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Lysophosphatidic Acid Decreases Epidermal Growth Factor Receptor Binding in Airway Epithelial Cells

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d) Abbreviations: LPA, lysophosphatidic acid; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; HASM, human airway smooth muscle; GPCR, G protein-coupled receptor; RTK, receptor tyrosine kinase; FBS, fetal bovine serum; Pen/Strep, penicillin/streptomycin; ether LPA, 1-O-octadecyl-2-hydroxy-sn-glycero-3-phosphate; hBECs, human bronchial epithelial cells; BSA, bovine serum albumin; S1P, sphingosine-1-phosphate; LPC, lysophosphatidylcholine; PA, phosphatidic acid; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; MMP, matrix metalloproteinase; PVDF, polyvinylidine fluoride; TBS, Tris-buffered saline; TBST, TBS + 0.1% Tween-20; HB-EGF, heparin-binding EGF; EC_{50}, effective concentration for 50% of maximal response; IC_{50}, effective concentration for inhibiting 50% of response; B_{max}, maximum number of receptor binding sites; K_d, concentration of agent that binds 50% of receptors.

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Abstract

We showed previously that treatment of human airway smooth muscle cells and lung fibroblasts with lysophosphatidic acid (LPA) increases the binding of epidermal growth factor (EGF) to its receptors (EGFRs). The purpose of this study was to determine if LPA also regulates EGFR binding in airway epithelial cells. Airway epithelial cells were incubated in the absence or presence of 10 μM LPA for increasing times, and binding of ^{125}I-EGF to intact cells on ice was measured. Exposure to LPA for only 15 min caused a 30-70% decrease in EGFR binding in a dose-dependent manner, depending on the cell line. This decrease in binding was sustained to at least 18 hr in BEAS-2B and primary human bronchial epithelial cells. In contrast, the LPA-induced decrease in binding reversed rapidly in two lung cancer epithelial cell lines, H292 and A549, returning to control levels within 3 hr. LPA increased phosphorylation of the EGFR in BEAS-2B cells, and this phosphorylation was inhibited by both AG1478 (EGFR tyrosine kinase inhibitor) and GM6001 (matrix metalloproteinase inhibitor) but not CRM197 (heparin-binding EGF inhibitor). AG1478 and GM6001 also inhibited the LPA-induced decrease in EGFR binding, but only by 50%, suggesting only partial involvement of EGFR transactivation in the decrease in EGFR binding. In summary, LPA stimulates a decrease in EGFR binding in airway epithelial cells that is sustained in normal cells but rapidly reverses in cancer cells. LPA-induced transactivation of EGFRs occurs and contributes to the decrease in EGFR binding, but additional pathway(s) may also be involved.

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Introduction

Asthma is a chronic inflammatory disease thought to result from a combination of increased susceptibility to bronchial epithelial injury and excess tissue repair leading to structural remodeling of the airway wall, perhaps due to an imbalance in responses to growth factors such as epidermal growth factor (EGF) (Holgate, et al., 2003; Munakata, 2006). This remodeling includes thickening of the lamina reticularis, mucus gland and smooth muscle cell hypertrophy and hyperplasia, and increased angiogenesis (Lazaar and Panettieri, Jr., 2003). These airway wall changes also contribute to the airway hyper-responsiveness that is the hallmark of asthma (Munakata, 2006).

The EGF receptor (EGFR) has been shown to be involved in many of the processes of airway remodeling and asthma, including mucin synthesis and secretion, goblet cell metaplasia, and sustaining a repair phenotype in the injured epithelium (Nadel, 2001; Holgate, et al., 2003; Puddicombe, et al., 2000). Asthmatic airways exhibit increased EGFRs in bronchial epithelium and smooth muscle as well as in the basement membrane and glandular cells, when compared to normal controls (Amishima, et al., 1998). Furthermore, the thickness of the lamina reticularis is positively correlated with EGFR expression (Puddicombe, et al., 2000). EGF has also been shown to stimulate proliferation of human airway smooth muscle (HASM) cells and lung fibroblasts (Ediger and Toews, 2000; Cerutis, et al., 1997).

The simple lipid mediator lysophosphatidic acid (LPA) stimulates proliferation, differentiation, tumor metastasis, and cytoskeletal rearrangement in multiple cell types (Zhao, et al., 2006; Wang, et al., 2003). LPA is released from activated platelets at sites of injury (Eichholtz, et al., 1993) where it then contributes to wound repair (Lee, et al., 2000), but it can also be synthesized by epithelial cells via the conversion of phosphatidic acid by phospholipase

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A_1/A_2 (Cummings, et al., 2002). LPA is increased in allergen-challenged lungs compared to saline controls (Georas, et al., 2007) and is 2- to 5-fold higher in injured, pre-fibrotic mouse lungs (Toews, et al., 2004). LPA also increases IL-8 production in human bronchial epithelial cells (hBECs) (Cummings, et al., 2004), which then acts as a neutrophil chemoattractant to enhance inflammation (Saatian, et al., 2006). LPA has been shown to enhance the contractility (Toews, et al., 1997) and to stimulate the proliferation of both airway smooth muscle cells (Cerutis, et al., 1997) and lung fibroblasts (Ediger and Toews, 2000) as well as to stimulate fibronectin secretion (Romberger, et al., 1993) and filopodia extension (Beckmann, et al., 1995) by airway epithelial cells. Together these effects led us to propose an important role for LPA in airway remodeling and disease (Toews, et al., 2002). Consistent with this hypothesis, three recent studies have reported significant protection of LPA receptor knockout mice from multiple components of airway disease (Tager AM, et al., 2007; Shea BS, et al., 2007; Zhao, et al., 2007).

Studies from our laboratory have shown that LPA stimulates HASM cell proliferation on its own, synergizes with EGF to greatly enhance HASM cell proliferation (Cerutis, et al., 1997; Ediger and Toews, 2000), and increases EGFR binding and protein expression (Ediger, et al., 2002). LPA does not stimulate transactivation of the EGFR in HASM cells (Ediger, et al., 2002) but does stimulate transactivation of the EGFR in primary human bronchial epithelial cells (Zhao, et al., 2006). Two pathways through which LPA can transactivate the EGFR have been established. "Ligand-dependent" transactivation involves the activation of matrix metalloproteinases (MMPs) (Shida, et al., 2005; Mori, et al., 2006), the cleavage of EGFR pro-ligands (Xu, et al., 2007; Zhao, et al., 2006), and the subsequent activation of the EGFR. "Ligand-independent" transactivation involves the intracellular activation of EGFRs by kinases such as Src (Daub, et al., 1997; Shah, et al., 2005).

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The purpose of this study was to examine LPA regulation of EGFR binding in airway epithelial cells and to determine whether LPA-mediated transactivation of the EGFR plays a role in this regulation. Because of the contribution of EGF and the EGFR to epithelial repair in asthma, determining how LPA and other factors that are elevated in asthmatic airways regulate the EGFR is critical for understanding the role of the EGFR in airway remodeling. These studies also define additional regulatory interactions between GPCRs and RTKs in airway epithelial cells. Importantly, these interactions are different for airway epithelial cells than for smooth muscle cells and fibroblasts, and they are also different between the normal and the lung cancer cell lines tested.

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Materials and Methods

Reagents. Cell culture medium, fetal bovine serum (FBS), penicillin/streptomycin (Pen/Strep), Tris-glycine gels, and polyvindylidine fluoride (PVDF)-FL membranes were obtained from Invitrogen (Grand Island, NY). Vitrogen was purchased from Angiotech Biomaterials (Palo Alto, CA). EGF was purchased from BioSource International (Camarillo, CA) and LPA, 1-O-octadecyl-2-hydroxy-sn-glycero-3-phosphate (ether LPA), phosphatidic acid, and lysophosphatidylcholine were from Avanti Polar Lipids (Alabaster, AL). GM6001 (N-[(2R)-2-(Hydroxamidocarbonylmethyl)-4-methylpentanoyl]-L-tryptophan methylamide) was from Biomol (Plymouth Meeting, PA), and AG1478 (4-(3-Chloroanalino)-6,7-dimethoxyquinazoline) and Ditheria toxin, CRM mutant (CRM197) were from Calbiochem (San Diego, CA). EGFR and phospho-tyrosine (pY99) antibodies were from Santa Cruz (Santa Cruz, CA). Goat anti-rabbit 800 antibody was from Rockland Immunochemicals (Gilbertsville, PA), and goat anti-mouse 680 antibody was from Molecular Probes (Carlsbad, CA). Other chemicals were from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Fairlawn, NJ). ^{125}I from Amersham (Piscataway, NJ) was used to synthesize ^{125}I-EGF by a chloramine T protocol, and the ^{125}I-EGF was purified on a Sephadex G-25 column (Rizzino, et al., 1988).

Cell culture. BEAS-2B, H292, and A549 cells were from ATCC (Manassas, VA). Primary cultures of human bronchial epithelial cells (hBECs) were kindly provided by Dr. Stephen Rennard (University of Nebraska Medical Center). BEAS-2B cells and hBECs were cultured in a 1:1 mixture of LHC-9 and RPMI-1640 (Lechner and Laveck, 1985). BEAS-2B cells and hBECs were cultured on 1% Vitrogen to enhance cell attachment. H292 lung cancer epithelial cells were cultured in RPMI-1640 with 10% FBS and 1% Pen/Strep, and A549 lung

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cancer epithelial cells were cultured in Ham's F-12 with 10% FBS and 1% Pen/Strep. All cells were maintained in a humidified 5% CO_2 incubator at 37°C and passaged weekly.

^{125}I-EGF binding assays. BEAS-2B cells and H292 cells were plated at 50,000 cells/well in six-well plates and grown to confluence. A549 cells were plated at 100,000 cells/well in six-well plates and grown to confluence. Cells were starved in the appropriate basal medium (without FBS or other growth factors) overnight (18-24 hr) and then treated with LPA or other agents for the indicated times, usually 15 min or 18 hr. Control wells received bovine serum albumin (BSA), because LPA was dissolved in 0.25% BSA. If cells were treated with an inhibitor, the inhibitor was added 30 min before stimulation with LPA. Cells were then washed once with 2 mL of 37ºC DMEM-HEPES with 0.1% BSA, washed once with 2 mL of ice-cold DMEM-HEPES with 0.1% BSA, and then incubated with ^{125}I-EGF for 4 hr on ice to selectively label cell surface EGFRs and to prevent EGFR endocytosis and EGF degradation. The ^{125}I-EGF solutions contained approximately 300,000 cpm/mL in DMEM-HEPES with 0.1% BSA. Nonspecific binding was defined by the addition of 300 ng/mL nonradioactive EGF. After 4 hr binding on ice, the cells were washed 4 times with 2 mL of ice-cold DMEM-HEPES with 0.1% BSA and dissolved in 1 mL 0.2 N NaOH; ^{125}I was then quantified in a gamma counter.

EGFR transactivation assays. BEAS-2B cells were plated on 60-mm dishes and grown to confluence. Cells were then starved in RPMI + Pen/Strep overnight. For experiments with inhibitors, cells were pretreated with the inhibitor for 30 min before stimulation with vehicle or 10 μM LPA for 2 min in the continued presence of inhibitor. Cells were then washed with PBS, incubated on ice for 10 min (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EGTA, 5 mM β-glycerophosphate, 1 mM $MgCl_2$, 1% Triton X-100, 1 mM sodium orthovanadate, and 10 μL/mL protease inhibitor cocktail), and lysed by scraping. The cell lysate was centrifuged for 5 min at

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15300 rpm at 4°C, and the supernatant was diluted in sample buffer (62.5 mM Tris pH 6.8, 2% SDS, 10% glycerol, 50 mM dithiothreitol, and 0.1% bromophenol blue) and boiled for 5 min. Samples were loaded on 6% Tris-glycine gels and electrophoresed for 110 min at 120 V. Gels for EGFR blots were transferred with a semi-wet apparatus to PVDF-FL membrane for 2 hr at 26 V. Membranes were then blocked for 1 hr with a 1:1 mixture of PBS:Odyssey Blocking Buffer (LiCor, Lincoln, NE). EGFR blots were incubated with pY99 (rabbit) and EGFR (mouse) antibodies overnight at 4°C. Blots were washed 5 times with TBS + 0.1% Tween-20 (TBST) for 5 min each, then incubated with secondary anti-rabbit (680 nm) and anti-mouse (800 nm) antibodies for 1 hr before washing again with TBST as above. Blots were then scanned and quantified using the Odyssey Infrared Imaging System (LiCor, Lincoln, NE). Data are expressed as the ratio of phosphorylated/total protein, and then further normalized as the ratio of treated/control cells.

Data analyses. All data are presented as means ± SEM. Data were analyzed using GraphPad Prism 4.00 (GraphPad Software, San Diego, CA). Unless otherwise indicated, all data shown are representative of at least three separate experiments. T-tests were used to determine statistical significance.

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Results

LPA induces a rapid and sustained decrease in EGFR binding in normal epithelial cells. BEAS-2B cells, which are virally transformed and immortalized but frequently used as a model for normal airway epithelial cells, and hBECs from primary cultures were treated with 10 μM LPA or with BSA-containing vehicle for various times and then incubated with ^{125}I-EGF on ice to quantify cell surface EGF receptor binding. In contrast to the up-regulation of EGFRs that occurs in airway smooth muscle cells (Ediger, et al., 2002), LPA induced a rapid decrease in EGFR binding in these epithelial cells, with the maximal decrease occurring by 15 min. After 15 min of treatment, BEAS-2B cells showed a decrease of 32 ± 2% and hBECs showed a decrease of 26 ± 14% (Fig. 1A). This decrease in binding was sustained through 18 hr of continuous LPA exposure, with decreases of 45 ± 5% and 44 ± 9% for BEAS-2B cells and hBECs respectively.

LPA induces a rapid but transient decrease in EGFR binding in lung cancer epithelial cells. H292 and A549 lung cancer epithelial cells were treated with 10 μM LPA for various times and then incubated with ^{125}I-EGF on ice as in the studies with normal cells above. These cancer cell lines showed a greater extent of rapid decrease at 15 min; LPA decreased EGFR binding by 54 ± 4% in H292 cells and 67 ± 2% in A549 cells after 15 min of treatment. However, in contrast to the normal epithelial cells, this decrease was not sustained (Fig. 1B). After only 1 hr of treatment with LPA, EGFR binding began to return to control levels, and by 18 hr, EGFR binding had returned to baseline in H292 cells (90 ± 3% of control) and was increased above baseline in A549 cells (113 ± 5% of control) (Fig. 1B). The reversal of the initial decrease in binding occurred somewhat more rapidly for H292 cells than for A549 cells.

Concentration dependence of the LPA-induced changes in EGFR binding. The LPA concentration dependence of the changes in EGFR binding was determined at both 15 min and

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18 hr for BEAS-2B, H292, and A549 cells (Fig. 2). In BEAS-2B cells, the LPA-induced decrease in EGFR binding at 15 min occurred with high potency; however, the dose-response curve was shifted to the right after 18 hr of treatment. The EC_{50} values were 3.8 ± 0.2 nM at 15 min (Fig. 2A) and 1.1 ± 0.1 μM at 18 hr (Fig. 2B). To test whether the lower potency at 18 hr was due to deacylation of LPA, we tested the effects of ether LPA, which has a stable ether bond between C1 of the glycerol backbone and the fatty acid chain, preventing phospholipase-mediated deacylation (Prestwich, et al., 2005). The dose-response curves for LPA and ether LPA were very similar at both 15 min and 18 hr, with EC_{50} values of 9.8 ± 0.8 nM at 15 min and 1.9 ± 0.1 μM at 18 hr for ether LPA (Fig. 2C). Thus phospholipase degradation of the LPA is apparently not the explanation for the lower potency of LPA for the 18 hr decrease.

Dose-response curves at 15 min for both H292 and A549 cells showed that the LPA-induced decrease in EGFR binding required higher concentrations of LPA in these cancer cells than in the BEAS-2B cells, with EC_{50} values of 170 ± 10 nM for H292 cells and 24 ± 3 nM for A549 cells (Fig. 2A). Though H292 cells showed no change in binding at 18 hr, the A549 cells consistently showed a modest increase in EGFR binding of 31 ± 8%, with an EC_{50} of 1.4 ± 0.4 μM after 18 hr of LPA treatment (Fig. 2B).

The decrease in binding results primarily from a decrease in B_{max}. To assess the nature of the decrease in EGFR binding, saturation and competition binding assays were performed following exposure to 10 μM LPA for 15 min for the two prototype cell lines, BEAS-2B for normal cells and H292 for cancer cells. Saturation curves with both cell lines revealed a prominent decrease in B_{max} after LPA treatment; a small increase in K_d was also observed, but not sufficient to be a major contribution to the decrease in binding (Fig. 3A,B). Competition

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curves similarly showed clear decreases in maximal EGFR binding after LPA treatment, with only small increases in IC_{50} values for both cell lines (Fig. 3C,D).

Effects of other GPCR agents on EGFR binding. The effects on EGFR binding were assessed for two additional GPCR agents, sphingosine-1-phosphate (S1P) and thrombin, at both 15 min and 18 hr. Both of these agonists are implicated in airway disease and can activate similar GPCR pathways as LPA (Jolly, et al., 2002; Rosenfeldt, et al., 2003; Kume, et al., 2007; Terada, et al., 2004). With 15-min treatments, the lysophospholipid mediator S1P was similarly potent but slightly more efficacious than LPA in both BEAS-2B and A549 cells, with decreases of 52 ± 4% and 69 ± 3% and EC_{50} values of 3.6 ± 0.6 nM and 35 ± 4 nM, respectively (Fig. 4A,C). S1P was less potent and equally efficacious as LPA in H292 cells, with a decrease of 38 ± 2% and an EC_{50} value of 2.0 ± 0.1 μM (Fig. 4B).

Thrombin was also able to decrease EGFR binding after 15 min of treatment, similar to the effects of LPA and S1P. As expected, the peptide thrombin was much more potent than LPA or S1P, with EC_{50} values of 6.3 ± 0.7 pM and 1.1 ± 0.1 nM in BEAS-2B and A549 cells, respectively (Fig. 4A,C); maximal effects of thrombin were similar to those with LPA and S1P. Thrombin appeared to decrease EGFR binding in H292 cells, but the potency was too low to establish a full dose-response curve and EC_{50} value (Fig. 4B).

S1P and thrombin also induced similar changes as LPA after 18 hr of treatment. In BEAS-2B cells, S1P was more potent but slightly less efficacious than LPA, with a decrease of 30 ± 4% and an EC_{50} value of 200 ± 40 nM (Fig. 5A). In A549 cells, both S1P and thrombin mimicked the small increase in EGFR binding seen after prolonged LPA treatment, with increases of 44 ± 5% and 35 ± 18%, respectively. Thrombin was the most potent agent for this effect, with an EC_{50} of 5.8 ± 0.4 nM, followed by S1P and LPA with EC_{50} values of 1.5 ± 0.3

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μM and 1.4 ± 0.4 μM, respectively (Fig. 5C, 2B). Similar to LPA, S1P and thrombin had no effect on EGFR binding after 18 hr of treatment in H292 cells (Fig. 5B).

Effects of LPA-related agents on EGFR binding. The specificity for LPA of these effects on EGFR binding was assessed by testing several structurally related compounds. Oleic acid is an 18:1 fatty acid identical to the single fatty acid chain on the 18:1 (oleoyl) LPA used in these studies; oleic acid had no effect on EGFR binding in any of the cell types tested at either 15 min or 18 hr (Table 1). Lysophosphatidylcholine (LPC) contains a choline head group attached to the phosphate rather than the free phosphate of LPA. LPC had no effect on any cell type after 18 hr of treatment, but showed weak effects in all cell types after 15 min of treatment (Table 1). Phosphatidic acid (PA) contains fatty acid chains on both C1 and C2 of the glycerol backbone instead of the single fatty acid on C1 for LPA. In contrast to oleic acid and LPC, PA did decrease EGFR binding in both BEAS-2B and A549 cells at 15 min, though with markedly lower potency than LPA. PA also exhibited a sustained decrease in EGFR binding in BEAS-2B cells with approximately the same potency as LPA. PA had no effect in H292 cells at 15 min or 18 hr and also had no effect on A549 cells at 18 hr (Table 1).

Effects of different fatty acid chain lengths. Oleoyl-LPA (18:1 LPA) is the most commonly studied form of LPA. We tested both stearoyl (18:0) LPA and palmitoyl (16:0) LPA to determine the LPA structure/activity relationship for the decrease in EGFR binding. 18:1 LPA was the most potent and most efficacious agent after 15 min of treatment, followed by 18:0 LPA (Table 1). 16:0 LPA was the least potent at 15 min, but was as effective at decreasing EGFR binding as 18:0 LPA in all cell types. Interestingly, 18:0 LPA was the most potent for the sustained decrease in binding after 18 hr of treatment in BEAS-2B cells, followed by 18:1 LPA and then 16:0 LPA (Table 1). This potency order was reversed in A549 cells, where EGFR

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binding increases rather than decreases after 18 hr of treatment, with 16:0 LPA being the most potent, followed by 18:1 LPA, and with 18:0 LPA being the least potent.

LPA stimulates phosphorylation of the EGFR in BEAS-2B cells. LPA transactivates the EGFR in many cells, including primary human bronchial epithelial cells (Zhao, et al., 2006). The ability of LPA to transactivate EGFRs in BEAS-2B cells was assessed by measuring EGFR phosphorylation (Fig. 6). BEAS-2B cells were treated for the indicated times in the presence of 10 μM LPA. LPA stimulated EGFR phosphorylation 1.7- to 2.0-fold, and this phosphorylation was sustained to at least 15 min. Though LPA stimulated EGFR phosphorylation much less than EGF itself, this magnitude is similar to what has been reported previously (Zhao, et al., 2006).

AG1478 and GM6001 inhibit EGFR phosphorylation in BEAS-2B cells. In order to assess the possible role of transactivation in the decrease in EGFR binding, inhibitors were used to block the LPA-stimulated transactivation. The EGFR tyrosine kinase inhibitor AG1478, the MMP inhibitor GM6001, and CRM197, a non-toxic mutant of diphtheria toxin that specifically binds heparin-binding (HB)-EGF, were used to test the mechanism of transactivation. BEAS-2B cells were pretreated with 2.5 μM AG1478, 25 μM GM6001, or 10 μg/mL CRM197 prior to cell treatment for 2 min with BSA or LPA. Western blotting for total and phospho-EGFR showed that both AG1478 and GM6001 completely inhibited phosphorylation of the EGFR by LPA (Fig. 7); thus MMP activation and the kinase activity of the EGFR itself are required for transactivation by LPA. However, CRM197 did not have any effect on the transactivation (Fig. 7), suggesting that an EGFR pro-ligand other than HB-EGF is cleaved to stimulate transactivation.

AG1478 and GM6001 inhibit the rapid decrease in binding by LPA in BEAS-2B cells. To determine if transactivation contributes to the LPA-induced decrease in EGFR binding,

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BEAS-2B cells were pretreated for 30 min with 2.5 μM AG1478 or 25 μM GM6001 prior to treatment for 15 min or 18 hr with 10 μM LPA in the continued presence of the inhibitor. Cells were then washed and incubated for 4 hr on ice with ^{125}I-EGF to label cell surface EGFRs. AG1478 caused an increase in EGFR binding of approximately 2-fold, similar to data previously reported with human airway smooth muscle cells (Ediger, et al., 2002). In the absence of AG1478 or GM6001, LPA significantly decreased EGFR binding by approximately 30% after 15 min. In the presence of AG1478 or GM6001, LPA only decreased binding by 14 ± 8% and 12 ± 3%, respectively (Fig. 8A,B), suggesting at least partial involvement of EGFR transactivation in the LPA-induced rapid decrease in EGFR binding. AG1478 and GM6001 had minimal effects on the sustained decrease in binding, suggesting that an additional mechanism is involved in the sustained decrease. The HB-EGF inhibitor CRM197 did not inhibit the LPA-induced decrease in binding at 15 min or 18 hr, consistent with to its inability to inhibit transactivation in BEAS-2B cells (Fig. 8C). To determine if Src-mediated ligand-independent transactivation might be responsible for the portion of the decrease in binding that is not mediated by ligand-dependent transactivation, BEAS-2B cells were treated with the Src inhibitors PP1 and PP2. Neither PP1 nor PP2 had any effect on the rapid or sustained decreases in binding (data not shown), indicating that Src-mediated ligand-independent transactivation is not involved in the LPA-induced decrease in EGFR binding in BEAS-2B cells.

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Discussion

The results presented here show that LPA regulates EGFR binding in human airway epithelial cells in ways that are both complex and markedly different from effects in airway mesenchymal cells. LPA caused a rapid decrease in cell surface EGFR binding in all of the airway epithelial cells tested, with maximal decreases occurring within 15 min. This decrease in binding was sustained to at least 18 hr in the two cell types used as models of normal airway epithelium, BEAS-2B cells and hBECs, but it reversed rapidly in the two lung cancer cell lines tested, H292 and A549 cells. Dose-response curves showed that the LPA-induced decrease in EGFR binding after 15 min of treatment occurs to a greater extent but with lower potency in the lung cancer epithelial cells compared to the normal epithelial cells. Whether these potency differences relate to different LPA receptor subtypes, differential metabolism of LPA, or differential regulation of EGFRs among these cells remains to be established.

Compounds with structural similarities to LPA, such as oleic acid and LPC, either had no effect on EGFR binding or were much less potent than LPA. PA also had either no effect or was much less potent than LPA, with the exception of 18-hr treatment of BEAS-2B cells, where PA and LPA had similar potency and efficacy. The difference in the dose-response curves to LPA at 15 min and 18 hr in BEAS-2B cells is not due to deacylation of LPA by phospholipases, but the decreased potency of LPA after 18 hr of treatment could result from the metabolism of LPA by other enzymes, thereby decreasing the amount of LPA available to regulate EGFR binding. The specificity of these effects suggests that the decreases in EGFR binding for LPA vs. structural analogs are mediated by LPA signaling through one or more of its specific GPCRs and not via a non-specific lipid-mediated effect.

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The decrease in EGFR binding is a result of a decrease in EGF binding sites after 15 min of LPA treatment, as indicated by both saturation and competition curves; there is also a small decrease in the affinity of EGF for its receptor, although this change in affinity contributes minimally to the decrease in EGFR binding. Because the binding studies were conducted with cells on ice to selectively label cell surface receptors, the decrease in binding could be due to receptor internalization or some other decrease in receptor accessibility, without an actual loss of EGFR protein. One obvious possibility is that the rapid decrease in binding is a result of transactivation and subsequent internalization of EGFRs induced by LPA. Previous studies have established that LPA transactivates the EGFR in primary human bronchial epithelial cells (Zhao, et al., 2006) and that transactivation of the EGFR results in receptor internalization and a decrease in ^{125}I-EGF binding on ice in some cell types (Olivares-Reyes, et al., 2005; Kim, et al., 2003; Pierce, et al., 2000). In rat hepatic C9 cells, angiotensin II stimulates a decrease in EGFR cell surface binding via transactivation and internalization of the EGFR (Olivares-Reyes, et al., 2005). Similar results were obtained with serotonin in rat renal mesangial cells (Grewal, et al., 2001). Therefore, transactivation and subsequent internalization of the EGFR provide one possible mechanism for the decrease in EGFR binding by LPA in these cells.

In agreement with these previous studies, LPA was also able to stimulate phosphorylation of the EGFR in BEAS-2B cells, and this transactivation was completely blocked by both GM6001 and AG1478. However, both GM6001 and AG1478 reduced the decrease in EGFR binding after 15 min of LPA treatment only partially. These data indicate that transactivation of the EGFR, presumably followed by internalization and/or degradation, may contribute to the decrease in EGFR binding after LPA treatment, but it is not the only mechanism through which LPA reduces EGFR binding in these cells. In spite of essentially complete inhibition of

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transactivation, AG1478 and GM6001 only inhibit the LPA-induced decrease in EGFR binding by about half. These results are very similar to those reported previously with primary hBECs, in which LPA-stimulated EGFR transactivation was completely blocked by AG1478 and GM6001 but the downstream response of IL-8 secretion was only inhibited by about half (Zhao, et al., 2006). However, whereas the HB-EGF inhibitor CRM197 partially inhibited transactivation in hBECs (Zhao, et al., 2006), it had minimal effect on LPA-induced transactivation in BEAS-2B cells, suggesting that BEAS-2B cells may use a different EGFR pro-ligand to stimulate transactivation.

Five LPA receptors have been identified to date, $LPA_{1\text{-}5}$; LPA_4 and LPA_5 have only been identified recently (Hecht, et al., 1996; An, et al., 1998; Bandoh, et al., 1999; Noguchi, et al., 2003; Lee, et al., 2006). The ability of LPA receptors to couple to multiple G proteins greatly expands the number of signaling pathways by which LPA can mediate its effects. LPA, S1P, and thrombin all stimulate common signaling pathways, including $G_{i/o}$, $G_{q/11}$, and $G_{12/13}$ pathways (Sorensen, et al., 2003; Radeff-Huang, et al., 2004). In addition, S1P and thrombin are mitogenic for HASM cells (Ediger and Toews, 2000) and have been implicated in airway disease similar to LPA (Kume, et al., 2007; Jolly, et al., 2002; Lan, et al., 2002). Both thrombin and S1P mimicked the effect of LPA to alter EGFR binding in airway epithelial cells; thrombin was much more potent but slightly less efficacious than LPA or S1P for decreasing EGFR binding, and LPA and S1P were comparable in both potency and efficacy. Unexpectedly, thrombin decreased EGFR binding at low concentrations after 18 hr of treatment in BEAS-2B cells, but this effect was lost at higher concentrations. The unique activation and complex regulatory mechanisms that are known to occur for thrombin receptors may contribute to these biphasic effects (Lan, et al., 2002).

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In all airway epithelial cell types tested, LPA induced a rapid decrease in EGFR binding, but a sustained decrease was seen only in a subset of these cells. This is markedly different from the response in airway mesenchymal cells, both smooth muscle cells and fibroblasts, in which LPA increases EGFR binding but only after 12- to 24-hr of exposure (Ediger, et al., 2002). The up-regulation of EGFRs in mesenchymal cells is via a transcriptional mechanism, leading to an increase in EGFR protein, and is blocked by both the transcription inhibitor actinomycin D and the translation inhibitor cycloheximide (Ediger, et al., 2002). Because of the rapid time course in the epithelial cells, it is unlikely that the initial decrease in binding is a result of transcriptional inhibition of EGFR synthesis, but rather to more rapid mechanisms such as transactivation, internalization, and perhaps degradation of EGFRs. Several studies have found that transactivation of the EGFR by LPA and other GPCR agents does not occur in HASM cells (Ediger, et al., 2002; Rakhit, et al., 1999; Capra, et al., 2003; Krymskaya, et al., 2000), and the lack of this response may be critical to the differences between regulation of EGFRs by LPA in epithelial *vs.* mesenchymal cells. For both the differences among epithelial cell lines and the differences between epithelial *vs.* mesenchymal cells, it is possible that one LPA receptor subtype and signaling pathway mediates one response while a different subtype and signaling pathway mediates the other response. Identification of specific LPA receptors and pathways for the rapid vs. sustained component of the changes in EGFRs in normal vs. cancer cells is an important goal of ongoing studies.

In summary, LPA rapidly decreases EGFR binding in airway epithelial cells, with a sustained decrease in two models of normal epithelial cells but only a transient decrease in two lung cancer cell lines. The rapid decrease in binding is partially mediated by transactivation of the EGFR, although an additional signaling pathway also appears to be involved. The decrease

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in EGFR binding is mimicked by other GPCR agents that are implicated in asthma and utilize signaling pathways similar to those for LPA, suggesting a broader relevance of these findings to other mediators of asthma and related airway diseases. These and further studies of the regulation of EGFR binding by LPA and other GPCRs and of the different effects in diverse types of airway cells will contribute to our understanding of multiple lung diseases and may also point to novel approaches for therapeutic intervention.

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References

Amishima M, Munakata M, Nasuhara Y, Sato A, Takahashi T, Homma Y and Kawakami Y (1998) Expression of epidermal growth factor and epidermal growth factor receptor immunoreactivity in the asthmatic human airway. *Am J Respir Crit Care Med* **157**:1907-1912.

An S, Bleu T, Hallmark OG and Goetzl EJ (1998) Characterization of a novel subtype of human G protein-coupled receptor for lysophosphatidic acid. *J Biol Chem* **273**:7906-7910.

Bandoh K, Aoki J, Hosono H, Kobayashi S, Kobayashi T, Murakami-Murofushi K, Tsujimoto M, Arai H and Inoue K (1999) Molecular cloning and characterization of a novel human G-protein-coupled receptor, EDG7, for lysophosphatidic acid. *J Biol Chem* **274**:27776-27785.

Beckmann JD, Romberger DJ, Rennard SI and Spurzem JR (1995) Induction of bovine bronchial epithelial cell filopodia by tetradecanoyl phorbol acetate, calcium ionophore, and lysophosphatidic acid. *J Cell Physiol* **164**:123-131.

Capra V, Habib A, Accomazzo MR, Ravasi S, Citro S, Levy-Toledano S, Nicosia S and Rovati GE (2003) Thromboxane prostanoid receptor in human airway smooth muscle cells: a relevant role in proliferation. *Eur J Pharmacol* **474**:149-159.

Cerutis D, Nogami M, Anderson JL, Churchill JD, Romberger DJ, Rennard SI and Toews ML (1997) Lysophosphatidic acid and EGF stimulate mitogenesis in human airway smooth muscle cells. *Am J Physiol* **273**:L10-L15.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 23, 2024

JPET Fast Forward. Published on July 19, 2007 as DOI: 10.1124/jpet.107.120584
This article has not been copyedited and formatted. The final version may differ from this version.

Cummings R, Parinandi N, Wang L, Usatyuk P and Natarajan V (2002) Phospholipase D/phosphatidic acid signal transduction: role and physiological significance in lung. *Mol Cell Biochem* **234-235**:99-109.

Cummings R, Zhao Y, Jacoby D, Spannhake EW, Ohba M, Garcia JG, Watkins T, He D, Saatian B and Natarajan V (2004) Protein kinase Cdelta mediates lysophosphatidic acid-induced NF-kappaB activation and interleukin-8 secretion in human bronchial epithelial cells. *J Biol Chem* **279**:41085-41094.

Daub H, Wallasch C, Lankenau A, Herrlich A and Ullrich A (1997) Signal characteristics of G protein-transactivated EGF receptor. *EMBO J* **16**:7032-7044.

Ediger TL, Danforth BL and Toews ML (2002) Lysophosphatidic acid upregulates the epidermal growth factor receptor in human airway smooth muscle cells. *Am J Physiol Lung Cell Mol Physiol* **282**:L91-L98.

Ediger TL and Toews ML (2000) Synergistic stimulation of airway smooth muscle cell mitogenesis. *J Pharmacol Exp Ther* **294**:1076-1082.

Eichholtz T, Jalink K, Fahrenfort I and Moolenaar WH (1993) The bioactive phospholipid lysophosphatidic acid is released from activated platelets. *Biochem J* **291 (Pt 3)**:677-680.

Georas SN, Berdyshev E, Hubbard W, Gorshkova IA, Usatyuk PV, Saatian B, Myers AC, Williams MA, Xiao HQ, Liu M and Natarajan V (2007) Lysophosphatidic acid is detectable in human bronchoalveolar lavage fluids at baseline and increased after segmental allergen challenge. *Clin Exp Allergy* **37**:311-322.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 23, 2024

JPET Fast Forward. Published on July 19, 2007 as DOI: 10.1124/jpet.107.120584
This article has not been copyedited and formatted. The final version may differ from this version.

Grewal JS, Luttrell LM and Raymond JR (2001) G protein-coupled receptors desensitize and down-regulate epidermal growth factor receptors in renal mesangial cells. *J Biol Chem* **276**:27335-27344.

Hecht JH, Weiner JA, Post SR and Chun J (1996) Ventricular zone gene-1 (vzg-1) encodes a lysophosphatidic acid receptor expressed in neurogenic regions of the developing cerebral cortex. *J Cell Biol* **135**:1071-1083.

Holgate ST, Davies DE, Puddicombe S, Richter A, Lackie P, Lordan J and Howarth P (2003) Mechanisms of airway epithelial damage: epithelial-mesenchymal interactions in the pathogenesis of asthma. *Eur Respir J Suppl* **44**:24s-29s.

Jolly PS, Rosenfeldt HM, Milstien S and Spiegel S (2002) The roles of sphingosine-1-phosphate in asthma. *Mol Immunol* **38**:1239-1245.

Kim J, Ahn S, Guo R and Daaka Y (2003) Regulation of epidermal growth factor receptor internalization by G protein-coupled receptors. *Biochemistry* **42**:2887-2894.

Krymskaya VP, Orsini MJ, Eszterhas AJ, Brodbeck KC, Benovic JL, Panettieri RA, Jr. and Penn RB (2000) Mechanisms of proliferation synergy by receptor tyrosine kinase and G protein-coupled receptor activation in human airway smooth muscle. *Am J Respir Cell Mol Biol* **23**:546-554.

Kume H, Takeda N, Oguma T, Ito S, Kondo M, Ito Y and Shimokata K (2007) Sphingosine 1-phosphate causes airway hyper-reactivity by rho-mediated Myosin phosphatase inactivation. *J Pharmacol Exp Ther* **320**:766-773.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 23, 2024

JPET Fast Forward. Published on July 19, 2007 as DOI: 10.1124/jpet.107.120584
This article has not been copyedited and formatted. The final version may differ from this version.

Lan RS, Stewart GA and Henry PJ (2002) Role of protease-activated receptors in airway function: a target for therapeutic intervention? *Pharmacol Ther* **95**:239-257.

Lazaar AL and Panettieri RA, Jr. (2003) Is airway remodeling clinically relevant in asthma? *Am J Med* **115**:652-659.

Lechner JF and Laveck MA (1985) A serum-free method for culturing normal human epithelial cells at clonal density. *J Tissue Cult Methods* **9**:43-48.

Lee CW, Rivera R, Gardell S, Dubin AE and Chun J (2006) GPR92 as a new G12/13- and Gq-coupled lysophosphatidic acid receptor that increases cAMP, LPA5. *J Biol Chem* **281**:23589-23597.

Lee H, Goetzl EJ and An S (2000) Lysophosphatidic acid and sphingosine 1-phosphate stimulate endothelial cell wound healing. *Am J Physiol Cell Physiol* **278**:C612-C618.

Mori K, Kitayama J, Shida D, Yamashita H, Watanabe T and Nagawa H (2006) Lysophosphatidic acid-induced effects in human colon carcinoma DLD1 cells are partially dependent on transactivation of epidermal growth factor receptor. *J Surg Res* **132**:56-61.

Munakata M (2006) Airway remodeling and airway smooth muscle in asthma. *Allergol Int* **55**:235-243.

Nadel JA (2001) Role of epidermal growth factor receptor activation in regulating mucin synthesis. *Respir Res* **2**:85-89.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 23, 2024

JPET Fast Forward. Published on July 19, 2007 as DOI: 10.1124/jpet.107.120584
This article has not been copyedited and formatted. The final version may differ from this version.

Noguchi K, Ishii S and Shimizu T (2003) Identification of p2y9/GPR23 as a novel G protein-coupled receptor for lysophosphatidic acid, structurally distant from the Edg family. *J Biol Chem* **278**:25600-25606.

Olivares-Reyes JA, Shah BH, Hernandez-Aranda J, Garcia-Caballero A, Farshori MP, Garcia-Sainz JA and Catt KJ (2005) Agonist-induced interactions between angiotensin AT1 and epidermal growth factor receptors. *Mol Pharmacol* **68**:356-364.

Pierce KL, Maudsley S, Daaka Y, Luttrell LM and Lefkowitz RJ (2000) Role of endocytosis in the activation of the extracellular signal-regulated kinase cascade by sequestering and nonsequestering G protein-coupled receptors. *Proc Natl Acad Sci U S A* **97**:1489-1494.

Prestwich GD, Xu Y, Qian L, Gajewiak J and Jiang G (2005) New metabolically stabilized analogues of lysophosphatidic acid: agonists, antagonists and enzyme inhibitors. *Biochem Soc Trans* **33**:1357-1361.

Puddicombe SM, Polosa R, Richter A, Krishna MT, Howarth PH, Holgate ST and Davies DE (2000) Involvement of the epidermal growth factor receptor in epithelial repair in asthma. *FASEB J* **14**:1362-1374.

Radeff-Huang J, Seasholtz TM, Matteo RG and Brown JH (2004) G protein mediated signaling pathways in lysophospholipid induced cell proliferation and survival. *J Cell Biochem* **92**:949-966.

Rakhit S, Conway AM, Tate R, Bower T, Pyne NJ and Pyne S (1999) Sphingosine 1-phosphate stimulation of the p42/p44 mitogen-activated protein kinase pathway in airway smooth muscle.

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JPET Fast Forward. Published on July 19, 2007 as DOI: 10.1124/jpet.107.120584
This article has not been copyedited and formatted. The final version may differ from this version.

Role of endothelial differentiation gene 1, c-Src tyrosine kinase and phosphoinositide 3-kinase. *Biochem J* **338 (Pt 3)**:643-649.

Rizzino A, Kazakoff P, Ruff E, Kuszynski C and Nebelsick J (1988) Regulatory effects of cell density on the binding of transforming growth factor beta, epidermal growth factor, platelet-derived growth factor, and fibroblast growth factor. *Cancer Res* **48**:4266-4271.

Romberger D, Plevin R, Adachi Y, Rennard SI, Nogami M and Toews ML (1993) Lysophosphatidic acid stimulates airway epithelial cell fibronectin release. *Clin Res* **41**:674A.

Rosenfeldt HM, Amrani Y, Watterson KR, Murthy KS, Panettieri RA, Jr. and Spiegel S (2003) Sphingosine-1-phosphate stimulates contraction of human airway smooth muscle cells. *FASEB J* **17**:1789-1799.

Saatian B, Zhao Y, He D, Georas SN, Watkins T, Spannhake EW and Natarajan V (2006) Transcriptional regulation of lysophosphatidic acid-induced interleukin-8 expression and secretion by p38 MAPK and JNK in human bronchial epithelial cells. *Biochem J* **393**:657-668.

Shah BH, Olivares-Reyes JA and Catt KJ (2005) The protein kinase C inhibitor Go6976 [12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo(2,3-a)py rrolo(3,4-c)-carbazole] potentiates agonist-induced mitogen-activated protein kinase activation through tyrosine phosphorylation of the epidermal growth factor receptor. *Mol Pharmacol* **67**:184-194.

Shea BS, LaCamera P, Poorvu EC, Hart WK, Chun J, Luster AD and Tager AM (2007) Pulmonary vascular leak following bleomycin lung injury is dependent on the lysophosphatidic acid receptor LPA1. *Am J Respir Crit Care Med* **175**:A237.

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JPET Fast Forward. Published on July 19, 2007 as DOI: 10.1124/jpet.107.120584
This article has not been copyedited and formatted. The final version may differ from this version.

Shida D, Kitayama J, Yamaguchi H, Yamashita H, Mori K, Watanabe T and Nagawa H (2005) Lysophosphatidic acid transactivates both c-Met and epidermal growth factor receptor, and induces cyclooxygenase-2 expression in human colon cancer LoVo cells. *World J Gastroenterol* **11**:5638-5643.

Sorensen SD, Nicole O, Peavy RD, Montoya LM, Lee CJ, Murphy TJ, Traynelis SF and Hepler JR (2003) Common signaling pathways link activation of murine PAR-1, LPA, and S1P receptors to proliferation of astrocytes. *Mol Pharmacol* **64**:1199-1209.

Tager AM, LaCamera P, Shea BS, Karimi-Shah BA, Campanella GS, Polosukhin V, Hart WK, Poorvu EC, Chun J, Blackwell TS, Xu Y and Luster AD (2007) Mice deficient for the lysophosphatidic acid receptor LPA1 demonstrate reduced fibroblast recruitment and reduced pulmonary fibrosis post-bleomycin injury. *Am J Respir Crit Care Med* **175**:A814.

Terada M, Kelly EA and Jarjour NN (2004) Increased thrombin activity after allergen challenge: a potential link to airway remodeling? *Am J Respir Crit Care Med* **169**:373-377.

Toews ML, Ediger TL, Romberger DJ and Rennard SI (2002) Lysophosphatidic acid in airway function and disease. *Biochim Biophys Acta* **1582**:240-250.

Toews ML, Ustinova EE and Schultz HD (1997) Lysophosphatidic acid enhances contractility of isolated airway smooth muscle. *J Appl Physiol* **83**:1216-1222.

Toews M, Moore BB, Saulnier-Blache JS, Thronson HL, Schulte NA and Toews G (2004) Elevated concentrations of lysophosphatidic acid in airway lavage fluids from mouse models of pulmonary fibrosis. *Am J Respir Crit Care Med* **169**:A300.

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JPET Fast Forward. Published on July 19, 2007 as DOI: 10.1124/jpet.107.120584
This article has not been copyedited and formatted. The final version may differ from this version.

Wang L, Cummings R, Zhao Y, Kazlauskas A, Sham JK, Morris A, Georas S, Brindley DN and Natarajan V (2003) Involvement of phospholipase D2 in lysophosphatidate-induced transactivation of platelet-derived growth factor receptor-beta in human bronchial epithelial cells. *J Biol Chem* **278**:39931-39940.

Xu KP, Yin J and Yu FS (2007) Lysophosphatidic acid promoting corneal epithelial wound healing by transactivation of epidermal growth factor receptor. *Invest Ophthalmol Vis Sci* **48**:636-643.

Zhao Y, Tong J, Gorshkova I, He D, Stern R, Berdyshev E, Pendyala S, Sperling A, Chun J and Natarajan V (2007) Lysophosphatidic acid receptor 1 and 2 null mice show resistance to airway inflammation induced by *Schistosoma mansoni* eggs. *Am J Respir Crit Care Med* **175**:A926.

Zhao Y, He D, Saatian B, Watkins T, Spannhake EW, Pyne NJ and Natarajan V (2006) Regulation of lysophosphatidic acid-induced epidermal growth factor receptor transactivation and interleukin-8 secretion in human bronchial epithelial cells by protein kinase Cdelta, Lyn kinase, and matrix metalloproteinases. *J Biol Chem* **281**:19501-19511.

Downloaded from jpet.aspetjournals.org at ASPET Journals on April 23, 2024

JPET Fast Forward. Published on July 19, 2007 as DOI: 10.1124/jpet.107.120584
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Footnotes

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Kassel KM, Parker SM, Danforth BL, Lanik AD, Mihaljevic JA, Meeks AS, Schulte NA, and Toews ML (2005) Lysophosphatidic acid decreases epidermal growth factor receptor binding in airway epithelial cells. 2005 Experimental Biology meeting abstract. *FASEB J*. 19:A527.

Toews ML, Parker SM, and Kassel KM (2006) Signaling pathways for lysophosphatidic acid (LPA) inhibition of epidermal growth factor receptor (EGFR) binding in lung epithelial cells. 2006 Experimental Biology meeting abstract. *FASEB J*. 20:A695.

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Legends for Figures

Figure 1. LPA decreases EGFR binding in airway epithelial cells. BEAS-2B, hBEC, H292, and A549 cells were starved for 24 hr in basal medium before treatment with BSA or 10 μM LPA for the indicated times (A) or with BSA or the indicated concentrations of LPA for 15 min (B) or 18 hr (C) followed by washing with DMEM-HEPES and binding of ^{125}I-EGF to intact cells on ice. Data represent the fold increase or decrease relative to binding in control cells treated with the BSA vehicle and are from at least 3 separate experiments, except for hBECs which are from 2 separate experiments.

Figure 2. LPA decreases binding in a dose-dependent manner. BEAS-2B cells were treated with BSA or the indicated concentrations of LPA for 15 min (A) or 18 hr (B) before incubation with ^{125}I-EGF on ice for 4 hr. (C) BEAS-2B cells were treated with the indicated concentrations of ether LPA for 15 min or 18 hr before incubation with ^{125}I-EGF on ice for 4 hr. Data represent the fold decrease relative to binding in control cells treated with BSA and are from at least 4 separate experiments.

Figure 3. LPA decreases B_{max} and affinity. BEAS-2B (A) and H292 (B) cells were treated with BSA or 10 μM LPA for 15 min. Cells were then incubated with the indicated concentrations of ^{125}I-EGF on ice for 4 hr to generate saturation curves. Data represent total ^{125}I-EGF binding from 2 separate experiments, except for the two highest concentrations, which were only tested once. BEAS-2B (C) and H292 (D) cells were treated with BSA or 10 μM LPA for 15 min. Cells were then incubated on ice for 4 hr with 300,000 cpm/mL ^{125}I-EGF plus the

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indicated amounts of non-radioactive EGF to generate competition curves. Data represent total ^{125}I-EGF binding and are from 2 separate experiments.

Figure 4. Other GPCR mitogens also decrease EGFR binding at 15 min. BEAS-2B (A), H292 (B), and A549 (C) cells were treated with vehicle (CTL) or the indicated concentrations of S1P or thrombin for 15 min. Cells were then washed with DMEM-HEPES and incubated with ^{125}I-EGF on ice for 4 hr. Data represent specific binding expressed as a fold of the appropriate control and are from 3 separate experiments.

Figure 5. GPCR mitogens decrease binding in BEAS-2B cells but not H292 or A549 cells at 18 hr. BEAS-2B (A), H292 (B), and A549 (C) cells were treated with vehicle (CTL) or the indicated concentrations of S1P or thrombin for 18 hr. Cells were then washed with DMEM-HEPES and incubated with ^{125}I-EGF on ice for 4 hr. Data represent specific binding expressed as a fold of the appropriate control and are from at least 3 separate experiments.

Figure 6. LPA transactivates the EGFR in BEAS-2B cells. BEAS-2B cells were treated with 10 μM LPA for the indicated times. Cells were then lysed, and cell lysate was separated by SDS-PAGE and blotted for total EGFR (green) and phospho-tyrosine (pY99 – red). Western blots were scanned and quantified using the Odyssey Infrared Imaging System. Data are shown as phospho-EGFR/total EGFR, then as a fold of time zero. The top panel contains a representative blot and the bottom panel is data from 3-4 separate experiments.

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Figure 7. LPA stimulates transactivation via MMPs in BEAS-2B cells. BEAS-2B cells were pretreated for 30 min with 2.5 μM AG1478, 25 μM GM6001, or 10 μg/mL CRM197, then treated with vehicle or 10 μM LPA for 2 min. Cells were then lysed, and cell lysate was separated by SDS-PAGE and blotted for total EGFR and phospho-tyrosine. Western blots were scanned and quantified using the Odyssey Infrared Imaging System. Data are shown as phospho-EGFR/total EGFR, then as a fold of vehicle-treated values and are from at least 4 separate experiments, except for CRM197 (n=2).

Figure 8. AG1478 and GM6001 inhibit the LPA-stimulated decrease in EGFR binding in BEAS-2B cells. BEAS-2B cells were pretreated for 30 min with (A) 2.5 μM AG1478, (B) 25 μM GM6001, or (C) 10 μg/mL CRM197 before treatment with 10 μM LPA for 15 min or 18 hr. Cells were then incubated with ^{125}I-EGF on ice for 4 hr. Data represent the fold increase or decrease relative to vehicle-treated cells and are from at least 3 separate experiments. *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$

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Table 1. Other LPA-related agents decrease EGFR binding in airway epithelial cells. The BEAS-2B, H292, and A549 cells were treated with the indicated agents for 15 min or 18 hr. Cells were then washed with DMEM-HEPES and incubated with ^{125}I-EGF on ice for 4 hr. Full dose-response curves were generated for agents that significantly decreased EGFR binding, with the highest concentration being 10 μM. EC_{50} values ± SEM are indicated. Full dose-response curves were not generated for agents that did not significantly change EGFR binding at the highest concentration, and the EC_{50} is marked NA for those agents. Data represent the maximal percent increase or decrease compared to control values and are from at least 3 separate experiments. *p≤0.05, **p≤0.01, ***p≤0.001 compared to control

	15 m		18 hr	
BEAS-2B	change in binding (% of CTL)	EC_{50} (nM)	change in binding (% of CTL)	EC_{50} (nM)
18:1 LPA	-38 ± 4 ***	4 ± 0.8	-47 ± 10 *	1100 ± 240
oleic acid	5 ± 4	NA	10 ± 5	NA
phosphatidic acid	-34 ± 3 **	120 ± 24	-72 ± 4 ***	900 ± 100
lysophosphatidyl choline	-20 ± 5 *	2600 ± 1800	-10 ± 5	NA
18:0 LPA	-33 ± 1 **	27 ± 8	-56 ± 7 **	580 ± 130
16:0 LPA	-33 ± 5 *	32 ± 8	-55 ± 8 **	1900 ± 500
H292	change in binding (% of CTL)	EC_{50} (nM)	change in binding (% of CTL)	EC_{50} (nM)

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18:1 LPA	-42 ± 3 ***	170 ± 30	-8 ± 2 **	1700 ± 1200
oleic acid	4 ± 3	NA	0.1 ± 2	NA
phosphatidic acid	-6 ± 7	NA	-9 ± 3 *	NA
lysophosphatidyl choline	-19 ± 4 *	>10000	1.0 ± 3	NA
18:0 LPA	-31 ± 3 **	250 ± 50	-7 ± 8	NA
16:0 LPA	-31 ± 5 **	1600 ± 600	-6 ± 6	NA
A549	change in binding (% of CTL)	EC_{50} (nM)	change in binding (% of CTL)	EC_{50} (nM)
18:1 LPA	-60 ± 3 **	24 ± 4	31 ± 8 *	1500 ± 940
oleic acid	-4 ± 3	NA	1 ± 4	NA
phosphatidic acid	-27 ± 2 **	530 ± 120	8 ± 3	NA
lysophosphatidyl choline	-20 ± 3 **	810 ± 370	10 ± 2 *	NA
18:0 LPA	-34 ± 2 ***	530 ± 140	26 ± 15	>10000
16:0 LPA	-27 ± 3 *	1200 ± 400	15 ± 5 *	860 ± 370

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Figure 1

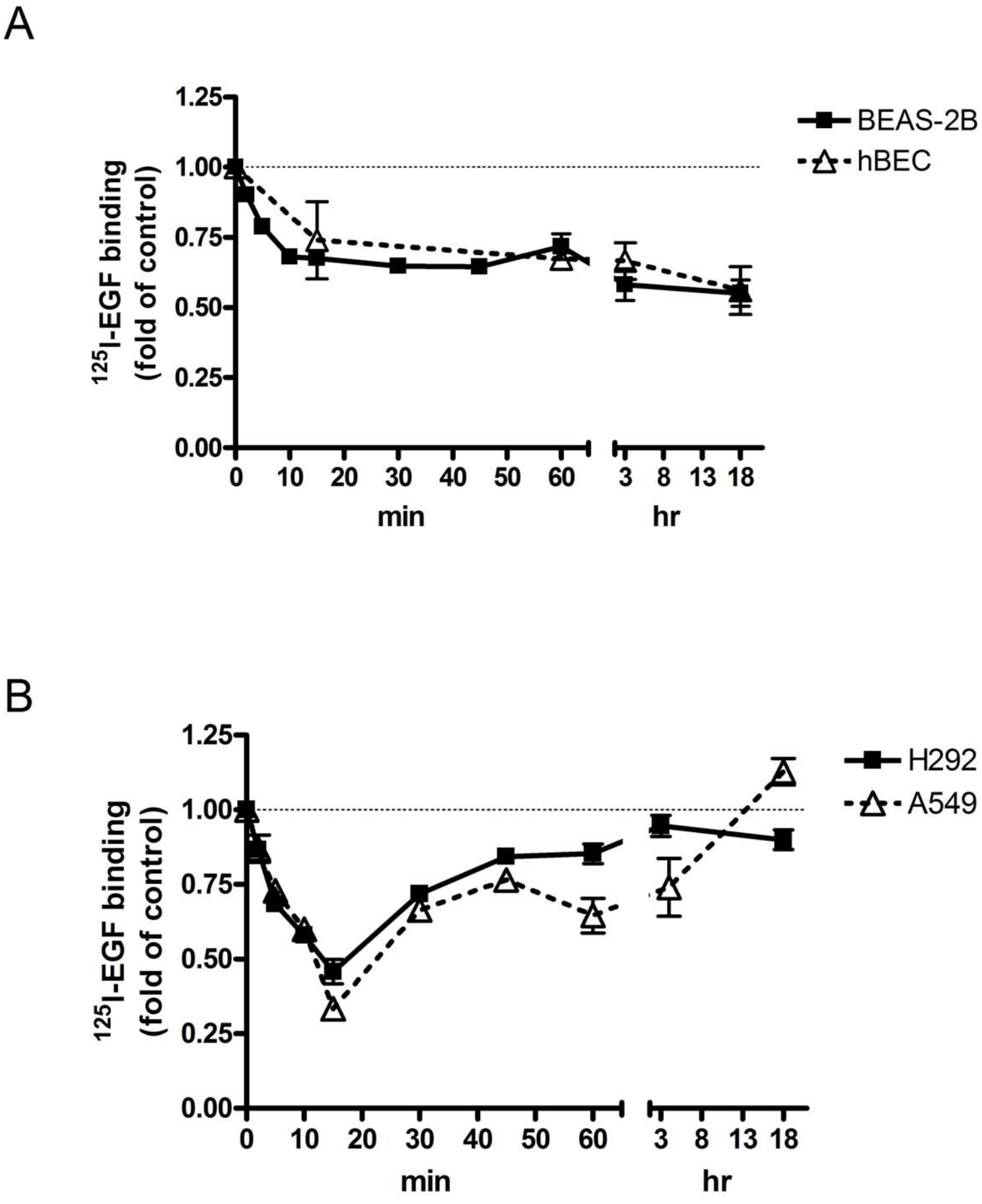

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Figure 2

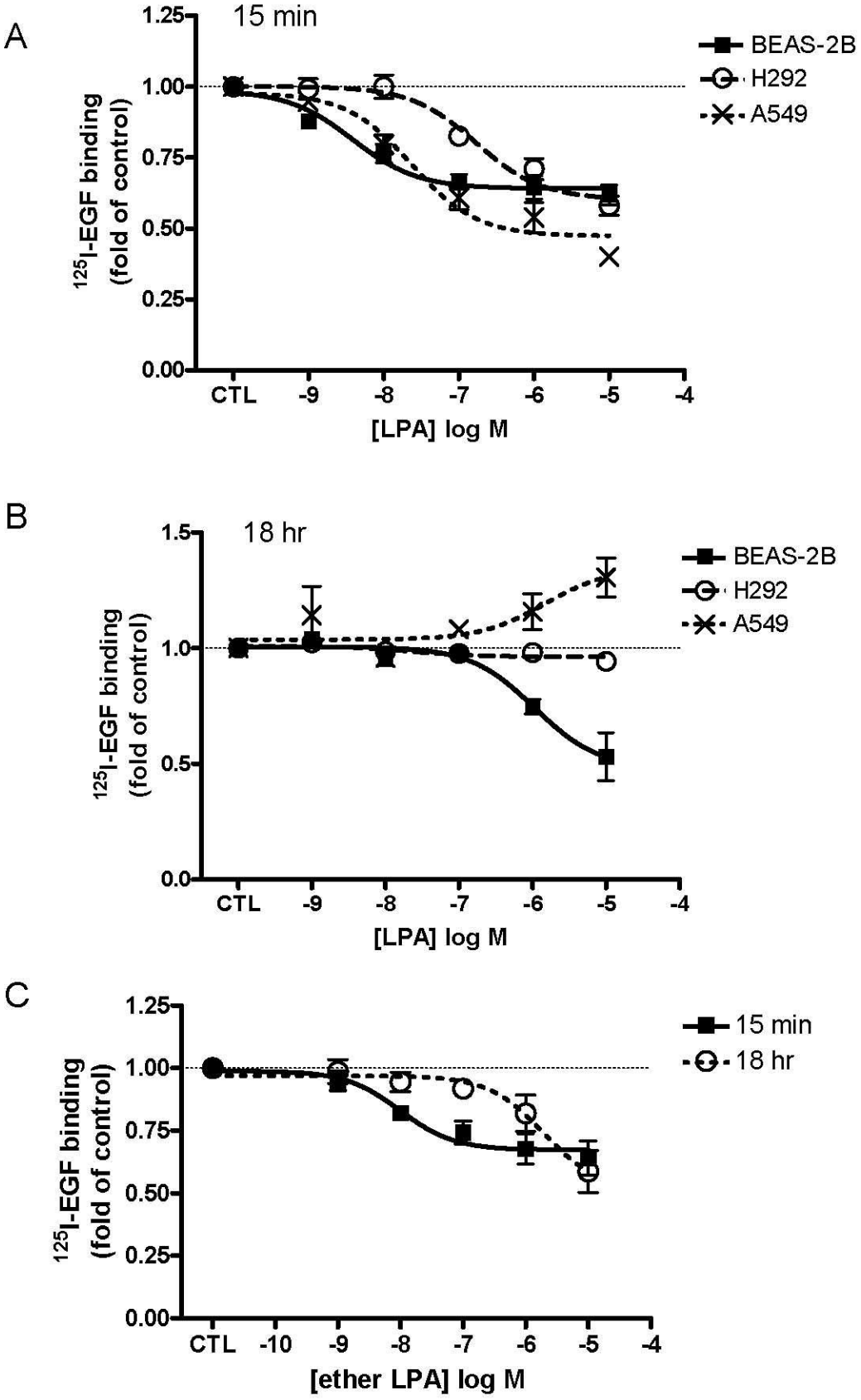

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Figure 3

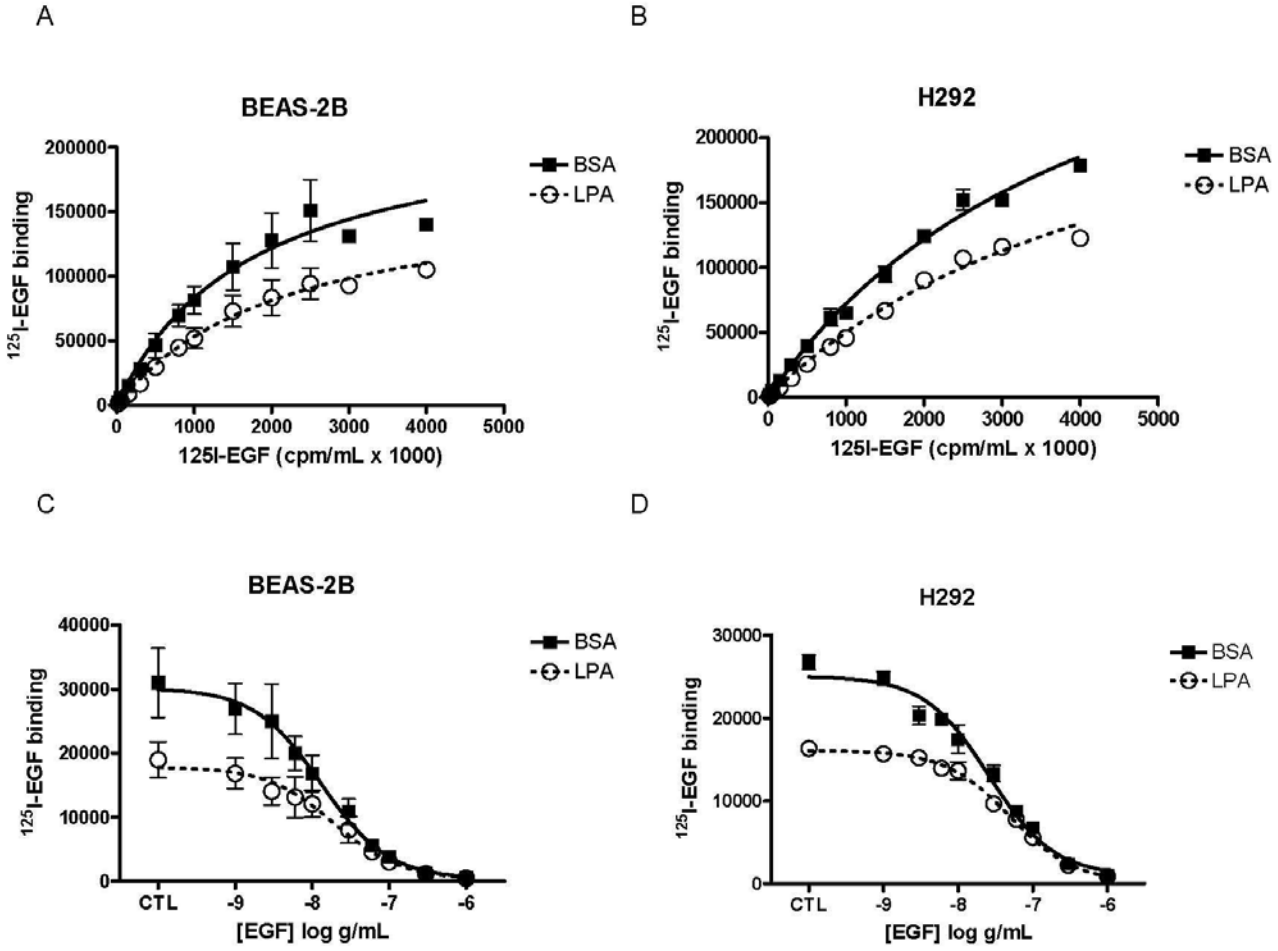

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Figure 4

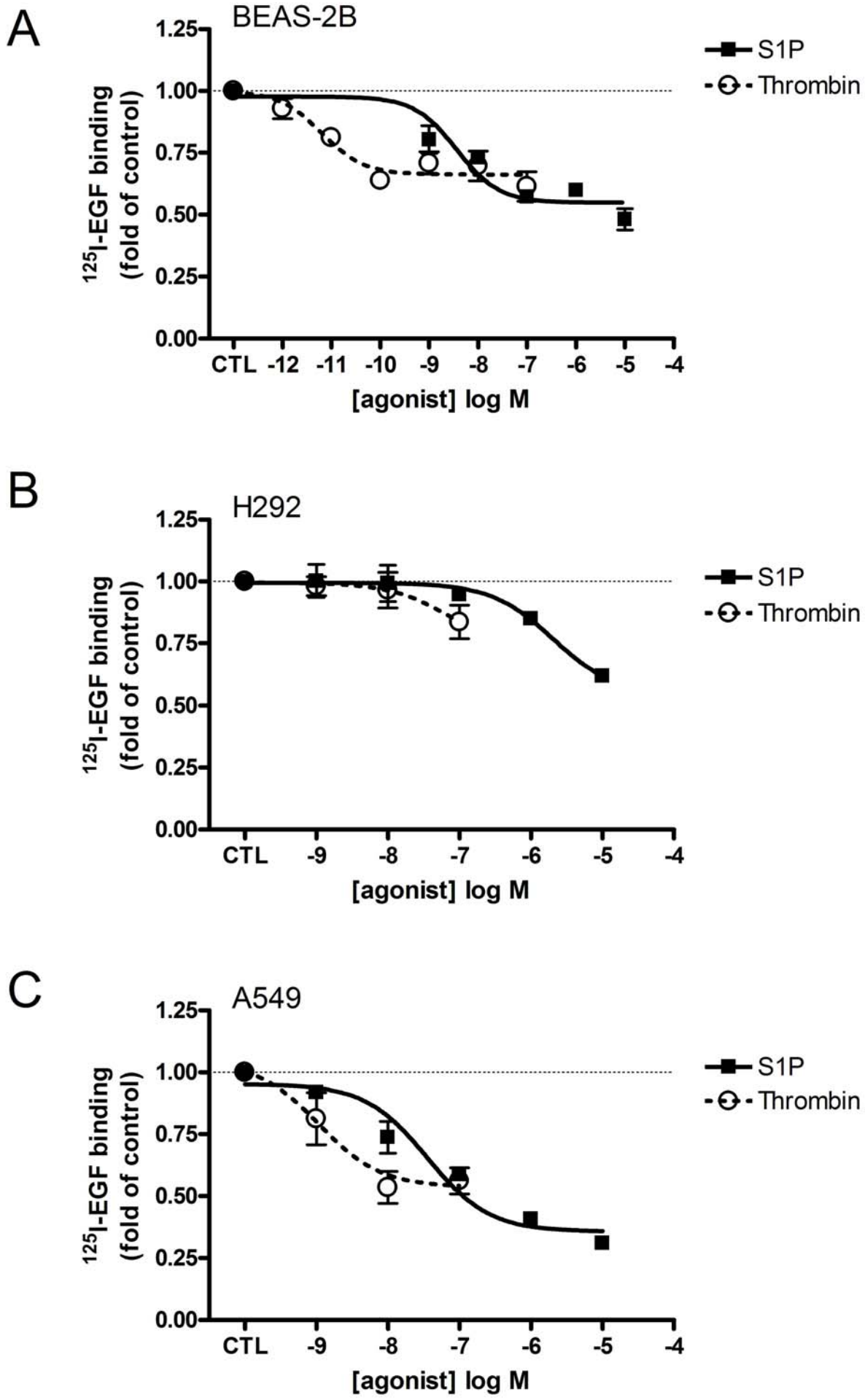

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Figure 5

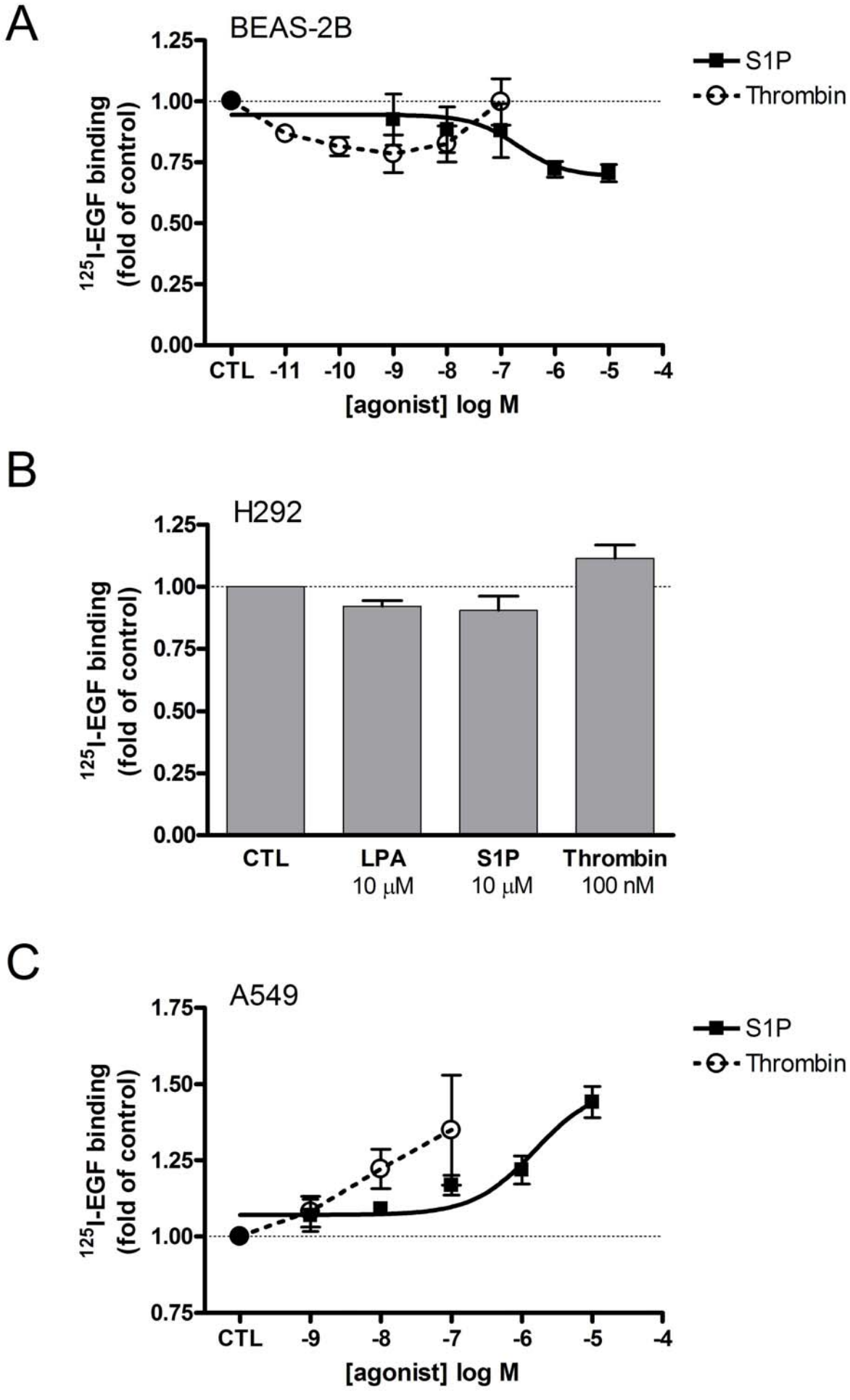

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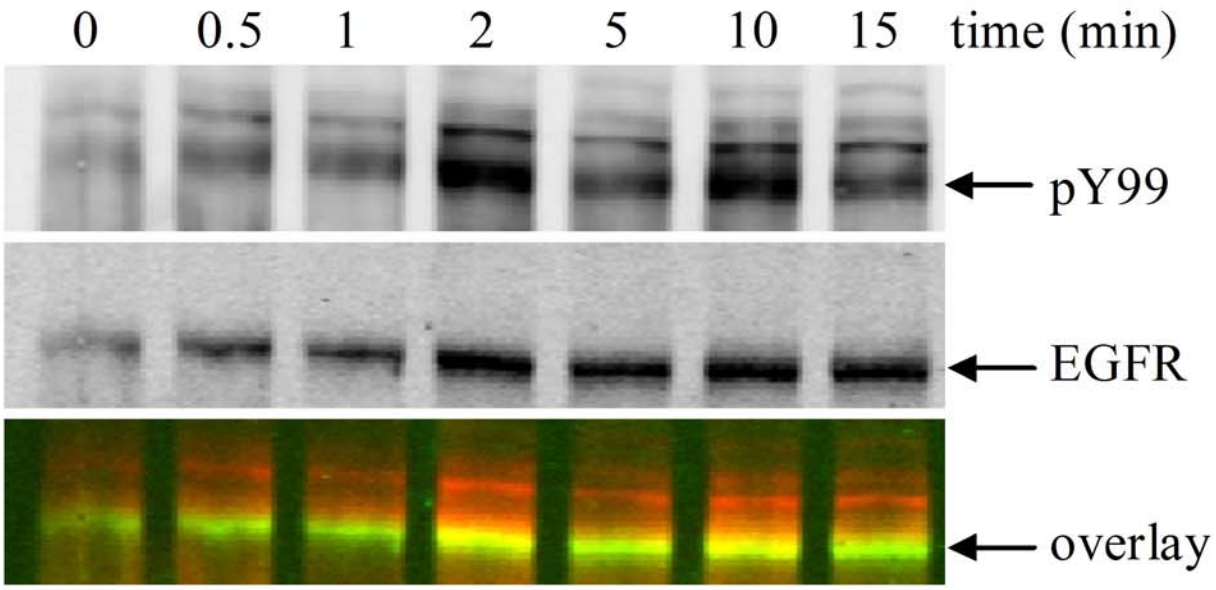
Figure 6



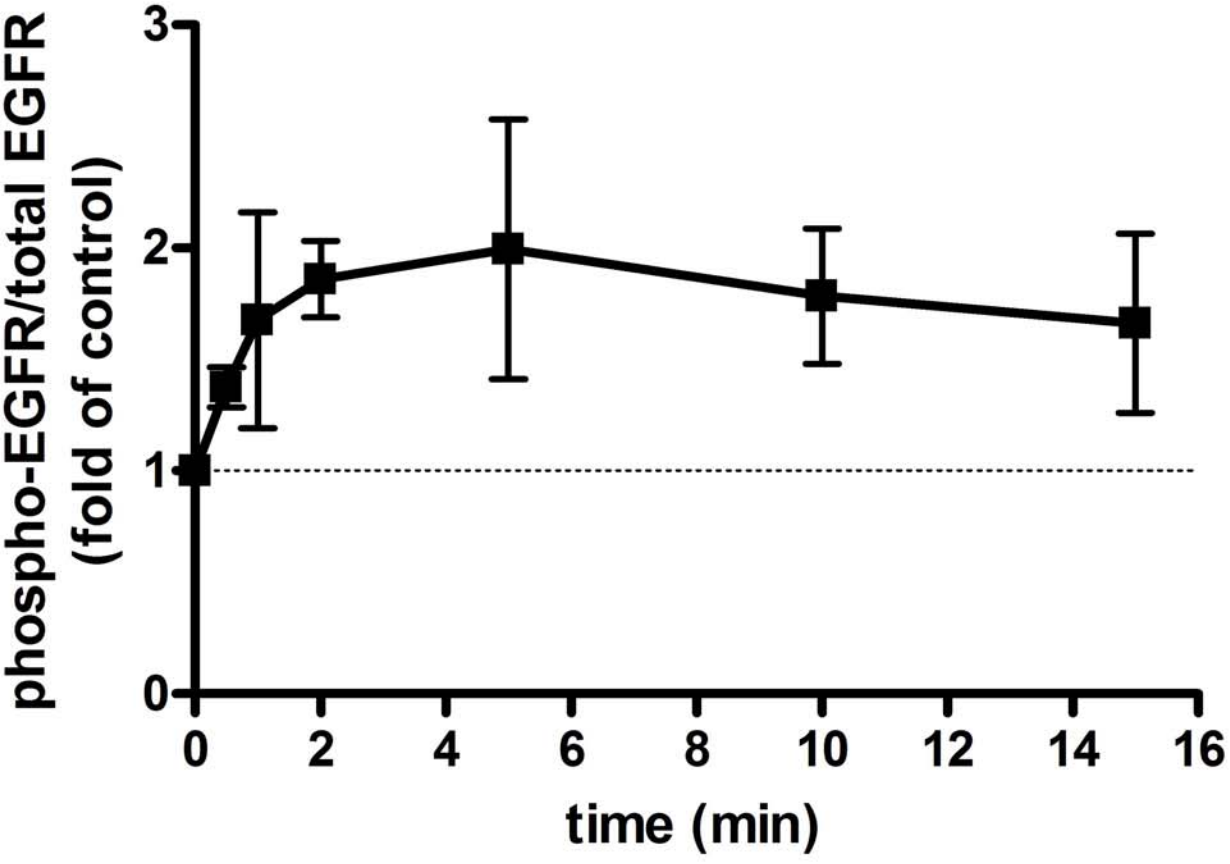


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Figure 7

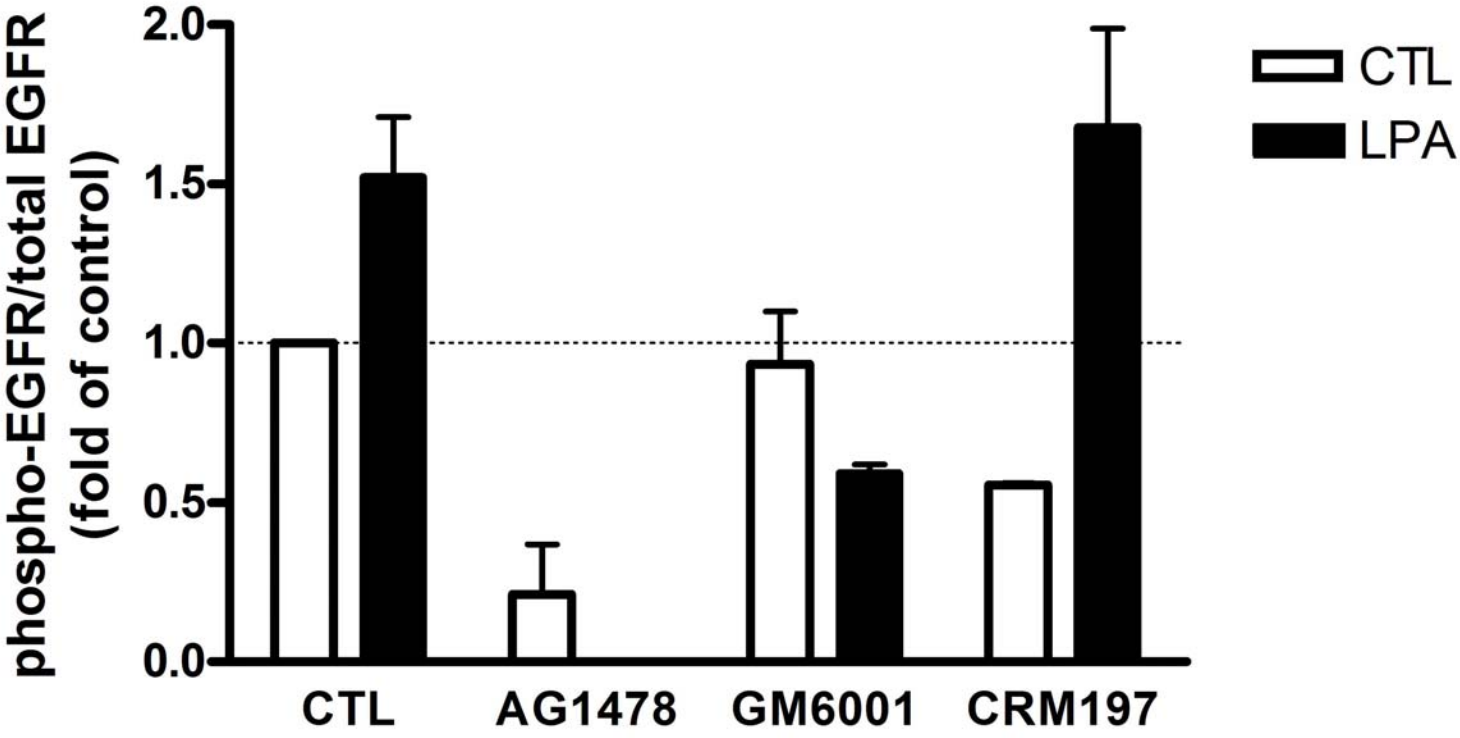

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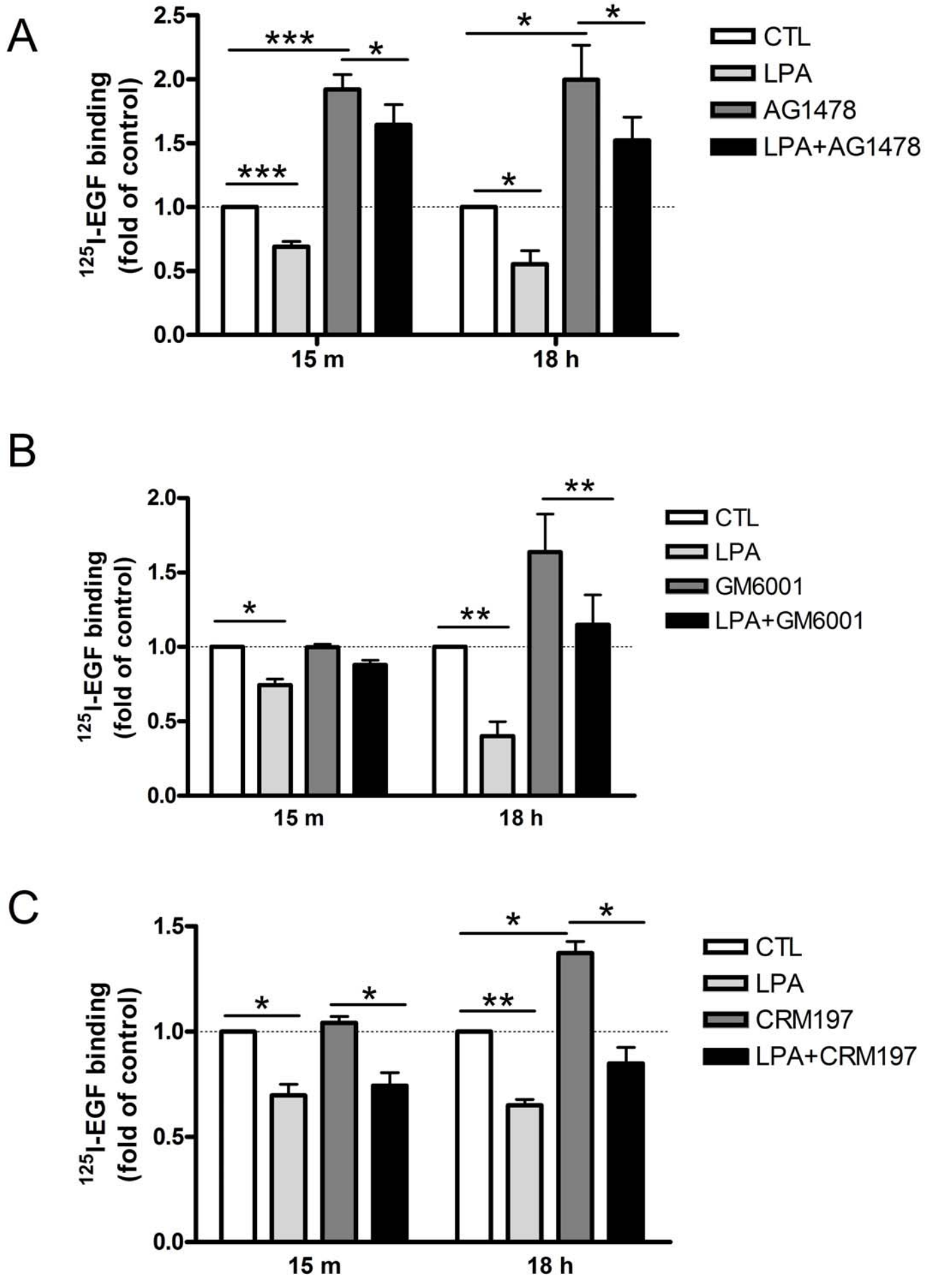
Figure 8



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