Lysophosphatidic acid upregulates the epidermal growth factor receptor in human airway smooth muscle cells

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Lysophosphatidic acid (LPA) is the simplest endogenous phospholipid, released from activated platelets and present in serum at concentrations of 2–20 μM (17). LPA has been shown to mimic the effects of serum in many systems (16). Recently, multiple GPCRs of the Edg or LPA receptor family have been cloned and shown to be LPA receptors (2, 3, 5, 13). LPA receptors can couple to G proteins of the G12/13 families; activation of LPA receptors can lead to proliferative signaling through the MAPK and Rho pathways as well as to structural changes related to migration and stress fiber formation through Rho-activated pathways (4).

Human airway smooth muscle (HASM) cells treated simultaneously with EGF and LPA exhibit a markedly synergistic activation of mitogenesis (6). This synergistic stimulation of proliferation may be of importance in the pathogenesis of asthma and other diseases of airway remodeling in which airway smooth muscle thickening is a hallmark feature (12, 20). Accordingly, we have explored the molecular mechanism(s) of synergism by investigating potential interactions between the pathways activated by LPA and EGF. Here we report that LPA treatment causes an upregulation of EGFR and investigate the signaling pathways and mechanisms involved. This EGFR upregulation may also be relevant to asthma because increased EGFR levels have recently been demonstrated in airway smooth muscle and epithelial cells in asthmatic tissue (1, 21).

METHODS

Reagents. Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum (FBS) were obtained from Life Technologies (Grand Island, NY). EGF was purchased from BIOSOURCE International (Camarillo, CA). LPA was obtained from American Peptide Co. (Sunnyvale, CA).

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LYSOPHOSPHATIDIC ACID UPREGULATES THE EGF RECEPTOR

Source International (Camarillo, CA) and 18:1(oleyl)-LPA was from Avanti Polar Lipids (Alabaster, AL). Actinomycin D was from BIOMOL (Plymouth Meeting, PA), pertussis toxin (PTX) was from List Biologicals (Campbell, CA), [3H]thymidine was from NEN (Boston, MA), and [125I]-EGF was from Amersham Pharmacia Biotech (Piscataway, NJ). Other chemicals were obtained from Sigma (St. Louis, MO).

Cell culture. HASM cells previously isolated from human trachea by enzymatic dissociation (19) were kindly provided by Dr. Michael Kotlikoff (University of Pennsylvania, Philadelphia, PA) (10, 11). Cells were cultured in high-glucose (4.5 g/l) DMEM with 10% FBS at 37°C in a humidified 5% CO2 incubator and passaged weekly, with cells used between passages 4 and 10. For assays of the amount of receptor expression per cell, cells were counted with a Coulter counter.

[3H]thymidine incorporation assays. [3H]thymidine incorporation assays were performed as previously described (9). Confluent HASM cells were starved in serum-free medium for 24 h before treatment with mitogens for 24 h. [3H]thymidine (2 μCi/ml) was added for the final 2 h of mitogen treatment. Cells were then washed once with PBS and twice with 10% trichloroacetic acid (one 10-min incubation followed by one wash). The precipitated DNA was dissolved with 0.2 N NaOH, and [3H]thymidine incorporation was quantitated by liquid scintillation counting.

Flow cytometric analysis of cell cycle. S phase analysis was performed as previously described (9). Confluent HASM cells were starved in serum-free medium for 24 h before treatment with mitogens for 24 h. Cells that were analyzed before confluence were also plated at 100,000 cells/60-mm dish but were treated with mitogens 2 days after plating, without starving. After treatment, cells were washed once with PBS, removed from the dish by trypsinization, and resuspended in Vindelov’s reagent [75 μg/ml of propidium iodide, 3.5 U/ml of ribonuclease A, and 0.1% Nonidet P-40 in Tris-buffered saline (3.5 mM Tris, pH 7.6, and 10 mM NaCl)]. Flow cytometric analysis was performed with a Becton Dickinson (San Jose, CA) FACSCalibur flow cytometer and modeled 10,000 events; data were analyzed with ModFit LT software from Verity Software House, Topsham, ME.

Second messenger assays. According to the cAMP accumulation assay method of Shimizu et al. (23), cells prelabeled with [3H]adenine were treated with agents that altered adenyl cyclase activity, and the conversion of [3H]ATP to [3H]cAMP was quantitated. Phosphoinositide hydrolysis was measured essentially as previously described (18). HASM cells were labeled with [3H]inositol; after stimulation, labeled compounds were extracted, and [3H]inositol phosphates were separated from [3H]phosphoinositides by ion-exchange chromatography.

[125I]-EGF binding. HASM cells were plated at 40,000 cells/well in six-well plates and grown to confluence. Cells were starved in serum-free medium for 24 h and then treated with growth factors and/or inhibitors for the indicated time period, typically 12 h. Cells were then washed once with 2 ml of 37°C DMEM-HEPES, washed once with 2 ml of ice-cold DMEM-HEPES, and then incubated with [125I]-EGF for 4 h on ice. The [125I]-EGF solutions contained 25,000–80,000 counts/min·1 ml−1 (~20–65 pM) in 0.1% BSA-containing DMEM-HEPES. Nonspecific binding was defined by the addition of 100 ng/ml (17 nM) of nonradioactive EGF. After 4 h on ice, the cells were washed four times with 2 ml of ice-cold DMEM-HEPES containing 0.1% BSA, dissolved in 0.2 N NaOH, and transferred to glass tubes to be counted in a gamma counter.

Western blotting for total and tyrosine-phosphorylated EGFR. HASM cells were plated at 250,000 cells/plate in 100-mm plates and grown to confluence. Cells were starved in serum-free medium for 24 h and then treated with LPA, EGF, LPA plus EGF, or the BSA vehicle for the indicated times before being harvested. Cells were then washed twice with ice-cold PBS and lysed in radioimmunoprecipitation assay (RIPA) buffer (0.1% SDS, 1% Nonidet P-40, 0.5% deoxycholate, 150 mM NaCl, 5 mM EDTA, and 50 mM Tris·HCl, pH 8.0). The lysates were collected and passed through a 25-gauge needle six to eight times. Proteins in the lysates were separated by SDS-PAGE on 7.5% acrylamide gels, transferred to nitrocellulose membrane, and blotted with an anti-EGFR antibody (Santa Cruz Biotechnology, Santa Cruz, CA) followed by chemiluminescence detection with LumiGLO (New England Biolabs, Beverly, MA). For phosphotyrosine analysis, lysates were immunoprecipitated with an anti-EGFR antibody conjugated to agarose beads (Santa Cruz Biotechnology); the immunoprecipitated EGFR was solubilized from the beads and then run on SDS-PAGE. Proteins were transferred to nitrocellulose membrane, blots were probed with the PY99 anti-phosphotyrosine antibody (Santa Cruz Biotechnology), and the signal was detected with LumiGLO chemiluminescence and exposure to film. Films were analyzed by scanning laser densitometry and analyzed with ImageQuant software (Molecular Dynamics/Amersham Pharmacia Biotech).

Data analysis and statistics. Data were analyzed with GraphPad Prism 3.0 software (GraphPad, San Diego, CA). Evaluations of significance employed the two-tailed paired t-test for the comparison of two values or one-way analysis of variance (ANOVA) followed by either the Bonferroni posttest for the comparison of multiple values in one group or Dunnett’s posttest for the comparison of values to control. Competition binding curves were analyzed with nonlinear regression analysis.

RESULTS

Lack of effect of EGF on LPA signaling pathways. To determine whether EGF changes functional responses to LPA, the effects of EGF pretreatment of HASM cells on LPA stimulation of inositol phosphate production and inhibition of cAMP accumulation were examined. Pretreatment of HASM cells with 60 ng/ml of EGF for either 4 or 12 h did not change the production of inositol phosphates by 10 μM LPA. Under control conditions, LPA stimulated inositol phosphate production by 3.9 ± 0.4-fold, similar to previous studies (18); with 4 h of EGF pretreatment, LPA stimulated inositol phosphate production by 3.9 ± 0.5-fold. Similarly, after 12 h, LPA stimulated inositol phosphate production by 3.1 ± 0.6-fold for control cultures and 3.3 ± 0.6-fold for cultures after EGF pretreatment. Additionally, no stimulation of inositol phosphate production by EGF alone was detected (0.9 ± 0.1- and 1.0 ± 0.1-fold after 4 and 12 h, respectively). Similarly, pretreatment of HASM cells with EGF did not change the effect of LPA to inhibit cAMP accumulation. LPA decreased forskolin stimulation of cAMP production in HASM cells by 43 ± 8%, similar to that seen in previous studies (18); after pretreatment of HASM cells with 60 ng/ml of EGF for 4 h, LPA decreased forskolin stimulation of cAMP production in HASM cells by 47 ± 10%.
Duration of mitogen treatment required for mitogenesis and synergism. To determine the duration of mitogen exposure required for mitogenic stimulation by LPA, EGF, and the combination of LPA plus EGF, a mitogen washout experiment was performed (Fig. 1). HASM cells that were treated with LPA for 6 or 8 h before the mitogen was removed showed stimulation of [3H]thymidine incorporation at the 24-h time point. Stimulation of [3H]thymidine incorporation by LPA appeared to plateau by 12 h of treatment. In contrast, stimulation of [3H]thymidine incorporation in response to EGF was not seen at 6 or 8 h but was present and maximal after 12 h of exposure. Similarly, synergism in response to LPA plus EGF was not seen until cells were treated with the combination for 12 h; at earlier time points, the results of stimulation by LPA plus EGF were the same as those of stimulation by LPA alone. These results suggested that 8–12 h of treatment with both mitogens together was required for the interaction of the LPA and EGF pathways that results in synergism. Additionally, the earlier effects of LPA in these mitogen washout experiments suggested that LPA might increase the capacity of the EGFR pathway to be activated in response to EGF.

Upregulation of EGFR by LPA. To investigate potential mechanisms by which LPA increases EGFR responsiveness, the effects of LPA pretreatment on EGFR binding were determined. LPA increased [125I]EGF binding to HASM cells by approximately twofold but only after 8 h or more of exposure to LPA (Fig. 2A). EGFR upregulation was nearly maximal at 12 h, with only a small further increase at 24 h. Thus LPA increased EGFR binding with a time course consistent with the effect of LPA in enhancing EGF-stimulated [3H]thymidine incorporation (Fig. 1). Upregulation was concentration dependent, with half-maximal upregulation at ~1 μM and nearly twofold upregulation at 10 μM (Fig. 2B). Further experiments to characterize LPA upregulation of EGFR levels focused on the 12-h time point and used 10 μM LPA. Treatment of HASM cells with the HASM cell mitogen thrombin (2 U/ml) also induced EGFR upregulation, similar to that seen with 10 μM LPA (Fig. 2B).

Competition binding analysis of upregulated receptors. To determine whether the increase in EGFR binding represented an increase in the total number of cell surface receptors or an increase in binding affinity of the existing receptors, competition binding assays were performed (Fig. 3). Competition binding analysis indicated single-site binding; pretreatment with LPA for 12 h resulted in a twofold increase in [125I]-EGF binding but did not significantly change the EC50 for EGF competition. The EC50 value for cells treated with
the vehicle BSA was 3.5 nM, whereas after LPA treatment, the EC50 value was 2.6 nM (P < 0.05 comparing best fit values by two-tailed t-test). Thus an increase in affinity is insufficient to explain the increased EGFR binding, suggesting that upregulation is due to an increase in total receptor number rather than an increase in binding affinity.

**Sensitivity of LPA-induced EGFR upregulation to cycloheximide and actinomycin D treatment.** To investigate whether the upregulated receptors represented newly synthesized receptors, the effects of the protein synthesis inhibitor cycloheximide and the transcription inhibitor actinomycin D on EGFR upregulation by LPA were assessed (Fig. 4). HASM cells treated with LPA in the presence of cycloheximide did not show EGFR upregulation. Inclusion of actinomycin D during the 12-h LPA treatment also blocked EGFR upregulation. Together, these results suggest that upregulation results from transcriptional activation and synthesis of additional EGFRs.

**Western blot analysis of upregulated receptors.** To confirm that the increase in EGFR levels seen by 125I-EGF binding resulted from an increase in the number of EGFR molecules, EGFR protein levels were assessed with Western blot analysis of whole cell lysates. LPA treatment of HASM cells for 12 h resulted in an increase in EGFR immunoreactivity of similar magnitude (1.8 ± 0.3-fold; Fig. 4C) to the increase seen in binding assays (Figs. 2–4). In contrast, cells treated with EGF for 12 h showed dramatically lower EGFR protein levels, 53 ± 16% of control levels, presumably due to EGF-stimulated downregulation of EGFR. EGFR levels in cells treated with LPA plus EGF were 94 ± 19% of those in control cells, greater than with EGF alone but less than with LPA treatment alone.

**Effects of signaling pathway inhibitors on LPA-induced EGFR upregulation.** Several other HASM cell responses to LPA are PTX sensitive, in particular, mitogenesis (6). The sensitivity of LPA-induced EGFR...
upregulation to treatment with PTX, which inactivates G\textsubscript{i}, was tested. Surprisingly, PTX pretreatment caused only a 30 \pm 4% inhibition of EGFR upregulation by LPA (Table 1), suggesting that G\textsubscript{i} activation is not the major mechanism for LPA-induced EGFR upregulation. To further identify LPA signaling pathways for EGFR upregulation, inhibitors of other pathways were also tested (Table 1). Both the MEK inhibitor U-0126 and rapamycin, which inhibits the activation of p70 ribosomal S6 kinase (p70\textsuperscript{S6K}), showed significant inhibition of LPA-induced EGFR upregulation, implicating both ERK and p70\textsuperscript{S6K} signaling. Adding both inhibitors simultaneously did not yield greater inhibition of EGFR upregulation (n = 2; P > 0.05 by ANOVA; data not shown). In contrast, neither the PI3K inhibitor LY-294002, the Src inhibitor PP1, nor the protein kinase C inhibitor calphostin C caused significant inhibition of EGFR upregulation by LPA.

Role of EGFR tyrosine kinase activity in LPA-induced EGFR upregulation. Because transactivation of the EGFR has recently been implicated in ERK activation by several GPCR signaling pathways (7, 8), we used the EGFR tyrosine kinase inhibitor AG-1478 to investigate whether the tyrosine kinase activity of the EGFR was required for its upregulation by LPA. However, analysis of the inhibitory effect of AG-1478 was complicated by the effect of AG-1478 itself to upregulate EGFR binding. EGFR binding values increased 4.5 \pm 0.3-fold after AG-1478 treatment in the absence of LPA (Fig. 5A), preventing a simple comparison of the fold increase by LPA in the absence and presence of AG-1478. However, when cells were treated with LPA in the presence of AG-1478, a further EGFR upregulation was observed (Fig. 5A), similar in magnitude to that seen in the absence of AG-1478, suggesting that LPA-induced EGFR upregulation does not require EGFR tyrosine kinase activity.

Western blot analysis with an anti-phosphotyrosine antibody was also performed to investigate EGFR transactivation in response to LPA treatment in these cells. EGF treatment for 5 min resulted in a strong stimulation of tyrosine phosphorylation of the EGFR (Fig. 5B); however, no EGFR tyrosine phosphorylation was detected in response to LPA plus EGF than that to EGF alone. No EGFR phosphorylation in response to LPA was seen at either 2 or 6 h of treatment (data not shown). Thus EGFR transactivation by LPA does not appear to occur in HASM cells, consistent with the inability of AG-1478 to block LPA-mediated EGFR upregulation.

### Table 1. Sensitivity of LPA-induced EGFR upregulation to inhibitors

<table>
<thead>
<tr>
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<tr>
<td>PTX (100 ng/ml)</td>
<td>70 \pm 4\textsuperscript{a}</td>
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<tr>
<td>U-0126 (10 \muM)</td>
<td>32 \pm 7\textsuperscript{?}</td>
</tr>
<tr>
<td>Rapamycin (1 \muM)</td>
<td>46 \pm 8\textsuperscript{†}</td>
</tr>
<tr>
<td>PP1 (1 \muM)</td>
<td>129 \pm 40</td>
</tr>
<tr>
<td>LY-294002 (10 \muM)</td>
<td>93 \pm 14</td>
</tr>
<tr>
<td>Calphostin C (1 \muM)</td>
<td>85 \pm 17</td>
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Values are averages \pm SE of 3–6 independent experiments calculated as multiple of increase of lysophosphatic acid (LPA) treatment over BSA treatment for each experiment. Data were normalized, with the fold increase seen on treatment with LPA without inhibitor taken as 100% and the BSA value taken as 0%. Confluent human airway smooth muscle (HASM) cells were starved for 24 h in serum-free medium before treatment with 10 \muM LPA or the vehicle BSA for 12 h in the absence and presence of 2.5 \muM AG-1478. EGFR levels were then assessed by \textsuperscript{125}I-EGF binding to intact cells on ice. Data are averages \pm SE of the fold of increase over vehicle control for 4 independent experiments. \*P < 0.05 vs. control. \*P < 0.05 for LPA + AG-1478 vs. LPA and AG-1478 alone (by 1-way ANOVA followed by Bonferroni posttest). A: sensitivity of LPA-induced EGFR upregulation to AG-1478. Confluent HASM cells were starved for 24 h in serum-free medium before treatment with 10 \muM LPA or the vehicle BSA for 12 h in the absence and presence of 2.5 \muM AG-1478. EGFR levels were then assessed by \textsuperscript{125}I-EGF binding to intact cells on ice. Data are averages \pm SE of the fold of increase over vehicle control for 4 independent experiments. \*P < 0.05 vs. control. \*P < 0.05 for LPA + AG-1478 vs. LPA and AG-1478 alone (by 1-way ANOVA followed by Bonferroni posttest). B: EGFR activation by EGF but not by LPA. Confluent HASM cells were starved for 24 h in serum-free medium before treatment with 10 \muM LPA, 60 ng/ml of EGF, 10 \muM LPA + 60 ng/ml EGF, or vehicle BSA for 5 min. EGFR protein was immunoprecipitated from cell lysates, separated by SDS-PAGE, transferred to nitrocellulose membrane, and blotted with an anti-phosphotyrosine antibody. One Western blot, representative of 3 independent experiments, is shown.

**Fig. 5. Lack of role for EGFR tyrosine kinase activity in upregulation.** A: sensitivity of LPA-induced EGFR upregulation to AG-1478. Confluent HASM cells were starved for 24 h in serum-free medium before treatment with 10 \muM LPA or the vehicle BSA for 12 h in the absence and presence of 2.5 \muM AG-1478. EGFR levels were then assessed by \textsuperscript{125}I-EGF binding to intact cells on ice. Data are averages \pm SE of the fold of increase over vehicle control for 4 independent experiments. \*P < 0.05 vs. control. \*P < 0.05 for LPA + AG-1478 vs. LPA and AG-1478 alone (by 1-way ANOVA followed by Bonferroni posttest). B: EGFR activation by EGF but not by LPA. Confluent HASM cells were starved for 24 h in serum-free medium before treatment with 10 \muM LPA, 60 ng/ml of EGF, 10 \muM LPA + 60 ng/ml EGF, or vehicle BSA for 5 min. EGFR protein was immunoprecipitated from cell lysates, separated by SDS-PAGE, transferred to nitrocellulose membrane, and blotted with an anti-phosphotyrosine antibody. One Western blot, representative of 3 independent experiments, is shown.

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**Effect of HASM cell confluence on synergism and on LPA-induced EGFR upregulation.** To investigate the effect of confluence on EGFR upregulation, subconfluent cells were assayed for LPA-induced EGFR upregulation. In these experiments with subconfluent cells, cells were not starved before treatment with LPA. HASM cells assayed on day 2 after being plated did not show EGFR upregulation in response
to LPA treatment; however, there was a progressive increase in EGFR upregulation with further time in culture (Fig. 6A). Similarly, subconfluent HASM cells showed only additive rather than synergistic mitogenic responses to treatment with LPA plus EGF, whereas confluent cells treated with LPA plus EGF showed strongly synergistic [3H]thymidine incorporation responses (Table 2). Thus both synergism and EGFR upregulation appear to be features of highly confluent cells. Interestingly, the increase in EGFR upregulation as cells became more confluent was accompanied by a decrease in the level of EGFR expression in control cells (Fig. 6B).

![Fig. 6](Fig. 6B)

Table 2. Effect of growth conditions on mitogenic stimulation by LPA plus EGF

<table>
<thead>
<tr>
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<th>Confluent Cells</th>
<th>Subconfluent Cells</th>
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<tbody>
<tr>
<td><strong>[3H]thymidine, fold increase</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BSA</td>
<td>1.0 ± 0.0</td>
<td>1.0 ± 0.0</td>
</tr>
<tr>
<td>LPA</td>
<td>5.4 ± 1.3</td>
<td>18.6 ± 9.1</td>
</tr>
<tr>
<td>EGF</td>
<td>3.7 ± 0.8</td>
<td>6.4 ± 1.6</td>
</tr>
<tr>
<td>LPA + EGF</td>
<td>23.4 ± 6.0</td>
<td>29.4 ± 12.8</td>
</tr>
<tr>
<td><strong>Flow cytometry, %S phase</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BSA</td>
<td>0.5 ± 0.1</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>LPA</td>
<td>2.2 ± 0.6</td>
<td>9.4 ± 1.9</td>
</tr>
<tr>
<td>EGF</td>
<td>1.6 ± 0.1</td>
<td>3.1 ± 0.8</td>
</tr>
<tr>
<td>LPA + EGF</td>
<td>11.9 ± 1.0</td>
<td>17.6 ± 2.1</td>
</tr>
<tr>
<td>1.5% FBS</td>
<td>1.3 ± 0.1</td>
<td>9.0 ± 0.7</td>
</tr>
<tr>
<td>5.0% FBS</td>
<td>5.9 ± 0.3</td>
<td>17.1 ± 1.6</td>
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</table>

Data are averages ± SE of 5 [3H]thymidine and 3 (flow cytometry) independent experiments. [3H]thymidine-incorporation experiments were performed in triplicate, and data are shown as fold increase for indicated treatment over BSA control. FBS, fetal bovine serum. Confluent and subconfluent HASM cells were treated with 10 μM LPA, 60 ng/ml of EGF, 10 μM LPA + 60 ng/ml of EGF, or the vehicle BSA for 24 h and assayed for [3H]thymidine incorporation or cell cycle analysis as described in METHODS. Data for cell cycle analysis represent percentage of cells in S phase.

**DISCUSSION**

Synergistic stimulation of HASM cell mitogenesis by the GPCR mitogen LPA and the receptor tyrosine kinase (RTK) growth factor EGF suggests that GPCR pathways and RTK pathways interact to enhance mitogenic signaling. LPA-activated second messenger pathways downstream of both Gi and Gq were not modulated by EGF pretreatment. In contrast, LPA pretreatment of HASM cells increased binding to cell surface EGFR, suggesting that one mechanism for LPA modulation of EGFR responsiveness is the upregulation of EGFR levels.

The increased EGFR binding after LPA treatment of HASM cells appeared to be due to an increase in EGFR synthesis because increased EGFR binding was sensitive to cycloheximide and actinomycin D, and Western blot analysis demonstrated increased EGFR protein. Both ERK and phospho-Erk play a role in EGFR upregulation by LPA, and a Gi-mediated signal may also be involved. EGFR upregulation by LPA does not require activation of protein kinase C, PI3K, or Src.

Interestingly, EGFR upregulation did not require the intrinsic tyrosine kinase activity of the EGFR, suggesting that transactivation of EGFR by LPA is not involved in EGFR upregulation. This observation is consistent with the lack of effect of the EGFR tyrosine kinase inhibitor AG-1478 on other LPA-mediated effects in these cells, including ERK activation and mitogenesis (T. L. Ediger and M. L. Toews, unpublished data). Additionally, we did not observe tyrosine phosphorylation of the EGFR upon LPA treatment. Together, these results suggest that LPA does not cause ligand-independent EGFR transactivation in HASM cells, consistent with the recently reported lack of EGFR transactivation by histamine, carbachol, and thrombin, other GPCR mitogens for HASM cells (14).
The EGFR tyrosine kinase inhibitor AG-1478 also induced a marked upregulation of cell surface EGFR binding. This AG-1478-induced upregulation is clearly distinct from that induced by LPA because it occurred within minutes of AG-1478 exposure, was insensitive to cycloheximide, and was not accompanied by an increase in EGFR protein (T. L. Ediger and M. L. Toews, unpublished data). Thus AG-1478 apparently increases EGFR binding activity by altering the properties of the existing receptors, whereas LPA increases binding by transcriptional mechanisms, leading to increased EGFR expression.

Western blot analysis showed an upregulation in HASM cells treated with LPA alone that was similar to that seen in radioligand binding assays with 125I-EGF, confirming that EGFR upregulation is due to new protein synthesis. In contrast, HASM cells treated with EGF alone showed a marked downregulation of EGFR levels. In the presence of both LPA and EGF, EGFR levels were greater than those with EGF alone, although clearly less EGFR protein was present with LPA plus EGF than with LPA alone. Thus the EGFR level seen when HASM cells were treated with the combination of LPA plus EGF most likely represents a balance between LPA-induced upregulation and EGF-induced downregulation. The ability of LPA to counteract the EGFR downregulation that occurs with EGF treatment provides one potential mechanism for the synergistic mitogenesis seen in HASM cells treated with LPA plus EGF.

The initial goal of these studies was to identify potential molecular mechanisms contributing to the strikingly synergistic mitogenesis observed when HASM cells are treated with LPA plus EGF. Multiple lines of evidence point to a link between EGFR upregulation and the synergism between LPA and EGF. Analysis of the concentration dependence of LPA-induced EGFR upregulation indicated that 10 μM LPA was required to see EGFR upregulation, consistent with previously published data (6) showing a similar LPA concentration dependence for both LPA-stimulated mitogenesis and for its synergism with EGF. More importantly, the time courses for synergism and EGFR upregulation in response to LPA were strikingly similar (Figs. 1 and 2A), with both processes becoming apparent only after 8–12 h of exposure to LPA. Additionally, high cell density was required for both synergism and LPA-induced EGFR upregulation. Finally, EGFR upregulation was also seen when HASM cells were treated with thrombin, a second GPCR mitogen that shows synergism with EGF for HASM cell mitogenesis (9, 14). Together, the correlation of LPA concentrations, time courses, cell growth conditions, and the similar effects of thrombin and LPA to evoke synergism and upregulate the EGFR all suggest a mechanistic link between the two phenomena.

The maximal mitogenic response to LPA plus EGF was not significantly different between confluent and subconfluent cells. Rather than larger responses to the combination of LPA plus EGF, confluent cells showed smaller responses to both LPA and EGF individually. Correspondingly, the occurrence of synergism with confluent cells may reflect the smaller responses to individual mitogens. Density-dependent downregulation of EGFR, platelet-derived growth factor receptors, fibroblast growth factor receptors, and transforming growth factor-β receptors have been reported in fibroblasts (22). Similarly, a decrease in the number of EGFRs per cell with increasing confluence was also observed for HASM cells in our studies. Perhaps confluent HASM cells undergo a generalized downregulation of GPCRs and growth factor receptors; synergism may thus occur when confluent, less responsive cells are simultaneously treated with a combination of mitogens, at least one of which may effect a generalized upregulation of growth factor-mediated responses by upregulating cell surface growth factor receptors.

Although upregulation of EGFR expression may represent one component of the mechanism for synergism, other potential mechanisms have been proposed. In another study (14) with HASM cells, greater activation of p70S6k was seen in response to thrombin plus EGF than to either agent alone, suggesting that a p70S6k-mediated response may be involved in synergistic proliferative signaling. In our studies, rapamycin sensitivity implicated p70S6k activation in EGF upregulation by LPA, suggesting EGFR upregulation as one potential mechanism by which p70S6k contributes to synergistic signaling.

Based on the known effects of LPA on HASM cell proliferation (6) and airway smooth muscle contractility (24), we hypothesized that LPA may be a mediator of asthma and airway remodeling. Because EGFR upregulation in epithelial and smooth muscle cells has been reported in asthmatic lung tissue (1, 21), the ability of LPA to upregulate the EGFR provides additional support for our hypothesis. More importantly, our results with both LPA and thrombin suggest GPCR-mediated transcriptional regulation as an important mechanism contributing to this increase in EGFR expression. Both LPA and thrombin are released from activated platelets and thus may be present at sites of injury and inflammation. Upregulation of smooth muscle cell EGFRs by these agents could potentially contribute to the increased smooth muscle mass and proliferation that occur in airway remodeling, and strategies targeted at preventing GPCR-mediated upregulation of EGFR may provide new avenues for treatment of airway remodeling and asthma.

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