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Adenosine monophosphate-activated protein kinase regulates platelet-derived growth factor-BB-induced vascular smooth muscle cell migration

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ABSTRACT

Migration of vascular smooth muscle cells (VSMCs) is essential for repair of vascular injury, development of atherosclerotic lesions and restenosis after angioplasty or by-pass graft surgery. It has been reported that platelet-derived growth factor (PDGF)-BB induces VSMC migration via the p44/p42 mitogen-activated protein (MAP) kinase pathway and the phosphatidylinositol 3 (PI3)-kinase/Akt pathway. Adenosine monophosphate-activated protein kinase (AMPK) is generally known to regulate multiple metabolic pathway. In the present study, we investigated the involvement of AMPK in PDGF-BB-induced migration of VSMCs using, a VSMC line, A10 cells. PDGF-BB induced phosphorylation of AMPK-α at Thr-172 residue. Treatment of A10 cells with compound C, an AMPK inhibitor, suppressed PDGF-BB-induced migration in a concentration-dependent manner (0.01–1 μM). Compound C truly attenuated PDGF-BB induced phosphorylation of acetyl-CoA carboxylase, a downstream substance of AMPK. Downregulation of AMPK-α expression by the siRNA appeared an anti-migratory effect on PDGF-BB-induced migration. PDGF-BB-induced phosphorylation of c-Raf, MEK1/2 or p44/p42 MAP kinase, and phosphorylation of PI3-kinase or Akt were markedly suppressed by compound C. In conclusion, our results strongly suggest that PDGF-BB induces activation of AMPK in VSMCs, and subsequently regulates the migration via both the p44/p42 MAP kinase pathway and the PI3-kinase/Akt pathway.

Introduction

It is generally recognized that vascular smooth muscle cells (VSMCs)[1] contribute the regulation of blood flow due to contraction of blood vessels [1]. VSMCs play important roles also in vascular injury repair, angiogenesis and atherosclerosis [2]. Among VSMC functions, VSMC migration is essential for vascular development, vascular injury repair, development of atherosclerotic lesions and restenosis after angioplasty or by-pass graft surgery [3]. VSMC protrudes leading edge to contact with an extracellular substance and binding the formation of focal adhesion complexes [4]. A cascade of intracellular signal transduction, including GTP-binding proteins and tyrosine kinases, results in actin filament alignment and myosin contraction within the leading edge, dissolution of adhesion complexes, so that movement can take place [4]. It has been shown that VSMC migration is regulated by a variety of factors, including platelet-derived growth factor (PDGF)-BB, basic fibroblast growth factor (bFGF), transforming growth factor-β (TGF-β), epidermal growth factor and insulin-like growth factor [3]. However, the details of VSMC migration remain to be clarified.

PDGF was first identified as a growth-promoting factor released by human platelets [5]. At the present, it is generally known that PDGF is produced by not only platelets, but also monocyte/macrophages, vascular endothelial cells and VSMCs [5]. PDGF is secreted at the site of wound and contributes to the healing [6]. It has been reported that PDGF is implicated in fibrotic diseases of several different organs, atherosclerosis and restenosis after intervention [7]. In the cardiovascular system, PDGF is the strongest chemoattractant for VSMC migration [8]. VSMC migration following vascular injury is dependent on PDGF release by monocyte/macrophages, vascular endothelial cells and VSMCs [9]. PDGF expression is dynamic and responsive to variety of stimuli, including hypoxia, thrombin, cytokines, and growth factors [7]. The PDGF family consists of four dimeric growth factors: PDGF-AA, -AB, -BB, and -DD [10]. Among them, PDGF-BB plays an important role in atherosclerosis, since PDGF-BB is expressed in macrophages and VSMCs within atherosclerotic lesions [10]. Basic expression level of PDGF receptors is low in VSMCs, but increased dramatically by several factors including TGF-β, estrogen, interleukin-1α, bFGF, tumor necrosis factor-α, and lipopolysaccharide [7].

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[1] *Abbreviations used*: VSMCs, vascular smooth muscle cells; PDGF, platelet-derived growth factor; bFGF, basic fibroblast growth factor; TGF-β, transforming growth factor-β; MAP, mitogen-activated protein; AMPK, Adenosine monophosphate-activated protein kinase.

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Regarding the intracellular signaling system, PDGF-BB signaling is engaged in several well-characterized cellular signaling pathways such as the p44/p42 mitogen-activated protein (MAP) kinase pathway, the phosphatidylinositol 3 (PI3)-kinase/Akt pathway and the phospholipase C (PLC)-protein kinase C (PKC) pathway, ultimately leading to cellular proliferation, differentiation, survival and migration [7]. As for PDGF-BB-induced VSMC migration, it has been reported that p44/p42 MAP kinase, stress-activated protein kinase/c-*Jun* N-terminal kinase (SAPK/JNK), PI3-kinase and PKC are involved in PDGF-BB-induced rat aortic VSMC migration [11,12]. In a rat VSMC line, A10 cells, it has been reported that PDGF-BB induces the migration through p44/p42 MAP kinase and PLCγ activation [13]. However, the exact mechanism of PDGF-induced VSMC migration has not yet been fully elucidated.

Adenosine monophosphate-activated protein kinase (AMPK) plays a key role in the regulation of energy homeostasis and monitors of cellular energy charge [14]. AMPK consists of three subunits such as α, β and γ [14]. Among the subunits, α-subunit is recognized as a catalytic site, whereas β and γ-subunits are considered as regulatory sites [14]. The activation of AMPK is mainly regulated by phosphorylation of AMPK-α at Thr-172 residue [15]. With regard to the role of AMPK in VSMCs, it has been reported that serum-induced VSMC migration is suppressed by 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranosyl 5′-monophosphate (AICAR), an activator of AMPK [16], and activation of AMPK strongly suppresses the proliferation in VSMCs [17]. However, the role of AMPK on PDGF-BB-induced VSMC migration has not yet been clarified. Therefore, in the present study, we investigated whether AMPK is involved in PDGF-BB-induced A10 cell migration and the detailed mechanism.

Materials and methods

Materials

PDGF-BB was obtained from R&D Systems, Inc (Minneapolis, MN). AICAR, was purchased from Sigma Chemical Co. (St. Louis, MO). Compound C, PD98059 and LY294002 were obtained from Calbiochem-Novabiochem (La Jolla, CA). Antibodies against phospho-specific AMPK-α (Thr-172), AMPK-α, phospho-specific acetyl-CoA carboxylase, phospho-specific c-Raf, phospho-specific MAP/extracellular signal-regulated kinase kinase (MEK) 1/2, MEK1/2, phospho-specific p44/p42 MAP kinase, p44/p42 MAP kinase, phosphor-specific Akt (Thr-308 or Ser-473) and Akt were obtained from Cell Signaling Technology, Inc (Beverly, MA). Antibodies against GAPDH and phospho-specific PI3-kinase p85 were purchased from Santa Cruz Biotechnology, Inc (Santa Cruz, CA). ECL Western blotting detection system was purchased from GE Healthcare (Buckinghamshire, UK). Other materials and chemicals were obtained from commercial sources. Compound C or AICAR was dissolved in dimethyl sulfoxide. The maximum concentration of dimethyl sulfoxide was 0.1%, which did not affect the cell migration assay or Western blot analysis.

Cell culture and treatments

Fetal rat aortic smooth muscle derived A10 cells were obtained from American Type Culture Collection (Rockville, MD). The cells were seeded into 35-mm (8×10^4 cells/dish) or 90-mm (4×10^5 -cells/dish) diameter dishes and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) at 37 °C under a humidified atmosphere of 5% carbon dioxide and 95% air. After 6 days, the medium was exchanged for serum-free DMEM. The cells (90-mm diameter dishes) were then used for Western blot analysis after 24 h. The cells were pretreated with compound C for 60 min before PDGF-BB or AICAR stimulation when indicated.

Cell migration assay

Cell migration was assessed in using Boyden chamber (polycarbonate membrane with 8-μm pores, Transwell®, Coring Costar Corp, Cambridge, MA). The cells were trypsinized, and seeded (3×10^4 cells/well) onto the upper chamber in serum-free DMEM. The cells were pretreated with compound C in lower chamber for 60 min at 37 °C. Then PDGF-BB or AICAR was added to lower chamber and incubated for 9 h at 37 °C. The cells on the upper surface of the membrane were mechanically removed. The migrated cells adherent to the underside of the membrane were fixed with 4% paraformaldehyde and stained with 4′,6-diamidino-2-phenylindole (DAPI) solution. The migrated cells were photographed and counted using fluorescent microscopy at a magnification of 20× by counting the stained cells from three randomly chosen high power fields.

Western blot analysis

The cultured cells (90-mm diameter dishes) were stimulated by 30 ng/ml PDGF-BB or 3 mM AICAR in serum-free DMEM for the indicated periods. The cells were washed twice with phosphate-buffered saline, and then lysed and sonicated in a lysis buffer containing 62.5 mM Tris/HCl (pH 6.8), 2% sodium dodecyl sulfate (SDS), 50 mM dithiothreitol and 10% glycerol. The sample was subjected to SDS–polyacrylamide gel electrophoresis (PAGE) that was performed using by the method of Laemmli [18]. Western blot analysis was performed using phospho-specific AMPK-α antibodies, AMPK-α antibodies, phospho-specific acetyl-CoA carboxylase antibodies, phospho-specific c-Raf antibodies, phospho-specific MEK1/2 antibodies, MEK1/2 antibodies, phospho-specific p44/p42 MAP kinase antibodies, p44/p42 MAP kinase antibodies, phospho-specific PI3-kinase antibodies, phospho-specific Akt antibodies, Akt antibodies or GAPDH antibodies with peroxidase-labeled anti-rabbit IgG antibodies used as secondary antibodies. Peroxidase activity on polyvinylidene difluoride membrane was visualized on X-ray film by means of the ECL Western blotting detection system.

Small interfering RNA transfection

The cells (35-mm diameter dishes) were seeded in DMEM containing 10% FBS and sub-cultured for 72 h. Predesigned small interfering RNAs (siRNAs) targeting rat AMPK-α (Prkaa1–5), and the negative control-siRNA (Silencer® Negative Control No.1) were purchased from Qiagen (Venlo, Netherlands), and Life technologies Co. (Carlsbad, CA), respectively. Transfection was performed according to the manufacturer's protocol (Bio-Rad, Tokyo, Japan). In brief, 5 μl of siLentFect (Bio-Rad, Tokyo, Japan) and finally 30 nM of AMPK-α-siRNA or negative control-siRNA were diluted with serum-free DMEM, preincubated at room temperature for 20 min. Cells were incubated at 37 °C for 72 h with siRNA–siLentFect complexes and subsequently harvested for cell migration assay or exchanged to serum-free DMEM for Western blot analysis.

Statistical analysis

The data were analyzed by ANOVA followed by Bonferroni's method for multiple comparisons between pairs. $P < 0.05$ was considered to be significant. All data are presented as the mean ± SD of triplicate determinations from three independent cell preparations. Each experiment was repeated three times with similar results.