Adhesion of Bovine Airway Smooth Muscle Cells Activates Extracellular Signal-regulated Kinases

Rita M. Heuertz, Kimm J. Hamann, Marc B. Hershenson, and Alan R. Leff

Departments of Medicine and Pediatrics, University of Chicago, Chicago, Illinois

Extracellular signal-regulated kinases (ERKs) phosphorylate and regulate cytoskeletal components of contractile cells and have been implicated in integrin-mediated adhesion. In this study, we examined the contributions of adherence, cell flattening, and cytoskeletal reorganization to adhesion-induced ERK activation in cultured bovine tracheal myocytes. We found, as evidenced by a reduction in electrophoretic mobility, that adhesion to fibronectin induced phosphorylation of both $p44^{ERK1}$ and $p42^{ERK2}$. In-gel kinase assays confirmed activation of both $p44^{ERK1}$ and $p42^{ERK2}$ in fibronectin-adherent cells, consistent with the notion that ligand–integrin binding is required for adhesion-induced ERK activation. However, ERK activation was maximal 2–4 h after plating, and adherence to either polystyrene or poly-L-lysine also caused ERK activation (fold increase 4 h after plating: fibronectin, 3.75 ± 0.33; polystyrene, 3.95 ± 0.78; poly-L-lysine, 2.14 ± 0.36). Inspection of myocytes following passage onto fibronectin showed near 100% adhesion and cell spreading after 4 h, whereas cells plated onto poly-L-lysine demonstrated adherence but minimal spreading. To test whether the cytoskeletal reorganization accompanying cell spreading is required for adhesion-induced ERK activation, we assessed ERK activity following pretreatment with cytochalasin D, an inhibitor of actin polymerization. Cytochalasin inhibited both cell spreading and ERK activation following adhesion to fibronectin, but had no effect on growth factor-induced ERK activation in adherent cells. We conclude that adhesion-induced ERK activation in bovine tracheal myocytes may occur independently of ligand–integrin binding and is primarily related to the cell spreading that follows adhesion. **Heuertz, R. M., K. J. Hamann, M. B. Hershenson, and A. R. Leff. 1997. Adhesion of bovine airway smooth muscle cells activates extracellular signal-regulated kinases. Am. J. Respir. Cell Mol. Biol. 17:456–461.**

The extracellular signal-regulated kinases (ERKs) are cytosolic serine/threonine kinases of the mitogen-activated protein kinase (MAPK) superfamily, which participate in the transduction of signals to the nucleus. Diverse signals activate ERK *in vivo*, including growth factors activating receptor tyrosine kinases (1, 2), hormones and neurotransmitters activating G protein-linked receptors (2–4), phorbol esters (5), calcium (6), and hydrogen peroxide (7). ERK activation may also be involved in cytoskeletal reorganization. ERKs have been shown to phosphorylate and regulate cytoskeletal components such as the microtubule-associated proteins (8). ERKs are activated and physically associated with microtubules in NIH 3T3 fibroblasts after mitogenic stimulation (9).

(*Received in original form July 3, 1996 and in revised form February 18, 1997*)

Address correspondence to: Alan R. Leff, M.D., Department of Medicine, University of Chicago, MC 6076, 5841 S. Maryland Avenue, Chicago, IL 60637-1470. E-mail: aleff@medicine.bsd.uchicago.edu

Abbreviations: extracellular signal-regulated kinase, ERK; focal adhesion kinase, FAK; mitogen-activated protein kinase, MAPK; myelin basic protein, MBP; MAPK/ERK kinase, MEK-1; platelet-derived growth factor, PDGF.

Am. J. Respir. Cell Mol. Biol. Vol. 17, pp. 456–461, 1997

Reports suggest that ERK activation may be involved in cell adhesion. Adhesion of cultured NIH 3T3 and Swiss 3T3 fibroblasts to fibronectin induces ERK activity whereas adhesion of these cells to polylysine does not (10, 11), suggesting that ligand–integrin binding is required for adhesion-induced ERK activation. However, it has been demonstrated that the time course of fibronectin-mediated ERK activation in cultured fibroblasts is related temporally to cell flattening rather than cell attachment per se (12). Thus, the proximal event responsible for ERK activation following cell adhesion (cell attachment versus flattening) is unclear. To our knowledge, studies addressing adhesion-mediated activation of ERKs in airway smooth muscle cells have not been reported previously.

In this study, we examined the roles of adherence, cell flattening and cytoskeletal reorganization of cultured airway smooth muscle cells in ERK activation. We found adhesion of bovine tracheal myocytes to fibronectin substantially increased ERK activation. However, activation of ERKs also occurred following adherence to polystyrene and, to a lesser degree, poly-L-lysine. ERK activation was temporally associated with cell flattening rather than attachment, and the magnitude of ERK activation corre-

sponded to the degree of cell flattening. Finally, both ERK activation and cell spreading were attenuated by cytochalasin D, an inhibitor of actin polymerization. Taken together, these data suggest that in airway smooth muscle, adhesion-induced ERK activation may occur independently of ligand–integrin binding and is primarily related to the cytoskeletal reorganization that follows cell attachment.

Materials and Methods

Materials

Nontreated, sterile six-well polystyrene plates were purchased from Corning Costar (Cambridge, MA). Human fibronectin was purchased from the New York Blood Center (New York, NY). Poly-L-lysine, cytochalasin D, myelin basic protein (MBP), anti-α-smooth muscle actin monoclonal antibody, and peroxidase-conjugated goat anti-rabbit IgG were purchased from Sigma Chemical Co. (St. Louis, MO). Ab283 antiserum, which recognizes both ERK1 and ERK2, was a gift from Dr. M. Rosner (University of Chicago, Chicago, IL) (13). Fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG was purchased from Becton Dickinson (Mountain View, CA). Enhanced chemiluminescence reagents and [γ-^{32}P]ATP were purchased from DuPont/NEN Research Products (Boston, MA). Platelet-derived growth factor (PDGF) was obtained from Upstate Biotechnology Incorporated (Lake Placid, NY). The synthetic MEK-1 inhibitor PD98059 was purchased from New England Biolabs (Beverly, MA).

Cell Culture

Bovine trachea smooth muscle cells were cultured as described previously (7) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, nonessential amino acids, penicillin, and streptomycin. Confluent flasks exhibited the typical "hill and valley" appearance under phase-contrast microscopy and exhibited specific immunostaining with an antibody against α-smooth muscle actin. The presence of the $\alpha_5\beta_1$ fibronectin receptor on these cells was confirmed by flow cytometry. Only low passage number cells ($\leqslant$ 5 passages) were studied.

Preparation of Adhesive Ligand-coated Plates

Substrata were prepared by allowing a 10-μg/ml solution of fibronectin or poly-L-lysine to adsorb overnight to wells of nontreated, sterile 6-well polystyrene plates at 4°C, followed by blocking with 0.5% heat-denatured bovine serum albumin (BSA) for 2 h at 37°C. The plates were rinsed twice with phosphate-buffered saline (PBS; 0.1 M sodium phosphate, pH 7.5) prior to use. Heat-denatured BSA was made by dissolving BSA in Hanks' balanced salt solution and placing it in a 75–80°C water bath for 30 min. The BSA was cooled immediately, sterile filtered, and stored at 4°C for use.

Cell Adhesion to Substrata and Preparation of Total Cell Lysates

Confluent cells were serum starved for 24 h and then washed twice with PBS and dissociated from culture flasks with 0.05% trypsin and 0.02% EDTA. The suspended cells were then treated with trypsin inhibitor (1 mg of inhibitor per milligram of trypsin) and centrifuged (5,000 rpm for 5 min). Trypsin–trypsin inhibitor was aspirated, and cells were suspended in DMEM. Approximately 10^5 cells were applied to culture wells with or without adhesive ligands. In some experiments, cells incubated in fibronectin-coated microwells also received 10 or 100 μM cytochalasin D. Cells were incubated at 37°C for 15 min, 1 h, 2 h, 4 h, 6 h, or 24 h. Following incubations, cells were observed for morphology and adhesiveness by phase microscopy. Adherent cells were washed once with ice-cold PBS and then incubated with lysis buffer consisting of 20 mM Tris (pH 7.4), 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 40 mM sodium chloride, 5 mM EDTA, 1% Nonidet P-40 (NP-40), 10 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM sodium vanadate, and 0.5% deoxycholic acid (15 min at 4°C). The wells were scraped, and cell lysates were collected in a microcentrifuge tube and centrifuged at 13,000 rpm for 1 min to remove cellular debris. The supernatant was transferred to a clean microcentrifuge tube. Cell lysates were frozen at −70°C. Nonadherent control cells (time 0) were collected, washed once with PBS, and then lysed and treated as described above. Data represent 10 experiments for fibronectin, and 4 experiments each for poly-L-lysine and polystyrene.

Immunoblot Analysis of ERKs

Cell lysates (10 μg of protein per lane) were resolved on a 10% separating gel by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to nitrocellulose membrane using a semidry transfer unit (Fisher Scientific, Pittsburgh, PA). Each membrane was stained with Ponceau S to verify efficient and equal transfer of protein samples. Membranes were blocked with 5% dry milk in Tris-buffered saline with Tween (1 mM Tris [pH 7.4]), 150 mM sodium chloride, 0.1% Tween 20) for 1 h at room temperature or overnight at 4°C. After blocking, Ab283 (rabbit anti-ERK) diluted 1:1,000 was used as primary antibody. The secondary antibody was peroxidase-conjugated goat anti-rabbit IgG diluted 1:1,000. To detect the ERK–antibody–peroxidase complex, enhanced chemiluminescence was used. A shift in the $p44^{ERK1}$ and $p42^{ERK2}$ bands toward a slower mobility reflected the phosphorylation of ERKs at threonine and tyrosine residues, which is required for enzyme activity (14).

Kinase Renaturation Assay

Because all isoforms of MAPK phosphorylate MBP *in vitro* (15), ERKs were renatured to their active forms and detected by phosphorylation of the substrate MBP after electrophoretic resolution on an MBP-impregnated polyacrylamide gel (1, 2, 6). Cell lysates (5–10 μg protein per lane) were resolved on a 10% SDS-polyacrylamide gel copolymerized with 0.1 mg/ml MBP. To dissolve SDS, the gel was washed twice with 20% isopropanol in 50 mM Hepes (pH 7.5) and 5 mM β-mercaptoethanol (1 h each at room temperature). After another wash in 50 mM Hepes and 5 mM β-mercaptoethanol (1 h), gels were incubated in 6 M guanidine-HCl plus 5 mM β-mercaptoethanol to denature proteins (two washes of 1 h each). Protein renaturation

was accomplished during two successive incubations at 4°C in a solution containing 50 mM Hepes, 5 mM β-mercaptoethanol, and 0.04% Tween 20 (total incubation time approximately 16 h). The gels were then incubated in a prephosphorylation buffer containing 25 mM Hepes (pH 7.5), 10 mM $MgCl_2$, 2 mM $MnCl_2$, 100 μM sodium vanadate, and 5 mM β-mercaptoethanol for 30 min at room temperature. The phosphorylation step was done by setting each gel in 10 ml of the prephosphorylation buffer containing 50 μM ATP and 250 μCi [γ-^{32}P]ATP for 1 h at 30°C. The reaction was stopped by extensive washing in 5% trichloroacetic acid and 10 mM sodium pyrophosphate.

To confirm that the 44- and 42-kD MBP kinases identified by kinase renaturation assays were indeed ERK1 and ERK2, the lysates of PDGF-stimulated cells were partially purified by Mono Q ion-exchange chromatography (16). The 44- and 42-kD MBP kinases were identified by anti-MAP kinase antiserum (Ab283) to be ERK1 and ERK2, respectively (data not shown).

Effect of Cytochalasin D on Cell Spreading and ERK Activation

To determine whether the cytoskeletal reorganization following cell adhesion might be the event most closely related to ERK activation, cells were pretreated with cytochalasin D, an inhibitor of actin filament formation (17), prior to plating on fibronectin. The effect of cytochaslasin D on PDGF-induced ERK activation in adherent cells was also assessed.

Effect of Synthetic MAPK/ERK Kinase Inhibitor PD98059 on Adhesion-induced ERK Activation

We have shown previously that MAPK/ERK kinase 1 (MEK-1) is required and sufficient for ERK activation in bovine tracheal myocytes (18). Therefore, to examine the precise role of ERK activation in bovine tracheal myocyte adhesion and spreading, we used a synthetic MEK inhibitor, PD98059 (18, 19). Cells were pretreated with PD98059 for 20 min and plated onto fibronectin-coated dishes.

Quantitation of Myelin Basic Protein Phosphorylation

Gels were stained with Coomassie blue, destained in 40% methanol with 10% acetic acid, and dried. Autoradiograms were developed by exposing film to the dried gel with an intensifying screen. Quantitation of MBP phosphorylation by ERK kinases was determined by digital optical scanning and image analysis (Jandel Scientific, San Rafael, CA).

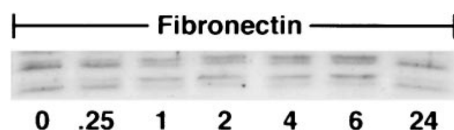

Figure 1. Effect of adherence to fibronectin on ERK phosphorylation in bovine tracheal myocytes. Anti-ERK immunoblots demonstrate two doublets per lane, the upper doublet representing ERK1, the lower doublet, ERK2. The lower band of each doublet represents unphosphorylated ERK; the upper band, phosphorylated ERK. After adherence to fibronectin, there is an increase in the ratio of phosphorylated to unphosphorylated ERKs. By contrast, nonadherent (time 0) cells demonstrate predominantly unphosphorylated ERKs.

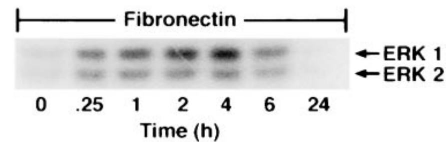

Figure 2. Effect of adherence to fibronectin on ERK activity. Confluent, quiescent bovine tracheal smooth muscle cells were plated onto fibronectin. Cell lysates were resolved on a 10% SDS-polyacrylamide gel copolymerized with MBP. After adherence to fibronectin, bands corresponding to $p44^{ERK1}$ and $p42^{ERK2}$ are present, indicating phosphorylation of MBP by ERKs. In suspended (time 0) cells, ERK activity is evident, but at a substantially lower level.

Statistical Analysis

All data are expressed as mean ± SEM. Group mean cumulative ERK activities for fibronectin-adherent, poly-L-lysine-adherent, polystyrene-adherent cells were compared by one-way analysis of variance (ANOVA). Significant differences identified by analysis of variance were distinguished by Student–Newman–Keuls multiple range test (20).

Results

Effect of Adhesion on ERK Activation

Bovine tracheal smooth muscle cells were plated on fibronectin-coated polystyrene dishes and incubated for 15 min to 24 h. ERK phosphorylation was examined by immunoblotting. At time 0, ERK1 and ERK2 were present primarily in the unphosphorylated forms (Figure 1). When cells adhered to fibronectin, there was a distinct increase in the amounts of phosphorylated ERKs relative to the unphosphorylated forms.

To establish that phosphorylation shifts corresponded to actual ERK activation, cell lysates from control and adherent cells were subjected to in-gel kinase renaturation assays. The lysates were separated on a polyacrylamide gel copolymerized with MBP. After the kinases were renatured to their active forms, they were identified by their ability to phosphorylate the substrate MBP. Nonadherent cells had minimal ERK1 and ERK2 activities whereas cells adherent to fibronectin demonstrated increased activity for both the 42- and 44-kD ERK homologs at 10 min to 6 h after plating (Figure 2). Peak activity was evident at 2–4 h following plating.

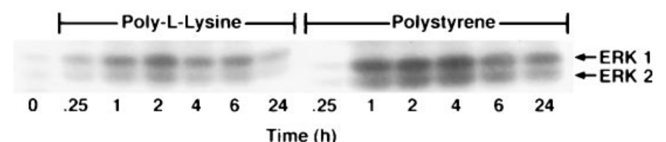

Figure 3. Adherence to poly-L-lysine and polystyrene activates $p44^{ERK1}$ and $p42^{ERK2}$. ERK activation was assessed by in-gel kinase renaturation assay.

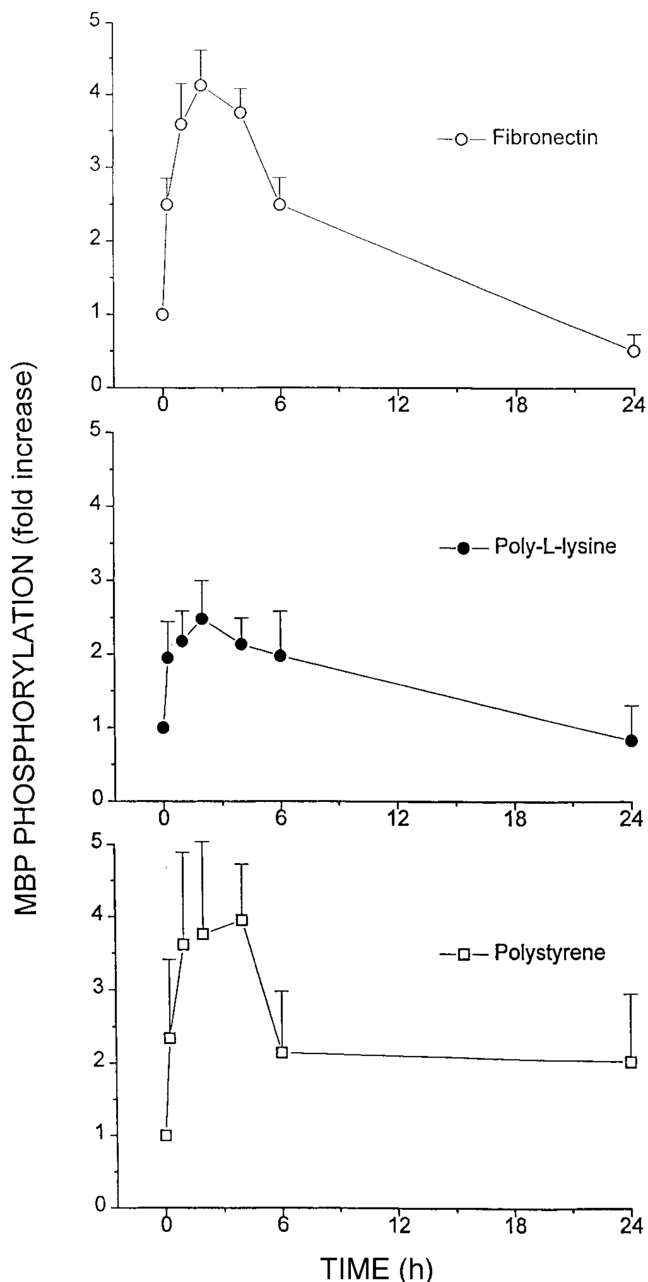

Figure 4. Time course of ERK activation in adherent cells, group mean data. In-gel kinase renaturation assays were performed to assess ERK activity. Resulting bands were scanned by densitometry. Total ERK (ERK1 plus ERK2) activity was quantified as fold increase over baseline (0 time point). *Open circles* = fibronectin; *closed circles* = poly-L-lysine; *open squares* = polystyrene.

Bovine tracheal smooth muscle cells were also plated on poly-L-lysine-coated and uncoated polystyrene dishes. Adhesion of cells to poly-L-lysine and polystyrene was also associated with ERK activation (Figure 3). Quantification of in-gel kinase renaturation assays by optical densitometry (Figure 4). Four hours after plating, total ERK activity (ERK1 + ERK2) for fibronectin-adherent myocytes was increased by (3.75 ± 0.33)-fold versus control activity (time 0). Adherence

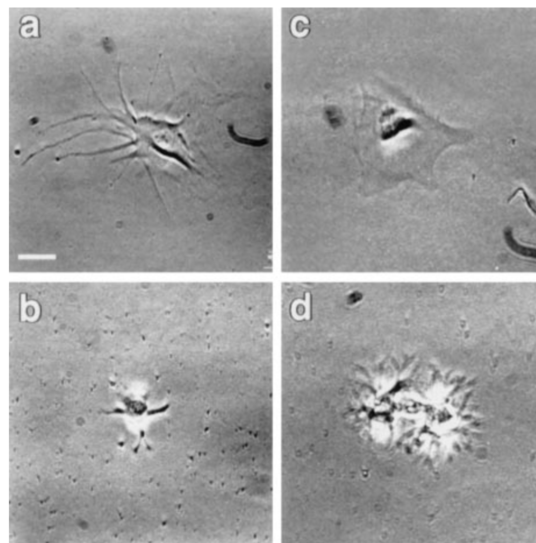

Figure 5. Differences in cell flattening with substratum. Confluent, serum-starved bovine tracheal smooth muscle cells were plated on fibronectin (*a*), poly-L-lysine (*b*), or polystyrene (*c*) and incubated at 37°C for 4 h. Cells adherent to fibronectin were flat 4 h after plating. Cells adherent to poly-L-lysine remained round at 4 h. Cells plated onto uncoated polystyrene wells were cuboidal in shape. Finally, treatment of cells plated on fibronectin with 10 μM cytochalasin D prevented cell flattening (*d*). Photographs were taken of representative fields. (*a*), (*b*), and (*c*) show one myocyte each; in (*d*) two cells are demonstrated. Bar: 10 μm.

to poly-L-lysine also caused ERK activation, but at a significantly lower level than that induced by adherence to fibronectin ([2.14 ± 0.36]-fold increase; $P = 0.039$; ANOVA). Adhesion to polystyrene induced a level of ERK activation comparable to fibronectin ([3.95 ± 0.78]-fold increase).

Association of Cell Adhesion with Cell Flattening

Cell morphology during adhesion differed depending upon the substrata to which the cells adhered (Figure 5). Cells adherent to all substrata were round at 15 min. Cells adherent to fibronectin and polystyrene were flat or cuboidal 4 h after plating. Cells adherent to poly-L-lysine remained round at 4 h. Thus, like adhesion-induced ERK activation, cell spreading was maximal with fibronectin and polystyrene and minimal with poly-L-lysine.

Effect of Cytochalasin D on ERK Activation

Because ERK activity corresponded to the degree of cell spreading, further studies were performed to determine whether the cytoskeletal reorganization following cell adhesion might be the event most closely related to ERK activation. Treatment of cells plated on fibronectin with cytochalasin D prevented cell flattening (Figure 5). Cytochalasin D treatment also inhibited the activation of both ERK1 and ERK2 (Figure 6). ERK activity was partially attenuated by 10 μM cytochalasin D and completely attenuated by 100 μM cytochalasin D. Treatment of adherent cells with 100 μM cytochalasin D did not block PDGF-induced ERK activation (Figure 7). Thus, the inhibitory effect of cytochalasin

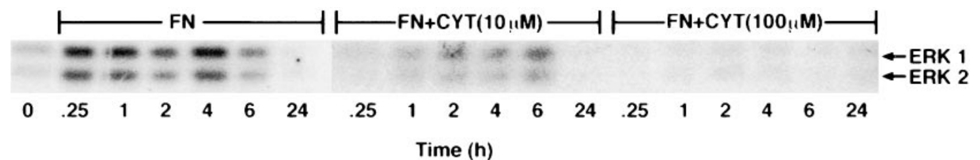

Figure 6. Effect of cytochalasin D on fibronectin-mediated ERK activation. Confluent, serum-starved bovine tracheal smooth muscle cells were treated with 10 or 100 μM cytochalasin D and plated onto fibronectin-coated wells. Treatment of smooth muscle cells with cytochalasin D substantially attenuated adhesion-induced activation of $p44^{ERK1}$ and $p42^{ERK2}$.

D on adherence-induced ERK activation was specific to cells undergoing the process of cell adhesion and spreading, and the concentration of cytochalasin D employed was nontoxic for bovine tracheal myocytes.

Effect of Synthetic MEK Inhibitor PD98059 on Bovine Tracheal Myocyte Adhesion-induced ERK Activation

Catalytic activation of ERKs was not required for adhesion and spreading. To examine the role of ERK activation in bovine tracheal myocyte adhesion and spreading, we employed a synthetic MEK inhibitor, PD98059. Cells were pretreated with PD98059 for 20 min and plated onto fibronectin-coated dishes. Although PD98059 inhibited adhesion-induced ERK activation in a concentration-dependent manner (Figure 8), there was no effect on cell adhesion and spreading (data not shown).

Discussion

The objective of this investigation was to determine the relationship between cellular adhesion to various substrata and ERK activation. Previous studies in fibroblastic cell lines have suggested that ERK activation following adhesion to fibronectin is directly related to ligand-specific adhesion (10, 11, 21) and is caused predominantly by specific ligation to β_1-integrin. These studies examined ERK activation over a short time frame (up to 60 min). Because sustained ERK activation may be required for DNA synthesis in airway smooth muscle (1), we examined ERK activation following adhesion of bovine tracheal myocytes to various substrata over a 24-h period. We confirmed that ERK activation occurred following adhesion to fibronectin. However, we also found that nonspecific adhesion to polystyrene or poly-L-lysine also caused ERK activation. Furthermore, the time course of ERK activation under these conditions was gradual, persisting for as long as 6 h after initial adhesion. Our findings suggest that while ligation interactions may well have a role in initial ERK activation, sustained activation is not exclusively or predominantly the result of ligand-mediated binding to a specific matrix.

We also found that the time course and magnitude of bovine tracheal myocyte ERK activation corresponded with the degree of cell contact with the substrata. Bovine tracheal myocytes adherent to fibronectin showed a flattened morphology, likely resulting in a large number of focal adhesion contacts between the cell and substratum. Cells adherent to poly-L-lysine demonstrated a rounded morphology, and therefore were likely to offer minimal points of contact with the substratum. Finally, addition of an inhibitor of actin polymerization, cytochalasin D, to cells plated on fibronectin attenuated both cell spreading and ERK activation. These data, which are consistent with the work of Zhu and Assoian (12) obtained in NIH 3T3 cells, suggest that the magnitude of ERK activation following adherence relates to the number of points of contact between the cell and substratum, not to cell attachment alone. Integrin binding likely increases ERK activation by increasing the cell surface area in contact with the substratum, but is not necessary for adhesion-induced ERK activation.

ERKs have been shown to phosphorylate and regulate cytoskeletal components such as the microtubule-associated proteins (8). It is therefore conceivable that cytochalasin attenuates ERK activation by disrupting cytoskeletal elements required for ERK function rather than by inhibiting cell spreading. However, pretreatment with cytochalasin did not inhibit PDGF-induced ERK activation in adherent cells, suggesting that the inhibitory effect of cytochalasin D on ERK activation is not caused by the nonspecific disruption of cytoskeletal elements.

To examine the precise role of ERK activation in bovine tracheal myocyte adhesion and spreading, cells were pretreated with a synthetic inhibitor of MEK, the dual function kinase required and sufficient for ERK activation in bovine tracheal myocytes (18, 19). Although PD98059

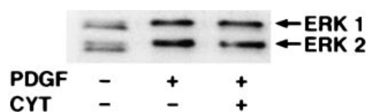

Figure 7. Anti-ERK immunoblot demonstrating phosphorylation of ERKs after treatment with PDGF. Pretreatment with 100 μM cytochalasin D had no inhibitory effect on ERK phosphorylation.

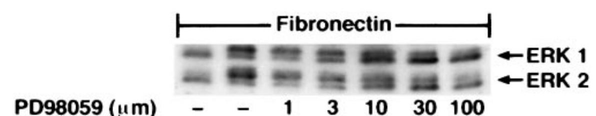

Figure 8. Anti-ERK immunoblot demonstrating inhibition of adhesion-induced ERK activation by the synthetic MEK inhibitor PD98059.

inhibited adhesion-induced ERK activation in a concentration-dependent manner, it had no apparent effect on cell adhesion and spreading. These data suggest that, while ERK activation is a consequence of cell adhesion and spreading, it is not required for these cellular events.

It is important to consider some limitations to our findings. While we were able to establish a relationship between degree of adhesive cell flattening and ERK activation, we did not identify the specific mechanism by which myocyte flattening activates ERKs. It has been demonstrated previously, however, that plating of NIH 3T3 cells on fibronectin elicits the successive activation of focal adhesion kinase (FAK) and the growth factor receptor-binding protein Grb2 (11). Grb2, in turn, mediates signal transduction from growth factor receptor tyrosine kinases to the Ras/MAPK pathway though interaction with the Ras GDP/GTP exchange factor Sos (22). We also did not establish the precise mechanism by which bovine tracheal myocytes adhere to the various substrata tested. Although our preliminary studies confirmed that these cells express the $\alpha_5\beta_1$ fibronectin receptor (*see* Materials and Methods), adhesion to fibronectin was not blocked with either the RGD peptide or blocking antibodies (data not shown), suggesting that nonspecific adhesion (as for polystyrene) is the predominant mechanism of ERK activation.

We have shown that adhesion to various substrata activates ERKs. Since a large proportion of the total cellular MAP kinase is associated with the microtubule cytoskeleton (9), activation of ERKs on cellular adhesion and spreading may be anticipated. On the other hand, it is conceivable that adhesion-induced ERK activation may be involved in the regulation of airway smooth muscle proliferation. For example, it has been shown that microinjection of endothelial cells with a kinase-inactive FAK reduces DNA synthesis (23), suggesting that adhesion-induced cellular signaling may influence cell growth. Further studies investigating the potential relationship between cell adhesion and proliferation may therefore be warranted.

Acknowledgments: The authors thank Jing Li and Pai Liu for technical support. These studies were supported by Grants AI34566 (to A.R.L. and K.J.H.), HL32654 (to K.J.H.), HL46368 and HL56399 (to A.R.L.), and HL02731, HL54685, and HL56578 (to M.B.H.).

References

1. Kelleher, M. D., M. K. Abe, T. S. Chao, M. Jain, J. M. Green, J. Solway, M. R. Rosner, and M. B. Hershenson. 1995. Role of MAP kinase activation in bovine tracheal smooth muscle mitogenesis. *Am. J. Physiol.* 268:L894–L901.
2. Hershenson, M. B., T. S. Chao, M. K. Abe, I. Gomes, M. D. Kelleher, J. Solway, and M. R. Rosner. 1995. Histamine antagonizes serotonin and growth factor-induced mitogen-activated protein kinase activation in bovine tracheal smooth muscle cells. *J. Biol. Chem.* 270:19908–19913.
3. Koide, M., Y. Kawahara, T. Tsuda, Y. Ishida, K. Shii, and M. Yokoyama. 1992. Endothelin-1 stimulates tyrosine phosphorylation and MAP kinase activity in cultured vascular smooth muscle cells. *J. Hypertension* 10:1173–1182.
4. Meloche, S., K. Seuwen, G. Pages, and J. Pouyssegur. 1992. Biphasic and synergistic activation of $p44^{mapk}$ (ERK1) by growth factors: correlation between late phase activation and mitogenicity. *Mol. Endocrinol.* 6:845–854.
5. Chao, T.-S. O., D. A. Foster, U. Rapp, and M. R. Rosner. 1994. Differential raf requirement for activation of mitogen-activated protein kinase by growth factors, phorbol esters and calcium. *J. Biol. Chem.* 269:7337–7341.
6. Chao, T. S., K. L. Byron, K. M. Lee, M. Villereal, and M. R. Rosner. 1992. Activation of MAP kinases by calcium-dependent and calcium-independent pathways: stimulation by thapsigargin and epidermal growth factor. *J. Biol. Chem.* 267:19876–19883.
7. Abe, M. K., T. S. Chao, J. Solway, M. R. Rich, and M. B. Hershenson. 1994. Hydrogen peroxide stimulates mitogen-activated protein kinase in bovine tracheal myocytes: Implications for human airway disease. *Am. J. Respir. Cell Mol. Biol.* 11:577–585.
8. Ray, L. B., and T. W. Sturgill. 1987. Rapid stimulation by insulin of a serine/threonine kinase in 3T3-L1 adipocytes that phosphorylates microtubule-associated protein 2 *in vitro. Proc. Natl. Acad. Sci. USA* 84:1502–1506.
9. Reszka, A. A., R. Seger, C. D. Diltz, E. G. Krebs, and E. H. Fischer. 1995. Association of mitogen-activated protein kinase with the microtubule cytoskeleton. *Proc. Natl. Acad. Sci. USA* 92:8881–8885.
10. Chen, Q., M. S. Kinch, T. H. Lin, K. Burridge, and R. L. Juliano. 1994. Integrin-mediated cell adhesion activates mitogen-activated protein kinases. *J. Biol. Chem.* 269:26602–26605.
11. Schlaepfer, D. D., S. K. Hanks, T. Hunter, and P. van der Geer. 1994. Integrin-mediated signal transduction linked to ras pathway by GRB2 binding to focal adhesion kinase. *Nature (London)* 372:786–791.
12. Zhu, X., and R. K. Assoian. 1995. Integrin-dependent activation of MAP kinase: a link to shape-dependent cell proliferation. *Mol. Biol. Cell* 6:273–282.
13. Chao, T. S., K. L. Byron, K. M. Lee, M. Villereal, and M. R. Rosner. 1992. Activation of MAP kinases by calcium-dependent and calcium-independent pathways: stimulation by thapsigargin and epidermal growth factor. *J. Biol. Chem.* 267:19876–19883.
14. Anderson, N. G., J. L. Maller, N. K. Tonks, and T. W. Sturgill. 1990. Requirement for integration of signals from two distinct phosphorylation pathways for activation of MAP kinase. *Nature (London)* 343:651–653.
15. Erickson, A. K., D. M. Payne, P. A. Martino, A. J. Rossomando, J. Shabanowitz, M. J. Weber, D. F. Hunt, and T. W. Sturgill. 1990. Identification by mass spectrometry of threonine 97 in bovine myelin basic protein as a specific phosphorylation site for mitogen-activated protein kinase. *J. Biol. Chem.* 265:19728–19735.
16. Ahn, N. G., J. E. Weiel, C. P. Chan, and E. G. Krebs. 1990. Identification of multiple epidermal growth factor-stimulated protein serine/threonine kinases from Swiss 3T3 cells. *J. Biol. Chem.* 265:11487–11494.
17. Franki, N., G. Ding, Y. Gao, and R. M. Hays. 1992. Effect of cytochalasin D on the actin cytoskeleton of the toad bladder epithelial cell. *Am. J. Physiol.* 263:C995–C1000.
18. Karpova, A. Yu., M. K. Abe, J. Li, P. Liu, J. M. Rhee, W. L. Kuo, and M. B. Hershenson. 1997. MEK-1 is required and sufficient for ERK activation and required for DNA synthesis in tracheal myocytes. *Am. J. Physiol. (Lung Cell Mol. Physiol.)* 16:L558–L565.
19. Dudley, D. T., L. Pang, S. J. Decker, A. J. Bridges, and A. R. Saltiel. 1995. A synthetic inhibitor of the mitogen-activated protein kinase cascade. *Proc. Natl. Acad. Sci. USA* 92:7686–7689.
20. Snedecor, G. W., and W. G. Cochran. 1980. Statistical Methods. Iowa State University Press, Ames, IA.
21. Morino, N., T. Mimura, K. Hamasaki, K. Tobe, K. Ueki, K. Kikuchi, K. Takehara, T. Kadowaki, Y. Yazaki, and Y. Nojima. 1995. Matrix/integrin interaction activates the mitogen-activated protein kinase, $p44^{ERK1}$ and $p42^{ERK2}$. *J. Biol. Chem.* 270:269–273.
22. Olivier, J. P., T. Raabe, M. Henkemeyer, B. Dickson, G. Mbamalu, B. Margolis, J. Schlessinger, E. Hafen, and T. Pawson. 1993. A *Drosophila* SH2–SH3 adaptor protein implicated in coupling the sevenless tyrosine kinase to an activator of Ras guanine nucleotide exchange, Sos. *Cell* 73:179–191.
23. Gilmore, A. P., and L. H. Romer. 1996. Inhibition of focal adhesion kinase (FAK) signaling in focal adhesions decreases cell motility and proliferation. *Mol. Biol. Cell.* 7:1209–1224.