Modulation of epidermal growth factor receptor binding to human airway smooth muscle cells by glucocorticoids and β2-adrenergic receptor agonists

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Kassel KM, Schulte NA, Toews ML. Modulation of epidermal growth factor receptor binding to human airway smooth muscle cells by glucocorticoids and β2-adrenergic receptor agonists. Am J Physiol Lung Cell Mol Physiol 296: L693–L699, 2009. First published February 6, 2009; doi:10.1152/ajplung.90446.2008.—EGF receptors (EGFRs) are increased in airway smooth muscle in asthma, which may contribute to both their hyperproliferation and hypercontractility. Lysophosphatidic acid (LPA) is a candidate pathological agent in asthma and other airway diseases, and LPA upregulates EGFRs in human airway smooth muscle (HASM) cells. We tested whether therapeutic glucocorticoids and/or β2AR agonists also alter EGFR binding in HASM cells. Exposure to glucocorticoids for 24 h induced a twofold increase in EGFR binding similar to that with LPA; fluticasone was markedly more potent than dexamethasone. The increase in EGFR binding by glucocorticoids required 24-h exposure, consistent with transcription-mediated effects. Although the increase in EGFR binding was blocked by the protein synthesis inhibitor cycloheximide for LPA, fluticasone, and dexamethasone, only LPA induced a significant increase in EGFR protein expression detected by immunoblotting. In contrast to the increased binding induced by the glucocorticoids, the β2AR agonists isoproterenol, albuterol, and salmeterol all induced a decrease in EGFR binding. β2AR agonist effects were multiphasic, with an initial decline at 2–4 h that reversed by 6 h and a second, somewhat greater decrease by 18–24 h. In cells pretreated with glucocorticoids, the decreases in EGFR binding by subsequent β2AR treatment were not statistically significant; glucocorticoid upregulation of EGFRs also prevented further increases by LPA. Similar increases by glucocorticoids and decreases by β2AR agonists were found in HFL-1 human lung fibroblasts. These complex and opposing effects of clinically relevant glucocorticoids and β2AR agonists on airway mesenchymal cell EGFRs likely contribute to their overall therapeutic profile in the diseased airway.

lyosphosphatidic acid; lung fibroblasts; combination therapy

INCREASED AIRWAY SMOOTH MUSCLE mass is a characteristic feature of asthma and chronic obstructive pulmonary disease (COPD) that contributes to both airway narrowing and hyperresponsiveness (8, 18, 26, 34). This remodeling is due at least in part to an imbalance of growth factors (19, 28), and increased expression of both EGF and the EGF receptors (EGFRs) occur in diseases characterized by airway remodeling (1, 20). EGF is an established mitogen for human airway smooth muscle (HASM) cells (7, 11, 24), and both the increased levels of EGF and the increased expression of airway smooth muscle EGFRs in asthma (1) are potential contributors to the pathological increase in airway smooth muscle mass. In fact, a recent study showed that inhibition of EGFRs completely blocked allergen-induced airway smooth muscle growth in a rat model of allergen-induced remodeling (37). These findings underscore the importance of studying how drugs used to treat asthma may regulate EGFR binding and expression to provide new clues to this aspect of airway disease pathology and/or new targets for therapeutic intervention.

The simple lipid mediator lysophosphatidic acid (LPA) has multiple effects on airway cells in culture that suggest its potential role in the pathology of asthma, COPD, and other airway fibroproliferative diseases (38). These effects include stimulation of airway mesenchymal cell proliferation (7, 11) and contraction (40) as well as effects on secretion of extracellular matrix components (33) and inflammatory mediators (43). Recent studies showing elevated levels of LPA in airway fluids in relation to lung disease in either human subjects (14, 36) or animal models (36, 39) further strengthen the evidence for LPA as a likely contributor to these diseases.

Our previous studies showed that LPA and EGF each stimulate proliferation of HASM cells on their own but that they also exhibit a markedly synergistic stimulation when added together (7, 10, 11). LPA mediates its effects by activating a family of G protein-coupled receptors (GPCRs), with the LPA1–5 subtypes now well-established (2, 4, 17, 23, 27, 30) and newer reports of likely additional subtypes (29, 31, 35). Many GPCR mitogens have been shown to mediate their effects by phosphorylation and transactivation of the tyrosine kinase activity of EGFRs (12, 13, 16). However, transactivation is not the mechanism for the synergism between LPA and EGF in HASM cells (9); in fact, multiple studies have shown that GPCR mitogens do not induce EGFR phosphorylation and transactivation in HASM cells (6, 9, 25). However, LPA does lead to an ∼2-fold increase in EGFR binding activity that is due to an increase in EGFR protein expression (9). Thrombin, which also exhibits synergism with EGF for stimulating mitogenesis (11), induces a similar increase in EGFR binding (9), consistent with EGFR upregulation as at least one component of the synergism of LPA and other GPCR mitogens with EGF for enhancing HASM cell proliferation.

LPA and β2-adrenergic receptor (β2AR) agonists have opposite effects on contractility of airway smooth muscle, with LPA enhancing contraction (40) and β2AR agonists being used clinically as bronchodilators to decrease contraction (3, 5). Intriguingly, similar opposing effects are also observed for airway smooth muscle cell proliferation, with LPA stimulating (7, 11) and β2AR agonists inhibiting proliferation (22, 41). Accordingly, we hypothesized that β2AR agonist effects on EGFR binding would also be opposite from those of LPA, with...
β2AR agonists causing a decrease rather than an increase. Because anti-inflammatory glucocorticoids are also used therapeutically for treating both asthma and COPD and are often used in combination with β2AR agonists (21), we hypothesized that these drugs would also alter EGFR binding. The studies presented here show that glucocorticoids induce an increase in EGFR binding similar to that with LPA in both HASM cells and lung fibroblasts, whereas β2AR agonists decrease binding to EGFRs, consistent with their other effects that are opposite to those of LPA.

MATERIALS AND METHODS

Drugs and other materials. Isoproterenol bitartrate (Iso) and dexamethasone (Dex) were purchased from Sigma (St. Louis, MO). Salmeterol xinafoate (Sal) and fluticasone propionate (Flu) were kindly provided by GlaxoSmithKline. Dex and Flu were dissolved in ethanol as 10 mM stock solutions and stored at −20°C. Sal was dissolved in 15 μl of glacial acetic acid and then diluted to 10 mM with PBS and stored at −20°C. Iso was prepared freshly for each experiment by dissolving in 100 mM ascorbic acid for stock solutions; the final concentration of ascorbic acid in all experiments with Iso was 1 mM or lower. 125I was obtained from Amersham Biosciences (Pittsburgh, PA) and used to radiodinate pure EGF obtained from BioSource International using the chloramine T method, and the 125I-EGF was then purified on a Sephadex G-25 column as previously described (32).

Cells and culture procedures. HASM cells prepared as previously described were obtained from either Dr. Michael Kotlikoff (then at University of Pennsylvania, currently at Cornell University) or Dr. Reynold A. Panettieri, Jr. (University of Pennsylvania). Cells were grown on six-well plates in DMEM containing 10% fetal bovine serum at 37°C in a humidified incubator at 5% CO2. Cells were plated and lung fibroblasts, whereas β2AR agonists decrease binding to EGFRs, consistent with their other effects that are opposite to those of LPA.

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Prepretreatments. Confluent cells were serum-starved for 18–24 h before being treated with the various drugs. Standard treatments were for 18–24 h for Dex and Flu and for either 3 or 18 h with Iso or Sal in most experiments. Drugs were added to the cells as 1:100 or 1:1,000 dilutions into the starvation medium, and control cells received appropriate vehicle treatment for the same time.

EGFR binding assays. Binding of 125I-EGF to intact cells on ice was assayed essentially as previously described (9). Cells were washed once with 2 ml of HEPES-buffered DMEM at 37°C to remove pretreatment drugs and then once with 2 ml at 4°C to chill the cells before initiating the binding assay. DMEM buffered to pH 7.4 with HEPES and containing 125I-EGF [1 ml per well, 200,000 counts per minute (cpm)/ml] was then added to each well, and binding was allowed to proceed at 4°C for 4 h. Nonspecific binding was determined in parallel wells containing 200 ng/ml nonradioactive EGF to block specific binding sites. At the end of the binding reaction, the cells were washed four times with 2 ml of HEPES-buffered DMEM to remove nonbound radioligand. The cells with bound radioligand were then dissolved in 1 ml of 0.2 N NaOH, and the samples were transferred to tubes. Radioactivity in each sample was determined in a gamma counter. In most experiments, total binding was measured in duplicate and nonspecific binding in only one well per condition. Experiments were repeated on at least 3 separate days. Data presentation and analyses were with GraphPad Prism.

Immunoblotting assays. Following the various pretreatments as described above, cells were washed once in PBS and then lysed in 200 μl of 2X sample loading buffer, and the samples were sonicated and boiled. Extracts were run on 7.5% polyacrylamide gels, and the separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes. The membranes were then blotted with primary rabbit anti-EGFR antibody sc-03 (Santa Cruz Biotechnology, Santa Cruz, CA) followed by secondary anti-rabbit antibody conjugated to horseradish peroxidase or with primary mouse anti-β-actin antibody AC-15 (Sigma) and secondary anti-mouse antibody conjugated to horseradish peroxidase. The labeled bands were visualized by ECL (Amersham Biosciences), and the density of the bands was quantified using NIH ImageJ software. Early studies showed that there were no changes in total cell protein due to LPA or glucocorticoid treatment, so immunoblotting for a housekeeping protein was not done routinely. Blotting for β-actin in two of the experiments confirmed equal protein loading and the lack of changes with LPA or glucocorticoid treatment.

Data analyses. For all experiments, data from treated cells are expressed as the fold change from vehicle-treated control cells. Statistical comparisons were by ANOVA with Dunnett’s post-test for significant differences from control.

RESULTS

Glucocorticoid-induced increase in HASM cell EGFR binding. Dex, the prototypical glucocorticoid, and Flu, a glucocorticoid used specifically in the treatment of asthma, were tested for their effects on EGFR binding in HASM cells. Pretreatment for 24 h with the glucocorticoids Dex or Flu led to a concentration-dependent increase of ~2-fold in EGFR binding, similar to that seen with LPA in previous studies. The maximal increase was 1.78 ± 0.01-fold for Dex and 1.92 ± 0.01-fold for Flu (Fig. 1). Flu was markedly more potent than Dex; half-maximal effects occurred with 22 ± 1 μM Flu but required 1,000 ± 40 μM Dex. The increases in EGFR binding with Dex and Flu required relatively long exposure, with no increases detected after 15 min or 4 h of treatment but large increases at the 24-h time point used routinely and further increases at 48 h (Fig. 2).

Molecular basis for the glucocorticoid-induced increase in HASM cell EGFR binding. The effects of the protein synthesis inhibitor cycloheximide (Chx) were tested to establish whether the increase in EGFR binding required ongoing protein synthesis (Fig. 3). Cells treated with LPA were included in these experiments as a positive control because our previous studies had documented that the LPA-induced increase in EGFR

![Fig. 1. Increase in EGF receptor (EGFR) binding by dexamethasone (Dex) and fluticasone (Flu) in human airway smooth muscle (HASM) cells. HASM cells were exposed to the indicated concentrations of Dex and Flu for 18 h, washed, and then assayed for 125I-EGF binding to intact cells on ice. Data are expressed as the fold of control and are the means ± SE from 6 experiments with duplicate assays in each experiment.](http://ajplung.physiology.org/)

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binding in these cells was inhibited by Chx and accompanied by an increase in EGFR protein (9). Chx prevented the increases in EGFR binding induced by both Dex and Flu, consistent with involvement of protein synthesis. In agreement with our previous study (9), Chx not only prevented the LPA-induced increase in EGFR binding, but also converted the LPA response to a decrease in binding instead. This decrease in EGFR binding in Chx-treated cells was not observed for the glucocorticoids.

EGFR protein expression was quantified by immunoblotting to determine the extent to which the glucocorticoid-induced increases in EGFR binding could be accounted for by increases in EGFR protein abundance (Fig. 4). Both Dex and Flu led to increases in EGFR band density after 24-h treatment, although these increases were smaller than for LPA, were not observed as consistently, and did not achieve statistical significance. This is in contrast to the EGFR binding data, where the increases induced by Dex and Flu were consistently as large or larger than that induced by LPA. The Chx data indicate that protein synthesis contributes to the increase in EGFR binding, but the immunoblotting data indicate that the increase in binding cannot be explained solely by a corresponding increase in EGFR protein.

\( \beta_2 \text{AR} \) agonist-induced decrease in EGFR binding. The effects of \( \beta_2 \text{AR} \) agonists on HASM cell EGFR binding were also assessed, including the prototypical \( \beta_1 \text{AR} \) and \( \beta_2 \text{AR} \) agonist Iso and two \( \beta_2 \text{AR} \)-selective agonists used to treat asthma, the rapid-acting agonist albuterol (Alb) and the long-acting agonist Sal. Treatment for 18 h with Iso, Alb, or Sal all induced concentration-dependent decreases in EGFR binding of 20–30% (Fig. 5). Iso decreased EGFR binding by 28 ± 2% with an EC\(_{50}\) of 4 ± 1 nM, Alb decreased binding by 24 ± 3% with...
an EC$_{50}$ of 90 ± 27 nM, and Sal decreased binding by 20 ± 3% with an EC$_{50}$ of 9 ± 5 nM.

The time courses for the decreases in EGFR binding induced by each β$_2$AR agonist were assessed (Fig. 6). The decrease in EGFR binding induced by Iso was complex, with a rapid but transient increase in binding consistently observed after 1 h of treatment, moderate but consistent decreases observed at 2 and 4 h of treatment, a return to the control level of binding at 6 h, and then more marked decreases observed at 18 and 24 h. The β$_2$AR-selective agonists Alb and Sal did not exhibit the transient increase seen at 1 h with Iso, but both did induce moderate decreases after 2 and 4 h of treatment, a return to the control level at 6 h, and more marked decreases at 18 and 24 h, similar to Iso. The decreases induced by these agents at 18 h in these time course experiments were ~30%, consistent with the data in Fig. 5.

Effects on EGFR binding of combinations of agents. Because glucocorticoids and β$_2$AR agonists are frequently used together in the therapy of asthma and COPD (21), including the specific combination of Sal with Flu in Advair, we tested the effects of treatment with these agents in combination on HASM cell EGFR binding (Fig. 7). The effects of both the glucocorticoids and the β$_2$AR agonists alone in these experiments were similar to those described above. In cells pretreated with Dex or Flu and then treated with β$_2$AR agonists, the values for EGFR binding were reduced, but the differences no longer achieved statistical significance. β$_2$AR agonists are thus less effective in reducing the elevated levels of EGFR binding induced by the glucocorticoids.

In cells pretreated with glucocorticoids, treatment with LPA did not induce any further increase in EGFR binding above the elevated level induced by the glucocorticoids. The values for fold increase were 3.06 ± 0.53 for Dex alone, 2.97 ± 0.31 for Dex plus LPA, 3.02 ± 0.63 for Flu alone, and 2.82 ± 0.58 for Flu plus LPA.

Effects of LPA, glucocorticoids, and β$_2$AR agonists on EGFR binding in HFL-1 lung fibroblasts. To determine whether the effects of these drugs on EGFRs in HASM cells were unique to smooth muscle cells or a more general feature of lung cells of mesenchymal origin, the effects of β$_2$AR agonists and glucocorticoids on EGFR binding were also assessed in the HFL-1 human fetal lung fibroblast cell line (Fig. 8). Glucocorticoid pretreatment of HFL-1 cells increased EGFR binding to HFL-1 cells similarly to their effects in HASM cells, with a 1.81 ± 0.22-fold increase by Dex and a 2.17 ± 0.31-fold increase by Flu. β$_2$AR agonists decreased EGFR binding in HFL-1 cells, with a 21 ± 1% decrease by Iso and a 20 ± 6% decrease by Sal.

DISCUSSION

Glucocorticoids and β$_2$AR-selective agonists are the two major drug classes used in the therapeutic management of both the acute and chronic components of asthma (21). The studies presented here document the ability of both of these drug classes to modulate binding to cell surface EGFRs of HASM cells. The well-established role of EGFRs in wound healing and cell proliferation (42) make the EGFR a particularly strong candidate for involvement in the excess repair and structural remodeling of the airway wall that are characteristic of asthma (1, 11, 24). Understanding the effects of asthma drugs on EGFRs is potentially important in terms of their clinically relevant therapeutic actions.
The studies presented here show that the anti-inflammatory glucocorticoids Dex and Flu induce an increase in EGFR binding, whereas the \( \beta_2 \)AR agonists used for bronchodilation induce a multiphasic decrease in EGFR binding. HPL-1 lung fibroblasts exhibited essentially identical increases in binding with Dex and Flu and decreases in binding with Iso and Sal, suggesting that these adaptive responses are a common feature of lung cells from the mesenchymal lineage.

Both Dex and Flu induced concentration-dependent increases in EGFR binding of \( \sim 2 \)-fold. Flu was \( \sim 100 \)-fold more potent for inducing this increase than Dex, as expected based on the known potencies of these 2 agents at the glucocorticoid receptor. The increase in EGFR binding was not observed after exposure for 4 h, became apparent \( \sim 12 \) h, and continued to rise at 24 and 48 h. This delayed response is consistent with the time course expected for effects mediated by the nuclear transcription factor receptor for glucocorticoids and with increased EGFR protein synthesis as a likely mechanism. The increase in EGFR binding was blocked by the protein synthesis inhibitor Chx, indicating that ongoing protein synthesis is in fact required. However, Western blotting for EGFR protein did not reveal a statistically significant increase in EGFR protein for cells pretreated with glucocorticoids. In contrast, the previously reported increase in EGFR protein induced by LPA (9), which was used as a positive control in these experiments, was consistently observed and statistically significant. Thus mechanisms other than simply increased EGFR protein expression appear to be involved in the glucocorticoid-induced increase in EGFR binding. The clear requirement for ongoing protein synthesis based on the Chx data could be explained by a requirement for some protein other than, or in addition to, the EGFR to achieve the full increase in EGFR ligand binding.

Treatment with Chx prevented the increase in binding induced by the glucocorticoids, whereas for LPA it not only prevented the increase, but also revealed an LPA-induced decrease in binding instead. These data are essentially identical to those in our initial studies of LPA-induced upregulation of EGFRs (9), and they show that the regulation of EGFRs by LPA is more complex than that for glucocorticoids. A likely explanation for these multiple effects of LPA is that different LPA receptor subtypes mediate different effects on EGFR binding, with one subtype mediating a protein synthesis-dependent increase in EGFR protein and binding and another subtype mediating an opposing decrease in binding. Treatment with Chx would eliminate the upregulation of EGFR protein mediated by one subtype and allow the decrease in binding induced by the other subtype to predominate. Further studies will be required to fully explain the more complex effects of LPA on EGFRs in these cells.

In contrast to the increase in EGFR binding induced by the anti-inflammatory glucocorticoids, all three of the \( \beta_2 \)AR agonist drugs that were tested led to a decrease in EGFR binding. The effect of the \( \beta_2 \)AR-selective agonist Alb was as great as that for the prototypical agonist Iso, which activates both \( \beta_1 \)ARs and \( \beta_2 \)ARs; thus the decrease in EGFR binding appears to be completely due to the \( \beta_2 \)AR, the prominent subtype expressed in these cells. Sal was significantly more potent than Alb for inducing this decrease, as expected based on their known potencies for other \( \beta_2 \)AR effects. In addition, the decrease induced by Sal was somewhat smaller in magnitude than those induced by Alb and Iso, likely due to the fact that Sal is only a partial agonist for increasing cAMP accumulation via \( \beta_2 \)ARs. However, it should be noted that involvement of cAMP in these changes in EGFR binding has not yet been established.

The time course for the \( \beta_2 \)AR agonist effects suggest that multiple changes in EGFR binding are induced by these agents. A transient increase in binding was consistently observed for Iso but not with either Alb or Sal, suggesting that this effect might be mediated by \( \beta_1 \)ARs. All three agents then induced a small decrease in binding at the 2- and 4-h time points, but this effect was no longer apparent at 6 h, suggesting that it is reversible, even in the continued presence of the inducing agent. Following 18- or 24-h incubation, a greater decrease in EGFR binding was observed for all three agents. Understanding the mechanisms for these multiphasic changes in EGFR binding in response to \( \beta_2 \)AR agonists will require further detailed studies; changes in EGFR protein expression, subcellular localization, and ligand binding properties could all be involved in various components of these changes.

The effects of \( \beta_2 \)AR agonists on EGFR regulation in HASM cells are opposite to those of LPA, consistent with their opposite effects on airway smooth muscle contraction reported previously (40). Our recent studies show that \( \beta_2 \)AR agonists also decrease HASM cell proliferation stimulated by either EGF or by LPA (22). Whether the ability of \( \beta_2 \)AR agonists to inhibit EGFR binding is related to their inhibition of proliferation remains to be established. Importantly, the inhibition of proliferation appears to be mediated by the exchange protein activated by cAMP (EPAC) rather than by the classic PKA pathway (22). The roles of cAMP and its PKA and EPAC target proteins in the multiphasic changes in EGFR binding induced by \( \beta_2 \)AR agonists and their relevance to \( \beta_2 \)AR inhibition of proliferation are important topics for future studies.

The effects of combinations of disease-relevant agents on EGFR binding were also assessed. Although LPA is often considered an inflammatory mediator (15), and glucocorticoids are clearly anti-inflammatory, both of these agents led to increases in both EGFR binding and EGFR protein. LPA did not further increase the upregulation of EGFR binding induced by pretreatment with glucocorticoids. These findings are consistent with the evidence that LPA and glucocorticoids act by similar protein synthesis-dependent upregulation mechanisms, which are apparently nonadditive. In contrast, the effects on EGFR binding of glucocorticoids and \( \beta_2 \)AR agonists, which are widely used in combination for multiple airway diseases, were in opposite directions. When cells pretreated with glucocorticoids were then exposed to \( \beta_2 \)AR agonists, the \( \beta_2 \)AR agonist effects were small and not statistically significant. The relatively large increase in EGFR expression induced by glucocorticoids thus appears to predominate over the smaller reductions in EGFR binding induced by the \( \beta_2 \)AR agonists.

In summary, the key findings of this study are that the two drug classes commonly used in the treatment of asthma, anti-inflammatory glucocorticoids and \( \beta_2 \)AR agonist bronchodilators, both alter EGFR binding to HASM cells but in opposite directions. The anti-inflammatory glucocorticoids increase EGFR binding, whereas \( \beta_2 \)AR agonist bronchodilators, both the rapid-acting Alb and the long-acting Sal, decrease EGFR binding. Similar changes occur in lung fibroblasts, which also contribute to the pathophysiology of asthma. These data provide new insight into adaptive changes induced by
these therapeutic drugs that are likely to be relevant to disease progression, and they also provide new approaches to modulate EGFR expression for potential experimental or therapeutic benefit.

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