Raf-1, actin dynamics and Abl in human airway smooth muscle cells

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ONLINE DATA SUPPLEMENT

Materials and Methods

Cell culture. Human airway smooth muscle (HASM) cells were obtained from laboratory of Dr. Reynold A. Panettieri in University of Pennsylvania. In addition, cells were prepared from human airway smooth muscle tissues that were obtained from the International Institute for Advanced Medicine. Briefly, muscle tissues were incubated for 10 min with dissociation solution [130 mM NaCl, 5 mM KCl, 1.0 mM $CaCl_2$, 1.0 mM $MgCl_2$, 10 mM Hepes, 0.25 mM EDTA, 10 mM D-glucose, 10 mM taurine, pH 7, 4.5 mg collagenase (type I), 10 mg papain (type IV), 1 mg tryosine inhibitor, 1 mg/ml BSA and 1 mM dithiothreitol]. All enzymes were obtained from Sigma-Aldrich. The tissues were then washed with Hepes-buffered saline solution (composition, in mM: 10 Hepes, 130 NaCl, 5 KCl, 10 glucose, 1 $CaCl_2$, 1 $MgCl_2$, 0.25 EDTA, 10 taurine, pH 7). The cell suspension was mixed with Ham's F12 supplemented with 10% (v/v) fetal bovine serum (FBS) and antibiotics (100 units/ml penicillin, 100 µg/ml streptomycin). Cells were cultured at 37°C in the presence of 5% CO_2 in the same medium. The medium was changed every 3–4 days until the cells reached confluence, and confluent cells were passaged with trypsin/EDTA solution.

Immunoblot analysis. Cells were lysed in SDS sample buffer composed of 1.5% dithiothreitol, 2% SDS, 80 mM Tris-HCl (pH 6.8), 10% glycerol and 0.01% bromophenol blue. The lysates were boiled in the buffer for 5 min and separated by SDS-PAGE. Proteins were transferred to a nitrocellulose membrane. The membrane was blocked with bovine serum albumin or milk for 1 h and probed with primary antibody followed by horseradish peroxidase (HRP)-conjugated secondary antibody (Fisher Scientific). Proteins were visualized by enhanced chemiluminescence (Fisher Scientific) using the LAS-4000 Fuji Image System. Raf-1 antibody was purchased from Santa Cruz Biotechnology and BD Biosciences. Phospho-ERK1/2 (Thr-

202/Tyr-204) antibody, total ERK1/2 antibody, phospho-MEK1/2 (Ser-218/Ser-222) antibody, and total MEK1/2 antibody were purchased from Santa Cruz Biotechnology and Cell Signaling. GAPDH antibody was purchased from Fitzgerald (MA, USA).

The levels of phosphoprotein and total protein were quantified by scanning densitometry of immunoblots (Fuji Multigauge Software). Changes in protein phosphorylation were expressed as a magnitude increase over levels of phosphorylation in unstimulated cells. The luminescent signals from all immunoblots were within the linear range.

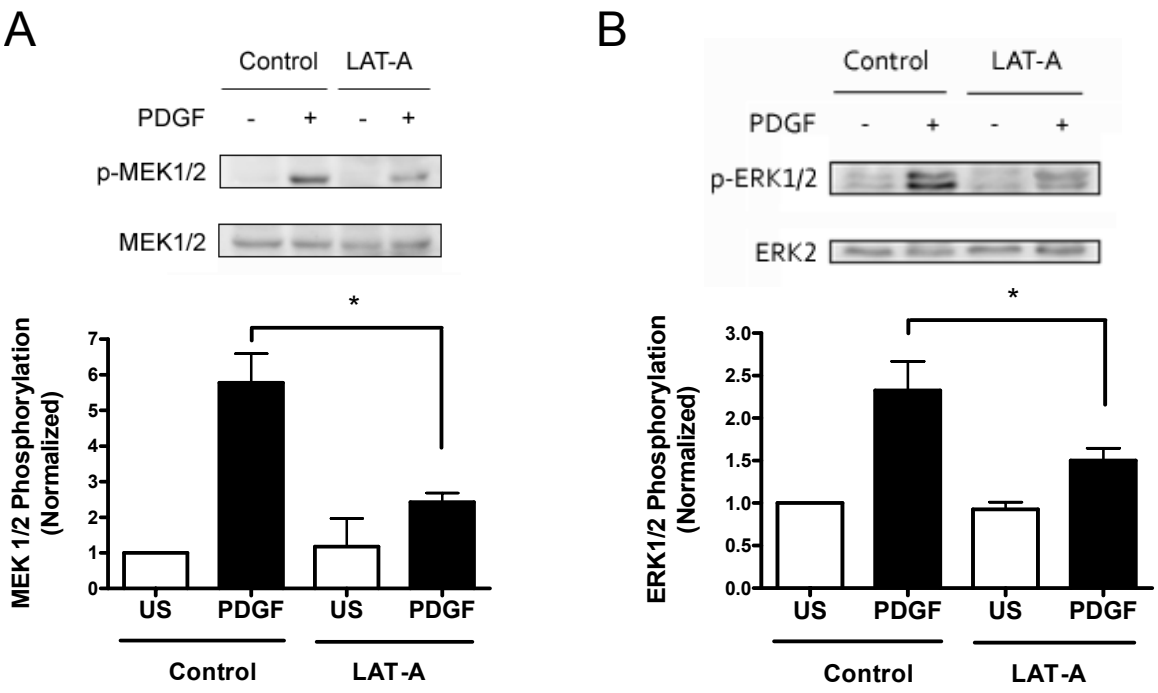
Immunofluorescence. Cells were plated in dishes containing coverslips and incubated in serum-free media for 1 day. After different treatments, these cells were fixed for 15 min in 4% paraformaldehyde, and were then washed three times in Tris-buffered saline (TBS) containing 50 mM Tris, 150 mM NaCl, and 0.1% NaN_3 followed by permeabilization with 0.2% Triton X-100 dissolved in TBS for 5 min. These cells were immunofluorescently stained using Raf-1 antibody followed by appropriate secondary antibody conjugated to Alexa 543 (Invitrogen). The cellular localization of fluorescently labeled proteins was viewed under a high resolution digital fluorescent microscopy (Leica). The time of image capturing, intensity gaining, and image black levels were optimally adjusted, and kept constant for all experiments to standardize the fluorescence intensity measurements among experiments. To evaluate Raf-1 membrane associated translocation, the fluorescent intensity was quantified for three line scans across the periphery of cells (excluding the nucleus) using NIH ImageJ software. Ratios of the grey value at the cell edge to the value in the cell interior were determined for each line scan as follows: ratios of the average maximal intensity at the cell periphery to minimal pixel intensity in the cell interior. The ratios of intensity at the cell border to that in the cell interior for all the line scans performed on a given cell were averaged to obtain a single value for the ratio of each cell.

Figure Legends

Figure S1. Inhibition of actin polymerization by latrunculin A (LAT-A) diminishes phosphorylation of MEK1/2 and ERK1/2 induced by PDGF. Control cells and cells treated with LAT-A (1 μM, 30 min) cells were stimulated with PDGF (10 ng/ml, 10 min), or left unstimulated (US). MEK1/2 phosphorylation (Ser-218/Ser-222) and ERK1/2 phosphorylation (Thr-202/Tyr-204) were evaluated by immunoblot analysis. Protein phosphorylation in cells induced by PDGF is normalized to the level in unstimulated cells. (A) Treatment with LAT-A attenuated PDGF-induced MEK1/2 phosphorylation (*, $P < 0.05$, n = 3-4). (B) PDGF-stimulated ERK1/2 phosphorylation was diminished in cells treated with LAT-A (*, $P < 0.05$, n = 4-5).

Figure S2. **Abl silencing and rescue affect MEK1/2 and ERK1/2 phosphorylation in response to activation with PDGF.** Control, Abl KD and rescue cells were stimulated with 10 ng/ml PDGF for 10 min. MEK1/2 phosphorylation (Ser-218/Ser-222) (A) and ERK1/2 phosphorylation (Thr-202/Tyr-204) (B) were then evaluated. Protein phosphorylation in cells induced by PDGF is normalized to the level in unstimulated cells. Phosphorylation levels in Abl KD cells are significantly reduced compared to control and rescue cells (* $P < 0.05$). Values represent mean ± SE (n = 3-4).

Fig. S1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PDGF
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MEK1/2
*
MEK 1/2 Phosphorylation (Normalized)
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1
2
3
4
5
6
7
US
PDGF
US
PDGF
Control
LAT-A
B
Control
LAT-A
PDGF
- + - +
p-ERK1/2
ERK2
*
ERK1/2 Phosphorylation (Normalized)
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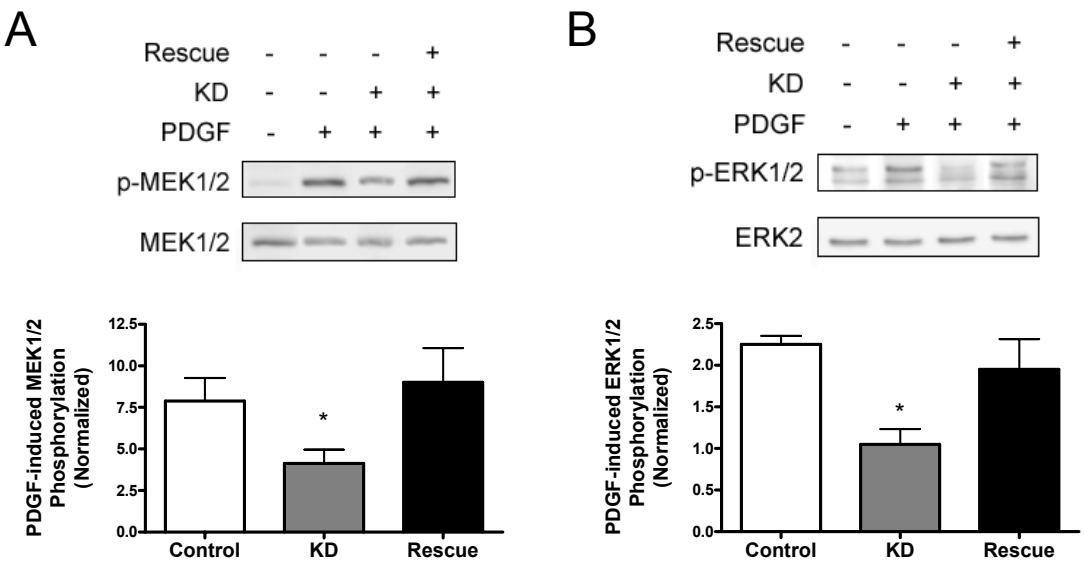
1.0
1.5
2.0
2.5
3.0
US
PDGF
US
PDGF
Control
LAT-A

Fig. S2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KD - - + +
PDGF - + + +
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MEK1/2
PDGF-induced MEK1/2 Phosphorylation (Normalized)
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10.0
7.5
5.0
2.5
0.0
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Control
KD
Rescue
B
Rescue - - - +
KD - - + +
PDGF - + + +
p-ERK1/2
ERK2
PDGF-induced ERK1/2 Phosphorylation (Normalized)
2.5
2.0
1.5
1.0
0.5
0.0
*
Control
KD
Rescue