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Dexamethasone rescues TGF- β 1-mediated β ₂-adrenergic receptor dysfunction and attenuates phosphodiesterase 4D expression in human airway smooth muscle cells

Elena Chung^{1,2}, Christie A. Ojiaku², Gaoyuan Cao², Vishal Parikh², Brian Deeney², Shengjie Xu^{1,2}, Serena Wang², Reynold A. Panettieri Jr^{2*} and Cynthia Koziol-White^{2†}

Abstract

Glucocorticoids (GCs) and β ₂-adrenergic receptor (β ₂AR) agonists improve asthma outcomes in most patients. GCs also modulate gene expression in human airway smooth muscle (HASM), thereby attenuating airway inflammation and airway hyperresponsiveness that define asthma. Our previous studies showed that the pro-fibrotic cytokine, transforming growth factor- β 1 (TGF- β 1) increases phosphodiesterase 4D (PDE4D) expression that attenuates agonist-induced levels of intracellular cAMP. Decreased cAMP levels then diminishes β ₂ agonist-induced airway relaxation. In the current study, we investigated whether glucocorticoids reverse TGF- β 1-effects on β ₂-agonist-induced bronchodilation and modulate *pde4d* gene expression in HASM. Dexamethasone (DEX) reversed TGF- β 1 effects on cAMP levels induced by isoproterenol (ISO). TGF- β 1 also attenuated G protein-dependent responses to cholera toxin (CTX), a G_os stimulator downstream from the β ₂AR receptor. Previously, we demonstrated that TGF- β 1 treatment increased β ₂AR phosphorylation to induce hyporesponsiveness to a β ₂ agonist. Our current data shows that expression of *grk2/3*, kinases associated with attenuation of β ₂AR function, are not altered with TGF- β 1 stimulation. Interestingly, DEX also attenuated TGF- β 1-induced *pde4d* gene expression. These data suggest that steroids may be an effective therapy for treatment of asthma patients whose disease is primarily driven by elevated TGF- β 1 levels.

Keywords: Glucocorticoids, Severe asthma, Bronchodilation, Airway remodeling

Clinical relevance

TGF- β 1 has been identified as pivotal in mediating airway remodeling and irreversible airway obstruction. Inhaled GCs and β ₂AR agonists are commonly used to treat asthma. Our study, however, demonstrates a

protective effect of dexamethasone on TGF- β 1-mediated attenuation of β ₂-agonist and G_os-dependent relaxation of airway smooth muscle.

Background

Asthma, a chronic inflammatory disease of the lungs manifests by several hallmarks: airway hyperresponsiveness, remodeling, and inflammation [1]. Airway smooth muscle (ASM) cells play an integral role in regulating bronchomotor tone in the asthma diatheses and are a

* Correspondence: rp856@rbhs.rutgers.edu

Reynold A. Panettieri Jr. and Cynthia Koziol-White shared Senior Authorship
²Rutgers Institute for Translational Medicine and Science, Rutgers, The State University of New Jersey, New Brunswick, NJ, USA

Full list of author information is available at the end of the article



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direct target of β_2 -agonists, a common therapy that promotes bronchodilation [2]. While β_2 -agonists evoke ASM relaxation, β_2 -agonists are not effective in all patients [3]. Patients who fall into the “severe” category of asthma are frequently hypo-responsive to bronchodilators [4]. Studies show that β_2 AR tolerance or desensitization occurs after repeated bronchodilator use that diminishes drug efficacy [3, 5–9].

TGF- β 1, a profibrotic cytokine whose levels are elevated in patients with asthma, augments human airway smooth muscle (HASM) cell stiffness and significantly increases myosin light chain (MLC) phosphorylation via Smad3 [10] that enhance contractile agonist-induced cell shortening and hyperresponsiveness. In addition to amplifying bronchoconstriction, we also demonstrated that TGF- β 1 blunts intracellular cAMP by upregulating *pde4d* expression that decreases β_2 -agonist-induced cAMP levels [11].

Signaling downstream of seven transmembrane G protein-coupled receptors (GPCRs) involves $G\alpha\beta\gamma$ trimer dissociation following receptor activation [12]. The G_α subunit family is comprised of $G_{\alpha i}$, $G_{\alpha q}$, and $G_{\alpha s}$, playing fundamental roles in regulating HASM relaxation and contraction [13]. Cholera toxin (CTX) catalyzes the ADP-ribosylation of $G_{\alpha s}$ that elicits adenylyl cyclase (AC) activation, causing the accumulation of intracellular cAMP and further activation of PKA to induce HASM relaxation [14]. CTX, and the β_2 -agonist isoproterenol (ISO), induce actin depolymerization in HASM in PKA-independent and -dependent pathways integrating activation of Src protein tyrosine kinases and $G_{\alpha s}$ protein [15], to promote smooth muscle relaxation. Whether TGF- β 1 directly modulates $G_{\alpha s}$ protein activation remains unknown.

Glucocorticoids (GCs) remain a cornerstone in the management of asthma. GC treatment alters gene expression in HASM, thereby modulating inflammation and airway reactivity. Others have demonstrated that dexamethasone, a glucocorticoid, can directly inhibit Smad3 activity [16, 17]. Our previous study showed that TGF- β 1 blunted the effects of β_2 agonist-induced reversal of carbachol-mediated phosphorylation of myosin light chain, a process which was Smad3-dependent [11]. Given this information, we posited that GC treatment would reverse TGF- β 1-induced hypo-responsiveness to β_2 -agonist. Our data demonstrate that dexamethasone (DEX) reverses TGF- β 1-induced attenuation of β_2 AR-induced signaling, rescuing β_2 -agonist- and $G_{\alpha s}$ -activator-mediated cAMP production by attenuating *pde4d* expression.

Methods

HASM cell culture

HASM cells from the National Disease Research Interchange (Philadelphia, PA, USA) and the International

Institute for the Advancement of Medicine (Edison, NJ, USA) were derived from trachea obtained from donors without chronic illness. All tissue was obtained from de-identified donors and was deemed non-human subjects research by the Rutgers University Institutional Review Board. Cells were cultured in Ham's F12 medium with 10% fetal bovine serum. The cells were incubated and grown at 37 °C in 5% CO₂. We have shown that isolated airway smooth muscle cells retain their phenotypic properties [18]. Primary HASM cells between passages 3–4 were used in all experiments. Donor demographics for the cell lines utilized in these studies are detailed in Table S1.

Western blot analysis

Primary HASM cells were serum deprived for 24 h prior to treatment. HASM cells were lysed with 0.6 N HClO₄, scraped, collected, and pelleted as previously described [19]. The membrane was blocked with ready-to-use Odyssey Blocking Buffer (LI-COR BioSciences) containing 0.1% sodium azide and probed for phospho-Smad3, pMLC, total MLC, and GAPDH.

Measurement of intracellular cAMP levels

Grown to 90% confluency on 24-well plates, HASM cells were stimulated and lysed using the Applied Biosystems cAMP-Screen Immunoassay System following the manufacturer instructions as previously described [11]. The cells were lysed and incubated for 30 min in 5% CO₂ and 37 °C. Conjugate Dilution Buffer, cAMP-AP Conjugate, anti-cAMP antibody, and the samples were added to pre-coated assay plate to incubate for 1 h on plate shaker. Plate was measured on luminometer after a 30-min incubation period with CSPD®/Sapphire-II RTU Substrate. Data was derived from standard curves and cAMP levels reported after using standard dilutions.

Quantification of *pde4d*, *grk2*, and *grk3* expression (RNA isolation and qPCR)

Following treatment with TGF- β 1 \pm dexamethasone, cells were suspended in TRIzol reagent, and total RNA were isolated following the manufacturer protocol. RNA was isolated and purified from HASM cells using the RNeasy Mini Kit and cDNA was created using SuperScript IV First-Strand Synthesis System. All reactions were performed in 20 μ L reaction volume in triplicate. For mRNA cDNA, PCR amplification consisted of 10 min of an initial denaturation step at 95 °C, followed by 40 cycles at 95 °C for 15 s, 60 °C for 60 s. Relative cDNA quantification was performed using TaqMan quantitative RT-PCR (Thermo Fisher Scientific) and the $\Delta\Delta C_t$ method, and *pde4d*,

grk2, and *grk3* expression were normalized to expression of endogenous β -actin.

Statistical analysis

Graphs were created and statistical analyses were conducted using GraphPad Prism 5.01 h software (La Jolla, Ca, USA) to determine statistical significance evaluated using two-tailed Student’s paired *t*-test for two groups. *P* values of <0.05 were considered significant. All results were confirmed by experiments in at least three unique cell lines. Data was fit to a normal distribution, and appropriate tests run to determine significance. For comparison of multiple conditions, one-way ANOVA was used with Bonferroni’s post-test. For the *pde4d* expression results, the differential expression analysis was performed under a negative binomial distribution model with DESeq2 (v.1.18.1), and the adjusted *p* values are noted.

Materials

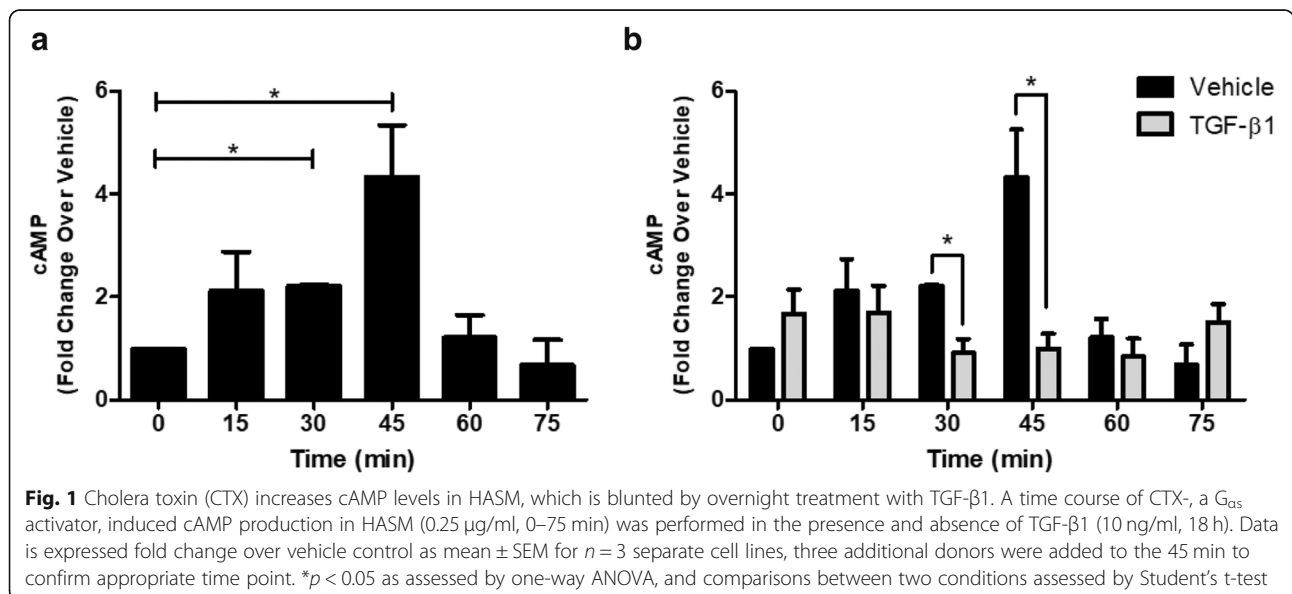
Compounds were purchased from the following vendors: R&D Systems (TGF- β 1; SB-431542), Sigma-Aldrich (albuterol [Alb], carbachol [Cch], cholera toxin [CTX], dexamethasone [DEX], isoproterenol [ISO]), Fisher BioReagents (Forskolin, [FSK]). Immunoblot antibodies were purchased from Abcam (phospho-Smad3; ab52903), Cell Signaling Technologies (phosphorylated myosin light chain pMLC, 3674S; GAPDH, 2118S; Tubulin, 3873S), and EMD Millipore (total myosin light chain [MLC, MABT180]). The following Taqman primer sets were purchased from Thermo Fisher Scientific: ACTB, actin beta, Hs01060665_g1; GRK2, G protein-coupled receptor kinase 2 Hs00176395_m1; GRK3, G protein-coupled receptor kinase 3, Hs00178266_m1; PDE4D, phosphodiesterase 4D, Hs01579625_m1.

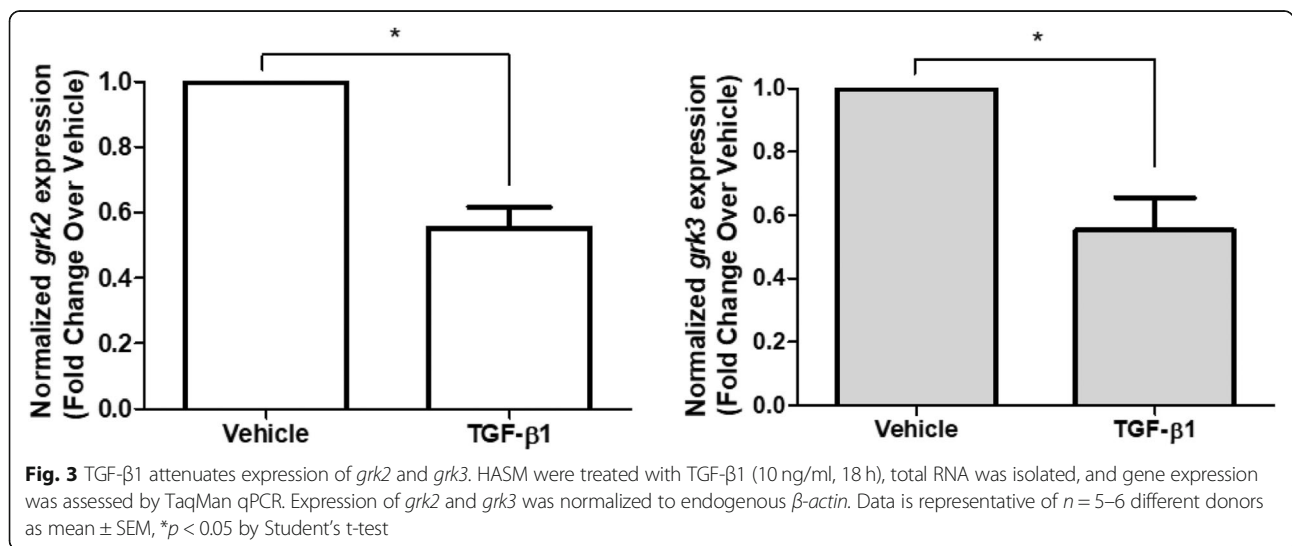
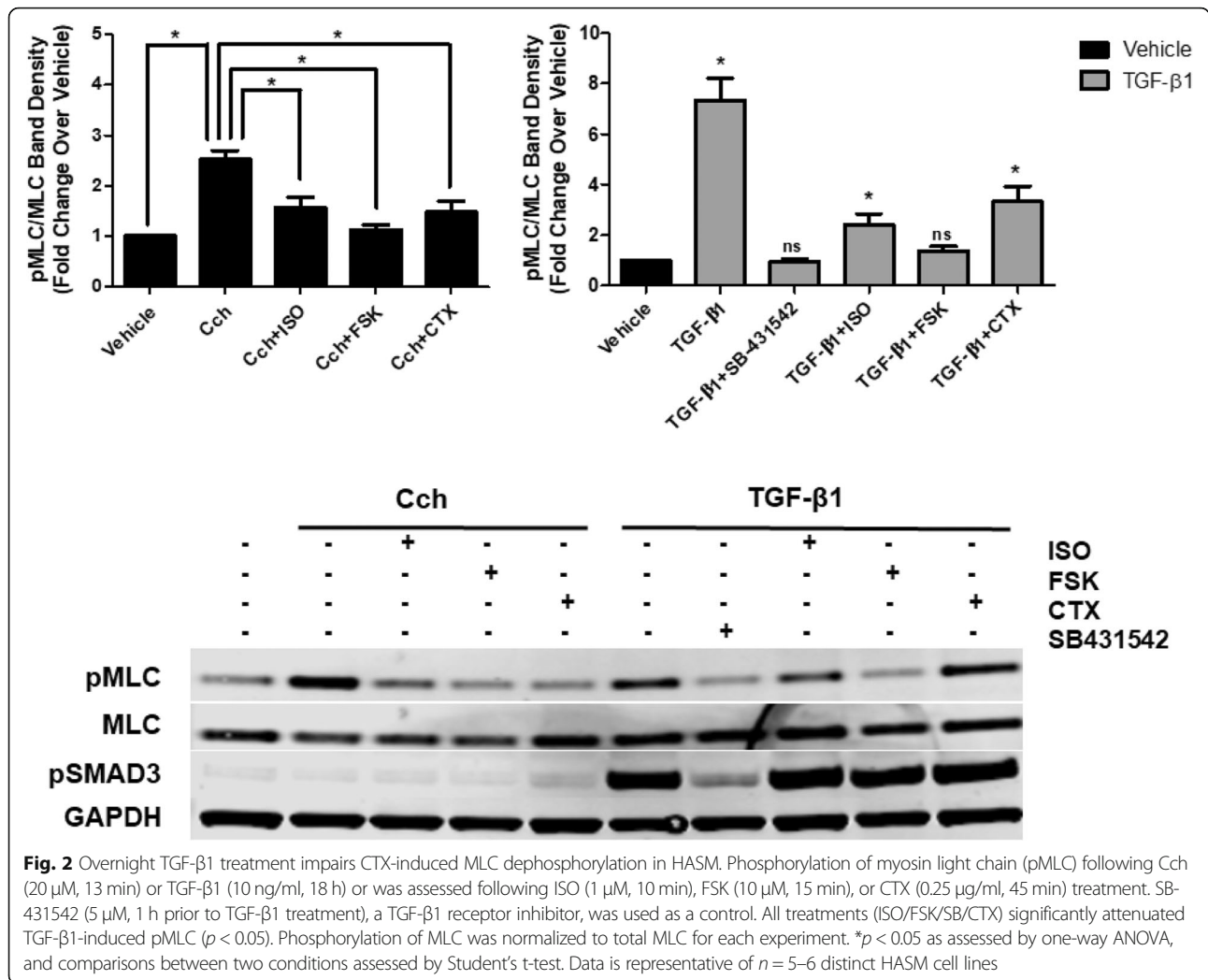
Results

TGF- β 1 attenuates $G_{\alpha s}$ -mediated cAMP production in HASM

Upon activation of β_2 AR, ADP-ribosylation of the α subunit of stimulatory G protein ($G_{\alpha s}$), stimulates adenylyl cyclase to increase intracellular cAMP [15, 19]. To further understand mechanisms underlying TGF- β 1-mediated hyporesponsiveness to a β_2 -agonist, intracellular cAMP levels were measured in TGF- β 1-treated HASM cells following stimulation with cholera toxin (CTX), a $G_{\alpha s}$ activator. Intracellular cAMP activity increased in a time-dependent manner following exposure to CTX, with the maximum level elicited at 45 min (Fig. 1a). Interestingly at 60 and 75 min, the CTX-induced cAMP levels extinguished. In TGF- β 1-treated cells, CTX-induced cAMP levels were completely abrogated compared to that of the diluent control (Fig. 1b).

We previously demonstrated that isoproterenol (ISO) decreased carbachol (Cch)- and TGF- β 1-induced phosphorylation of myosin light chain (pMLC); TGF- β 1, however, decreased the ability of a β_2 -agonist to abrogate Cch-induced pMLC [11]. Cch-induced pMLC was inhibited by ISO, forskolin (an adenylyl cyclase activator) and CTX to comparable levels, as shown in Fig. 2. Interestingly, TGF- β 1-induced pMLC was also decreased with CTX, forskolin, ISO and SB-431542, a TGF- β 1 receptor antagonist. However, ISO and CTX-induced inhibition was less effective as compared with that of SB-431542 or forskolin in blocking TGF- β -induced pMLC (Fig. 2, upper right). Phosphorylation of SMAD3 (pSMAD3) induced by TGF- β 1 confirmed engagement of TGF- β 1 receptors and activation of downstream signaling pathways (Fig. 2, lower panel). Collectively, these data suggest that TGF- β 1 inhibits the ability of β_2 AR or $G_{\alpha s}$





activation, but not forskolin, to increase cAMP levels and diminish pMLC.

TGF-β1 attenuates *grk2* and *grk3* expression in HASM

One mechanism by which inflammatory mediators can attenuate signaling downstream of the β₂AR is through phosphorylation of the receptor [20–23]. Evidence suggests that GRK2 and 3 are associated with the β₂AR, mediating desensitization through phosphorylation of intracellular portions of the receptor [24, 25]. We next examined the effect of TGF-β1 on expression of GRK2 and 3. As shown in Fig. 3, we show that TGF-β1 attenuates, rather than augments, *grk2* and *grk3* expression. Despite our previous findings showing that β₂AR phosphorylation is increased following TGF-β1 exposure [26], we determined that the increase in TGF-β1-induced β₂AR phosphorylation is not due to increased expression of GRK2/3.

Dexamethasone rescues TGF-β1-induced decreases in cAMP levels induced by ISO or CTX

Glucocorticoids remain a cornerstone in the management of asthma by decreasing airway inflammation and reversing β₂AR desensitization [27, 28]. To address whether the steroids also modulate TGF-β1 effects on cAMP levels, HASM cells were pretreated with dexamethasone (DEX) in the presence and absence of TGF-β1 and then treated with either ISO or CTX. As shown in Fig. 4a, TGF-β1 treatment attenuated ISO-induced cAMP by 47.3% and DEX pretreatment significantly rescued TGF-β1’s effect by 35.7%. Similarly, TGF-β1 treatment attenuated CTX-induced cAMP by 61.7%, and DEX pretreatment significantly reversed TGF-β1’s effect

by 43.8% (Fig. 4b). These data demonstrate partial steroid responsiveness of TGF-β1-mediated hypo-responsiveness to β₂AR-induced cAMP levels in HASM.

Dexamethasone attenuates TGF-β1-induced *pde4d* expression in HASM

Previously, we determined that the blunted cAMP response to ISO induced by TGF-β1 was dependent upon increased *pde4d* expression [11]. Given that DEX rescued TGF-β1-induced attenuation of ISO- and CTX-induced cAMP production, we posited that DEX pretreatment would attenuate TGF-β1-mediated *pde4d* expression. In a dose-dependent manner, DEX pretreatment significantly attenuated TGF-β1-induced *pde4d* expression in HASM as shown in Fig. 5.

Discussion

Insensitivity to current therapeutics occurs in some patients with severe asthma. Evidence suggests that β₂AR dysfunction can manifest in an inflammatory milieu due to inflammatory cytokines associated with severe asthma and airway remodeling such as TGF-β1, IL-13, and TNF-α [11, 19, 29]. Additionally, TGF-β1 stimulation has been shown to attenuate β adrenergic receptor signaling in other cell types [30, 31] in addition to HASM. We examined whether generation of cAMP levels in HASM cells after CTX-treatment occurred in the presence and absence of TGF-β1 (Fig. 1b). As we had previously described, β₂-agonist-induced responses were blunted following TGF-β1 treatment. To characterize mechanisms by which TGF-β1 diminishes β₂AR responses, the effects of TGF-β1 on G_{αs}-induced reversal

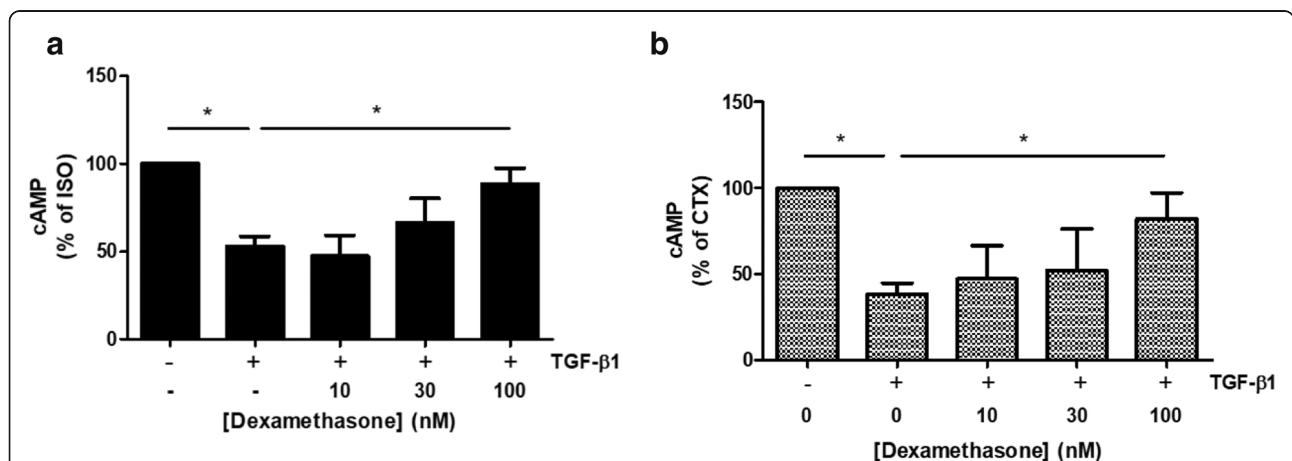
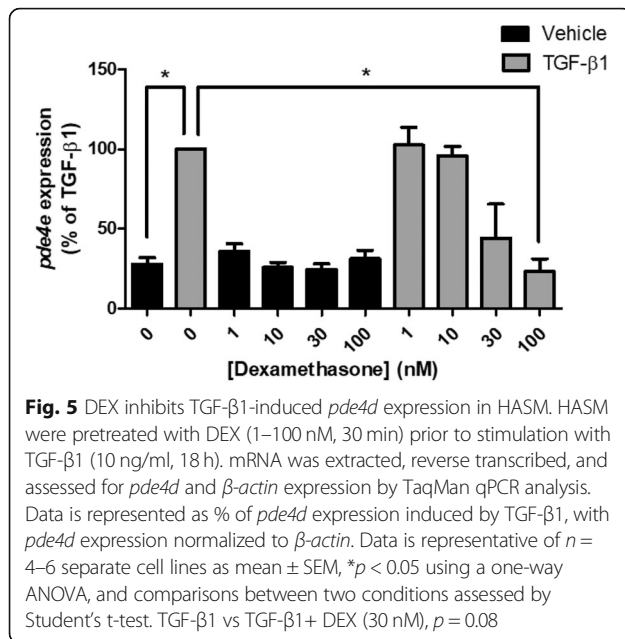


Fig. 4 Dexamethasone (DEX) rescues TGF-β1-mediated attenuation of ISO- and CTX-induced cAMP production in HASM. HASM were pretreated with (a, b) DEX (10–100 nM, 30 min) prior to TGF-β1 (10 ng/ml, 18 h) stimulation. HASM were subsequently stimulated with (a) ISO (1 μM, 5 min) or (b) CTX (0.25 μg/ml, 45 min) and assessed for cAMP generation. Data is expressed % of max cAMP produced either by ISO (a) or CTX (b). Data is representative of (a) n = 6–13 (b) or n = 4–8 separate cell lines as mean ± SEM, *p < 0.05 comparing control/ISO/CTX to TGF-β1/ISO/CTX, and TGF-β1/ISO/CTX to TGF-β1/ISO/CTX ± DEX using a one-way ANOVA, and comparisons between two conditions assessed by Student’s t-test

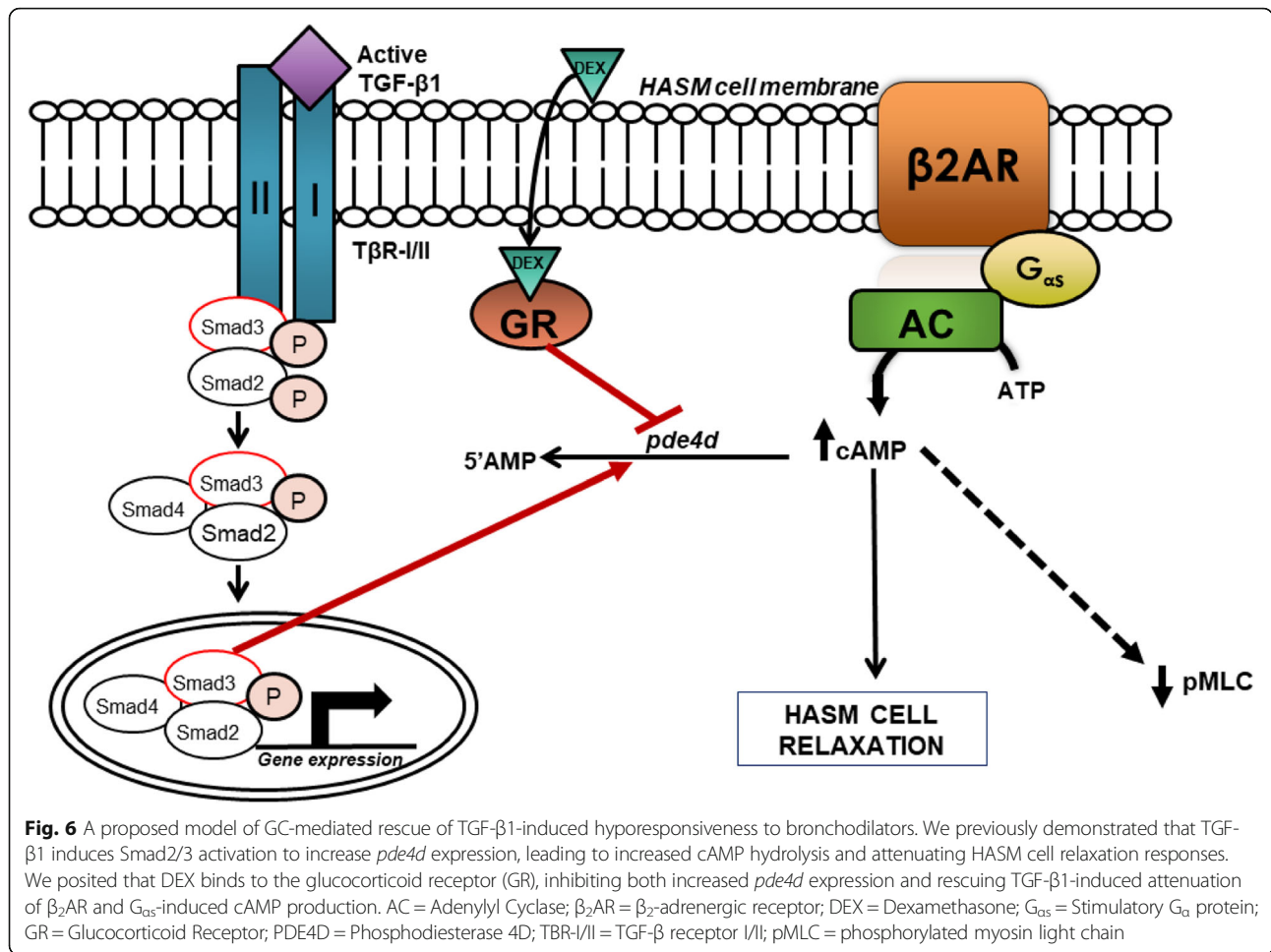


of agonist-induced HASM cell contractile signaling was examined. We extended our previous findings that phosphorylation of MLC, an important element of agonist-induced HASM contraction, induced by Cch or TGF-β1 was reversed by activation of the β_2 AR and G_{α_s} (Fig. 2). Given these data and our previously published results, these data suggest that the effects of TGF-β1 are more prominent at the receptor and G_{α_s} level rather than downstream at the level of adenylyl cyclase, as evidenced by the lack of effect of TGF-β1 on forskolin-induced increases in cAMP levels.

Attenuation of β_2 AR signaling occurs after phosphorylation of the receptor, promoting internalization and desensitization of the β_2 AR [32–34]. Activation of GRK2 and GRK3, members of the GRK (G protein-coupled receptor kinase) family, provide a mechanism by which β_2 AR desensitization occurs. We previously demonstrated that TGF-β1 stimulation induces β_2 AR phosphorylation that is consistent with desensitization of the receptor [26]. To assess a potential role for GRKs in attenuating β_2 -agonist-induced relaxation of HASM, *grk2* and *grk3* expression were assessed with overnight TGF-β1 treatment. In Fig. 3, we demonstrate that TGF-β1 treatment decreased *grk2* and *grk3* expression. Given these data, it is highly unlikely that upregulation of GRK2/3 expression contributes the hyporesponsiveness to bronchodilators induced by TGF-β1. Current evidence and our previous studies suggest that TGF-β1 likely modulates activity of these types of kinases, rather than modulating GRK expression to induce hyporesponsiveness to β_2 -agonists.

Currently, glucocorticoids like dexamethasone are used to attenuate inflammation associated with asthma as well as restore β_2 AR responsiveness [3, 19, 35], and reverse the effects of inflammatory mediator-induced β_2 AR dysfunction. In osteoblasts, it has been demonstrated that dexamethasone suppresses the transcriptional activity of, but not expression, Smad3, attenuating TGF-β1-induced alkaline phosphatase activity and type I collagen expression [16]. Additionally, dexamethasone also repressed transcriptional activation of PAI-1 through inhibition of Smad3/4 by direct interaction between the glucocorticoid receptor and Smad3 [17]. We previously showed that TGF-β1-induced attenuation of ISO reversal of Cch-induced pMLC was Smad3-dependent in HASM. We also showed that TGF-β1 treatment increased expression of *pde4d*, suggesting a role for phosphodiesterases in TGF-β1-induced hyporesponsiveness to bronchodilators. Given our data and others' observations concerning the effects of glucocorticoids on TGF-β1/Smad3-dependent signaling, we posited that dexamethasone would thereby reverse TGF-β1- and Smad3-induced attenuation of ISO-induced cAMP accumulation and *pde4d* expression in HASM. Figure 6 depicts a model of mechanisms underlying glucocorticoid-mediated rescue of TGF-β1-induced β_2 AR hyporesponsiveness. We demonstrate that TGF-β1-induced attenuation of β_2 -agonist- and G_{α_s} -induced cAMP accumulation in HASM can be rescued by treatment with dexamethasone (Fig. 4a & b). Our data also show that DEX treatment reversed TGF-β1-induced *pde4d* expression (Fig. 5). Consistent with these data, we previously demonstrated that DEX (1 μ M, 18 h) stimulation alone has little effect *pde4d* expression [36]. We recognize that changes in transcript expression do not necessarily translate to changes in protein expression or PDE4D activity. Previous work in HASM has demonstrated that PDE4D5 is expressed and, in part, controls cAMP production following β_2 agonist stimulation [37, 38]. As in our study, Billington et al. demonstrated transcript expression, but not protein expression or enzymatic activity, of the specific PDE isoform. Interestingly, Niimi et al. demonstrated that transcript expression of PDE4D isoforms in HASM was altered, but not protein or enzymatic activity [39]. Extensive work by the Houslay group has shown the difficulty of isolating specific isoforms and splice variants of PDE enzymes, as well as the difficulty of enzymatic activity assessment [40]. Given the findings of Billington, Niimi, and Triani, and the complexities of the assays performed by the Houslay group, we recognize the limitations to our work, but feel that our results likely translate to functional outcomes affecting bronchomotor tone in the context of TGF-β1-induced hyporesponsiveness to β_2 agonists and cAMP mobilizers.

Despite the fact that we showed that TGF-β1 attenuates CTX-induced cAMP production, and that DEX



rescues TGF-β1-mediated attenuation of both ISO- and CTX-induced cAMP production, it remains to be seen if these mechanisms are operative in vivo. Despite this limitation, we have shown effects of TGF-β1 on HASM to be recapitulated in small airways derived from human lungs [10]. Additionally, while it would be interesting to study this phenomenon in asthma-derived HASM, we and others have demonstrated that β₂-agonist-induced cAMP production in these cells is already blunted due partially to increased PDE expression [26, 38]. Therefore, exposure of asthma-derived HASM to TGF-β1 will likely have little effect on modulating β₂-agonist-induced cAMP production.

Conclusion

Regardless of evidence that steroids may not reverse the TGF-β1-induced remodeling effects [41] in asthma, our findings suggest that in asthma patients with high levels of TGF-β1, steroids may be an effective treatment to reverse β₂AR hyporesponsiveness observed in these patients.

Supplementary information

Supplementary information accompanies this paper at <https://doi.org/10.1186/s12931-020-01522-w>.

Additional file 1: Table S1. Donor demographics for cAMP and *pde4d* expression studies. All cells were derived from subjects with no history of chronic disease.

Abbreviations

TGF-β1: Transforming growth factor β1; GC: Glucocorticoid; β₂AR: Beta 2 adrenergic receptor; HASM: Human airway smooth muscle; PDE4D: Phosphodiesterase 4D; DEX: Dexamethasone; ISO: Isoproterenol; CTX: Cholera toxin; GRK: G protein receptor-coupled kinase; ASM: Airway smooth muscle; Smad3: Mothers against decapentaplegic homolog 3; cAMP: Adenosine 3',5'-cyclic monophosphate; GPCR: G protein-coupled receptor; ADP: Adenosine monophosphate; PKA: Protein kinase A; AC: Adenylyl cyclase; PBS: Phosphate buffered saline; pMLC: Phosphorylated myosin light chain; MLC: Myosin light chain; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; cAMP-AP: Adenosine 3',5'-cyclic monophosphate-alkaline phosphatase; CSPD: Chemiluminescent alkaline phosphatase substrate; RNA: Ribonucleic acid; qPCR: Quantitative polymerase chain reaction; mRNA: Messenger ribonucleic acid; cDNA: Complementary deoxyribonucleic acid; ANOVA: Analysis of variance; Alb: Albuterol; Cch: Carbachol; FSK: Forskolin; IL-13: Interleukin 13; TNFα: Tumor necrosis factor α

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None.

Authors' contributions

E. Chung – study design, data acquisition, data analysis and interpretation, drafting and editing of the manuscript, final approval of publication; C.A. Ojiaku - study design, data acquisition, data analysis and interpretation, drafting and editing of the manuscript, final approval of publication; G. Cao – study design, data acquisition, data analysis and interpretation, drafting and editing of the manuscript, final approval of publication; V. Parikh - data acquisition, data analysis and interpretation, drafting and editing of the manuscript, final approval of publication; B. Deeney - data acquisition, data analysis and interpretation, drafting and editing of the manuscript, final approval of publication; S. Xu –data analysis and interpretation, drafting and editing of the manuscript, final approval of publication; S. Wang - data acquisition, data analysis and interpretation, drafting and editing of the manuscript; R. Panettieri - drafting and editing of the manuscript, final approval of publication; C. Koziol-White - drafting and editing of the manuscript, final approval of publication

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Availability of data and materials

All data and materials that support the findings of this study are available from the corresponding author upon reasonable request.

Ethics approval and consent to participate

Human tissue was acquired through the National Disease Research Interchange (NDRI) and the International Institute for the Advancement of Medicine (IIAM) and was obtained commercially from anonymous donors and according to the procedures approved by the Rutgers University IRB. As such, the human tissue is exempt from requiring IRB approval. Although information concerning the cause of death, gender, race, age, body weight & height of the donor is available, there are no unique identifiers that can link the subject's identification to the tissue sample.

Consent for publication

Not applicable.

Competing interests

All authors declare that they have no competing interests.

Author details

¹Department of Pharmacology and Toxicology, School of Pharmacy, EOHSI, Rutgers University, Piscataway, NJ, USA. ²Rutgers Institute for Translational Medicine and Science, Rutgers, The State University of New Jersey, New Brunswick, NJ, USA.

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