ERK activation and mitogenesis in human airway smooth muscle cells

JIN-HEE LEE,1 PETER R. A. JOHNSON,1 MICHAEL ROTH,1 NICHOLAS H. HUNT,2 AND JUDITH L. BLACK1

Departments of 1Pharmacology and 2Pathology, University of Sydney, New South Wales 2006, Australia

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Lee, Jin-Hee, Peter R. A. Johnson, Michael Roth, Nicholas H. Hunt, and Judith L. Black. ERK activation and mitogenesis in human airway smooth muscle cells. Am J Physiol Lung Cell Mol Physiol 280: L1019–L1029, 2001.—Asthmatic airways are characterized by an increase in smooth muscle mass, due mainly to hyperplasia. Many studies suggest that extracellular signal-regulated kinases 1 and 2 (ERK1 and ERK2, respectively), one group of the mitogen-activated protein (MAP) kinase superfamily, play a key role in the signal transduction pathway leading to cell proliferation. PGE2 and forskolin inhibited mitogen-induced ERK activation. Inhibition of MAP kinase kinases 1 and 2 (MEK1 and MEK2, respectively), which are upstream from ERK, with the specific MEK inhibitor U-0126 blocked both cell proliferation and ERK activation. In addition, U-0126 inhibited mitogen-induced activation of p90 ribosomal S6 kinase and expression of c-Fos and cyclin D1, all of which are downstream from ERK in the signaling cascade that leads to cell proliferation. Antisense oligodeoxynucleotides directed to ERK1 and -2 mRNAs reduced ERK protein and cell proliferation. These results indicate that ERK is required for human airway smooth muscle cell proliferation. Thus targeting the control of ERK activation may provide a new therapeutic approach for hyperplasia seen in asthma.

asthma; antisense extracellular signal-regulated kinase; p90 ribosomal S6 kinase; c-Fos; cyclin D1

Asthma is a chronic inflammatory disease characterized by an increase in smooth muscle cell mass and airway hyperresponsiveness. The increase in muscle mass is due mainly to an increase in cell number (hyperplasia) (11).

Various types of mitogens, such as epidermal growth factor, both AB and BB forms of platelet-derived growth factor (PDGF), the serine proteinase thrombin, and the inflammatory mediators endothelin-1, histamine, and mast cell tryptase, have been shown to cause human airway smooth muscle (HASM) cell proliferation (3, 15, 19, 34, 35). Elevation of cAMP with prostaglandin E2 (PGE2), forskolin, or salbutamol inhibits mitogenesis in HASM cell cultures (22, 47). The signal transduction pathways underlying these factors are not fully understood.

Mitogens can be categorized into two groups, those that activate intrinsic tyrosine kinase and those that activate receptors coupled to G proteins (34). Thrombin, for example, acts via a G protein-coupled receptor (proteinase-activated receptor-1) (49). PDGF acts via receptor tyrosine kinase (RTK) (20, 21). In HASM cells, thrombin activates G proteins that inhibit cAMP mobilization (35). Both thrombin and PDGF may activate p21ras (1, 12, 35). Active p21ras binds to GTP and leads to activation of Raf-1 (53), and Raf-1 then translocates to the plasma membrane where it phosphorylates mitogen-activated protein (MAP) kinase kinases (MEK1 and MEK2, respectively), which, in turn, activate extracellular signal-regulated kinases (ERK1 and ERK2, respectively) (53). Activated ERK activates numerous transcription factors such as Elk-1, c-Jun, c-Fos, and c-Myc in the nucleus (8, 21, 36, 42, 53). It also can activate c-Fos or via phosphorylation of p90 ribosomal S6 kinase (p90rsk) in 3T3 cells (7). In addition, ERK activation leads to elevated activator protein (AP)-1 (c-Fos and c-Jun) activity via c-Fos induction (23), suggesting a central role for ERK in the control of cell proliferation.

Three distinct groups of MAP kinases have been identified in mammalian cells: ERK, c-Jun NH2-terminal kinase (JNK), and p38 (53). MAP kinases are proline-directed serine/threonine kinases that are activated by dual phosphorylation of threonine and tyrosine residues in response to a wide array of extracellular stimuli. MAP kinases are known to play a role in growth, differentiation, and gene expression. Phosphorylated ERK translocates into the nucleus and activates transcription factors needed for the induction of proliferation (8, 21, 36, 42, 53). The transcription factors, in turn, regulate the expression of genes required for DNA synthesis, such as cyclin D1 (4). However, the other MAP kinases are stimulated by assorted cytokines, hormones, various forms of stress such as osmotic stress, and ultraviolet irradiation and are involved in programmed cell death (53).

The signaling cascade described above can be blocked by increased intracellular cAMP levels, pre-

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venting the Ras-dependent activation of Raf-1. Abrogation of Raf-1 blocks the MEK-ERK pathway in Rat-1 cells (9, 34), Chinese hamster ovary cells, and human aortic smooth muscle cells (5, 14). In contrast, in bovine tracheal cells, NIH/3T3 CCL39 fibroblasts, and vascular smooth muscle cells, phosphorylation of the MAP kinases ERK1 and ERK2 was not affected by increased cAMP (30, 38, 41, 43), suggesting that the effects of cAMP on MAP kinase activity are cell-type specific. Therefore, it is important to investigate the role of ERK in the signaling pathway leading to cell proliferation specifically in HASM cells.

In this study, we used PDGF, thrombin, and fetal bovine serum (FBS) to induce ERK activation and, subsequently, HASM cell proliferation. The effects of elevation of cAMP by PGE2 and forskolin on ERK activation and proliferation were also investigated. In addition, we examined the effects of the specific MEK inhibitor U-0126 on ERK activation, p90RSK activation, and c-Fos and cyclin D1 expression and proliferation. Finally, we examined the effects of an antisense oligodeoxynucleotide (ODN) directed to ERK1 and -2 on ERK1 and -2 protein expression and cell proliferation. We compared the role of ERK in the signaling cascade induced by PDGF acting via RTK and that of thrombin, involving G protein-coupled receptors.

METHODS AND MATERIALS

Cell Culture

Primary cultures of HASM were established as previously reported (22). Human lung was obtained from patients undergoing either surgical resection for carcinoma or lung transplantation. The collection and use of lung specimens was approved by the Human Ethics Committee of the University of Sydney (New South Wales, Australia). Large bronchi (5- to 15-mm internal diameter) were dissected from the surrounding parenchyma in ice-cold carbogenated Krebs-Henseleit solution, pH 7.4 (composition in mM: 118 NaCl, 4.7 KCl, 2.5 CaCl2, 1.2 MgSO4, 1.2 NaH2PO4, 25.5 NaHCO3, and 11.1 g-glucose). The epithelium was removed, and the bands of airway smooth muscle (ASM) were gently separated from the underlying connective tissue in small bundles. ASM bundles, termed explants, were washed three times with DMEM containing 20 U/l of penicillin, 20 μg/ml of streptomycin, and 10% FBS. The explants were incubated at 37°C in air with 5% CO2, and 1% FBS for 24 h. The cells were stimulated with 10% FBS, 20 ng/ml of PDGF, or 10 U/ml of thrombin in DMEM containing 1% FBS alone or in combination with U-0126 (0.05, 0.25, 0.5, 2.5, or 5 μM) for 5 min. Untreated cells were used as a control. In some experiments, the cells were pretreated with 1 μM PGE2 for 40 min or 10 μM forskolin for 15 min and were then stimulated with the mitogen.

For the time course of ERK activation, cells were left untreated or were stimulated with 10% FBS in DMEM, 40 ng/ml of PDGF, or 10 U/ml of thrombin for 2, 5, 10, or 30 min or 1 or 2 h. The cells were washed in ice-cold PBS and harvested by cell scraping.

For protein extraction, cells were incubated for 5 min in 50 μl of lysis buffer A containing HEPES (20 mM), KCl (25 mM), MgCl2 (1.5 mM) dithiothreitol (DTT; 1 mM), aprotinin (1% wt/vol), leupeptin (1% wt/vol), phenylmethylsulfonyl fluoride (2% wt/vol), NaF (20 mM), and Triton X-100 (0.2% vol/vol). The samples were sonicated for 20 s to break the nuclear membrane and centrifuged at 14,000 rpm at 4°C for 5 min. The supernatants were collected and used as whole cell fractions. To obtain cytosolic and nuclear fractions, HASM cells were seeded as above in 175-cm² tissue culture flasks in DMEM containing 10% FBS and incubated for 7 days. After the cells were equilibrated for 24 h in 1% FBS, they were stimulated with 10% FBS for 2, 5, 10, or 30 min or 1 or 2 h, washed in ice-cold PBS, and harvested by cell scraping. For some experiments, the cells were stimulated with one of the mitogens with and without U-0126 for 4 or 24 h. To isolate cytosolic protein, the cells were incubated with 50 μl of buffer A, the samples were centrifuged at 7,000 rpm for 5 min, and the supernatants were collected as cytosolic fractions. The remaining pellet was resuspended in 40 μl of buffer B containing HEPES (20 mM), KCl (0.4 mM), MgCl2 (1.5 mM), EDTA (1 mM), DTT (1 mM), glycerol (50% vol/vol), aprotinin (1% wt/vol), leupeptin (1% wt/vol), phenylmethylsulfonyl fluoride (2% wt/vol), and NaF (20 mM). After 30 min, the cells were centrifuged at 14,000 rpm at 4°C for 5 min, and the supernatant was collected as the nuclear fraction and stored at -20°C.

Effect of the MEK Inhibitor U-0126 on Phosphorylation of p90RSK

Cells were seeded in six-well plates at a density of 1×10⁴ cells/cm², grown for 7 days, and arrested with 0.5% FBS for 48 h to reduce background phosphorylation of p90RSK. Cells were treated with 10% FBS with and without 5 μM U-0126 for 15 min. The cells were then washed twice with ice-cold PBS, and SDS loading buffer (4% SDS, 15% glycerol, 62.5 mM Tris-HCl, pH 6.8, 0.005% bromphenol blue, and 200 mM DTT) was directly added. The samples were then boiled for 5 min and subjected to Western blotting.

Western Blotting

Protein extracts (10–40 μg) were combined with a gel loading buffer [SDS (2% wt/vol), glycerol (7.5% vol/vol), Tris-HCl (31.25 mM, pH 6.8), bromphenol blue (0.0025% wt/vol), and DTT (200 mM)] and heated to 100°C for 2 min. Proteins were subjected to SDS-polyacrylamide gel electrophoresis (12% wt/vol) at 120 V for 2 h with a buffer consisting of 1% SDS, 25 mM Tris, and 200 mM glycine, pH 8.4, and then transferred onto a nitrocellulose membrane. The membrane was blocked with Tris-buffered saline (TBS; 20 mM Tris and 137 mM NaCl, pH 7.6) containing 0.1% Tween 20 (TBS-T) and 5% nonfat milk at room temperature for 2 h. After a brief rinse with TBS-T, the membrane was incubated with a monoclonal or polyclonal antibody (1:588–1,000 in
of the inhibitors. For the final 5 h, 1 μCi/well of [methyl-3H]thymidine was added to measure DNA synthesis. Cell proliferation was arrested by freezing, and the cells were harvested with distilled water onto Packard fiber filters with primary antibody for 3 h at room temperature followed by a wash with a solution of PBS with 0.1% Tween 20. Incubation with the secondary antibody and visualization were performed as described above.

Proliferation Assay

\[ ^{3}H \text{thymidine incorporation} \]

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was used as an indicator of cell proliferation. Cells were plated at 1 × 10^4 cells/cm² in 96-well cluster plates and incubated for 7 days to achieve cell confluence. The cells were equilibrated by incubation with 1% FBS in DMEM for 24 h. Mitogen-dependent cell proliferation was examined on cells exposed to 10% FBS in DMEM, 20 ng/ml of PDGF, or 10 U/ml of thrombin. The effects of the MEK inhibitor U-0126 (0.05, 0.25, 0.5, 2.5, or 5 μM), PGE₂ (1 μM), and forskolin (10 μM) on cell proliferation were examined. The cells were incubated at 37°C for 24 h with mitogens alone or in the presence of one of the inhibitors. For the final 5 h, 1 μCi/well of [methyl-3H]thymidine was added to measure DNA synthesis. Cell proliferation was arrested by freezing, and the cells were harvested with distilled water onto Packard fiber filters with a Packard cell harvester. The fiber filters were dried and placed on Packard 96-well plates, and 20 μl scintillation fluid/well were added. The samples were counted with a Packard top plate counter.

Viable cell count. HASM cells were seeded in gelatin-covered 12-well plates and treated with ODNs as described in Liposomal transfection of antisense ODNs. After 48 h of incubation with ODN and 2% FBS, the cells were washed twice with HBSS, harvested with trypsin (0.5% (wt/vol) in HBSS) containing 1 mM EDTA, and counted with a hemacytometer. Cell viability was determined with trypan blue exclusion; cells that stained blue were counted as nonviable.

Antisense ERK1 and -2 Treatment of HASM Cells

Confirmation of the transfection with ODN. Cells were grown to ~70% confluence on chamber slides and were treated with 2 μM FITC-labeled ODN prepared in Opti-MEM. The ODNs were mixed with Lipofectin at ratios of 1:3, 1:2, and 1:4 antisense-Lipofectin and incubated for 6 h. The cells were then washed twice with ice-cold PBS and fixed with 50% methanol (in PBS). To recognize cell nuclei, Hoechst stain was applied to the cells. ODN uptake was visualized with fluorescence microscopy (Olympus).

Liposomal transfection of antisense ODNs. The antisense 3'-end phosphorothioate-modified ODN to ERK was an 18-mer (5'-AGC AGA TAT GGT CAT TGC-3'). This sequence is shared by human ERK1 (bp 377–394) and ERK2 (bp 488–505) mRNAs. Scrambled ODN (5'-TGCT TCT ATAT CCA GTA ACG-3') was used as a control. Cells at ~70% density in 12-well plates were treated with an ODN-Lipofectin mixture at a ratio of 1:2 (0.2 and 0.5 μM ODN) prepared in Opti-MEM and incubated for 6 h. The cells were then washed with Opti-MEM to remove the ODN-Lipofectin mixture and incubated in DMEM containing 2% (vol/vol) FBS and the appropriate concentration of ODN alone. After 18 h, the cells were supplemented with the same concentration of ODN and then incubated for a further 24 h. The cells were then washed twice with ice-cold PBS. SDS loading buffer was added, and the cell lysates were harvested and boiled for 5 min. The samples were then subjected to Western blotting.

Materials

All tissue culture reagents (DMEM, HBSS, penicillin, streptomycin, Opti-MEM, and trypan blue) except FBS (CSL, Sydney, Australia) were from Gibco BRL (Sydney, Australia). Tissue culture flasks and 96-well plates were obtained from Becton Dickinson (Sydney, Australia); chamber slides were obtained from Nunc (Naperville, IL); EDTA was purchased from Ajax Chemicals (Sydney, Australia); human recombinant PDGF-AB was purchased from Upstate Biotechnology (Lake Placid, NY); thrombin was purchased from Parke Davis (Auckland, New Zealand); trypsin, monoclonal anti-β-actin, and PGE₂ were from Sigma (St. Louis, MO); and methyl-3H]thymidine was from NEN Research Products (Boston, MA). Polyclonal phosphorylated p90 rac, monoclonal anti-phosphorylated ERK, and horseradish peroxidase-conjugated anti-mouse IgG antibody were from New England Biolabs (Beverly, MA). The enhanced chemiluminescence detection kit and molecular weight markers were purchased from Amersham (Little Chalfont, UK), and polyclonal anti-phosphorylated ERK and U-0126 were from Promega (Madison, WI). Monoclonal anti-cyclin D1, polyclonal anti-c-Fos, and polyclonal anti-ERK were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated goat anti-rabbit IgG antibody and ammonium persulfate were obtained from Bio-Rad (Sydney, Australia). Lipofectin was from Life Technologies (Palo Alto, CA). ODNs were synthesized at CybeSym (Lenni, PA).

Statistical Analysis

All experiments were performed on cells from at least three different patients. A mean value for the density of the protein in the Western blots was calculated from all experiments. The density of the control was taken as 100%. The results are expressed as means ± SE. Results of individual treatments from the [3H]thymidine assay were obtained in quadruplicate and are expressed as counts per minute (cpm). The percent change from the response to 10% FBS for each individual result was then calculated. An overall mean (±SE) counts per minute was then calculated from all patients. Statistical significance was determined with ANOVA and Fisher's protected least significant difference test. Differences were considered significant at P ≤ 0.05.

RESULTS

Effects of FBS, Thrombin, and PDGF-AB on Phosphorylation of ERK1 and -2 in HASM Cells

Equilibrated HASM cells were treated with 10% FBS, 10 U/ml of thrombin, and 40 ng/ml of PDGF-AB at the indicated time points (Fig. 1A). Activation of ERK1 and -2 by these mitogens occurred after 2 min. PDGF-induced ERK activation reached a maximum at 10 min and declined thereafter. The maximal activation of ERK-1 and -2 by PDGF was 10-fold over the
control value (Fig. 1A). Thrombin achieved maximal activation of ERK at 2 min, and this decreased within 10 min. Compared with control value, thrombin induced a fivefold maximal activation of ERK. In the presence of 10% FBS, ERK1 and -2 were maximally activated after 5 min, with a 6.8-fold increase over the control value, and the activation persisted for 30 min (Fig. 1A).

Activation or phosphorylation of ERK resulted in translocation from the cytosol into the nucleus. As shown in Fig. 1B, phosphorylated ERK1 and -2 were observed as early as 2 min in both the cytosol and nucleus after the addition of 10% FBS. However, translocation of phosphorylated ERK from the cytosol to the nucleus was maximal at 10 min, with the amount of ERK in the nucleus declining rapidly thereafter (Fig. 1B).

Figure 1C depicts the effect of the three mitogens (FBS, thrombin, and PDGF) on [3H]thymidine incorporation 24 h after addition to HASM cells. The potency of the mitogens correlated with their effect on ERK activation and duration (Fig. 1A). Although thrombin induced a twofold increase in [3H]thymidine incorporation, 10% FBS induced a fourfold increase and PDGF caused an eightfold increase.

**Effect of cAMP Elevation on Cell Proliferation and ERK Activation**

To investigate the role of cAMP on ERK activation and proliferation, we repeated the previous experiments in the presence of PGE2 and forskolin. Both PGE2 (1 μM) and forskolin (10 μM) inhibited FBS-induced ERK activation by 53.5 and 70.1%, respectively (P < 0.05; n = 3 experiments; Fig. 2A). Similarly, both substances reduced FBS-induced [3H]thymidine incorporation by 35.4 and 55.1%, respectively (Fig. 2B). When ERK was activated by thrombin (10 U/ml), PGE2 decreased this effect by 42.03% and forskolin by 71.68% (Fig. 2A). Figure 2B shows similar effects of PGE2 and forskolin on thrombin-induced [3H]thymidine incorporation. PDGF-dependent ERK activation was inhibited in the presence of PGE2 by 32.4% and in the presence of forskolin by 34.3% (P < 0.05; n = 3 experiments; Fig. 2A). Similarly, PGE2 decreased PDGF-induced [3H]thymidine incorporation by 27.4% and forskolin reduced the effect of PDGF by 36.4% (P < 0.05; n = 3 experiments; Fig. 2B).

**Effects of U-0126 on ERK Activation and Proliferation**

As shown in Fig. 3A, the specific MEK inhibitor U-0126 inhibited ERK activation induced by FBS in a dose-dependent manner. At a concentration of 5 μM U-0126, FBS-induced ERK activation was reduced to control levels (Fig. 3A). A similar inhibitory effect of
ERK activation and mitogenesis in HASM cells

U-0126 was observed when ERK activation was induced by thrombin (Fig. 3A). The effect of U-0126 was again dose dependent, and ERK activation returned to control levels at a concentration of 5 μM U-0126. PDGF-induced ERK activation was also decreased in the presence of U-0126 in a dose-dependent manner (Fig. 3A).

\[ ^{[3]H} \text{thymidine incorporation was also inhibited by U-0126 in a dose-dependent manner. As depicted in Fig. 3B, thymidine uptake induced by 10\% FBS (22,602 ± 2,494 cpm) was reduced by 65.5\% (7,793 ± 608 cpm; } P \leq 0.05; n = 3 \text{ experiments). Thrombin-induced } ^{[3]H}\text{thymidine incorporation (15,052 ± 1,039 cpm) was reduced to the control level (3,424 ± 573 cpm; } P \leq 0.05; n = 3 \text{ experiments) in the presence of 0.5 \mu M U-0126. PDGF-induced } ^{[3]H}\text{thymidine incorporation (29,270 ± 902 cpm) was also inhibited by 5 \mu M U-0126 to the control level (7,212 ± 508 cpm; } P \leq 0.05; n = 3 \text{ experiments).}

Inhibitory Effect of U-0126 on Activation of p90\textsuperscript{rsk}, c-Fos, and Cyclin D1 Induction

We investigated the effect of U-0126 on activation of p90\textsuperscript{rsk}, c-Fos, and cyclin D1 induction. U-0126 (5 μM) reduced the activation of p90\textsuperscript{rsk} induced by 10% FBS by 68.2% (P ≤ 0.05; n = 4 experiments; Fig. 4). Based on this finding, we determined the effect of U-0126 on c-Fos and cyclin D1 induction. As shown in Fig. 5, unstimulated cells (1% FBS) did not show c-Fos or cyclin D1 protein in the nuclear fraction. DMSO (2.5%), which was used to dissolve U-0126, did not induce c-Fos or cyclin D1 protein expression (Fig. 5). All three mitogens (10% FBS, 10 U/ml of thrombin, and 20 ng/ml of PDGF) induced c-Fos and cyclin D1 accumulation in the nucleus, indicating activation of the two factors (Fig. 5). Maximal induction of c-Fos was achieved 4 h after addition of one of the stimuli. Cyclin D1 induction
was maximal after 24 h of stimulation. The following experiments determined the effect of U-0126 at the time point of maximal induction. In the presence of U-0126, c-Fos induction by FBS was reduced by 58.3% ($P < 0.05$; $n = 3$ experiments), thrombin-dependent c-Fos induction was reduced by 54.2%, and PDGF-stimulated c-Fos induction was reduced by 42.4% (Fig. 5). In parallel, U-0126 diminished FBS-induced cyclin D1 induction by 50.5%. Thrombin-stimulated cyclin D1 expression was reduced by 80.8% and PDGF-dependent cyclin D1 expression was reduced by 72% ($P < 0.05$; $n = 3$ experiments; Fig. 5).

Effect of ERK Antisense ODN on ERK1 and ERK2 Protein Expression and Cell Proliferation

We examined the effects of Lipofectin at concentrations from 5 to 100 μg/ml and of ERK antisense from 0.2 to 10 μM. The maximal concentration used was 20 μg/ml of Lipofectin and 0.5 μM antisense (ratio 1:2). At this combination, we observed no decrease in cell viability as assessed with manual cell counts with trypan blue exclusion.

The uptake of the 18-mer ERK ODN was titrated with FITC-labeled ODN together with 1:0, 1:2, and 1:4 ratios of Lipofectin-antisense (positive and negative charge, respectively) to find the effective concentration of Lipofectin and ODN. As shown in Fig. 6, the uptake of ODN was Lipofectin dose dependent (A–C) and ODN did not enter the nucleus of the transfected cells (Fig. 6D).
ERK antisense ODN inhibited the FBS-dependent expression of both ERK1 and -2 protein in a dose-dependent manner (P < 0.05; n = 3 experiments; Fig. 7A). Antisense ERK (0.5 μM) downregulated synthesis of ERK1 and -2 almost completely. Scrambled ODN (0.5 μM) had no effect on ERK1 or ERK2 expression. β-Actin was used as the internal control for the specificity of action of ERK antisense ODN and was not altered as shown in Fig. 7A. The inhibitory effect of ERK antisense ODN on ERK expression was paralleled by inhibition of cell proliferation determined as absolute cell numbers after an incubation time of 48 h (Fig. 7B). ERK antisense ODN (0.5 μM) inhibited cell proliferation stimulated by FBS (2%; chosen to give a slight increase in cell proliferation) by 45.5% (cell number for 2% FBS plus Lipofectin control, 29.5 ± 5.1 × 10^4 cells/well; P < 0.05; n = 3 experiments; Fig. 7B), whereas scrambled ODN had no effect.

**DISCUSSION**

In this study, we found that FBS, thrombin, and PDGF activated ERK within 2 min in HASM cells. The known inhibitors of HASM cell proliferation, PGE2 and forskolin, inhibited mitogen-induced ERK activation. Inhibition of MEK1 and -2, which are upstream from ERK, by the specific MEK inhibitor U-0126 blocked both cell proliferation and ERK activation. In addition, U-0126 inhibited mitogen-induced activation of p90rsk and expression of c-Fos and cyclin D1, all of which are downstream from ERK in the signaling cascade for cell proliferation. We also used antisense ODNs directed to ERK1 and -2 mRNAs to reduce ERK protein expression. ERK-specific ODN inhibited cell proliferation. Thus our results suggest that ERK is crucial for the regulation of HASM cell proliferation.

A critical role for ERK activation in cell proliferation has been suggested in many cell types. ERK activation is elicited by various mitogens such as PDGF, epidermal growth factor, insulin-like growth factor I, and 5-hydroxytryptamine in ASM cells (25, 32). In animal studies, growth factor-stimulated c-Fos, c-Jun, and cyclin D1 expression as well as cell proliferation were abolished with a dominant negative mutant ERK (51).
ERK antisense ODN (33), or the synthetic MEK inhibitors PD-98059 (24, 51, 52) and U-0126 (13).

The relationship between duration of ERK activation and mitogenesis has been investigated in many cell types (25, 29, 32, 51). Stimulation of ERK activation by the three mitogens in our study occurred within 2 min, similar to the results in other cell types (18, 40). In some studies, ERK activation was sustained after treatment with mitogens but was transient after treatment with nonproliferative stimuli (25, 32). In contrast, prolonged activation and nuclear retention of ERKs also has been implicated in differentiation in PC12 cells (29). In the present study, PDGF-induced ERK activation was sustained for up to 2 h, whereas that induced by 10% FBS and thrombin declined after 30 min (Fig. 1, A and C). Both transient and sustained ERK activation were associated with cell proliferation. PDGF induced a high level of ERK activation, and this was reflected in the extent of cell proliferation. Thus, in HASM cells, both the duration and the efficacy of ERK activation correlate with efficacy in cell proliferation.

We used a specific antibody for the phosphorylated forms of ERK1 and -2, which recognizes the enzyme only when it is phosphorylated at Thr183 and Tyr185, which are required for full enzymatic activity (2). Although we found transient ERK activation associated with HASM cell proliferation (Fig. 1, A and C), Orsini et al. (32), using the phospho-specific ERK antibody that is directed to Thr202/Tyr204, reported that only sustained ERK activation leads to HASM cell proliferation. FBS induced the translocation of phosphorylated ERK into the nucleus (Fig. 1B), implicating its role in the control of transcription factors.

The present study revealed that mitogens acting via both G protein-coupled receptors and RTK induce cell proliferation through an ERK-dependent pathway. It is well documented that mitogens induce successive activation of Raf-1, MEK, and ERK (34, 53). Elevation of cAMP can block activation of Raf-1 via inhibition of protein kinase A activation (18). Thus, to block the mitogen-induced ERK signaling cascade, we used agents that elevate intracellular cAMP, the specific MEK inhibitor U-0126 and ERK antisense ODN, which target Raf-1 and MEK and ERK, respectively.

PGE2 and forskolin are known to cause inhibition of HASM cell proliferation (22, 47) and are known to elevate cAMP levels in HASM cells (17). Elevation of cAMP has been reported to either inhibit or stimulate ERK activation depending on the cell type studied (5, 14, 30, 34, 38, 41, 43). In HASM cells, we found that PGE2 and forskolin inhibited both cell proliferation and ERK activation. In vascular smooth muscle cells, cAMP-mediated inhibition of thrombin-induced growth was mediated not via inhibition of ERK but rather of JNK (38). However, in rat tracheal smooth muscle cells, cAMP inhibits thrombin-induced proliferation via inhibition of both ERK activation and JNK activation (44). Also, in bovine tracheal myocytes, cAMP, although significantly reducing Raf-1 activity, did not inhibit PDGF-induced activation of ERK (18). The present study showed that in HASM cells, cAMP inhibits cell proliferation via an ERK-dependent mechanism.

In the current study, we used U-0126 to inhibit MEK1 and -2. U-0126 is a specific inhibitor of ERK...
activation in that it does not inhibit other MAP kinases such as JNK or p38. This is because it has low affinity for their kinases upstream, namely MEK kinases 3, 4, and 6 (2, 13). In our study, the inhibition of ERK activation and cell proliferation by U-0126 were well correlated ($r^2 = 0.990$ for 10% FBS; $r^2 = 0.999$ for 10 U/ml of thrombin; $r^2 = 0.875$ for 20 ng/ml of PDGF). These data support the reliance of HASM cell proliferation on ERK activation. However, even the highest concentration of U-0126, which inhibited ERK activation to below control levels, could not completely abolish the mitogen-induced DNA synthesis, implying that there might be other pathways leading to cell proliferation. The same observation was made in human cardiac fibroblasts (16).

In a recent study by Krymskaya et al. (26), the phosphatidylinositol 3-kinase inhibitors wortmannin and LY-294002 inhibited proliferation of HASM cells by epidermal growth factor and thrombin. Inhibition of phosphatidylinositol 3-kinase inhibits activation of 70-kDa S6 kinases that are required for cell cycle progression in the G1 phase. ERK activity was not affected, whereas cell proliferation was inhibited by those inhibitors. This provides evidence for an alternate, ERK-independent pathway leading to HASM cell proliferation.

To investigate further the mechanism of mitogen-induced, ERK-dependent cell proliferation, we studied the effects of U-0126 on mitogen-induced activation of p90rsk, c-Fos, and cyclin D1 protein induction. We found that p90rsk, c-Fos, and cyclin D1 all lie downstream from ERK in HASM cells, suggesting a role for ERK in regulating the cell cycle.

Little is known about ERK-induced phosphorylation of p90rsk in HASM cells. Activated ERK phosphorylates p90rsk, which, in turn, activates transcription factors such as c-Fos (7), suggesting that p90rsk plays a role in transcriptional regulation. This is the first study to show that p90rsk is present and downstream from ERK activation in HASM cells.

Activated ERK may activate numerous transcription factors such as Elk-1, c-Jun, c-Fos, and c-Myc in the nucleus (8, 28, 36, 42). AP-1 is a sequence-specific transcriptional activator composed of members of the Jun and Fos families. ERK activation leads to elevated AP-1 activity, which results in increased synthesis of c-Fos (23). In the present study, we found that U-0126 decreased c-Fos protein induction. Thus the downregulation of AP-1 activity by U-0126 as measured by luciferase activity in HASM cells (32) is partly due to the downregulation of c-Fos protein induction.

Mitogen-induced AP-1 has been shown to regulate cyclin D1 expression in JEG-3 trophoblasts and COS cells (23). Cyclins D and E, with their catalytic partners cyclin-dependent kinase (CDK) 4 and CDK6, respectively, are required for G1 progression. CDK inhibitors such as p27kip1 inhibit the activity of CDKs. The tumor suppressor protein retinoblastoma protein inhibits specific transcription factors that encode proteins that are essential for cell cycle regulation when cells are quiescent (45). Phosphorylation of retinoblastoma protein by assembly of newly synthesized cyclin D and CDK4 allows cells to express proteins that are essential for entry into the S phase. The increase in cyclin D-CDK activity is a result of both an increase in cyclin D and a decrease in G1 CDK inhibitor expression (50). Regulation of cyclin D1-associated factors such as CDK4 or CDK inhibitor seems to be unrelated to the ERK pathway (50, 51). Cyclin D1 expression is ERK dependent for mitogen-induced cell proliferation. A MEK inhibitor and a dominant negative mutant of MEK1 or ERK abolished PDGF-induced cyclin D1 promoter activity or cyclin D1 expression by blocking G1 progression (37, 50) in bovine airway smooth muscle cells and Chinese hamster embryo fibroblasts. In our study, U-0126 reduced cyclin D1 expression stimulated by FBS, thrombin, or PDGF, suggesting ERK-dependent regulation of cyclin D1 in HASM cells.

In contrast, Hawker et al. (17) found that in HASM cells, PGE2 and forskolin did not significantly inhibit FBS-stimulated cyclin D1 expression at the 5-h time point, but in another study, thrombin-stimulated cyclin D1 expression as well as ERK activation was downregulated by albuterol, 8-bromo-cAMP, or PGE2 (46). The differences in cyclin D1 regulation by cAMP-elevating agents in HASM cells may reflect differences in cross talk between the cAMP system and tyrosine kinase pathways. In rat tracheal smooth muscle cells, only thrombin-induced ERK activation was suppressed by cAMP, whereas that induced by PDGF had no effect (44). However, in the present study, in HASM cells, FBS-, thrombin-, or PDGF-induced ERK activation were all downregulated by forskolin and PGE2. Thus the dissociation of mitogen-induced ERK activation and cyclin D1 expression may be simply because of the different time points chosen to detect cyclin D1 in the two studies or different levels of cAMP produced by different agents.

Many studies (13, 24, 32, 51, 52) have used a MEK inhibitor to inhibit the MEK-ERK pathway because there is no direct inhibitor of ERK. However, we cannot exclude the possibility of nonspecific effects of inhibitors. Thus our use of an antisense ODN against ERK mRNA provided a critical tool to determine the specific role of ERK in HASM cell proliferation. Several studies using antisense ODN directed to ERK observed that ERK is critical for cell proliferation induced by PDGF (39) or angiotensin (54) in rat aortic smooth muscle cells and by Hex A in bovine airway smooth muscle (27). We designed and synthesized an antisense directed to the homologous part of ERK1 and ERK2. The antisense that was used for rat aortic smooth muscle cells had a homologous sequence with human translation initiation factor 3 (eIF3), which may well be activated by ERK in the relationship with eIF4E (31).

In the case of HASM cells, ERK1 and -2 protein expression stimulated by FBS (2%) was abolished by 0.5 μM antisense ERK1 and -2. Others have used 10 or 0.4 μM antisense ERK to inhibit PDGF (39)- or angiotensin (54)-stimulated ERK expression. We used a 3′ end phosphorothioate modification, which is resistant to nuclease digestion, especially on the site where the
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enzyme binds (6, 48), and may result in higher efficacy of ERK ODN. The fact that antisense ERK1 and -2, but not the scrambled ODN, inhibited expression of ERK1 and -2 and cell proliferation shows that this is a specific effect of the ERK antisense ODN. Moreover, the antisense ERK did not affect protein levels nonspecifically as evidenced by the fact that levels of β-actin did not change. This is the first study that provides direct evidence for the involvement of ERK1 and -2 in HASM cell proliferation.

In summary, this study has shown that ERK represents a critical point in the signaling pathway for proliferation of HASM cells in culture. Regulation of ERK activation either by growth-promoting factors such as PDGF, thrombin, and FBS or by inhibiting factors such as PGE_2 should result in the regulation of hyperplasia. However, whether this is also true in vivo requires further investigation.

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