Regulation of airway smooth muscle α-actin expression by glucocorticoids

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Regulation of airway smooth muscle α-actin expression by glucocorticoids. Am J Physiol Lung Cell Mol Physiol 292: L99–L106, 2007. First published September 15, 2006; doi:10.1152/ajplung.00269.2006.—Airway smooth muscle hypertrophy appears to be present in severe asthma. However, the effect of corticosteroids on airway smooth muscle cell size or contractile protein expression has not been studied. We examined the effects of dexamethasone, fluticasone, and salmeterol on contractile protein expression in transforming growth factor (TGF)-β-treated primary bronchial smooth muscle cells. Dexamethasone and fluticasone, but not salmeterol, each reduced expression of α-smooth muscle actin and the short isoform of myosin light chain kinase. Steady-state α-actin mRNA level and stability were unchanged, consistent with posttranscriptional control. Fluticasone significantly decreased α-actin protein synthesis following treatment with the transcriptional inhibitor actinomycin D, indicative of an inhibitory effect on mRNA translation. Fluticasone also significantly increased α-actin protein turnover. Finally, fluticasone reduced TGF-β-induced incorporation of α-actin into filamentous actin, cell length, and cell shortening in response to ACh and KCl. We conclude that glucocorticoids reduce human airway smooth muscle α-smooth muscle actin expression and incorporation into contractile filaments, as well as contractile function, in part by attenuation of mRNA translation and enhancement of protein degradation.

corticosteroids; fluticasone; hypertrophy; salmeterol; transforming growth factor-β

Recent studies have demonstrated that glucocorticoids may affect diverse functions of airway smooth muscle (11, 26). Perhaps the most well known of these effects is the enhancement of β2-adrenoreceptor number and function, especially following chronic administration of β2-adrenoceptor agonists. Other actions include the suppression of agonist-induced increases in intracellular calcium levels, downregulation or uncoupling receptors linked to airway smooth muscle contraction, inhibition of cyclooxygenase (COX)-2 expression and consequent release of arachidonic acid metabolites, reduction of cytokine production, and attenuation of airway smooth muscle cell proliferation and migration.

The effects of glucocorticoids on airway smooth muscle function depend in large part on the regulation of gene expression. Glucocorticoids influence gene expression by multiple mechanisms (1). First, the glucocorticoid receptor, upon ligand binding, can influence transcription by binding to a consensus sequence, the glucocorticoid response element, present within the 5′-promoter regions of target genes. Such binding induces the transcription of genes encoding proteins with anti-inflammatory effects. Second, glucocorticoids may inhibit gene transcription via interactions between the activated glucocorticoid receptor and transcription factors or cofactors, such as activator protein-1 and nuclear factor-κB family transcription factors, which are critical for the transcription of genes encoding proteins with proinflammatory effects. Through this interaction, glucocorticoids interfere with the binding of the trans-acting factor to its consensus sequence within the promoter of the target genes, thereby inhibiting transcription.

Glucocorticoids may also have posttranscriptional effects, reducing protein abundance by regulating mRNA stability and translation (33). Glucocorticoid-mediated inhibition of mitogen-activated protein kinase signaling, particularly p38, decreases the stability of mRNAs with 3′-untranslated regions bearing adenylate/uridylate-rich elements. A number of genes that are posttranscriptionally repressed by glucocorticoid, such as IL-1, vascular endothelial cell growth factor, and tumor necrosis factor-α, IL-6, IL-8, and COX-2, are also regulated by p38. In skeletal muscle cells, glucocorticoids have been demonstrated to retard translation by interfering with the phosphorylation of proteins regulating translation initiation and capacity, namely eukaryotic translation initiation factor 4E-binding protein 4E-BP1 and p70 ribosomal S6 kinase (17, 18, 29–32). Finally, glucocorticoids may reduce muscle protein abundance by post-translational mechanisms. Glucocorticoids stimulate skeletal muscle myofibrillar protein breakdown in vivo (13, 14).

The effects of β-adrenergic agonists on airway smooth muscle gene expression have been less well studied. Alterations in cAMP levels could alter the expression of genes driven by cAMP response elements (CREs) (15, 28), including COX-2, IL-6, and the gene for the β2-adrenergic receptor itself. β-Agonists induce IL-6 secretion from airway smooth muscle via activation of the promoter CRE region (2). β-Agonists inhibit expression of cyclin D1, likely via a similar mechanism (21).

We examined the effects of the glucocorticoids dexamethasone and fluticasone, in combination with the long-acting β2-adrenoceptor agonist, salmeterol, on airway smooth muscle contractile protein expression in primary bronchial smooth muscle cells stimulated with transforming growth factor (TGF)-β. We selected TGF-β because it is increased in the airways of severe asthmatics, some of whom demonstrate airway smooth muscle hypertrophy (4), compared with patients with less severe disease (20, 22, 23, 36). We have previously shown that TGF-β increased airway smooth muscle cell size, total protein synthesis, contractile protein expression, formation of actomyosin filaments, and cell shortening to ACh (10). Furthermore, TGF-β increased airway smooth muscle α-actin synthesis in the presence of the transcriptional inhibitor actinomycin D, evidence that translational control is a physiologically important element of the observed hypertrophy. In the

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present study, we found that fluticasone reduces airway smooth muscle α-actin expression, incorporation of α-actin into contractile filaments, and contractile function by attenuating mRNA translation and enhancing protein degradation.

MATERIALS AND METHODS

Cell culture. Primary human bronchial smooth muscle cells, isolated from unacceptably lung donor tissue (supplied by Dr. Julian Solway, Univ. of Chicago), were studied. Use of lung specimens was approved by the Institutional Review Boards of the Universities of Chicago and Michigan. Briefly, bronchus segments were dissected free from surrounding parenchyma, minced with scissors, and enzymatically dispersed for 60 min at 37°C. Cells were seeded on uncoated plastic culture plates in DMEM with 10% fetal bovine serum and penicillin-streptomycin. Passage 2–5 cells were used in the proposed studies.

For experiments, primary bronchial smooth muscle cells were seeded on uncoated plastic culture plates at ~50% confluence and incubated overnight. After serum deprivation for 24 h, 1 ng/ml TGF-β was added. All experiments were performed in the absence of serum.

Experimental protocol. Cells were incubated with either dexamethasone (0.1–1 μM), fluticasone (1–100 nM), and/or salmeterol (1–10 nM), or an equal volume of carrier (dimethyl sulfoxide) for up to 8 days. Fluticasone and salmeterol concentrations were based on the tissue levels achieved in vivo (M. Johnston, GlaxoSmithKline, personal communication). In most experiments, cells were incubated with drug from the time of TGF-β treatment. For experiments measuring α-smooth muscle actin synthesis in the presence of the transcriptional inhibitor actinomycin D (see below), cells were treated with TGF-β for 24 h before incubation with the above compounds.

Immunoblotting. Immunoblotting was performed as previously described (43). Equal amounts of protein were loaded per lane. Cellular proteins were probed with mouse anti-α-smooth muscle actin (Caltibiochem, San Diego, CA), mouse anti-myosin light chain kinase (MLCK, Sigma Chemical), or anti-β-actin (Sigma).

Northern analysis. Steady-state mRNA levels were measured as described (43). A 240-bp fragment from human airway smooth muscle α-smooth muscle actin (position 1057–1297) was amplified by RT-PCR to make the α-smooth muscle actin probe. mRNA half-life was assessed by examining the level of α-smooth muscle actin mRNA following incubation with actinomycin D, a transcriptional inhibitor (5 μg/ml for 8–24 h).

α-Smooth muscle actin protein synthesis and turnover. To measure α-smooth muscle actin protein synthesis, human bronchial smooth muscle cell lines were treated with TGF-β for 24 h. Next, cells were incubated with [35S]methionine (100 μCi for 24 h; PerkinElmer, Boston, MA) and treated with either carrier, dexamethasone, or fluticasone. Four to eight hours later, cell lysates were immunoprecipitated for α-smooth muscle actin (BioGenex, San Ramon, CA). Samples were resolved by SDS-PAGE, transferred to nitrocellulose, and exposed to film or a PhosphorImager screen.

To measure α-smooth muscle actin protein turnover, primary human bronchial smooth muscle cells were treated with TGF-β for 24 h in the presence of [35S]methionine (100 μCi). Twenty-four hours later, cells were incubated in standard medium and treated with TGF-β and either carrier, dexamethasone, or fluticasone. Four to eight days later, cell lysates were immunoprecipitated for α-smooth muscle actin. Samples were resolved by SDS-PAGE, transferred to nitrocellulose, and exposed to film or a PhosphorImager screen.

Fluorescence immunocytochemistry. Cells were grown on collagen-coated sterile glass slides (BD Biosciences, Bedford, MA). Cells were fixed for 20 min in 1% paraformaldehyde, exposed to 1% Triton X-100 for 15 min, and washed in PBS supplemented with 0.1% Tween 20 (PBS-T). Non-specific binding was blocked with PBS-T/3% BSA. Cells were washed with PBS-T and incubated overnight in mouse anti-α-smooth muscle actin-Cy3 (Sigma Chemical) in PBS-T at a concentration of 1:500. To stain filamentous actin, slides were incubated with Alexa Fluor 488-conjugated phalloidin (Molecular Probes, Eugene, OR).

Cell number. Cells were plated into six-well plates at a concentration of 10⁶ cells/well. After 24 h of serum deprivation, primary cells were treated with TGF-β in the presence or absence of glucocorticoids and salmeterol for 48 h. Cells were harvested and counted in a hemocytometer.

Cell length. The individual length of cells stained with phalloidin was determined with NIH ImageJ software using a hemocytometer grid. Length of the cells was defined as the longest linear dimension through the nucleus. Twenty-five to 46 cells per condition were studied.

Cell shortening. Cells were seeded in 100-mm dishes and grown to confluence in TGF-β (1 ng/ml) in the absence or presence of fluticasone for a total of 6 days. Cells were then scraped off with a rubber policeman and allowed to float freely for 1 h in a standing flask to prevent settling and sticking to the bottom of the flask (27). Aliquots of cultured cell suspension (2.5 × 10⁶ cells/0.5 ml) were stimulated with ACh (10⁻⁸ M) or KCl (75 mM). The reaction was allowed to proceed for 5 min and stopped by the addition of 1% glutaraldehyde. After 1 h, cells were centrifuged for 5 min at 800 rpm to concentrate and mixed with 10% polyvinyl alcohol and 10% glycerol in PBS for mounting. Cells were photographed, and cell length was measured with NIH ImageJ software using a hemocytometer grid. Average length of cells before or after addition of test agents was obtained from 10–22 cells encountered in successive microscopic fields.

Statistical analysis. Data are described as means ± SE. Statistical significance was assessed by one-way ANOVA using repeated measures when appropriate. Differences between groups were pinpointed using Newman-Keuls multiple range test.

RESULTS

Effect of corticosteroids and salmeterol on human bronchial smooth muscle contractile protein abundance. As previously shown, α-smooth muscle actin protein abundance increased after TGF-β treatment (Fig. 1, A and B). For MLCK, there was a shift in expression from the long isoform, which has been associated with reduced contractile function (3), to the short isoform. Treatment with dexamethasone and fluticasone for 6 days each significantly reduced TGF-β-induced α-actin protein abundance. Dexamethasone and fluticasone also reversed the TGF-β-induced shift in MLCK expression. Salmeterol had modest inhibitory effects on the protein abundance of α-smooth muscle actin and the short form of MLCK, but these effects were not statistically significant. Larger doses of salmeterol significantly reduced α-smooth muscle actin expression, but these effects were not completely reversed by propranolol, suggesting a nonspecific effect (not shown). The observed effects of corticosteroids on α-actin expression were not due to changes in cell number (Fig. 2).

Effect of corticosteroids on α-smooth muscle actin mRNA steady-state level and stability. We tested whether corticosteroid-induced changes in protein abundance were accompanied by similar changes in mRNA expression. Cells were harvested for RNA extraction 48 h after TGF-β treatment. Decrements in α-smooth muscle actin protein abundance were not accompanied by a reduction in mRNA level, suggesting a posttranscriptional mechanism (Fig. 3A). To check whether a reduction in mRNA stability might account for the observed reduction in α-smooth muscle actin protein abundance, we examined the effects of corticosteroids on the rate of mRNA degradation.
following treatment of cells with actinomycin D (5 \(\mu\)g/ml), a transcriptional inhibitor (Fig. 3B). There was no significant effect of corticosteroids on \(\alpha\)-actin mRNA level 8 or 24 h after actinomycin D treatment.

Corticosteroids decrease \(\alpha\)-smooth muscle actin synthesis in the presence of actinomycin D. We asked whether corticosteroids regulate mRNA translation. We examined \(\alpha\)-smooth muscle actin synthesis in the presence of actinomycin D. Cells were treated with TGF-\(\beta\) for 24 h and then incubated with \([^{35}\text{S}]\)methionine, actinomycin, and either dexamethasone or fluticasone. After an additional 24 h, immunoprecipitated \(\alpha\)-smooth muscle actin was resolved by SDS-PAGE, proteins were transferred to nitrocellulose, and the membranes were exposed to film. Addition of fluticasone significantly reduced the abundance of \(^{35}\text{S}\)-labeled \(\alpha\)-actin protein (Fig. 4, A and B). Since gene transcription is prevented in the presence of actinomycin D, these data suggest that, in the presence of fluticasone, \(\alpha\)-actin mRNA translation is reduced.

Effect of corticosteroids on \(\alpha\)-smooth muscle actin turnover.

To measure \(\alpha\)-smooth muscle actin protein turnover, primary human bronchial smooth muscle cells were treated with TGF-\(\beta\) and \([^{35}\text{S}]\)methionine. After 24 h, the medium was replaced...
with cold methionine, and cells incubated for an additional 8 days with dexamethasone or fluticasone. Cell lysates were immunoprecipitated for \( ^{35}S \)-labeled \( \alpha \)-smooth muscle actin protein (Fig. 5, A and B).

**Effect of fluticasone on incorporation of \( \alpha \)-smooth muscle actin into filaments.** We examined individual cells for \( \alpha \)-smooth muscle actin expression by fluorescent microscopy. Addition of fluticasone appeared to reduce the \( \alpha \)-actin expression of individual cells (Fig. 6). Although individual \( \alpha \)-actin filaments extended to the periphery of the cells, fluticasone-treated cells showed a concentration of \( \alpha \)-actin toward the cell center. Finally, there was less colocalization of \( \alpha \)-actin with phalloidin, indicating a reduction in the amount of \( \alpha \)-actin incorporated into contractile filaments.

**Effect of fluticasone on the length of adherent primary human bronchial smooth muscle cells.** The individual length of phalloidin-stained cells was determined with NIH ImageJ software using a hemacytometer grid. Twenty-five to 46 cells per condition were studied. Fluticasone significantly reduced the length of adherent airway smooth muscle cells (Fig. 7 A, \( P \leq 0.05 \), ANOVA).

**Effect of fluticasone on primary bronchial smooth muscle cell shortening.** To determine whether fluticasone treatment alters contractile function, cells were seeded in 100-mm dishes and grown to confluence in either serum-free medium with TGF-\( \beta \) (1 ng/ml) or TGF-\( \beta \) with fluticasone. After 6 days, cells were then scraped off and allowed to float freely for 1 h in a standing flask. Aliquots of cell suspension were stimulated with ACh (10^{-4} M) or KCl (75 mM). The reaction was allowed to proceed for 5 min and was stopped by the addition of 1% glutaraldehyde. Cells were photographed, and cell...
length was measured with NIH ImageJ software using a hemacytometer grid. Fluticasone (1 nM) reduced the starting length of nonadherent TGF-β/H9252-treated human bronchial smooth muscle cells from 88 ± 9 to 50 ± 5 μm (P < 0.001, ANOVA). Consequently, fluticasone-treated cells shortened less than untreated cells (Fig. 7B). Responses to ACh and KCl were similar, consistent with the notion that the responses were dependent on the contractile apparatus rather than receptor expression. Note that cells in suspension were markedly shorter than adherent cells, suggesting that they may not have been fully relaxed at the time of study.

**DISCUSSION**

We have found that the corticosteroids and specifically, fluticasone, have significant inhibitory effects on airway smooth muscle contractile protein expression. In TGF-β-treated primary human bronchial smooth muscle cells, dexamethasone and fluticasone each significantly inhibited α-smooth muscle actin protein abundance and shifted MLCK expression from a less contractile long form to a shortened form. Fluticasone decreased α-smooth muscle actin synthesis in the presence of actinomycin D, suggesting that this treatment inhibits protein expression at least in part by reducing the translation of contractile apparatus mRNA. Fluticasone also increased α-actin degradation. In individual cells, fluticasone reduced α-smooth muscle actin expression, length, and incorporation of α-actin into filaments. Finally, fluticasone reduced the shortening of TGF-β-treated cells in response to ACh and KCl. Together, these results suggest that treatment with corticosteroids may reduce airway smooth muscle contractile protein abundance and that these changes may be functionally significant. Fluticasone appeared to have greater inhibitory effects on α-actin expression than dexamethasone, perhaps because of its higher potency (12).

TGF-β has been shown to increase contractile protein abundance by increasing gene transcription (8, 25) and, more recently, mRNA translation (9). Furthermore, we have shown that TGF-β-induced airway smooth muscle hypertrophy requires phosphorylation of 4E-BP, release of eukaryotic translation initiation factor 4E (eIF4E), and cap-dependent mRNA translation (9). We have found a similar requirement in con-
Glucocorticoids also activate the ATP-ubiquitin-dependent protease, calpain, leading to the cleavage of myofibrillar proteins. Glucocorticoids may regulate protein abundance by multiple mechanisms, including the regulation of gene expression, mRNA stability and translation, and protein turnover. Dexamethasone has been shown to reduce α-smooth muscle actin mRNA expression in rat Achilles tendon cells (39). Dexamethasone acutely attenuates rat skeletal muscle protein synthesis via the impairment in translation initiation (30). As noted in the Introduction, dexamethasone treatment results in the dephosphorylation of 4E-BP1 and p70 ribosomal S6 kinase (17, 18, 29–32). Thus dexamethasone may reverse the permissive effect of TGF-β on translation initiation.

Corticosterone treatment induces skeletal muscle myofibrillar proteolysis (13, 14) through at least two cellular mechanisms. In chick embryo myoblasts, corticosterone increases cellular calcium uptake (19). The resulting elevation in intracellular [Ca²⁺] may activate the nonlysosomal cysteine protease, calpain, leading to the cleavage of myofibrillar proteins. Glucocorticoids also activate the ATP-ubiquitin-dependent proteolytic system in skeletal muscle (40). Triamcinolone, but not prednisolone, induces rat diaphragmatic atrophy, suggesting that specific corticosteroid preparations may affect contractile function differently (6). We now, for the first time, show that corticosteroids reduce abundance of contractile proteins in cultured smooth muscle cells, at least in part, by inhibiting mRNA translation and enhancing protein degradation.

On the other hand, β₂-adrenergic agonists are potent muscle growth promoters in many species. In a rat cancer cachexia model, the β₂-adrenergic agonist clenbuterol prevents skeletal muscle breakdown by restoring protein degradative rates to control values through effects on the ubiquitin-proteasome pathway (5). In human airway smooth muscle cells, salmeterol treatment had no effect on TGF-β-induced α-smooth muscle actin protein abundance.

Glucocorticoids have antiproliferative activity against a broad spectrum of airway smooth muscle mitogens (34). Glucocorticoids attenuate expression of cyclin D1 (9), a key cell cycle intermediate required for airway smooth muscle G1 traversal (41). Similarly, β₂-adrenergic agonists such as salmeterol have anti-mitogenic effects that are exerted via an increase in intracellular cAMP (37). Alterations in cyclin D1 transcription (21) and proteosomal degradation (35) have also been implicated in this process. It is therefore conceivable that the observed reduction in contractile protein expression relates to an inhibition of cell proliferation. We do not think this is the case, however. First, immunocytochemical studies revealed that fluticasone reduces the α-smooth muscle actin staining of individual cells. Second, since fluticasone reduced cell size, immunoblot samples from corticosteroid-treated cells showing reduced α-smooth muscle actin protein abundance, which included equal amounts of protein per lane compared with untreated samples, were likely to represent more, not fewer, smooth muscle cells. Finally, our studies showed that TGF-β is not a mitogen for human bronchial smooth muscle cells in this system and, in the absence of mitogenic stimulation, glucocorticoids and salmeterol had no effect on cell number. Together, these points strongly argue against reduced cell proliferation as the cause of the observed reductions in protein abundance.

Airway smooth muscle hypertrophy appears to be present in severe asthma. Ebina and coworkers (7) found two subtypes in fatal asthma, one with airway smooth muscle hypertrophy throughout the airways and another with hyperplasia in central bronchi. Benayoun and colleagues (4) found that patients with severe asthma had increased airway smooth muscle cell diameter and expression of α-smooth muscle actin and MLCK. Treatment with inhaled corticosteroids reduces subepithelial thickening in asthma (24, 38), and corticosteroids prevent airway smooth muscle DNA synthesis in allergen-sensitized rats (16). The effect of corticosteroids on airway smooth muscle cell size or contractile protein expression has not been directly studied in vivo. However, Benayoun and colleagues (4) concluded from a subanalysis of their data that steroid use reduces thickness of the subepithelium in patients with severe asthma but has no effect on airway smooth muscle mass. Additional work is needed to determine whether treatment with inhaled corticosteroids and salmeterol reduces airway smooth muscle remodeling in asthma.

We conclude that glucocorticoids reduce human airway smooth muscle α-smooth muscle actin protein abundance and incorporation into contractile filaments, leading to functional effects. These effects appear to be mediated, at least in part, via the attenuation of mRNA translation and enhancement of protein degradation.

Fig. 7. Effect of fluticasone on cell length and contractile function. A: the individual length of phalloidin-stained cells was determined with NIH ImageJ software using a hemacytometer grid (n = 25–46, *different from TGF-β, P < 0.001, ANOVA). B: to determine whether fluticasone treatment alters contractile function, cells were grown to confluence in either serum-free medium with TGF-β (1 ng/ml) or TGF-β with fluticasone. Cells were then scraped off and allowed to float freely for 1 h in a standing flask. Aliquots of cell suspension were stimulated with ACh (10⁻⁴ M) or KCl (75 mM). The reaction was allowed to proceed for 5 min and was stopped with the addition of 1% glutaraldehyde. Cells were photographed, and cell length was measured with NIH ImageJ software using a hemacytometer grid (n = 10–22, *different from TGF-β, P < 0.001, ANOVA).
GRANTS
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DISCLOSURES
GlaxoSmithKline did not have any role in the study design; collection, analysis, or interpretation of data; the writing of the manuscript; or the decision to submit the manuscript for publication.

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