Original Paper

Crosstalk between PI3 Kinase/PDK1/Akt/Rac1 and Ras/Raf/MEK/ERK Pathways Downstream PDGF Receptor

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Key Words

PDGF- $\beta\beta$ receptor • Akt • Rac1 • ERK • Crosstalk

Abstract

Background/Aims: Our earlier studies suggested crosstalk between IRS/PI3 kinase/PDK1/Akt/Rac1/ROCK and (Shc2/Grb2/SOS)/Ras/Raf/MEK/ERK pathways downstream PDGF- $\beta\beta$ receptor responsible for chemotaxis and proliferation of malignant mesothelioma cells. The present study was conducted to obtain evidence for this. **Methods:** To assess activation of Akt, MEK, and ERK, Western blotting was carried out on MSTO-211H malignant mesothelioma cells using antibodies against phospho-Thr308-Akt, phospho-Ser473-Akt, Akt, phospho-MEK, MEK, phospho-ERK1/2, and ERK1/2. To knock-down Akt, PI3 kinase, PDK1, and Rac1, siRNAs silencing each-targeted gene were constructed and transfected into cells. To monitor Rac1 activity, FRET monitoring was carried out on living and fixed cells. **Results:** ERK was activated under the basal conditions in MSTO-211H cells, and the activation was prevented by inhibitors for PI3 kinase, PDK1, Akt, and Rac1 or by knocking-down PI3 kinase, PDK1, Akt, and Rac1. Akt was also activated under the basal conditions, and the activation was suppressed by a MEK inhibitor and an ERK1/2 inhibitor. In the FRET analysis, Rac1 was activated under the basal conditions, and the activation was inhibited by a MEK inhibitor and an ERK1/2 inhibitor. **Conclusion:** The results of the present study show that ERK could be activated by PI3 kinase, PDK1, Akt, and Rac1 and that alternatively, Akt and Rac1 could be activated by MEK and ERK in MSTO-211H cells.

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Introduction

Platelet-derived growth factor (PDGF) promotes proliferation of malignant mesothelioma cells as well as other cancer types of cells [1-6]. The PDGF family includes PDGF-A, -B, -C and -D. PDGF-A and -B are secreted as active dimers composed of single-domain protein chains (PDGF-AA and -BB), but otherwise PDGF-C and -D, which contain an N-terminal CUB and a conserved C-terminal growth factor domain, are secreted as a latent dimeric factor and undergo proteolytic processing at the hinge region between the CUB domain and the growth factor domain to produce the active form of PDGF-CC and -DD through tissue plasminogen activator (tPA) and urokinase-type plasminogen activator (uPA), respectively [7-9]. PDGF receptors consist of the PDGF- α and/or - β subunit such as $\alpha\alpha$ homodimer, $\alpha\beta$ heterodimer, and $\beta\beta$ homodimer [7]. PDGF- $\alpha\alpha$ and - $\alpha\beta$ receptors are activated by PDGF-AA, -BB, and -CC, and PDGF- $\beta\beta$ receptor is activated by PDGF-DD [8, 9].

In our earlier studies, PDGF-D, endogenously secreted, facilitated chemotaxis and promoted proliferation of malignant mesothelioma cells through PDGF- $\beta\beta$ receptor [10, 11]. PDGF- $\beta\beta$ receptor is a receptor tyrosine kinase, which phosphorylates the receptor by itself and insulin receptor substrate protein (IRS). IRS, in turn, recruits and activates phosphoinositide 3-kinase (PI3 kinase), to produce phosphatidylinositol (3,4,5)-trisphosphate [PI (3,4,5) P_3] from phosphatidylinositol (4,5)-biphosphate [PI (4,5) P_2]. PI (3,4,5) P_3 binds to and activates phosphoinositide-dependent kinase-1 (PDK1), which phosphorylates and activates Akt. Akt activates the Rho family Rac1/Cdc42, followed by activation of the effector Rho-associated coiled-coil forming protein kinase (ROCK). PDGF- $\beta\beta$ receptor, alternatively, phosphorylates Shc2, which forms a complex of Shc2/Grb2/SOS to activate Ras. Ras subsequently activates Raf followed by the sequent phosphorylation and activation of mitogen-activated protein (MAP) kinase cascades such as MAP kinase kinase (MEK) and extracellular signal-regulated kinase (ERK). Spontaneous proliferation of malignant mesothelioma cells was clearly suppressed by knocking-down PDGF-D and PDGF- $\beta\beta$ receptor [11]. The proliferation was significantly inhibited by the Akt inhibitor MK2206, and the ROCK inhibitor Y27632, but amazingly the PI3 kinase inhibitor wortmannin, the PDK1 inhibitor BX912, or the Rac1 inhibitor NSC23766 had no effect [11]. This suggests that Akt or ROCK is not activated along a PDGF- $\beta\beta$ receptor/PI3 kinase/PDK1/Akt/Rac1/ROCK axis. Moreover, proliferation of MSTO-211H cells was still inhibited by the MEK inhibitor PD98059 and the ERK1/2 inhibitor FR180204. Then, we hypothesized that crosstalk between IRS/PI3 kinase/PDK1/Akt/Rac1/ROCK and (Shc2/Grb2/SOS)/Ras/Raf/MEK/ERK pathways underlies downstream PDGF- $\beta\beta$ receptor in MSTO-211H cells.

To prove this hypothesis, the present study monitored activities of Akt and MEK/ERK by Western blotting and Rac1 using a plasmid encoded f $\ddot{o}$ erster resonance energy transfer (FRET) probe in MSTO-211H cells, a biphasic human malignant mesothelioma cell line. We show here that PI3 kinase, PDK1, Akt, and Rac1 could activate ERK in MSTO-211H cells and that MEK and ERK could otherwise activate Akt and Rac1.

Materials and Methods

Cell culture

MSTO-211H cell line was purchased from American Type Culture Collection (Manassas, VA, USA). Cells were grown in RPMI-1640 medium supplemented with 10% (v/v) heat-inactivated FBS, 0.003% (w/v) L-glutamine, penicillin (final concentration, 100 U/ml), and streptomycin (final concentration, 0.1 mg/ml), in a humidified atmosphere of 5% CO₂ and 95% air at 37 °C.

Western blotting

MSTO-211H cells were untreated and treated with a variety of inhibitors or transfected with small interfering RNAs (siRNAs), and then lysed in a lysate solution [150 mM NaCl, 20 mM Tris, 0.1% (v/v) Tween-20 and 0.1% (w/v) sodium dodecyl sulfate (SDS), pH 7.5] containing 1% (v/v) protease inhibitor

cocktail and 1% (v/v) phosphatase inhibitor cocktail. The lysates were centrifuged at 3,000 rpm for 5 min at 4 °C. Proteins were separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) using a TGX gel (BioRad; Hercules, CA, USA) and then transferred to polyvinylidene difluoride (PVDF) membranes. Blotting membranes were blocked with TBS-T [150 mM NaCl, 0.1% (v/v) Tween-20 and 20 mM Tris, pH 7.5] containing 5% (w/v) bovine serum albumin and subsequently reacted with antibodies against phospho-Thr308-Akt (pT308)(Cell Signaling Technology, Inc.; Danvers, MA, USA), phospho-Ser473-Akt (pS473)(Cell Signaling Technology), Akt (Cell Signaling Technology), phospho-MEK (pMEK)(Cell Signaling Technology), MEK (Cell Signaling Technology), phospho-ERK (pERK)(Santa Cruz Biotechnology; Santa Cruz, CA, USA), ERK (Santa Cruz Biotechnology), PI3 kinase (Sigma-Aldrich; St. Louis, MO, USA), PDK1 (Sigma-Aldrich), Rac1 (Sigma-Aldrich), and β -actin (Sigma-Aldrich). After washing, membranes were reacted with a horseradish peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG antibody. Immunoreactivity was detected with an ECL kit (GE healthcare; Piscataway, NJ, USA) and visualized using a chemiluminescence LAS-4000mini detection system (GE healthcare). Protein concentrations for each sample were determined with a BCA protein assay kit (Thermo Fisher Scientific; Waltham, MA, USA).

Construction and transfection of siRNA

The siRNA to silence the Akt1/2-targeted gene (Akt siRNA) was purchased from Santa Cruz Biotechnology. The siRNAs to silence the PI3 kinase p110 δ -targeted gene (PI3K KD), the PDK1-targeted gene (PDK1 KD), the Rac1-targeted gene (Rac1 KD) were obtained from Ambion (Carlsbad, CA, USA). The sequences of siRNAs used were 5'-GUGAGAAUUGAACGGUUt -3' and 5'-AACCGUUCAAAUUUCACActa-3' for PI3 kinase p110 δ ; 5'-GGACCAUCCGUUCAAUUt-3' and 5'-AAUUGAACGGAUGGUGUCt-3' for PDK1; and 5'-CUACUGUCUUUGACAAUUt-3' and 5'-UAAUUGUCAAGACAGUAGgg-3' for Rac1. Each negative control siRNA (NC siRNA)(Ambion) had the scrambled sequence, the same GC content, and nucleic acid composition. siRNAs were transfected into cells using a Lipofectamine reagent (Invitrogen; Carlsbad, CA, USA). Cells were used for experiments 48 h after transfection.

Monitoring of Rac1 activity

FRET probe containing Raichu-Rac1 with EV linker was kindly gifted from Dr. Matsuda (Kyoto university)[12, 13]. MSTO-211H cells were transfected with the FRET probe using an X-tremeGENE HP DNA transfection reagent (Roche, Mannheim, Germany). Twenty-four h after transfection fluorescent signals in living cells were monitored at 485-nm argon laser for cyan fluorescent protein (CFP) and 517-nm argon laser for yellow fluorescent protein (YFP) in the presence and absence of inhibitors with a Zeiss LSM510 META inverted microscope (Oberkochen, Germany). In a different set of experiments, cells were untreated and treated with inhibitors for periods of time, and then fixed with formaldehyde [final conc. 3.7 (v/v) %] for 30 min. Then, CFP and YFP signals were monitored. The background was subtracted and the FRET ratio (YFP signal intensity/CFP signal intensity) was calculated using an ImageJ software (National Institutes of Health, USA).

Statistical analysis

Statistical analysis was carried out using unpaired *t*-test and Dunnett's test.

Results

PI3 kinase, PDK1, Akt, and Rac1 activates ERK

ERK was phosphorylated in the absence of inhibitors in MSTO-211H cells (Fig. 1A-E), indicating that ERK is spontaneously activated under the basal conditions. The PI3 kinase inhibitor wortmannin (10 μ M) [14] reduced ERK phosphorylation (Fig. 1A). Expression of PI3 kinase was suppressed in MSTO-211H cells transfected with the PI3 kinase siRNA (Fig. 4A), confirming knocking-down PI3 kinase. ERK phosphorylation was significantly prevented by knocking-down PI3 kinase (Fig. 4C). It is indicated from these results that PI3 kinase has the potential to activate ERK.

The PDK1 inhibitor BX912 (100 nM) [15] significantly reduced ERK phosphorylation (Fig. 1B). Expression of PDK1 was suppressed in MSTO-211H cells transfected with the PDK1

Fig. 1. ERK activation via a PI3 kinase/PDK1/Akt/Rac1 pathway. MSTO-211H cells were untreated (-) and treated (+) with wortmannin (WM)(10 μ M)(A), BX912 (BX)(100 nM)(B), MK2206 (MK)(5 μ M)(C), NSC23766 (NSC)(1 μ M)(D), or Y27632 (10 μ M)(E) for 10 min, and then, Western blotting was carried out using antibodies against pERK and ERK. In the graphs, each column represents the mean ($\pm$ SEM) ratio of pERK signal intensity/ERK signal intensity ($n=4$ independent experiments). P values, Dunnett's test.

Fig. 2. Akt activation due to MEK and ERK. MSTO-211H cells were untreated (-) and treated (+) with wortmannin (WM)(10 μ M)(A), BX912 (BX)(100 nM)(B), MK2206 (MK)(5 μ M)(C), PD98059 (PD)(50 μ M)(D), or FR180204 (FR)(10 μ M)(E) for 10 min, and then, Western blotting was carried out using antibodies against pT308, pS473, and Akt. In the graphs, each column represents the mean ($\pm$ SEM) ratio of pT308 signal intensity or pS473 signal intensity/Akt signal intensity ($n=4$ independent experiments). P values, Dunnett's test.

siRNA (Fig. 4D), confirming knocking-down PDK1. ERK phosphorylation was significantly prevented by knocking-down PDK1 (Fig. 4F). Collectively, these results indicate that PDK1 has the potential to activate ERK.

The Akt inhibitor MK2206 (5 μ M) [16] significantly reduced ERK phosphorylation (Fig. 1C). Expression of Akt was significantly reduced in MSTO-211H cells transfected with the Akt siRNA (Fig. 3A), confirming Akt knocking-down. MEK phosphorylation was not attenuated by knocking-down Akt (Fig. 3B). Phosphorylation of ERK, on the other hand, was significantly

Fig. 3. Akt-mediated ERK activation. (A) Western blotting was carried out in MSTO-211H cells using antibodies against Akt and β -actin 48 h after transfection with the NC siRNA (NC) or the Akt siRNA. Signal intensities for Akt were normalized by those for β -actin. In the graph, each column represents the mean ($\pm$ SEM) normalized expression of Akt ($n=4$ independent experiments). P value, unpaired t -test. In different sets of experiments, Western blotting was carried out using antibodies against pMEK and MEK (B) or pERK and ERK (C) 48 h after transfection with siRNAs. In the graphs, each column represents the mean ($\pm$ SEM) ratio of pMEK signal intensity/MEK signal intensity or pERK signal intensity/ERK signal intensity ($n=4$ independent experiments). KD, knock-down. P value, Dunnett's test.

prevented by knocking-down Akt (Fig. 3C). These results interpret that Akt activates ERK directly, but not through MEK activation.

The Rac1 inhibitor NSC23766 (1 μ M) [17] reduced ERK phosphorylation (Fig. 1D). Expression of Rac1 was suppressed in MSTO-211H cells transfected with the Rac1 siRNA (Fig. 4G), confirming knocking-down Rac1. ERK phosphorylation was significantly prevented by knocking-down Rac1 (Fig. 4I). These results indicate that Rac1 has the potential to activate ERK.

In contrast, the ROCK inhibitor Y27632 (10 μ M) [18] had no effect on ERK phosphorylation (Fig. 1E), indicating no implication of ROCK in ERK activation. Taken together, these results imply that ERK could be still activated through a pathway along a PDGF- β receptor/PI3 kinase/PDK1/Akt/Rac1 axis.

MEK and ERK activate Akt and Rac1

Akt was phosphorylated at Thr308 and Ser473 in the absence of inhibitors in MSTO-211H cells (Fig. 2A-E), indicating that Akt is spontaneously activated under the basal conditions. Phosphorylation of Akt at Thr308 and Ser473 was inhibited by wortmannin (10 μ M) (Fig. 2A) and BX912 (100 nM) (Fig. 2B). In addition, Akt phosphorylation at Thr308 and Ser473 was clearly inhibited by knocking-down PI3 kinase (Fig. 4B) and PDK1 (Fig. 4E). These results imply that Akt is activated through a pathway along a PDGF- β receptor/PI3 kinase/PDK1/Akt axis. Interestingly, Akt phosphorylation was also inhibited by MK2206 (5 μ M) (Fig. 2C). This suggests that Akt might be activated through its autophosphorylation. Moreover, Akt phosphorylation was significantly inhibited by knocking-down Rac1 (Fig. 4H). This suggests that Rac1 is capable of activating Akt, i.e., Rac1 serves as a positive feedback activator of Akt.

Phosphorylation of Akt at Thr308 and Ser473 was also prevented by the MEK inhibitor PD98059 (50 μ M) [19] (Fig. 2D) and the ERK1/2 inhibitor FR180204 (10 μ M) [20, 21] (Fig. 2E). This indicates that MEK and ERK have the potential to activate Akt.

We finally monitored Rac1 activity in living and fixed MSTO-211H cells using a FRET probe. In the FRET analysis, increasing and reducing FRET ratio (YFP signal intensity/CFP signal intensity) correspond to activation and inactivation of Rac1, respectively. For living cells, the FRET ratio was apparently reduced by MK2206 (5 μ M), while the ratio was not altered in the absence of inhibitors (Fig. 5A). This indicates that Rac1 is activated in an Akt-dependent manner under the basal conditions. The FRET ratio was also diminished by FR180204 (10 μ M), but to a lesser extent than that for MK2206 (Fig. 5A), suggesting ERK-mediated Rac1 activation. For fixed cells, the FRET ratio was clearly inhibited by wortmannin

Fig. 4. ERK activation due to PI3 kinase, PDK1, and Rac1. Expression of PI3 kinase (A), PDK1 (D), and Rac1 (G) in MSTO-211H cells transfected with the NC and each siRNA. Signal intensities were normalized by those for β -actin. In the graphs, each column represents the mean ($\pm$ SEM) normalized expression of PI3 kinase, PDK1, and Rac1 ($n=4$ independent experiments). P values, unpaired t -test. Akt activity in cells transfected with the NC siRNA, the PI3 kinase siRNA (B), the PDK1 siRNA (E), and the Rac1 siRNA (H). In the graphs, each column represents the mean ($\pm$ SEM) ratio of pT308 signal intensity or pS473 signal intensity/Akt signal intensity ($n=4$ independent experiments). P values, Dunnett's test. ERK activation in cells transfected with the NC siRNA, the PI3 kinase siRNA (C), the PDK1 siRNA (F), and the Rac1 siRNA (I). In the graphs, each column represents the mean ($\pm$ SEM) ratio of pERK signal intensity/ERK signal intensity ($n=4$ independent experiments). P values, Dunnett's test. PI3K, PI3 kinase; KD, knock-down.

(10 μ M), BX912 (100 nM), and MK2206 (5 μ M)(Fig. 5B), confirming Rac1 activation along a PI3 kinase/PDK1/Akt axis. The FRET ratio was still reduced by PD98059 (50 μ M) and FR180204 (10 μ M)(Fig. 5B). This indicates that Rac1 could be activated by MEK or ERK.

Discussion

PDGF- β receptor is implicated in two main pathways; an IRS/PI3 kinase/PDK1/Akt/Rac1/ROCK pathway and a (Shc2/Grb2/SOS)/Ras/Raf/MEK/ERK pathway (Fig. 6). In the present study, activation of Akt and ERK was found under the basal conditions in MSTO-211H biphasic human malignant mesothelioma cells. This, in the light of the fact that spontaneous proliferation of malignant mesothelioma cells was inhibited by knocking-down PDGF-D and PDGF- β receptor [11], indicates that Akt and ERK are activated via two main pathways linked to PDGF- β receptor, which is activated by PDGF-D endogenously secreted in MSTO-211H cells.

Fig. 5. Rac1 activation due to MEK and ERK. (A) FRET monitoring was carried out on living MSTO-211H cells in the absence (Control) and presence of MK2206 (5 μ M) or FR180204 (10 μ M), and the FRET ratio (YFP signal intensity/CFP signal intensity) was calculated. The FRET ratio images are shown in the upper panel and time-course FRET ratio profiles are shown in the graph. Note that a similar result was obtained with 6 independent experiments. (B) Cells were untreated and treated with wortmannin (10 μ M), BX912 (100 nM), MK2206 (5 μ M), PD98059 (50 μ M), or FR180204 (10 μ M) for periods of time as indicated. Subsequently, cells were fixed followed by FRET monitoring. In the graphs, each point represents the mean ($\pm$ SEM) FRET ratio ($n=5-10$ independent experiments).

An established pathway is that ERK is activated through a pathway along a (Shc2/Grb2/SOS)/Ras/Raf/MEK/ERK axis as mediated by receptor tyrosine kinase including PDGF- β receptor. In the present study, ERK phosphorylation in MSTO-211H cells was prevented by inhibitors for PI3 kinase, PDK1, Akt, and Rac1 or by knocking-down PI3 kinase, PDK1, Akt, and Rac1. This indicates that ERK could be still activated through a pathway along a PDGF- β receptor/IRS/PI3 kinase/PDK1/Akt/Rac1 axis, regardless of a PDGF- β receptor/ (Shc2/Grb2/SOS)/Ras/Raf/MEK/ERK axis (Fig. 6). In other words, this shows a crosstalk activation of ERK in PDGF- β receptor signaling pathways (Fig. 6).

It is well-recognized that Akt is activated through a pathway along an IRS/PI3 kinase/PDK1/Akt axis as mediated by receptor tyrosine kinase including PDGF- β receptor. In support of this pathway,

Fig. 6. A schematic diagram for crosstalk in PDGF- β receptor signaling pathways.

Akt phosphorylation in MSTO-211H cells was inhibited by inhibitors for PI3 kinase and PDK1 or by knocking-down PI3 kinase and PDK1. Notably, Akt phosphorylation was still inhibited by an Akt inhibitor, suggesting that Akt might be activated through its autophosphorylation. Of particular interest is the finding that Akt phosphorylation was suppressed by knocking-down Rac1. This raises the possibility that Rac1 serves as a positive feedback activator of Akt.

Akt phosphorylation was also prevented by a MEK inhibitor or an ERK inhibitor. This indicates that MEK and ERK could activate Akt via a pathway along a (Shc2/Grb2/SOS)/Ras/Raf/MEK/ERK axis as mediated by receptor tyrosine kinase including PDGF- $\beta\beta$ receptor (Fig. 6). In the FRET analysis, Rac1 was activated under the basal conditions in MSTO-211H cells. Expectedly, Rac1 activation was suppressed by a PI3 kinase inhibitor, a PDK1 inhibitor, and an Akt inhibitor. This provides further evidence for Rac1 activation through a pathway along a PDGF- $\beta\beta$ receptor/IRS/PI3 kinase/PDK1/Akt/Rac1 axis (Fig. 6). Moreover, Rac1 activation was also inhibited by a MEK inhibitor and an ERK1/2 inhibitor. This indicates that MEK and ERK could also activate Rac1 via a PDGF- $\beta\beta$ receptor/(Shc2/Grb2/SOS)/Ras/Raf/MEK/ERK signaling pathway (Fig. 6). Overall, these results may represent fresh insight into activation of Akt/Rac1 and ERK through crosstalk between PDGF- $\beta\beta$ receptor-linked two signaling pathways.

In summary, the results of the present study show that ERK could be activated through a pathway along a PDGF- $\beta\beta$ receptor/IRS/PI3 kinase/PDK1/Akt/Rac1 axis and that alternatively, Akt and Rac1 could be activated through a pathway along a PDGF- $\beta\beta$ receptor/(Shc2/Grb2/SOS)/Ras/Raf/MEK/ERK axis in MSTO-211H cells.

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