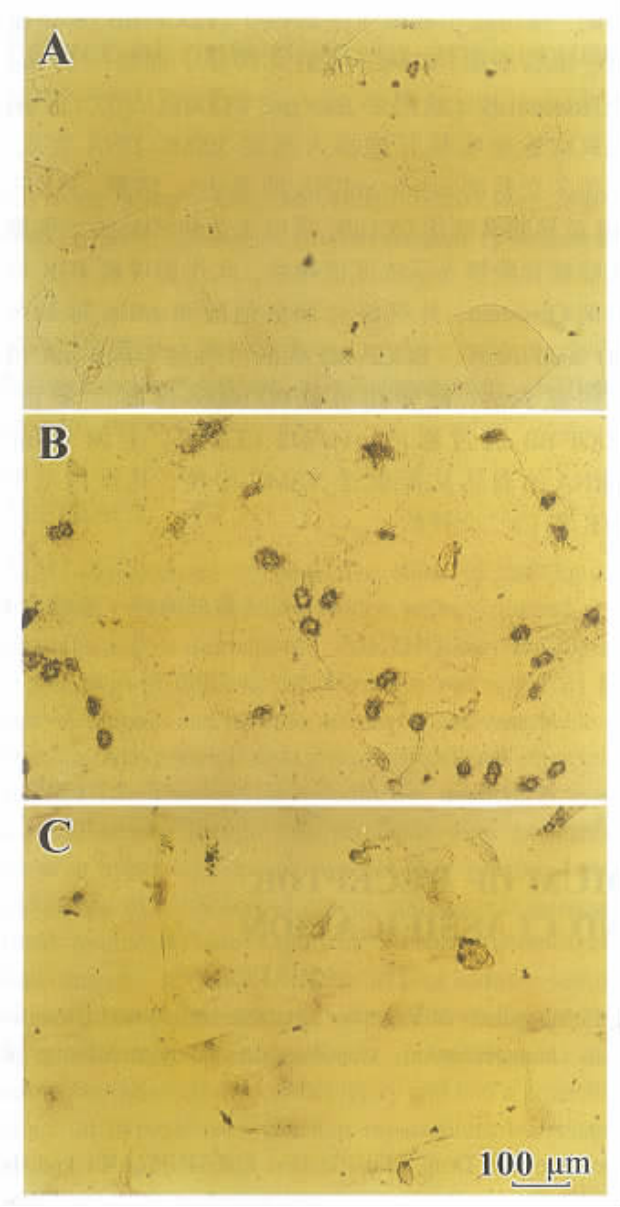

Fig 3. Effect of PD 98059 on PDGF-BB-stimulated *c*-myc mRNA expression in VSMC by *in situ* hybridization. A) Control VSMC without treatment; B) VSMC stimulated by PDGF-BB 2 μg·L^{-1} for 30 min; C) PD 98059 50 μmol·L^{-1} treated VSMC stimulated by PDGF-BB (2 μg·L^{-1}). (×100).

A number of agents activate p42/p44 CCDPK by stimulation of receptors with intrinsic tyrosine kinase activity or G-protein-coupled receptors[11]. The present study showed that genistein, a PTK inhibitor, inhibited PDGF-BB-induced activation of p42/p44 CCDPK, egtazic acid, an extracellular calcium chelator, had the same effect. These results suggest that tyrosine kinases and calcium may be upstream activators of the p42/p44 CCDPK pathway.

The c-*myc* oncogene has been shown to play an important role in the control of cell growth and cellular differentiation. Our study revealed that PDGF-BB induced a high level of c-*myc* mRNA expression, which was inhibited by PD 98059, suggesting that c-*myc* gene expression was consistent with celluar proliferation patterns and might be regulated by p44/p42 CCDPK.

In conclusion, the results of the present study demonstrated that PDGF-BB stimulated VSMC proliferation by activation of p44/p42 CCDPK, which was mediated by Ca^{2+}, PTK, and up-regulation of c-*myc* mRNA expression. Understanding the intracellular mechanism responsible for growth of SMC will potentially lead to preventing or controlling SMC proliferation and intimal hyperplasia.

REFERENCES

1 Ross R. Rous-Whipple Award Lecture. Atherosclerosis: A defense mechanism gone awry. Am J Pathol 1993; 143: 987 – 1002.
2 Ross R. The pathogenesis of atherosclerosis: a perspective for the 1990s. Nature 1993; 362: 801 – 9.
3 Schwartz SM, Reidy M. Common mechanisms of proliferation of smooth muscle in atherosclerosis and hypertension. Hum Pathol 1987; 18: 240 – 7.
4 Schwartz SM, Campbell GR, Campbell JH. Replication of smooth muscle in vascular disease. Circ Res 1986; 58: 427 – 44.
5 Jawien A, Bowen-Pope DF, Lindner V, Schwartz SM, Clowes AW. Platelet-derived growth factor promotes smooth muscle migration and intimal thickening in a rat model of balloon angioplasty. J Clin Invest 1992; 89: 507 – 11.
6 Sale EM, Atkinson PG, Sale GJ. Requirement of MAP kinase for the differentiation of fibroblasts to adipocytes, for insulin activation of p90 S6 kinase and for insulin or serum stimulation of DNA synthesis. EMBO J 1995; 14: 674 – 84.
7 Eguchi S, Hirata Y, Imai T, Kanno K, Marumo F. Phenotypic change of endothelial receptor subtype in cultured rat vascular smooth muscle cells. Endocrinology 1994; 134: 222 – 8.
8 Lucchesi PA, Bell JM, Wills LS, Byron KL, Corson MA, Berk BC. Ca^{2+}-dependent mitogen-activated protein kinase activation in spontaneously hypertensive rat vascular smooth muscle defines a hypertensive signal transduction phenotype. Circ Res 1996; 78: 962 – 70.
9 Bradrord MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 1976; 72: 248 – 54.
10 Huang SL, Ding B, Yu QS, Guo ZG. Effect of antisense mitogen-activated protein kinase oligonucleotides on rat vascular smooth muscle cell proliferation induced by EGF *in vitro*.

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E-mail aps@server.shcnc.ac.cn Phn/Fax 86-21-6474-2629

Acta Pharmacol Sin 1998；19：489－93.
11 Denhardt DT. Signal-transducing protein phosphorylation cascades mediated by Ras Rho protein in the mammalian cell：the potential for multiplex signalling. Biochem J 1996；318：729－47.

血小板源生生长因子刺激血管平滑肌细胞增殖及其分子机制

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关键词 Ca^{2+}-钙调蛋白依赖性蛋白激酶；血小板源生生长因子；血管平滑肌；培养的细胞；蛋白质印迹；原位杂交

目的：探讨血小板源生生长因子（PDGF-BB）刺激血管平滑肌细胞（VSMC）增殖及其分子机制. 方法：用 Western Blot 法测定 p44/p42 CCDPK 活性. [^{3}H] 脱氧胸腺嘧啶核苷酸掺入测定 VSMC DNA 合成. 原位杂交检测 c-*myc* mRNA 的表达. 结果：PDGF-BB 诱导的磷酸化 CCDPK 蛋白表达和 [^{3}H] 脱氧胸腺嘧啶核苷酸掺入呈浓度依赖性，此作用可被 PTK 抑制剂 Genistein，外钙络合剂依他酸和 MEK 抑制剂 PD 98059抑制. PDGF-BB 刺激可引起 c-*myc* mRNA 的明显表达，此作用可被 PD 98059 抑制. 结论：PDGF-BB 通过激活 p44/p42 CCDPK，上调 c-*myc* mRNA 的表达从而促进 VSMC 增殖，其作用是由 PTK 和 Ca^{2+} 介导的.

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