CHARACTERIZATION OF TRANSFORMING GROWTH FACTOR-β1 IN β2 ADRENERGIC RECEPTOR DYSFUNCTION AND SIGNALING MECHANISMS IN HUMAN AIRWAY SMOOTH MUSCLE

By

ELENA CHUNG

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ABSTRACT OF THE THESIS

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Thesis Director: DR. REYNOLD A. PANETTIERI

Underlying source(s) of airway smooth muscle hypercontractility and hyporesponsiveness to bronchodilatory therapy in the context of inflammatory diseases of the lung remain unclear. In asthma and chronic obstructive pulmonary disease (COPD), it is unclear if the airway dysfunction is due to intrinsic genetic or epigenetic contribution that precipitate increased basal tone and enhanced contractile responses, or attenuate bronchodilation capability of the airways. Airway smooth muscle (ASM) cells are primary cells modulating bronchomotor tone, but that alterations in the cells may be a primary contributor to airway dysfunction associated with asthma and COPD. Studies suggest that β-adrenergic receptor (β2AR) dysfunction in the inflammatory environment occurs via exposure to inflammatory cytokines including exposure to IL-1β, IL-13, TNFα, and TGF-β, and lipid mediators. The purpose of this thesis was to characterize β2AR dysfunction within the context of TGF-β1

exposure, as well as to characterize responsiveness to dexamethasone, a glucocorticoid. Modulation of gene expression, cyclic AMP (cAMP) levels, and myosin light chain (MLC) phosphorylation have been posited to play important roles in regulating both bronchoconstriction and bronchodilation in asthma. Furthermore, β2AR agonists (β2-agonists), and both direct and indirect signaling components downstream of the receptor, are effective treatments for asthma and other inflammatory conditions to induce relaxation of the airways. However, persistent usage of these therapeutics causes desensitization and downregulation of the receptor. Overall, the focus of this thesis is examining the efficacy of bronchodilators in the context of TGF-β1 exposure, as well as the effects of dexamethasone in modulating the effects of TGF-β1.

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CHAPTER ONE

Literature Review of the Role of β2 Adrenergic Receptor and Signaling in Inflammatory Respiratory Diseases (Asthma and COPD)

1.0. GENERAL INTRODUCTION

1.1. Asthma and Chronic Obstructive Pulmonary Disease (COPD)

Asthma is the most prevalent chronic respiratory disease worldwide; chronic COPD is another respiratory disorder that is less prevalent but equally detrimental (1, 2). Patients with asthma and COPD rely heavily on β2-agonist bronchodilators to counterbalance bronchospasm that is a major characteristic of both diseases. β2-agonists are currently the mainstay therapy used for both acute and long-term control of pulmonary diseases and exacerbations of the diseases, and increasing evidence supports that frequent β2-agonist use leads to resistance and deterioration of asthma control (3–5). Additionally, the inflammatory environments of the lung in these diseases may contribute to decreased efficacy of β2 agonists (6–9).

1.2. β 2 Adrenergic Receptor (β2AR)

1.2.1. Characterizing β2AR Desensitization

β2-agonists target β2AR, a seven-transmembrane G protein coupled receptor, to activate downstream signaling to induce relaxation of the airways. The $G_{\alpha s}$ protein activates adenylyl cyclase (AC) and generates cAMP; increased levels of cAMP then activate protein kinase A (PKA) (10). In addition to $G_{\alpha s}$, the β2AR is coupled to the $G_{\alpha i}$ protein which releases the activated $G_{\alpha i}\alpha$ subunit, reducing AC activity (11, 12); the β2AR is coupled to βγ subunits that are known to activate mitogen-activated protein kinases

(MAPK) (10). Figure 1-1 pictorially represents the integration of β2AR signaling and its downstream responses.

G proteins, like $G_{\alpha i}$, are coupled to contractile receptors and are typically associated with the reduced $G_{\alpha s}$-stimulated AC activity thus, decreasing cAMP generation (13). The activation of these contractile receptors attenuate the actions of the β2AR through the activation of $G_{\alpha i}$ (14).

There are some evidence that there is variability in β2AR responses between asthmatic and non-asthmatic subjects (15, 16). Human data concerning β2AR function is highly variable, in part due to β2AR expression and in part due to variation in functional coupling to AC between individuals. A defect in asthmatic human airway smooth muscle (HASM) results in approximately 50% less cAMP production in response to different β2-agonists and approximately a 2-fold increased expression and activity of (phosphodiesterase 4) PDE4 in comparison to non-asthmatic HASM (17). In healthy and non-smoking subjects, the half maximal effective concentration (EC50) of the cAMP response to a β2-agonist varied by 39-fold and maximum specific binding (B_{max}) varied by 9-fold (18). β2AR is also highly expressed on epithelial cells from normal adults, but the receptor expression and its functional coupling to AC varies inter-individually (18).

β2AR desensitization has been shown to reduce β2-agonist therapeutic effects and has been associated with both airway inflammation and chronic β-agonist use. Mechanisms underlying desensitization of β2AR can be classified into 3 main types of processes: (A) phosphorylation, (B) sequestration, and (C) receptor downregulation.

1.2.1.1. Phosphorylation

Phosphorylation of the β2AR leads to receptor desensitization through direct interference with receptor coupling to $G_{\alpha s}$. In cells, phosphorylation activity is mediated by two distinct classes of serine/threonine kinases, which are second messenger-dependent such as protein kinase A (PKA) and protein kinase C (PKC), and -independent kinases such as GPCR kinases (GRK) (19). GPCR kinase 2 (GRK2), and GPCR kinase 3 (GRK3) are expressed in HASM, regulating receptor responsiveness (20). GRKs are ubiquitously expressed kinases that are part of a family of cAMP-dependent enzymes. They alter structural conformation of the receptor, reducing receptor interactions with $G_{\alpha s}$ and diminishing the affinity of receptors for ligands, shifting the β2AR towards an inactive state (21). Specifically, GRK2/3 may induce desensitization in airway smooth muscle, thereby attenuating β2AR signaling (20).

1.2.1.1.1. Single Nucleotide Polymorphisms Influence Protein Phosphorylation

β2AR, binds to catecholamines in various tissues (22). It is abundantly expressed in ASM cells and increasing evidence has reported that the ADRB2 gene is associated to airway hyperresponsiveness and is an associated response with available therapeutics (23, 24). *ADRB2* polymorphisms, commonly located at four types of point mutations to codons 16 and 34, have been postulated to be associated with prevalence of asthma as well as with severity of disease (20, 25). β2AR polymorphisms may influence β2-agonist-stimulated-desensitization (26) and common *adrb2* variants associated with asthma-related phenotypes are Arg16Gly, and Gln27Glu, Thr164Ile, and Val34Met amino acid positions, altering bronchial hyperresponsiveness (23). Interestingly, in a study with transfected

Chinese hamster fibroblast cells, Ile164 variant receptors displayed lower affinity to β2-agonist ligand binding properties causing desensitization in comparison to their controls without the inserted sequence at the N terminus, while Met34 β2AR variant presented with normal agonist (ISO) binding (27), suggesting distinguished properties within these potential regions participating in agonist-promoted downregulation. Ser165 involved with β2AR ligand binding, forms a hydrogen bond with β-hydroxyl group of catecholamine (28), and Ile164 variants do not contain β-hydroxyl groups, thus this suggests a perturbed specific interaction at Ser165 (28). Conformational changes are assumed to occur at other sites in the Ile64 variant receptor because antagonists bind at different sites after competing for the same binding site on the receptor. In CHO cells expressing Ile164, basal adenylyl cyclase (AC) activity was lowered with no agonist present, suggesting that the receptor did not go through as much receptor sequestration with saturating concentrations of the agonist.

The other common variants, including Gly16, have been linked to steroid insensitivity following long-acting β-agonist (LABA) use (25). In addition, Glu27 variant gives a bronchoprotective effect as it is associated with decreased hyperreactivity of the lower airways in asthmatics (25). Despite some evidence, there have been limited studies focusing on whether these and other variants contribute to asthmatic phenotypes due to few patients with these polymorphisms entering clinical studies. Genetic polymorphisms affect asthma and COPD susceptibility thereby reconfiguring β2AR signaling.

1.2.1.1.2. β2AR Sites Phosphorylated by GRKs and Recruitment of β-arrestin

Phosphorylation of the β2AR by GRK2/3 causes conformational changes that are induced or stabilized by agonist occupancy of the receptor, subsequently resulting in the

binding to another protein, β-arrestin (21). This form of the receptor is strictly agonist-dependent, and it is referred to as homologous desensitization state where PKA and PKC are activated via receptor stimulation (29). β-arrestin regulates silencing and trafficking of the receptor while also interacting with various signaling proteins for signal transduction (30). Hence, specific active receptor conformations are essential for β2AR phosphorylation and β-arrestin binding, and desensitization occurs by sterically blocking $G_{\alpha s}$ binding, facilitating receptor internalization for the coupling of β-arrestin (31). The previously mentioned kinases, GRK2/3 and PKA are two different signaling mechanisms by which desensitization of β2AR occurs, where β-arrestins serve important roles both pathways.

Another type of desensitization, termed heterologous desensitization of β2AR is an independent G protein uncoupling phenomenon catalyzed by other GPCR kinases (32). Phosphorylation of the receptors within the third intracellular loop sites is "turned off" leading to a desensitizing response (10). The work presented in this thesis examines the role of these types of pathways in downregulation of β2AR signaling following TGF-β1 exposure.

1.2.1.2. Sequestration

Sequestration is another mechanism of β2AR desensitization. Sequestration is an agonist-induced process where there is a rapid reduction in the number of the unliganded β2AR on the cell surface (33, 34), thereby leaving the receptor unavailable to signal. A study in β2-agonist-treated lymphoma cells demonstrated rapid sequestration of the β2AR from the cell surface, contributing to a homologous desensitization process where there was an uncoupling of components in the AC system, physically dividing them from the

receptor (35). The study noted β2AR binding is assessed based on its high- or low- affinity state and this determines agonist-induced stimulation enzyme activity (35). There is reduced hydrophilic ligand binding and hydrophilic agonist affinity of binding subsequent to homologous desensitization and thus, demonstrating characteristics of sequestration (35). Sequestration also occurs in heterologous desensitization as seen in human airway epithelial BEAS-2B cells after β2-agonist and an EP2 receptor-agonist exposure (36); the sequestration activates the PKA and AC pathways, thereby decreases downstream signaling activity (36).

In addition, evidence has shown that activated ERK, a downstream component coupled to serine/threonine kinases, attenuates the steady-state cell surface expression of various GPCRs, suggesting sequestration is essential in ERK1/2 activation and in pathways regulating responses to β2AR stimulation (37). β-arrestins, providing a scaffold for ERK1/2 binding and activity for those kinases, can prevent dimerization in the cytoplasm and prolong MAPK/ERK kinase 1 (MEK1) interaction, thus impeding on nuclear translocation (37). Together, β2AR internalization and the process of receptor sequestration promotes agonist-dependent desensitization.

1.2.1.3. Downregulation

Downregulation with prolonged agonist exposure results in β2AR desensitization as well. There are at least two pathways for β2AR downregulation: 1. reduction in receptor mRNA levels from transcript destabilization (38); and 2. loss of pre-existing receptor binding sites (39, 40). The total receptor density is diminished through

compartmentalization, decreased transcription, enhanced mRNA degradation, and augmented receptor protein degradation following prolonged agonist exposure (41). Downregulation of microRNA (miRNA) *let-7f* establishes a baseline level of expression of β2AR; with prolonged exposure of a β2-agonist, *let-7f* levels are decreased, promoting *adrb2* gene silencing and leading to a reduction in agonist-induced via downregulation (42). Furthermore, β2AR downregulation is crucial because variants of the human β2AR are associated with downregulation of the receptor β2-agonist responses and attenuated β2-agonist-induced responses (38).

1.3. The Effects of Inflammatory Mediators on Signaling Cascades Downstream of the β2AR

1.3.1. Dysfunction of the β2 Adrenergic Receptor

Desensitization to β2-bronchodilators is associated with dysfunction of the β2 adrenergic receptor (β2AR)/adenylyl cyclase (AC) pathway that mediates airway dilation (43). β2-agonists facilitate airway smooth muscle relaxation through the cyclic adenosine monophosphate (cAMP)-protein kinase A (PKA) phosphorylation cascade via stimulation of AC enzyme activity (44). Inflammatory mediators including cytokines, chemokines, growth factors, and lipid mediators are released by cells such as eosinophils, lymphocytes, mast cells, and phagocytes (45, 46). All of these inflammatory mediators are released by airway smooth muscle (47, 48) and airway epithelial cells (49–51). These mediators activate signaling through GPCRs, growth factor receptors, and cytokine receptors that may alter the ability of the β2AR to induce dilation of the airways (52). Thus, elucidating

how these mediators alter the molecular signal transduction mechanisms leading to dysfunction of the β2AR will improve the efficacy of the therapeutics available for the world's most prevalent airways diseases.

1.3.2. Inflammatory Mediators in Lung Diseases

Inflammatory mediators including histamine, prostaglandins (PGs), chemokines, and cytokines, are released by a number of different cell types in the airways (53). Some products released from these cells evoke bronchospasm, while others damage the epithelium and induce airway smooth muscle cell (ASM) proliferation (54). Some of these inflammatory mediators can regulate β2AR responsiveness via multiple mechanisms in bronchial ASM cells (55, 56) and in epithelial cells (6, 8).

1.3.3. Interleukin-13

Interleukin-13 (IL-13), a T helper 2 cytokine highly expressed in the lungs of asthmatics, impairs the ability of β2-agonists to induce dilation of the airways (57). Production of inflammatory lipid mediators, has been shown to positively correlate with IL-13-stimulated phosphorylation of both β2AR and GRK2 (58). IL-13-mediated phosphorylation attenuated cAMP production after β2-agonist stimulation. In human asthmatic epithelial cells and tissue, 15LO1, an enzyme elevated in asthmatics, binds to PEBP1 and is associated with diminished GRK binding to phosphatidylethanolamine-binding protein 1 (PEBP1) (58). In this study, 15LO1 binding to PEBP1 dissociated from Raf-1 and activated MAPK/(Extracellular Signal-Regulator Kinase) ERK (59), attenuating cAMP production in the presence of IL-13 (58). These data demonstrate β2AR

internalization after receptor stimulation and β-arrestin binding following treatment with IL-13, suggesting a GRK2-dependent mechanism that results in phosphorylation and desensitization of the β2AR. IL-13 can also promote agonist-induced calcium signaling, evoking ASM responsiveness and modulating contractility in HASM (60). This mechanism by which IL-13 modulates ASM function with intracellular calcium levels can be linked to PKA activation and may offer therapeutic approaches to treat asthma-associated hyperresponsiveness.

1.3.4. Tumor Necrosis Factor α

Tumor Necrosis Factor alpha (TNFα), produced mainly by Immunoglobulin E (IgE)-dependent macrophages- and mast cell- activation in allergic responses (61), is augmented in the airways of asthma and COPD subjects (62). Evidence suggests that expression of TNFα is associated with increased airway obstruction (63, 64). TNFα may elicit β2AR desensitization via prostaglandin E_2 receptor 2 (EP2) receptor- and PKA-dependent mechanisms in ASM. The absence of EP2 receptor in mice tracheal rings allowed the airways to be resistant to TNFα-mediated β2AR desensitization (63, 64).

Additionally, others have demonstrated that engagement of the p55 receptor, a subunit of NF-κB, or tumor necrosis receptor 1 (TNFR1) may mediate attenuation of β2AR-induced signaling. Activated TNFR1, expressed by all human tissues (65), is coupled to downstream transducer tumor necrosis factor-associated factor 2 (TRAF2). TRAF2 stimulates NF-κB and modulates Ca^{2+} homeostasis (66), possibly targeting at the level of the G protein and phospholipase C in ASM. Parallel studies have demonstrated

TNFα-induced G_q and G_i protein expression in HASM cells, further linking smooth muscle hyporesponsiveness to β2-agonists in the presence of TNFα (67). While anti-TNFα therapy to improve the condition of steroid-dependence in asthmatic patients appears to be promising, (68, 69), there is a lack of studies linking anti-TNFα treatment to the improvement of lung function with bronchodilator therapy.

1.3.5. Interleukin-1 beta

Interleukin-1 beta (IL-1β) is pro-inflammatory cytokine released by human airway epithelial cells (HAEC), and regulates mucosal inflammation (70) and modulates cell stiffness (71). In HAEC, IL-1β attenuates mucociliary clearance by impairing cAMP expression in response to β2-agonists (53), and also mediates cell migration via NF-κB signaling (72). Increased expression of IL-1β in the airways resulted in decreased β2AR-mediated relaxation of rat, guinea pig, rabbit and dog ASM (73, 74). IL-1β binds to its receptor IL-1R1 and induces cyclooxygenase-2 (COX-2) and microsomal prostaglandin synthase-1 (mPGES-1) expression, augmenting the levels of production and secretion of EP2. EP2 agonists subsequently activates receptor EP2 which stimulates cAMP production, ultimately activating PKA (55, 75). IL-1β reduced agonist-induced cAMP generation via cyclooxygenase (COX)-2 stimulation and prostanoid release (76, 77). These studies indicate that prostanoid release by IL-1β contributes to β2AR hyporesponsiveness and that heterologous desensitization induced by EP2 is due to the attenuation of β2-agonist-induced cAMP.

1.3.6. Transforming Growth Factor Beta 1

Transforming growth factor beta 1 (TGF-β1), a pro-inflammatory cytokine that augments airway contractile responses (55) and contributes to remodeling of the airways, plays an extensive role in the pathogenesis of asthma and fibrotic lung diseases (78). Previous studies suggest that TGF-β1 modulates β2AR-mediated responses via a protein synthesis-dependent mechanism (79, 80) and attenuates β2AR responsiveness in HASM cells (81, 82). TGF-β1 reduced β2-agonist-evoked bronchodilation demonstrated in human precision cut lung slices (PCLS) displaying similar effects by other cytokines such as IL-1β and IL-13 and altering β2AR phosphorylation like fibroblast growth factor (81).

TGF-β1 modulates Rho kinase-dependent contractile signaling by augmenting MLC phosphorylation in HASM cells (81, 83). The effects by TGF-β1 may also upregulate protein ARHGEF1 expression further altering RhoA-dependent phosphorylation (84). Interestingly, ARHGEF1, coupled to contractile signaling pathways, may be activated by β-arrestins through downregulation of β2AR thus attenuating the effects of a bronchodilator through the activation of Rho kinase leading to contraction of HASM (85).

Our recent studies demonstrate that TGF-β1 decreases β2-agonist downstream cAMP relaxation responses via a Smad2/3-dependent mechanism in HASM cells through upregulation of PDE4D expression (86). Our current studies show that TGF-β1 attenuates intracellular cAMP levels in HASM through the modulation of G_s-dependent signaling, as well as attenuated reversal of agonist-induced myosin light chain phosphorylation in the presence of TGF-β1 (86). The results and our previous data suggest that TGF-β1 modulates not only remodeling associated with asthma, but also attenuates the actions of bronchodilators used in asthma therapy.

1.3.7. Modulation of β2AR function by Glucocorticoids

Glucocorticoids (GC), a class of corticosteroids, influences function of the β2AR, including β2-agonist-induced bronchial hyporesponsiveness. Inhaled glucocorticoids, the a very effective anti-inflammatory class of medication have variable effects in asthmatic patients compared to non-asthmatic subjects. The administration of corticosteroids to asthmatic patients reduces their chronic airway inflammation via the action of activated glucocorticoid receptors (GR), and has been shown to contribute to the restoration of β2AR responsiveness (87), although exact mechanisms by which they restore β2AR responsiveness are unclear.

Glucocorticoids primarily mediate their effects on the synthesis of cytokines and other inflammatory mediators through GR, a ligand-activated transcriptional factor belonging to the superfamily of nuclear hormone receptors (88). Without GCs, the GR and other proteins remains inactive in the cytoplasm until the ligand binds. Activated GR translocates from the cytoplasm to the nucleus where it functions as a homodimer, binding GC response elements (GREs) to regulate gene expression. Inflammatory signaling cascades are attenuated through GR-dependent transrepression (89). The transrepression mediates anti-inflammatory action with GR and antagonizes DNA-bound transcription factors such as NK-κB and activator protein-1 (AP-1) (90). The C-terminal domain that binds to the steroid, also contains binding sites for heat shock protein (hsp) 90. When the steroid-binding domain is removed, the active GR molecule becomes a transcriptional repressor.

Corticosteroids are lipophilic and accumulate in the lipid membranes, consequently altering membrane fluidity and may cause dysfunction of embedded proteins such as ion

channels and receptor proteins (91). In immune cells, GC interactions with plasma membranes lead to rapidly reduced calcium and sodium cycling across the membrane, contributing to immunosuppression and reduction of inflammation (92, 93). Additionally, glucocorticoids have been also shown to increase high-affinity β2-agonist binding in human neutrophils, and neutrophils can exchange information with macrophages, dendritic cells and other cells in the adaptive immune system (94). Given this information, there is a capacity for GCs to regulate β2AR signaling independently of the canonical effects on gene expression (95).

1.3.8. Interactions Between Glucocorticoids and β2-Agonists in Inflammatory Diseases

Resensitization of the β2AR may occur by regulating the coupling of receptor G protein and AC activation (19). β2AR desensitization causes rapid uncoupling of second messengers by phosphorylation of the receptor (29), and glucocorticoids (GC) can modulate pathways that have been downregulated with chronic β2-agonist exposure (96).

The combination therapy of inhaled glucocorticoids and β2-agonists synergistically inhibit the effects caused by proinflammatory signaling pathways (97). Additionally, the combination therapy decreases the development of tolerance with chronic β2-agonist therapeutic use in asthma and COPD patients (98, 99). Specifically, dexamethasone (DEX), a steroid used to treat airway inflammatory and autoimmune conditions, augments cAMP production by airway epithelial cells and bovine smooth muscle cells in response to β2-agonist, isoproterenol (ISO) (100). Additionally, $G_{\alpha s}$-knockdown in steroid-treated HASM

cells diminished agonist-induced cAMP production, suggesting that glucocorticoids stimulate $G_{\alpha s}$ protein and induce cAMP generation (101).

Glucocorticoids have been indicated to augment β2AR expression in some systems (102); literature supports glucocorticoids activate genomic signaling (103) and may enhance bronchodilation via non-genomic signaling (101, 104). Acute β2-agonist treatment combined with budesonide, another GC, augments bronchodilation and attenuates contractile agonist-induced phosphorylation of myosin light chain (105). Thus, it is critical to study the relationship of impaired β2AR function by exposure to inflammatory cytokines.

1.3.9. Glucocorticoid Insensitivity in Smooth Muscle Cells and Epithelial Cells

Inflammatory conditions in a number of organs have been shown to exhibit corticosteroid-resistant properties (106, 107). A subset of patients with COPD have been shown to be unresponsive GCs (108). This hyporesponsiveness may occur due to oxidative or nitrative stress-induced histone deacetylase 2 activity, an enzyme that plays an important role in regulation of gene expression and transcriptional repression (109).

In asthma subjects, there is a high incidence of glucocorticoid insensitivity. A study suggests an imbalance in expression of GRα and GRβ (isoforms of the GR), with increased expression of GRβ competing with GRα, impeded ability of GR to interact with GREs (110). It has been shown that decreased GRα nuclear translocation in GC-insensitive subjects is present in airway cells from bronchoalveolar lavage (BAL) (111). In addition to GR's role in transcription, impaired GR phosphorylation may reduce GC-induced functions in HASM cells, mediating TNFα-induced sensitivity (111) and IL-1β-induced

GR insensitivity in rodent bronchial smooth muscle. In HASM, GC insensitivity is also mediated by impaired GR site-specific phosphorylation (111) and promoted by the transcription factor interferon regulatory factor 1 (IRF-1). Furthermore, it is important to study the potential mechanisms underlying corticosteroid insensitivity observed in HASM and HAE from patients with severe asthma.

1.4. Conclusion and Future Perspectives

Agonists of the β2AR have been a mainstay of asthma and COPD therapy used to relieve bronchospasm in these lung disorders. However, the inflammatory environment, as well as genetic contributions, alter responsiveness to β2-agonists and attenuate the bronchodilation they elicit. Possible mechanisms by which the inflammatory environment alter these responses are discussed, and these mechanisms provide novel and important points for therapeutic intervention in inflammatory lung diseases.

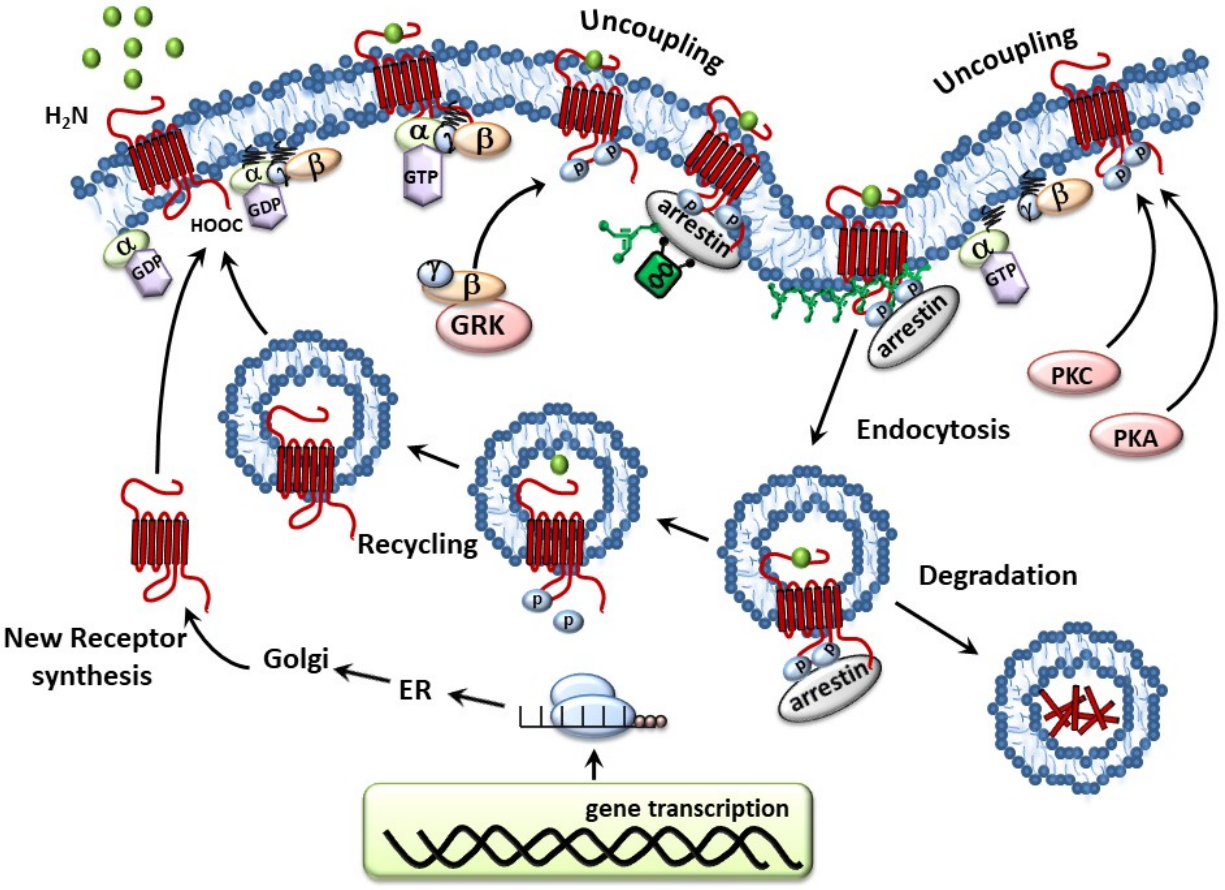
Uncoupling
Uncoupling
H_2N
HOOC
GDP
GTP
GRK
arrestin
arrestin
PKC
PKA
Endocytosis
Recycling
Degradation
New Receptor synthesis
Golgi
ER
gene transcription

Figure 1-1. Schematic illustration of the signaling pathways elicited by the β2AR dysfunction in HASM. Shown is a representation of molecular signaling mechanisms mediating dilation of the airways and desensitization of the β2AR. Inflammatory mediators and their effects may alter receptor expression and signaling downstream signaling of the receptor. Steroids and other therapeutics utilized in respiratory diseases like asthma and COPD can target the β2AR, providing ways to improve disease management. (This diagram is by courtesy of William Jester)

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CHAPTER TWO

TGF-β1 Decrease β2-Agonist-Induced Relaxation in Human Airway Smooth Muscle

Adapted from Ojiaku CA, **Chung E**, Parikh V, Williams JK, Schwab A, Fuentes AL, Corpuz ML, Lui V, Paek S, Bexiga NM, Narayan S, Nunez FJ, Ahn K, Ostrom RS, An SS, and Panettieri RA. Transforming Growth Factor-β1 Decreases β2-Agonist–induced Relaxation in Human Airway Smooth Muscle. Am J Respir Cell Mol Biol 2019; 61:209–218.

I provided Figures 2-1B, 2-2B, 2-4B-C, 2-E2 for the manuscript as well as facilitated with manuscript revisions.

2. TGF-β1 DECREASE β2-AGONIST-INDUCED RELAXATION IN HUMAN AIRWAY SMOOTH MUSCLE

2.2. Abstract

Helper T effector cytokines implicated in asthma modulate the contractility of human airway smooth muscle (HASM) cells. We have reported recently that a profibrotic cytokine, transforming growth factor beta 1 (TGF-β1), induces HASM cell shortening and airway hyper-responsiveness (AHR). Here we assessed whether TGF- β1 affects the ability of HASM cells to relax in response to β2-agonists, a mainstay treatment for AHR in asthma. Overnight TGF-β1 treatment significantly impaired isoproterenol (ISO)-induced relaxation of carbachol-stimulated isolated HASM cells. This single-cell mechanical hypo-responsiveness to ISO was corroborated by sustained increases in myosin light chain (MLC) phosphorylation. In TGF- β1 treated HASM cells, ISO evoked markedly lower levels of intracellular cAMP. These attenuated cAMP levels were, in turn, restored with pharmacological and siRNA inhibition of PDE4 and Smad3, respectively. Most strikingly, TGF-β1 selectively induced PDE4D gene expression in HASM cells in a Smad2/3-dependent manner. Together these data suggest that TGF-β1 decreases HASM cell β2-agonist relaxation responses by modulating intracellular cAMP levels via a Smad2/3-dependent mechanism. Our findings further define the mechanisms underlying β2-agonist hypo-responsiveness in asthma, and suggest TGF-β1 as a potential therapeutic target to decrease asthma exacerbations in severe and treatment-resistant asthma.

2.3.Introduction

β2-agonist bronchodilators are a mainstay therapeutic used for acute and long-term control of asthma exacerbations. However, patients with severe asthma often respond poorly to β2-agonists, and increasing evidence demonstrates that frequent β2-agonist use leads to resistance and deterioration of asthma control (1, 2). Therefore, understanding the mechanisms mediating β2-agonist resistance is important for decreasing asthma-related morbidity and mortality.

Evidence suggests a link exists between β2-adrenergic receptor (β2AR) hypo-responsiveness and airway hyper-responsiveness (AHR), where increased levels of bronchoconstriction can decrease bronchodilator responsiveness (2, 3). Unsurprisingly, several cytokines modulate hyper-responsiveness and β2-agonist resistance in human airway smooth muscle (HASM), the main regulator of bronchomotor tone (4, 5). We have previously reported that transforming growth factor β1 (TGF-β1)–a pro-fibrotic cytokine elevated in the airways of patients with asthma–augments agonist-induced contractile responses in HASM via a Smad3-dependent pathway (6). However, the role of TGF-β1 in modulating β2-agonist-induced relaxation responses in HASM remains unknown.

β2-agonists induce airway relaxation by binding to β2-adrenergic G-protein coupled receptors (GPCRs) on HASM cells, stimulating adenylyl cyclase (AC) enzyme activity (7). AC activation by the β2AR G_s alpha subunit elevates intracellular cyclic adenosine monophosphate (cAMP) levels, and increased cAMP leads to subsequent HASM cell relaxation by antagonizing HASM cell contractile pathways. HASM cell relaxation responses are also regulated by the action of prostaglandin E2 (PGE2), an arachidonic acid-derived mediator that exerts its effects via prostanoid EP receptors from the GPCR family (8). Stimulation of the G_s-coupled EP2 and EP4 receptor subtypes elevates intracellular

cAMP levels via activation of AC, with EP4 receptor stimulation selectively leading to HASM cell relaxation (9, 10).

Intracellular cAMP levels in HASM cells are regulated by the balance between AC activation and cAMP-hydrolyzing phosphodiesterase (PDE) activity. While HASM cells express multiple PDE isoforms (11), functional studies have established PDE3 and PDE4 as the major cAMP hydrolyzing enzymes (12–14). PDE4, in particular, plays a pivotal role in HASM cell cAMP degradation and is more widely studied as a therapeutic target in airway disease (15). Of the four PDE4 encoding genes (16), evidence supports a critical role for PDE4D in mediating HASM cell contractile and relaxation responses (17–20). Increased PDE4D activity and expression is associated with decreased β2-agonist-induced cAMP generation in HASM from subjects with asthma (20). Mice deficient in PDE4D also exhibit a loss of responsiveness to cholinergic stimulation (10), suggesting the therapeutic potential of PDE4D inhibitors in asthma.

Previous studies investigating the role of TGF-β1 in decreased β2AR responses have been purely biochemical in nature and largely limited to human tracheal smooth muscle cells and human lung embryonic fibroblasts (21, 22). As these studies were conducted in the presence of PDE inhibitors, neither study assessed the potential of TGF-β1 to modulate downstream components of the cAMP signaling pathway via PDE4. Therefore, we aimed to elucidate the mechanisms by which TGF-β1 modulates β2-agonist-induced relaxation responses in HASM cells.

2.4.Methods

2.4.1. Human Airway Smooth Muscle (HASM) Cell Culture

Human lungs from otherwise healthy, aborted transplant donors were received from the International Institute for the Advancement of Medicine (IIAM; Edison, NJ, USA) and the National Disease Research Interchange (NDRI; Philadelphia, PA, USA). HASM cells were isolated from the trachea and cultured as previously described (23).

2.4.2. Immunoblot Analysis

Confluent HASM cells were serum starved overnight prior to treatment and collected as previously described (24).

2.4.3. Magnetic Twisting Cytometry (MTC)

Dynamic changes in cell stiffness were measured as an indicator of the single-cell contraction and/or relaxation of isolated HASM cells as previously described (25, 26). Briefly, RGD-coated ferrimagnetic microbeads bound to the cytoskeleton were magnetized horizontally and then twisted in a vertically-aligned homogeneous magnetic field that varied sinusoidally in time (27). The ratio of specific torque to bead displacements is expressed here as the cell stiffness in units of Pascal per nm (Pa/nm).

2.4.4. Small Interfering RNA (siRNA) Transfection

In vitro siRNA knockdown was performed using a reverse transfection procedure as previously described (28). HASM cells were seeded onto cell culture plates for a final siRNA concentration of 10 μM.

2.4.5. Measurement of Cyclic AMP Levels

Following stimulation, cAMP levels were measured in lysed HASM cells using the Applied Biosystems cAMP-Screen® ELISA system according to manufacturer protocol. For kinetic measurement of cAMP production in live cells, HASM cells were infected with a recombinant BacMam virus expressing the cADDis cAMP sensor (Montana Molecular, Bozeman, MT) as previously described (29). Cells were stimulated with agonist then fluorescence measured at 30 second intervals for 30 minutes. Data were fit to a single-site decay model using GraphPad Prism 7.0 (GraphPad Software Inc., San Diego, CA). Concentration-response curves were generated from each decay curve by multiplying the kinetic rate constant, k, with the plateau.

2.4.6. Quantitation of Phosphodiesterase (PDE) Gene Expression

RNA was isolated from HASM cells using the RNeasy Mini Kit (Qiagen Sciences, Inc., Germantown, MD, USA). cDNA was generated using SuperScript™ IV First-Strand Synthesis System (Thermo Fisher Scientific, Waltham, MA, USA). Relative cDNA quantification was performed using TaqMan quantitative RT-PCR (Thermo Fisher Scientific, Waltham, MA, USA) and the $\Delta\Delta C_t$ method, and expression was normalized to β-actin control.

2.4.7. Statistical Analysis

Unless otherwise stated, statistical analysis was conducted using GraphPad Prism software (La Jolla, CA, USA), with significance evaluated at a p-value of < 0.05. Significance was determined using Fisher's Least Significant Differences tests or multiple

t-tests with Holm-Sidak correction. For MTC experiments involving multiple lung donor cell responses, statistical analysis was conducted using mixed effect models using SAS V.9.2 (SAS Institute Inc., Cary, NC) (30).

2.5.Materials

Compounds were purchased from Sigma Aldrich (St. Louis, MO, USA) [isoproterenol, prostaglandin E2, carbachol, perchloric acid], Selleck Chemicals (Houston, TX, USA) [roflumilast], Cayman Chemicals (Ann Arbor, MI, USA) [3-isobutyl-1-methylxanthine (IBMX)], and R&D Systems (Minneapolis, MN, USA) [TGF-β1, SB-431542]. Immunoblot antibodies were purchased from Cell Signaling Technologies (Danvers, MA, USA) [pMLC(3674S)] and EMT Millipore (Billerica, MA, USA) [MLC(MABT180)]. siRNA was purchased from Thermo Fisher Scientific (Waltham, MA, USA) [Smad3(VHS41114)] and Dharmacon (Lafayette, CO, USA) [Smad2(L-003561-00), Non-targeting Pool(D-001810-10-05)].

2.6.Results

2.6.1. TGF-β1 Decreases β2-Agonist-Induced Relaxation in HASM Cells

To determine the extent to which TGF-β1 mediates resistance to β2AR-induced relaxation in HASM cells, we investigated contractile outcomes in TGF-β1-pretreated HASM cells stimulated acutely with the β-agonist isoproterenol (ISO) (Fig. 2-1). Single-cell relaxation responses were determined using magnetic twisting cytometry (MTC), a technique that measures changes in cell stiffness as a surrogate for agonist-induced force

generation (26). TGF-β1 or vehicle pretreated cells were pre-contracted to carbachol and stimulated acutely with ISO. TGF-β1 significantly impaired ISO-induced single-cell relaxation in basal and carbachol-stimulated HASM cells as compared to vehicle control (Fig. 2-1A). No significant changes in cell stiffness were observed in non-stimulated vehicle controls for the duration of our measurements (data not shown) (25, 26). To further confirm TGF-β1's effects on HASM cell contractile responses, we investigated the phosphorylation of MLC–an essential component of agonist-induced HASM cell contraction–following overnight TGF-β1 treatment. TGF-β1 augmented basal and agonist-induced MLC phosphorylation in a similar manner to previously published literature (6). Following stimulation with ISO, MLC phosphorylation in TGF-β1-treated HASM cells remained significantly higher than that of vehicle control (Fig. 2-1B). Notably, the addition of the contractile agonist carbachol to TGF-β1 and ISO-treated HASM cells significantly increased MLC phosphorylation to levels above that in TGF-β1 and ISO-treated HASM cells.

2.6.2. TGF-β1 Blunts Agonist-Induced cAMP Levels

To elucidate the mechanism by which TGF-β1 reduces HASM cell relaxation responses, total cAMP levels were measured in lysed TGF-β1-treated HASM cells. In TGF-β1 treated cells, ISO- and PGE2-induced cAMP levels were decreased versus that of respective control (Fig. 2-2A). TGF-β1 treatment did not alter forskolin-stimulated cAMP levels (Fig. 2-2B), suggesting that AC function was not negatively affected by TGF- β1; there were no significant differences in forskolin-evoked cAMP levels in vehicle control and TGF- β1 treated HASM cells.

To further confirm these results, cAMP levels were monitored in live HASM cells pretreated with either vehicle or TGF-β1. In TGF-β1 treated cells, ISO-induced cAMP responses were 2.6-fold less potent and 1.7-fold less efficacious compared to the vehicle-treated control (Fig. 2-2C, 2-E1A-B). TGF-β1 treatment appeared to decrease the potency of PGE2-stimulated cAMP responses, although this increase did not reach significance due to large variation of PGE2 responses between donors (Fig. 2-2D, 2-E1C-D). Forskolin-stimulated cAMP responses were unaffected by TGF-β1 treatment in live HASM cells (Fig. 2-3E, 2-E1E-F).

2.6.3. PDE Inhibition Rescues ISO-Stimulated Responses in TGF-β1-Treated HASM Cells

Intracellular cAMP levels are primarily reduced via hydrolysis–an effect mediated by the action of PDEs in HASM cells (31). To determine whether TGF-β1 mediates β2-agonist hypo-responsiveness by modulating PDE-mediated cAMP hydrolysis, MLC phosphorylation and cAMP levels were measured in TGF-β1 and ISO-treated HASM cells in the presence or absence of the pan-PDE inhibitor IBMX (Fig. 2-3, 2-E2A).

MLC phosphorylation in HASM cells was increased following TGF-β1 treatment, and levels remained higher than vehicle control following ISO stimulation (Fig. 2-3A, *left*). Treatment with IBMX, however, reduced MLC phosphorylation in TGF-β1-pre-treated, ISO-stimulated HASM cells to a level similar to that of vehicle control (Fig. 2-3A, *left*). In ISO-stimulated HASM cells, MLC phosphorylation levels were increased in TGF-β1 and carbachol-treated cells above those in TGF-β1-treated cells alone (Fig. 2-3A, *right*). IBMX

treatment decreased MLC phosphorylation in TGF-β1 and carbachol-treated cells to a level similar to that of vehicle control (Fig. 2-3A, *right*).

We next investigated the role of PDE activity in TGF-β1-mediated decreases in ISO-induced cAMP (Fig. 2-3B). Vehicle or TGF-β1-treated HASM cells were pre-treated with IBMX prior to ISO stimulation. IBMX pretreatment significantly elevated ISO-induced cAMP levels in TGF-β1-treated HASM cells (Fig. 2-3B).

2.6.4. TGF-β1 Induces PDE4D Gene Expression in a Concentration-Dependent Manner

To determine the extent to which PDEs contribute to β2-agonist hypo-responsiveness in TGF-β1-treated HASM cells, we investigated the expression of HASM cell-specific PDEs in TGF-β1-treated HASM cells (Fig. 2-E3) (29). TGF-β1 selectively increased PDE4D gene expression in a concentration-dependent manner (Fig. 2-4A, 2-E3). Furthermore, inhibition of TβR-I receptor signaling with SB-431542 pretreatment blocked increased PDE4D gene expression evoked by TGF-β1.

To further determine the extent to which TGF-β1 modulates PDE4D to decrease β2-agonist-induced relaxation responses, cAMP accumulation, MLC phosphorylation, and cell stiffness were measured in HASM cells treated with the PDE4 inhibitor roflumilast (Fig. 2-4B-D). Roflumilast pretreatment rescued blunted ISO-stimulated cAMP levels in TGF-β1-treated cells (Fig. 2-4B). In the presence of roflumilast, TGF-β1-induced MLC phosphorylation in ISO-stimulated cells showed little increase over vehicle control (Fig. 2-4C, 2-E2B). Additionally, roflumilast pretreatment decreased augmented HASM cell stiffness in TGF-β1 and ISO-stimulated HASM cells (Fig. 2-4D).

2.6.5. TGF-β1 Decreases β2-Agonist-Induced Relaxation Responses in a Smad2/3-Dependent Manner

The canonical TGF-β1 signaling pathway involves the activation of Smad2/3–intracellular signaling proteins that mediate a variety of TGF-β1's effects on HASM cell signaling in asthma (32). To determine the role of Smad proteins in TGF-β1-mediated inhibition of HASM cell relaxation responses, we investigated TGF-β1's modulation of ISO-induced cAMP levels in Smad2/3 siRNA-transfected cells (Fig. 2-5). ISO-induced cAMP was significantly increased in Smad3 siRNA-transfected cells in the presence and absence of TGF-β1 treatment (Fig. 2-5A). TGF-β1 blunted ISO-induced cAMP levels in HASM cells transfected with non-targeting and Smad2 siRNA, but had little effect on ISO-induced cAMP levels in Smad3 siRNA-transfected HASM cells.

To determine the role of Smad signaling in TGF-β1-mediated induction of PDE4D gene expression, PDE4D gene expression was investigated in Smad2 or Smad3 siRNA-transfected HASM cells following overnight TGF-β1 treatment (Fig. 2-5B). Smad2 and Smad3 knockdown reduced PDE4D gene expression induced by TGF-β1 treatment of HASM cells (Fig. 2-5B).

2.7.Discussion

In the present study, we demonstrate that TGF-β1 attenuates β2-agonist-induced relaxation responses in HASM cells. To date, TGF-β1 has been shown to negatively modulate β-adrenergic responses in multiple cell types (21, 22, 33, 34). Here, we demonstrate that TGF-β1 treatment – in the presence or absence of the contractile agonist

carbachol – significantly attenuates ISO-induced HASM cell relaxation via increased cell stiffness and MLC phosphorylation (Fig. 2-1). Importantly – as β1 agonists have little bronchodilator effect in humans and HASM cell beta receptors are solely of the β2 subtype – this study selectively demonstrates the effects of TGF-β1 and the β-agonist ISO on β2AR-induced relaxation (35, 36). While previous studies suggest that TGF-β1 modulates β2AR-mediated responses through a protein synthesis-dependent mechanism, the details by which this modulation occurs is not fully understood (21, 22). For the first time, we demonstrate that TGF-β1's effects on HASM cell relaxation responses occur via a Smad2/3 pathway that upregulates the expression of PDE4D. Collectively, our findings further establish TGF-β1 as a mediator of bronchodilator resistance via modulation of downstream cAMP pathway effects.

Previous studies suggest that TGF-β1 attenuates ISO-induced cAMP accumulation by negatively regulating β2AR number, protein, and gene expression (21, 22). However, our data suggest yet an additional mechanism for the attenuation of cAMP by TGF-β1. In our study, TGF-β1 blunted cAMP induced by both ISO and PGE2, a mediator that binds to the G_s/(G_i)-associated prostaglandin EP2 and EP4 G protein-coupled receptors to elevate intracellular cAMP levels (Fig. 2-2A, 2-2C, 2-2D) (37). Little is known regarding TGF-β1's effects on EP receptor expression in HASM, and it is unlikely that TGF-β1 blunts HASM cell cAMP by decreasing the expression of two independent G_s-coupled receptors.

Interestingly, other studies suggest a role for TGF-β1 in modulating G protein function. Treatment with pertussis toxin, an irreversible G_i inhibitor, blocked TGF-β1-induced PGE2 production in human lung fetal fibroblasts (38). Additionally, a report demonstrating an augmentation of cholera and pertussis toxin-induced ADP-ribosylation

in TGF-β1-treated rat osteoblast-like cells suggests that TGF-β1 alters the abundance of both G_s and G_i proteins (39). TGF-β1 also modulates the expression of guanine nucleotide exchange factors (GEF) – proteins that regulate the activity of small G proteins – in various cells (40, 41). A study in murine fibroblasts suggests that TGF-β1 increases GTPase activity via a pertussis-sensitive mechanism (42). Further studies will be needed to investigate whether TGF-β1 modulates G protein expression or activity in HASM cells, and whether this potential modulation further affects HASM cell relaxation responses. However, our present results suggest that TGF-β1 – in addition to attenuating β2AR function – works downstream of the receptor level to impair ISO-stimulated cAMP levels.

We used forskolin – a direct activator of AC – as a tool to further investigate TGF-β1's downstream effects on the cAMP signaling pathway (43). In this study, TGF-β1 did not significantly alter forskolin-stimulated cAMP levels in HASM cells (Fig. 2-2B, 2-2E). Current literature suggests an unclear role for cytokines in modulating AC activity. In previous reports using human and guinea pig airway smooth muscle, TGF-β1 treatment induced little or modest reductions in forskolin-stimulated cAMP accumulation (21, 34). Curiously, other reports demonstrate that chronic cytokine treatment sensitizes AC in HASM (44). In these studies, chronic incubation of HASM cells with the cytokine IL-1β or TNF-α caused a 2- to 3-fold increase in forskolin-stimulated cAMP (44, 45). It is posited that AC sensitization may be a feedback response to upregulate relaxation pathways in the face of cytokine-induced airway hyperresponsiveness (45). While TGF-β1 induces hyperresponsiveness in HASM cells (6), we did not find significant alteration of forskolin-stimulated cAMP in TGF-β1-treated HASM cells, (Fig. 2-2B). Thus, further studies will be needed to determine the effect of TGF-β1 on AC activation.

As TGF-β1 did not negatively regulate AC function in HASM cells, we next investigated the role of cAMP-hydrolyzing PDE enzymes in TGF-β1's attenuation of HASM cell relaxation responses. Previous reports suggest that TGF-β1 modulates PDE4 expression and activity. In human alveolar epithelial cells, TGF-β1 upregulated PDE4 mRNA, protein expression, and total cAMP-PDE activity (46). TGF-β1 has also been shown to mediate fibronectin, collagen I, and connective tissue growth factor induction in bronchial rings via a PDE4D-dependent mechanism (47). In human fetal lung fibroblasts, TGF-β1-mediated collagen gel contraction, fibronectin release, and fibroblast chemotaxis was inhibited in the presence of PDE4 pharmacological inhibitors (48). Therefore, we aimed to further investigate the role of PDE4 in the attenuation of ISO-induced cAMP by TGF-β1.

We demonstrate that TGF-β1 selectively induces PDE4D gene expression in HASM cells, and that PDE4D inhibition rescues attenuated ISO-induced cAMP levels in HASM cells (Fig. 2-E3, 2-4A, 2-4B). While roflumilast only modestly enhanced ISO-mediated decreases in TGF-β1-induced MLC phosphorylation (Fig. 2-4C), roflumilast significantly enhanced ISO-induced, single-cell relaxation in TGF-β1-treated HASM cells (Fig. 2-4D). While discrepancies between biochemical and cell stiffness measurements in roflumilast-treated HASM cells are puzzling, studies suggest that both actomyosin cross-bridge cycling – regulated by MLC phosphorylation - and actin polymerization (49, 50) mediate HASM cell contractile responses. Reports demonstrate that TGF-β1 induces both MLC phosphorylation (6, 40) and actin polymerization (51, 52) in HASM cells. While the individual contributions of these pathways to HASM cell shortening remain unclear, both pathways are modulated by cAMP signaling (31, 53). Evidence suggests that PDEs shape

compartmentalized cAMP signaling in the cell, where subcellular PDE localization mediates variations in cAMP-stimulated responses (16, 29, 54). As both PDE3 and PDE4 hydrolyze cAMP in HASM, the observed discrepancy may result from the relative contribution of cAMP signaling to each pathway, driven by the spatially-mediated effects of PDE isoforms.

To further determine the mechanism by which TGF-β1 attenuates ISO-induced responses, we investigated the role of the canonical TGF-β1 signaling pathway via Smad2/3 in HASM cells (Fig. 2-5). In non-targeting and Smad2 siRNA-transfected cells, ISO-stimulated cAMP was decreased following TGF-β1 treatment (Fig. 2-5A). In Smad3 siRNA-transfected cells – however – TGF-β1 had little effect on ISO-induced cAMP. Surprisingly, ISO stimulation induced significantly higher cAMP levels in Smad3 siRNA-transfected cells than those observed in non-targeting siRNA-transfected cells.

This increase in cAMP may indicate that Smad3 knockdown attenuates baseline TGF-β1 receptor activity following the release of biologically active TGF-β1 in HASM cells (55). Alternatively, it is possible that Smad3 knockdown augments basal cAMP levels through its association with HASM cell microtubules. Smad3 has been reported to bind directly to microtubules in the absence of TGF-β1 signaling (56), and TGF-β1 has been shown to induce microtubule stability in a variety of cell types (57, 58). Therefore, impaired TGF-β1 signaling via Smad3 knockdown may exert destabilizing effects on microtubule stability.

Microtubule destabilization has been correlated with impaired cAMP accumulation in multiple cell types. The microtubule assembly inhibitor colchicine has been shown to induce cAMP generation in human leukocytes in a concentration-dependent manner (59).

In human leukocyte and S49 lymphoma cell studies, multiple microtubule assembly inhibitors enhanced β-adrenergic and prostaglandin-stimulated cAMP accumulation in a time- and concentration-dependent manner, potentially by acting on microtubules that inhibit AC activity (60, 61). However, further studies are needed to determine the significance of the interaction between Smad3 and microtubules in HASM cells, and how this interaction may affect microtubule stability and cAMP generation.

In addition to modulating HASM cell cAMP levels, Smad2/3 knockdown also decreased TGF-β1-stimulated PDE4D gene expression (Fig. 2-5B). These findings were mirrored by a decrease in TGF-β1-stimulated PDE4D gene expression in HASM cells pre-treated with the TβR-I receptor inhibitor SB-431542 (Fig. 2-4A). SB-431542 is a highly selective inhibitor of the TβR-I receptor ALK5 (IC_{50} = 94 nM), and – to a lesser extent – the activin type I receptor ALK4, and the nodal type I receptor ALK7, which share highly-related kinase domains and Smad2/3 proteins as substrates (62). SB-431542 selectively inhibits TGF-β1 signaling in HASM at concentrations as high as 10 μM – and exerts little effect on more divergent ALK family members that recognize bone morphogenic proteins – suggesting it to be an effective and selective inhibitor of Smad2/3 signaling in HASM (6, 62, 63). Together, these experiments suggest that TGF-β1-induced PDE4D gene expression is Smad2/3 activation-dependent.

In both Smad2 and Smad3 siRNA-transfected HASM cells, PDE4D gene expression in TGF-β1-treated cells was not significantly increased over vehicle control (Fig. 5B). These results are surprising given that Smad2 and Smad3 exert differential effects on β2-agonist-induced cAMP in TGF-β1-treated cells (Fig. 2-5A). However, these results support previous studies demonstrating that Smad2 and Smad3 can exert differential effects on cell

function (6, 64, 65). It is possible that Smad3 selectively modulates PDE4D activity, while Smad2/3 mediate induction of PDE4D expression by TGF-β1. However, more studies will be needed to assess the potential role of Smad2/3 in PDE4D activation. Nonetheless, our collective findings demonstrate a role for TGF-β1 and Smad2/3 signaling in decreased HASM cell relaxation responses.

Due to the breadth and complexity of TGF-β1 signaling, there may be additional pathways by which TGF-β1 attenuates HASM cell cAMP levels that we did not investigate in this study. Other cytokines that attenuate HASM cell relaxation responses – such as IL-1β – attenuate ISO-induced cAMP via COX-2 induction and prostanoid release (66, 67). As TGF-β1 induces COX-2 expression in HASM cells (68), it is possible that prostanoid induction contributes to TGF-β1's impairment of relaxation responses. Further studies will be needed to determine the contribution of potential TGF-β1 signaling pathways in HASM cell relaxation responses.

In conclusion, our study further establishes TGF-β1 as a mediator of bronchodilator resistance in asthma via a Smad3-dependent pathway (Fig. 2-6). In light of our previous work on TGF-β1-induced hyperresponsiveness in HASM, these results further suggest TGF-β1 to be a promising therapeutic target to increase bronchodilator sensitivity and attenuate airway obstruction in asthma.

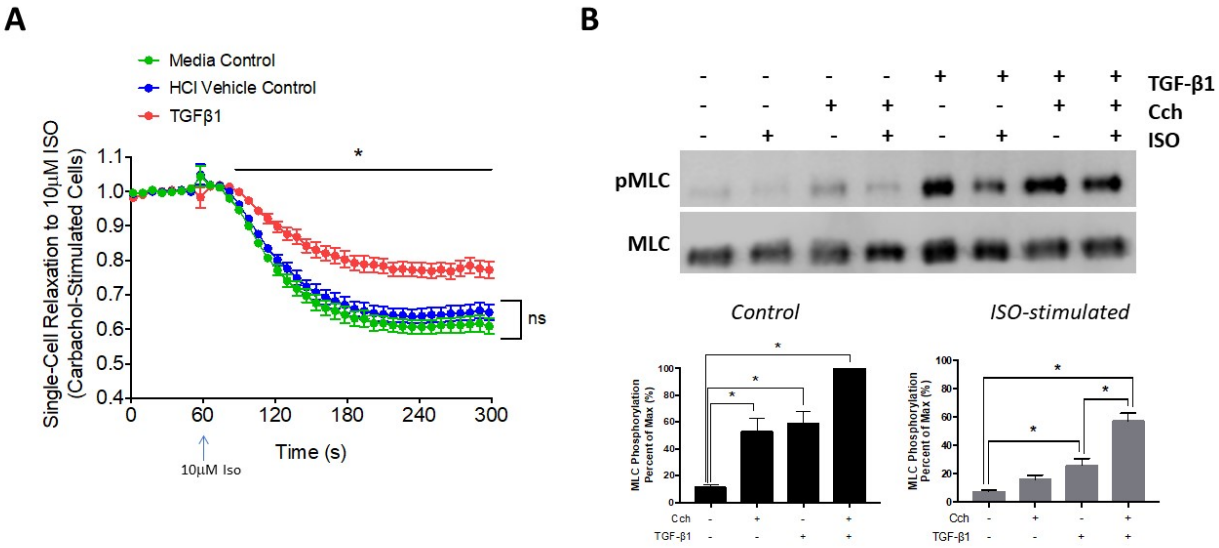
A
Media Control
HCl Vehicle Control
TGFβ1
Single-Cell Relaxation to 10μM ISO
(Carbachol-Stimulated Cells)
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
*
ns
0
60
120
180
240
300
Time (s)
10μM Iso
B
TGF-β1
Cch
ISO
pMLC
MLC
Control
ISO-stimulated
MLC Phosphorylation
Percent of Max (%)
100
80
60
40
20
0
Cch
TGF-β1

Figure 2-1. TGF-β1 Decreases β2-Agonist-Induced Relaxation in HASM Cells. A) Single-cell relaxation of isoproterenol (ISO)-stimulated HASM cells in the presence or absence of TGF-β1 (10 ng/mL, 18 h) (N= 3 donors ± SEM). HASM cells were contracted with carbachol (CCh) for 5 min and subsequently relaxed with isoproterenol. CCh-stimulated stiffness was measured for the first 0-60 s, and changes in cell stiffness in response to ISO were measured continuously up to the indicated time (60-300 s). For each cell, stiffness was normalized to CCh-stimulated stiffness before ISO stimulation. **B)** Phosphorylated MLC following TGF-β1 (10 ng/mL, 18 h), CCh (20 μM; *bottom left*), and/or isoproterenol (ISO, 1 μM; *bottom right*) treatment (N=4-7 ± SEM). *Representative immunoblot of seven separate experiments.* $*P \leq 0.05$

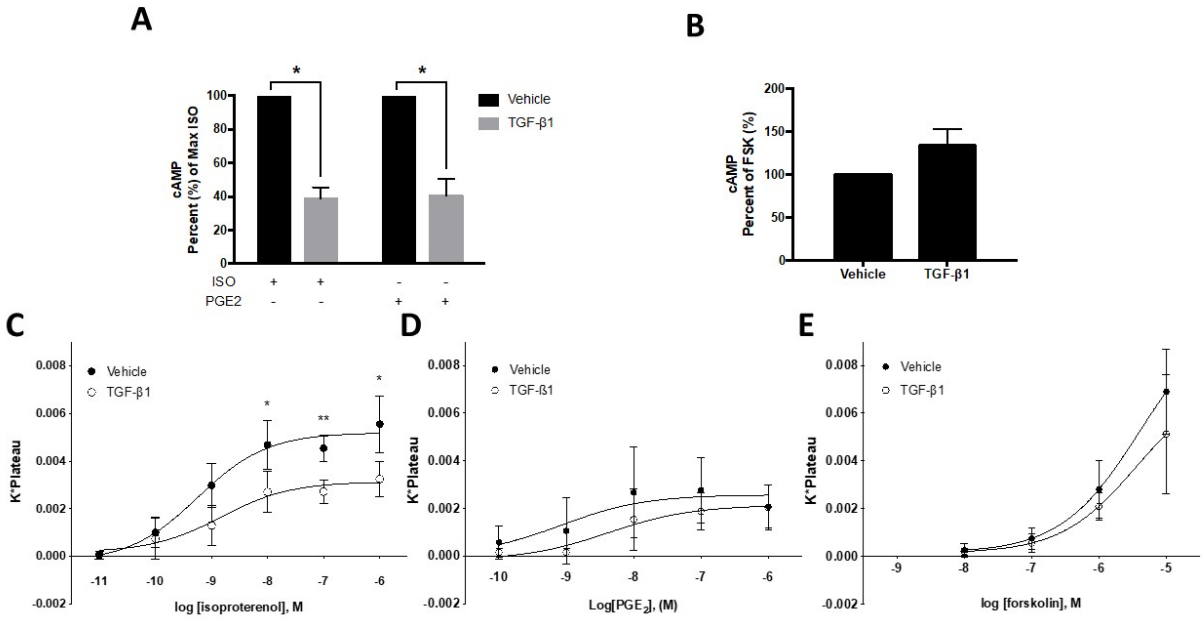
A
B
C
D
E
cAMP
Percent (%) of Max ISO
Vehicle
TGF-β1
ISO
PGE2
cAMP
Percent of FSK (%)
K⁺Plateau
log [isoproterenol], M
Log[PGE2], (M)
log [forskolin], M

Figure 2-2. TGF-β1 Blunts Agonist-Induced cAMP Levels. A) HASM cells were pre-treated with TGF-β1 (10 ng/mL) overnight and acutely stimulated with ISO (1 μM, 5 min) (N=7 ± SEM; ISO 1 μM: 3684.2 ± 1170.0 pmol/well)**,** PGE2 (100 nM; 5 min) (N=4 donors ± SEM; PGE2: 40270.4 ± 25537.2 pmol/well), or **B)** Forskolin (10 μM; 15 min) (N=3 donors ± SEM; FSK 10 μM: 7192.4 pmol/well ± 3244.3) prior to lysis for cAMP level determination. **C)** Live HASM cells were pre-treated with TGF-β1 (10 ng/mL) overnight then acutely stimulated with various concentrations of this indicated drug and cAMP levels monitored using cADDis. Isoproterenol (vehicle $logEC_{50}$ -9.25 ± 0.258, E_{max} 0.0052 ± 0.00035; TGF-β1 $logEC_{50}$ -8.83 ± 0.433, E_{max} 0.0031 ± 0.00038). **D)** PGE2 (vehicle $logEC_{50}$ -9.08 ± 1.798, E_{max} 0.0026 ± 0.00065; TGF-β1 $logEC_{50}$ -8.37 ± 0.647, E_{max} 0.0021 ± 0.00048). **E)** Forskolin (vehicle $logEC_{50}$ -5.40 ± 1.547, E_{max} 0.010 ± 0.0012; TGF-β1 $logEC_{50}$ -5.44 ± 2.31, E_{max} 0.0074 ± 0.0132). Data is expressed as mean ± SEM of N=5 donors. *$*P \leq 0.05$ **$P \leq 0.01$; ***$P \leq 0.001$.*

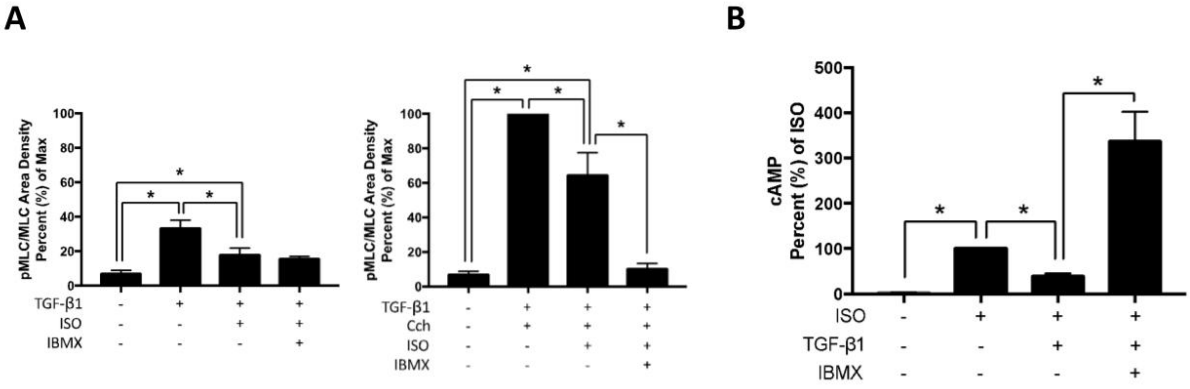
A
pMLC/MLC Area Density
Percent (%) of Max
100
80
60
40
20
0
*
*
*
TGF-β1 - + + +
ISO - - + +
IBMX - - - +
pMLC/MLC Area Density
Percent (%) of Max
*
*
*
*
TGF-β1 - + + +
Cch - + + +
ISO - - + +
IBMX - - - +
B
cAMP
Percent (%) of ISO
500
400
300
200
100
0
*
*
*
ISO - + + +
TGF-β1 - - + +
IBMX - - - +

Figure 2-3. PDE Inhibition Rescues ISO-Stimulated Responses in TGF-β1-Treated HASM Cells. A) MLC phosphorylation in HASM cells pre-treated with vehicle or TGF-β1 (10 ng/mL; 18h) and/or IBMX (500 μM, 30 min) prior to stimulation with CCh (20 μM; 12 min) and/or ISO (1 μM, 10 min) (N=4 ± SEM; Max: 23.2 fold change over vehicle ± 9.4). **B)** cAMP levels in TGF-β1 (10 ng/mL; 18h)-treated HASM cells pre-treated with vehicle (N=7± SEM; ISO 1 μM: 3684.2 ± 1170.0 pmol/well) or IBMX (500 μM, 30 min) (N=6± SEM; IBMX 1 μM ISO: 11927.4 ± 1599.3 pmol/well) prior to ISO (1 μM, 5 min) stimulation. N=4 donors ± SEM. *$P \leq 0.05$

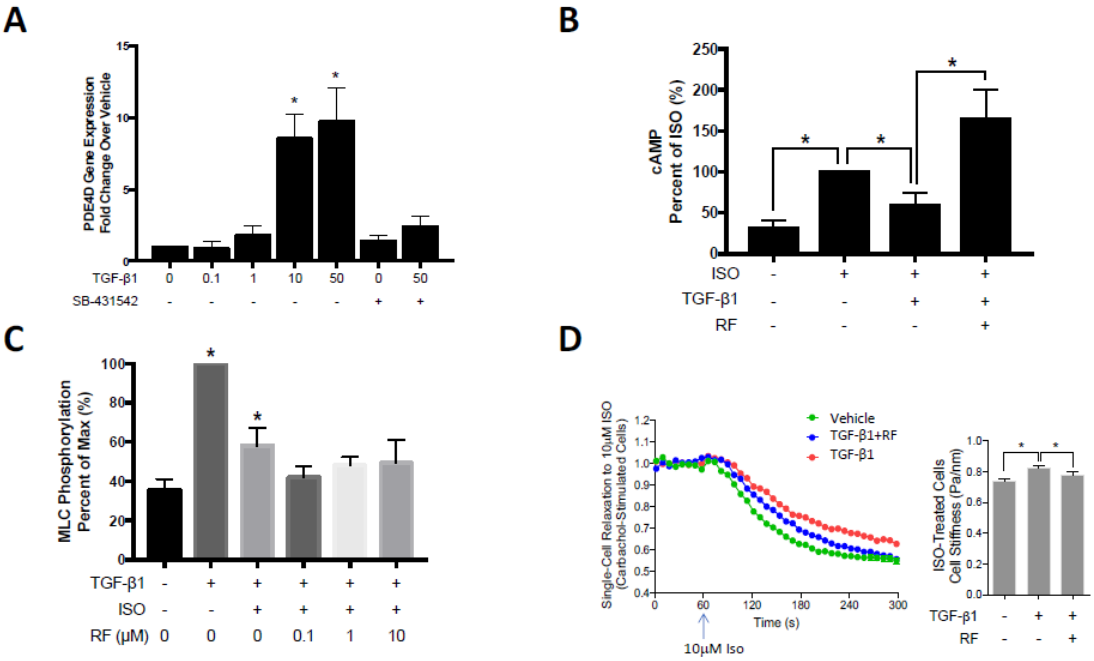
A
PDE4D Gene Expression
Fold Change Over Vehicle
TGF-β1 0 0.1 1 10 50 0 50
SB-431542 - - - - - + +
B
cAMP
Percent of ISO (%)
ISO - + + +
TGF-β1 - - + +
RF - - - +
C
MLC Phosphorylation
Percent of Max (%)
TGF-β1 - + + + + +
ISO - - + + + +
RF (μM) 0 0 0 0.1 1 10
D
Single-Cell Relaxation to 10μM ISO
(Carbachol-Stimulated Cells)
Vehicle
TGF-β1+RF
TGF-β1
Time (s)
10μM Iso
ISO-Treated Cells
Cell Stiffness (Pa/nm)
TGF-β1 - + +
RF - - +

Figure 2-4. TGF-β1 Induces PDE4D Gene Expression in a Concentration-Dependent Manner. A) PDE4D gene expression in TGF-β1-treated (10 ng/ml; 18 h) HASM cells in the presence or absence of SB-431542 (5 μM; 1 h pretreatment) (N=3 donors ± SEM). **B)** cAMP levels in ISO-stimulated HASM cells treated with TGF-β1 (10 ng/mL; 18h) in the presence or absence of roflumilast (RF; 10 μM, 30 min) pretreatment (N=6 ± SEM; ISO μM**:** 1281.1 ± 406.6 pmol/well). **C)** MLC phosphorylation in TGF-β1 (10 ng/ml; 18 h)-treated HASM cells in the presence of roflumilast (RF; 10 μM, 30 min), CCh (20 μM, 12 min) and/or ISO (1 μM, 10 min) stimulation (N=6 donors ± SEM). **D)** Single-cell relaxation of TGF-β1 (10 ng/ml 18 h)-treated HASM cells in the presence or absence of roflumilast *(*RF; 10 μM, 30 min) (N=1 donor; N=223 ± SEM). **P* $\leq$ *0.05; relative to control unless otherwise shown.*

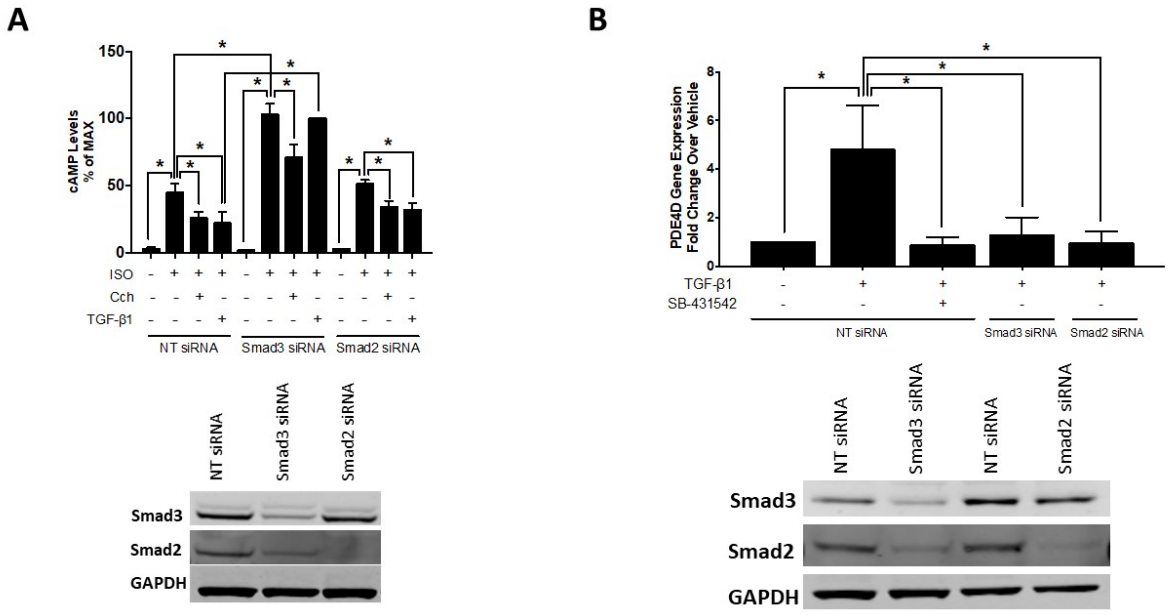
A
cAMP Levels
% of MAX
150
100
50
0
ISO
Cch
TGF-β1
NT siRNA
Smad3 siRNA
Smad2 siRNA
NT siRNA
Smad3 siRNA
Smad2 siRNA
Smad3
Smad2
GAPDH
B
PDE4D Gene Expression
Fold Change Over Vehicle
8
6
4
2
0
TGF-β1
SB-431542
NT siRNA
Smad3 siRNA
Smad2 siRNA
NT siRNA
Smad3 siRNA
NT siRNA
Smad2 siRNA
Smad3
Smad2
GAPDH

Figure 2-5. TGF-β1-Decreases β2-Agonist-Induced Relaxation Responses in a Smad2/3-Dependent Manner. A) *Top:* cAMP levels in non-targeting (NT) or Smad2/3 siRNA-transfected HASM cells pre-treated with TGF-β1 (10 ng/mL, 18 h) and stimulated with CCh (20 μM; 10 min) and/or ISO (1 μM, 5 min) (N=4 donors ± SEM; Max: 15397.2 ± 3010.4 pmol/well). *Bottom:* Representative immunoblot of total Smad3 (*left,* 16% of NT siRNA control ± 15%, N=3) and total Smad2 (*right,* 10.7% of NT siRNA control ± 22.7%, N=3) protein expression in Smad2/3 siRNA transfected HASM cells. **B)** *Top:* PDE4D gene expression in non-targeting (NT)- or Smad2/3 siRNA-transfected HASM cells pre-treated with SB-431542 (5 μM, 30 min) prior to TGF-β1 (10 ng/mL) overnight treatment (N=3-4 donors ± SEM). *Bottom:* Representative immunoblot of total Smad3 (*left,* 20.3% of NT siRNA control ± 4.2%, N=3) and total Smad2 (*right,* 38.1% of NT siRNA control ± 23.4%, N=3) in Smad2/3 siRNA transfected HASM cells. $*P \leq 0.05$

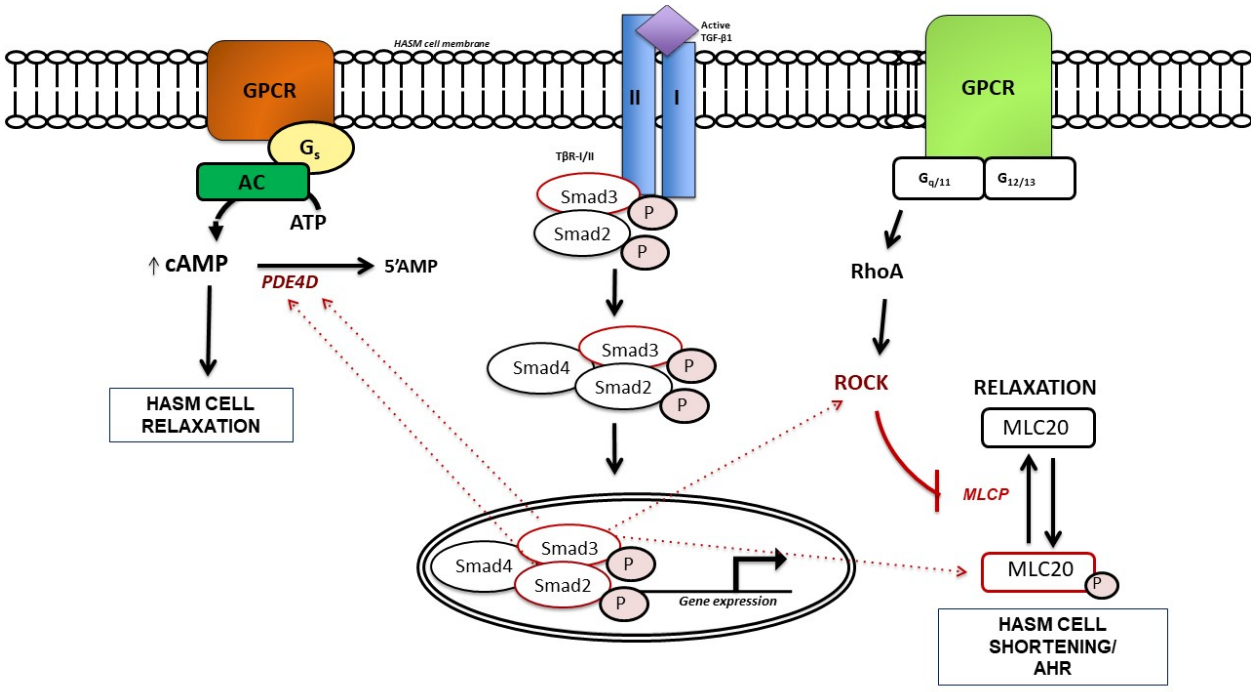
HASM cell membrane
GPCR
G_s
AC
ATP
↑cAMP
5'AMP
PDE4D
HASM CELL RELAXATION
Active TGF-β1
II
I
TβR-I/II
Smad3
P
Smad2
P
Smad4
Smad3
P
Smad2
P
Smad4
Smad3
P
Smad2
P
Gene expression
GPCR
$G_{q/11}$
$G_{12/13}$
RhoA
ROCK
MLCP
RELAXATION
MLC20
MLC20
P
HASM CELL SHORTENING/ AHR

Figure 2-6. Proposed Role of TGF-β1 in HASM Cell Contractile Responses in Asthma. TGF-β1 signaling augments basal and HASM cell shortening through a Smad3, ROCK-dependent pathway as previously described (6). In addition to modulating HASM cell contractile responses, Smad2/3 activation increases PDE4D gene expression, leading to increased cAMP hydrolysis and blunted HASM cell relaxation responses. TGF-β1, transforming growth factor beta 1; TβR-I/II, TGF-β receptor I/II; ROCK, rho-associated protein kinase; RhoA, Ras homolog gene family, member A; MLCP, myosin light-chain phosphatase; MLCK, myosin light chain kinase; MLC20, 20-kDa myosin light chain 20; cAMP, cyclic adenosine monophosphate; 5'AMP, 5' adenosine monophosphate; PDE4D, phosphodiesterase 4D.

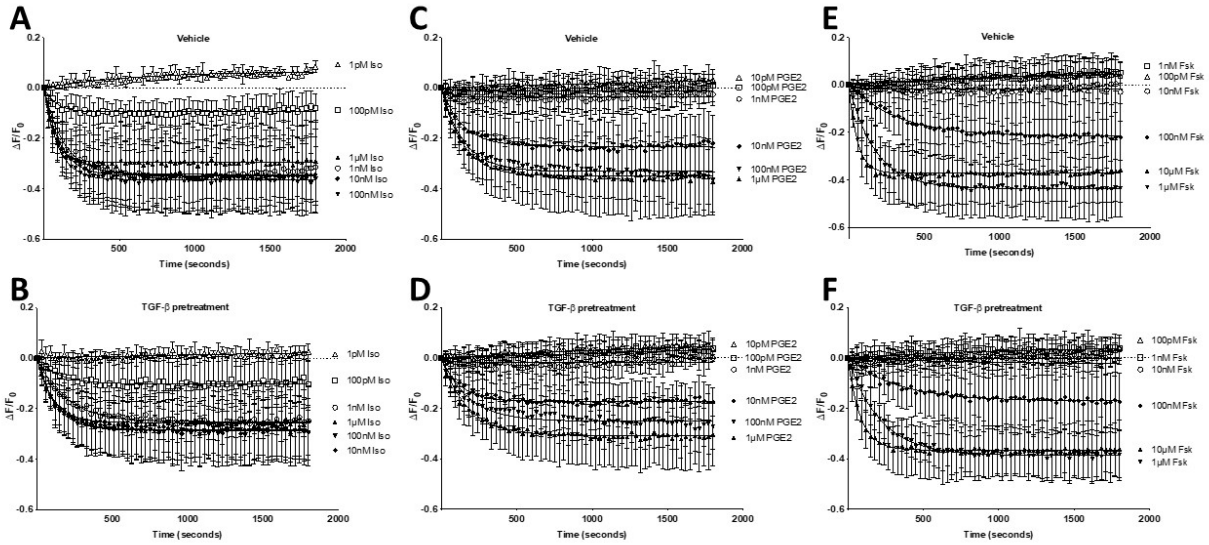
A
Vehicle
1pM Iso
100pM Iso
1µM Iso
1nM Iso
10nM Iso
100nM Iso
B
TGF-β pretreatment
1pM Iso
100pM Iso
1nM Iso
1µM Iso
100nM Iso
10nM Iso
C
Vehicle
10pM PGE2
100pM PGE2
1nM PGE2
10nM PGE2
100nM PGE2
1µM PGE2
D
TGF-β pretreatment
10pM PGE2
100pM PGE2
1nM PGE2
10nM PGE2
100nM PGE2
1µM PGE2
E
Vehicle
1nM Fsk
100pM Fsk
10nM Fsk
100nM Fsk
10µM Fsk
1µM Fsk
F
TGF-β pretreatment
100pM Fsk
1nM Fsk
10nM Fsk
100nM Fsk
10µM Fsk
1µM Fsk
ΔF/F0
Time (seconds)

Figure 2-E1. cAMP sensor (cADDis) fluorescence decay curves in live HASM cells pretreated with vehicle or TGF-ß1 then stimulated with ISO, PGE2 or forskolin. Data is plotted as the mean ± SEM of the change in fluorescence over the initial fluorescence ($\Delta F/F_0$) of n=5 donors for each data point collected (reads were performed every 30 sec in each sample following addition of drug). No PDE inhibitor was present in this assay. Non-linear regression analysis fit the data using a single site decay model (as indicated by lines) and the decay constant (k) was multiplied by the plateau to generate the concentration-response curves presented in Figure 2.

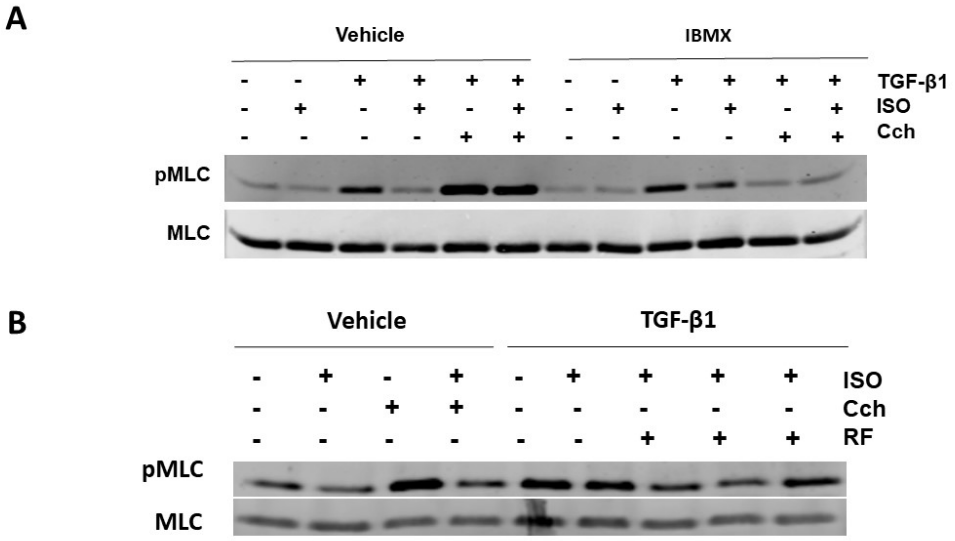
A
Vehicle
IBMX
TGF-β1
ISO
Cch
pMLC
MLC
B
Vehicle
TGF-β1
ISO
Cch
RF
pMLC
MLC

Figure 2-E2. Effect of PDE Inhibition on TGF-β1-Mediated MLC Phosphorylation in ISO stimulated HASM Cells. Representative immunoblot of MLC phosphorylation in HASM cells pre-treated with A) IBMX (500 μM, 30 min) or B) Roflumilast (RF; 10 μM, 30 min) prior to TGF-β1 (10ng/ml 18 h) and/or ISO (1 μM, 5 min) stimulation.

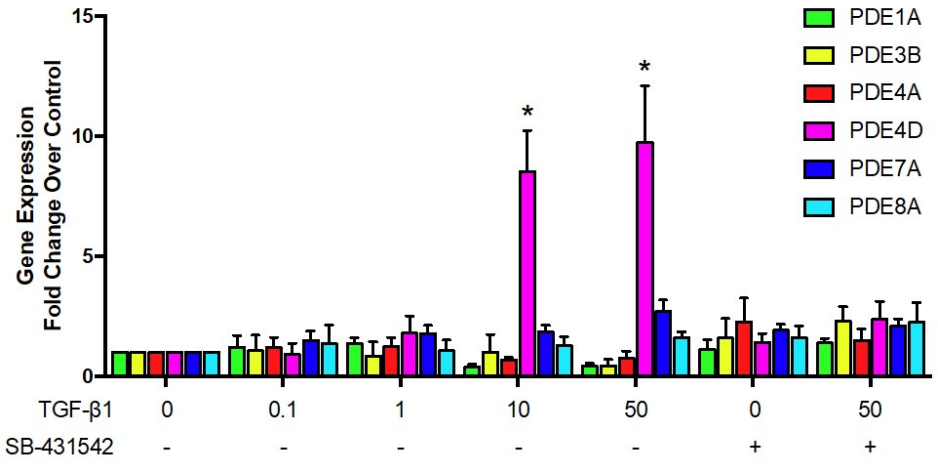
Gene Expression
Fold Change Over Control
15
10
5
0
*
*
PDE1A
PDE3B
PDE4A
PDE4D
PDE7A
PDE8A
TGF-β1 0 0.1 1 10 50 0 50
SB-431542 - - - - - + +

Figure 2-E3. PDE Gene Expression in TGF-β1-treated HASM Cells. PDE gene expression in TGF-β1-treated (10 ng/ml; 18 h) HASM cells in the presence or absence of SB-431542 (5 μM; 1 h pretreatment) (N=3 donors ± SEM).

DONOR CHARACTERISTICS

Sex, M/F	16/10
Age, yr	30.54 (13.82)
Race, C/B/H/NA	17/5/3/1
BMI, kg/m^2	28.96 (8.42)

Data are means (SD); *n* = 26 donors. M, male; F, female; C, Caucasian; B, Black; H, Hispanic; NA, Native American; BMI, body mass index

Figure 2-E4. **Characteristics of the Non-Asthma Human Lung Donors in the Study.**

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CHAPTER THREE

Dexamethasone rescues TGF-β1-mediated β_2-Adrenergic Receptor (β2AR) dysfunction and attenuates phosphodiesterase 4D (*pde4d*) expression in human airway smooth muscle cells

Chung E, Ojiaku CA, Cao G, Parikh V, Deeney B, Xu S, Wang S, Himes BE, Panettieri RA, Koziol-White C. Manuscript Submitted.

3.1. Abstract

Glucocorticoids (GCs) and β_2-adrenergic receptor (β_2AR) agonists decrease asthma symptoms 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- β1 (TGF-β1) 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-β1-effects on β_2-agonist-induced bronchodilation and modulates *pde4d* gene expression in HASM. Dexamethasone (DEX) reversed the TGF-β1 effects on cAMP levels induced by isoproterenol (ISO). TGF-β1 also attenuates G protein-dependent responses to cholera toxin (CTX), a $G_{\alpha s}$ stimulator downstream from the β_2AR receptor. Interestingly, DEX also attenuated TGF-β1-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-β1 levels.

3.2. Introduction

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 asthma diatheses and are a direct target of β_2-agonists, one of the most common therapies that promote bronchodilation (2). While β_2-agonists promote ASM relaxation, β2-agonists are not

effective in all patients (3, 4). Patients who fall into the "severe" category of asthma are frequently hyporesponsive to bronchodilators (5). Studies show that β_2AR tolerance or desensitization occurs after repeated bronchodilator use that diminishes drug efficacy (3, 6–10).

TGF-β1, 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 (11), enhancing agonist-induced cell shortening and hyperresponsiveness. In addition to amplifying bronchoconstriction, we also demonstrated that TGF-β1 blunts intracellular cAMP by upregulating *pde4d* expression that decreases β_2-agonist-induced cAMP levels (12).

Signaling downstream of seven transmembrane G protein-coupled receptors (GPCRs) evokes the membrane Gαβγ trimer to dissociate following receptor activation (13). 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 (14). Cholera toxin (CTX) catalyzes the ADP-ribosylation of $G_{\alpha s}$, elicits adenylyl cyclase (AC) activation, causing the accumulation of intracellular cAMP and further signaling of PKA to induce HASM relaxation (15). 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 (16), thereby contributing to smooth muscle relaxation. Whether TGF-β1 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. Our previous study showed that TGF-β1 blunted the effects of β_2 agonist-

induced intracellular cAMP production to induce bronchodilation by enhancing cAMP breakdown through increased expression of phosphodiesterase 4D (PDE4D) (12). Given this information, we posited that GC treatment would reverse TGF-β1-induced hyporesponsiveness to β_2-agonist, and attenuate *pde4d* expression. Our data demonstrate that DEX reverses TGF-β1-induced attenuation of β_2AR-induced signaling, rescuing β_2-agonist- and $G_{\alpha s}$-activator-mediated cAMP production by attenuating *pde4d* expression.

3.3. Methods

3.3.1. 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 subject research by the Rutgers University Institutional Review Board. Cells were routinely cultured in Dulbecco's modified Eagle medium with 10% fetal bovine serum. The cells were incubated and grown at 37°C in 5% CO_2. Studies have shown that cell culture in this manner will retain phenotypic properties in isolated airway smooth muscle cells (17). 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.

3.3.2. Western Blot Analysis

Primary HASM cells were serum deprived for 24 hours prior to treatment. HASM cells were lysed with 0.6 N $HClO_4$, scraped, collected, and pelleted as previously described (18). The membrane was blocked with ready-to-use Odyssey Blocking Buffer (PBS) (LI-COR BioSciences) containing 0.1% sodium azide and probed for phospho-Smad3, pMLC, total MLC, and GAPDH.

3.3.3. 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 (12). The cells were lysed and incubated for 30 minutes in 5% CO_2 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 hour on plate shaker. Plate was measured on luminometer after a 30-minute incubation period with CSPD®/Sapphire-II RTU Substrate. Data was derived from standard curves and cAMP levels reported after using standard dilutions.

3.3.4. Quantification of *pde4d* Expression (RNA isolation and qPCR)

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 µL 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* expression was normalized in relation to expression of endogenous *β-actin*.

3.3.5. Statistical Analysis

Graphs were created and statistical analyses were conducted using GraphPad Prism 5.01h 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.

3.3.6. Materials

Compounds were purchased from the following vendors: R&D Systems (TGF-β1; SB-431542), Sigma-Aldrich (albuterol [Alb], carbachol [Cch], cholera toxin [CTX], dexamethasone [DEX], isoproterenol [ISO]), Fisher BioReagents (Forskolin, [FSK]), Cayman Chemical Company (3-isobutyl-1-methylxanthine [IBMX]). 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)

3.4. Results

3.4.1. TGF-β1 attenuates $G_{\alpha s}$-mediated cAMP production in HASM

Upon activation of β_2AR, ADP-ribosylation of the α subunit of stimulatory G protein ($G_{\alpha s}$), followed by the stimulation of adenylyl cyclase increases intracellular cAMP (15, 19). To further understand mechanisms underlying TGF-β1-mediated hyporesponsiveness to a β_2-agonist, intracellular cAMP levels were measured in TGF-β1-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 minutes (Figure 3-1A). Interestingly at 60 and 75 min, the CTX-induced cAMP levels extinguished. In TGF-β1-treated cells, CTX-induced cAMP levels were completely abrogated compared to that of the diluent control (Figure 3-1B).

We previously demonstrated that isoproterenol (ISO) decreased carbachol (Cch)- and TGF-β1-induced phosphorylation of myosin light chain (pMLC); TGF-β1, however, decreased the ability of a β_2-agonist to abrogate Cch-induced pMLC (12). Cch-induced pMLC was inhibited by ISO, forskolin (an adenylyl cyclase activator) and CTX to comparable levels, as shown in Figure 3-2A. Interestingly, TGF-β1-induced pMLC was also decreased with CTX, forskolin, ISO and SB-431542, a TGF-β1 receptor antagonist. However, ISO and CTX-induced inhibition was less effective as compared with that of SB-

431542 or forskolin in blocking TGF-β1-induced pMLC (Figure 3-2A, upper right). Phosphorylation of SMAD3 (pSMAD) induced by TGF-β1 confirmed engagement of TGF-β1 receptors and activation of downstream signaling pathways (Figure 3-2, lower panel). Collectively, these data suggest that TGF-β1 selectively inhibits the ability of β2AR or $G_{\alpha s}$ activation but not forskolin to increase cAMP levels and diminish pMLC.

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

One mechanism by which inflammatory mediators have been shown to attenuate signaling downstream of the β_2AR is through phosphorylation of the receptor (20–23). It has been demonstrated that GRK2 and 3 are associated with the β_2AR, mediating desensitization through phosphorylation of intracellular portions of the receptor (24, 25). Therefore, we examined the effect of TGF-β1 on expression of GRK2 and 3. As shown in Figure 3-3, we demonstrate that TGF-β1 attenuates *grk2* and *grk3* expression rather than augmenting it. Despite our previous findings showing that β_2AR phosphorylation is increased following TGF-β1 exposure (26), we determined that the increase in TGF-β1-induced β_2AR phosphorylation is not due to increased expression of GRK2/3.

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

Previously, we determined that the blunted cAMP response to ISO induced by TGF-β1 was dependent upon increased *pde4d* expression (7). 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. As shown in Figure 3-

5, DEX pretreatment significantly attenuated TGF-β1-induced *pde4d* expression in HASM.

3.4.4. 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 (12). 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 Figure 3-5.

3.5.Discussion

Insensitivity to current therapeutics occurs in some patients with severe asthma. Evidence suggests that β_2AR dysfunction can manifest in an inflammatory milieu due to inflammatory cytokines impugned in severe asthma and airway remodeling such as TGF-β1, IL-13, and TNF-α (12, 18, 29). We investigated generation of cAMP levels in HASM cells after CTX-treatment in the presence and absence of TGF-β1 (Figure 3-1B). As we had previously described, β_2-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 $G_{\alpha s}$-induced reversal of agonist-induced HASM cell contractile signaling was examined. We confirmed and 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 β2AR and $G_{\alpha s}$ (Figure 3-2A).

Given these data and our previously published results, this suggests that the effects of TGF-β1 are more prominent at the receptor and $G_{\alpha s}$ level rather than downstream at the level of adenylyl cyclase, as evidenced by the lack of effect of TGF-β1 on FSK-mediated cAMP production.

Attenuation of β_2AR signaling occurs phosphorylation of the receptor, leading to internalization and desensitization (30–32). GRK2 and GRK3, members of the GRK (G protein-coupled receptor kinase) family, are components of one mechanism mediating desensitization of the β_2AR. We previously demonstrated that TGF-β1 stimulation induces β_2AR phosphorylation that is consistent with desensitization of the receptor (26). To assess a potential role for GRKs in this phosphorylation attenuation of β_2-agonist-induced relaxation of HASM, *grk2* and *grk3* expression was assessed with overnight TGF-β1 treatment. In Figure 3-3, 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. Our previous data, and these data, suggest that TGF-β1 likely modulates activity of these types of kinases, rather than modulating their expression to induce hyporesponsiveness to β_2-agonists.

Currently, glucocorticoids like dexamethasone are used to attenuate inflammation associated with asthma as well as to help restore β_2AR responsiveness (3, 18, 33), and reverse the effects of inflammatory mediator release on $\beta_2$agonist dysfunction. We demonstrate that TGF-β1-induced attenuation of β_2-agonist- and $G_{\alpha s}$-induced bronchodilation responses in HASM can be rescued by treatment with dexamethasone (Figure 3-4A & B). We previously showed that TGF-β1 treatment increased expression of

pde4d, suggesting a role for phosphodiesterases in TGF-β1-induced hyporesponsiveness to bronchodilators. Given this data, we examined whether steroid treatment prior to TGF-β1 exposure modulated *pde4d* expression, finding that DEX reversed TGF-β1-induced *pde4d* expression (Figure 3-5). Consistent with these data, we previously demonstrated that DEX (1 uM, 18 hr) stimulation alone has little effect *pde4d* expression (34).

Despite the fact that we show that TGF-β1 attenuates CTX-induced cAMP production, and that DEX rescues TGF-β1-mediated attenuation of both ISO- and CTX-induced cAMP production, we don't show a direct physiologic effect on intact airways. Despite this limitation, we have shown effects of TGF-β1 on HASM to be recapitulated in small airways derived from human lungs (11). Additionally, while it would be interesting to study this phenomenon in asthma-derived HASM, we and others have demonstrated that β_2-agonist-induced cAMP production in these cells is already blunted because partially due to increased PDE expression (26, 35). Therefore, exposure of asthma-derived HASM to TGF-β1 will likely have little effect on modulating β_2-agonist-induced cAMP production. Figure 3-6 depicts a model of mechanisms underlying glucocorticoid-mediated rescue of TGF-β1-induced β_2AR hyporesponsiveness.

Regardless of evidence that steroids may not reverse the remodeling effects (36) observed in asthma that may elicited by TGF-β1, our findings suggest that in asthma patients with high levels of TGF-β1, that steroids are an effective treatment to reverse any β_2AR hyporesponsiveness observed in these patients.

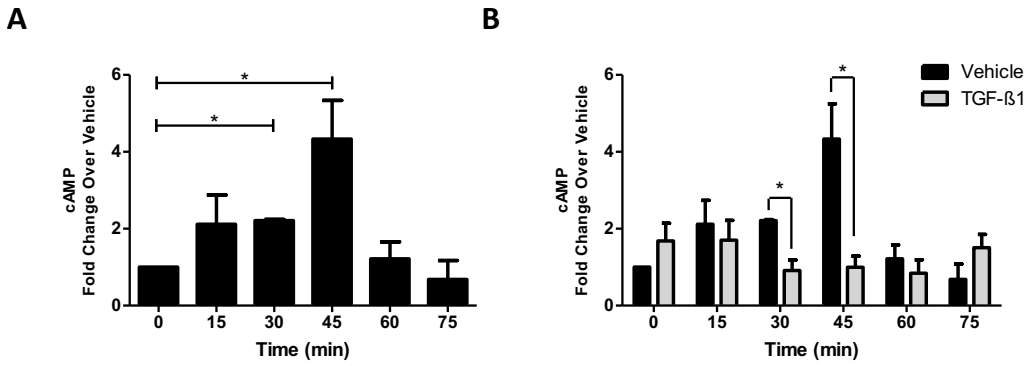

A
B
cAMP
Fold Change Over Vehicle
Time (min)
0
15
30
45
60
75
2
4
6
*
Vehicle
TGF-ß1

Figure 3-1. Cholera toxin (CTX) increases cAMP levels in HASM, which is blunted by overnight treatment with TGF-β1. (A) Time course of CTX-, a $G_{\alpha s}$ activator, induced cAMP production in HASM (0.25 μg/ml, 0-75 min). (B) TGF-β1 (10 ng/ml, 18 hr) treatment attenuates CTX-induced cAMP production. Data is expressed fold change over vehicle control as mean ± SEM for n=3 separate cell lines, three additional donors were added to the 45 min to confirm appropriate time point. *$p<0.05$ as assessed by one-way ANOVA, and comparisons between two conditions assessed by Student's t-test.

A

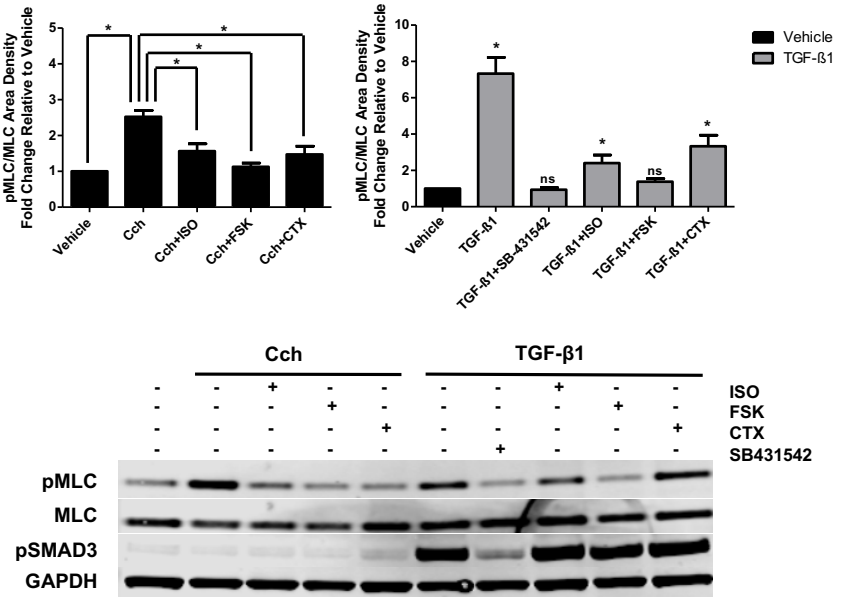
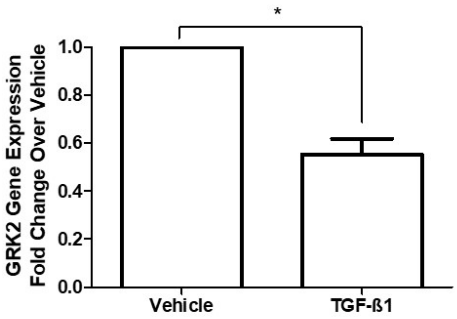

pMLC/MLC Area Density
Fold Change Relative to Vehicle
Vehicle
Cch
Cch+ISO
Cch+FSK
Cch+CTX
TGF-ß1
TGF-ß1+SB-431542
TGF-ß1+ISO
TGF-ß1+FSK
TGF-ß1+CTX
ns
Cch
TGF-β1
ISO
FSK
CTX
SB431542
pMLC
MLC
pSMAD3
GAPDH

Figure 3-2: Overnight TGF-β1 treatment impairs CTX-induced MLC dephosphorylation in HASM. (A) Phosphorylation of myosin light chain (pMLC) following Cch (20 μM, 13 min) or TGF-β1 (10 ng/ml, 18 hr) or was assessed following ISO (1 μM, 10 min), FSK (10 μM, 15 min), or CTX (0.25 μg/ml, 45 min) treatment. SB-431542 (5 μM, 1 hr prior to TGF-β1 treatment), a TGF-β1 receptor inhibitor, was used as a control. All treatments (ISO/FSK/SB/CTX) significantly attenuated TGF-β1-induced pMLC ($p<0.05$).


GRK2 Gene Expression
Fold Change Over Vehicle
1.0
0.8
0.6
0.4
0.2
0.0
*
Vehicle
TGF-ß1

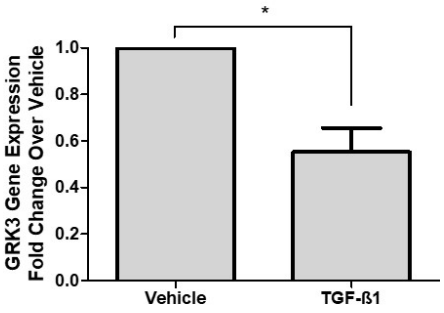

GRK3 Gene Expression
Fold Change Over Vehicle
1.0
0.8
0.6
0.4
0.2
0.0
*
Vehicle
TGF-ß1

Figure 3-3: TGF-β1 attenuates expression of *grk2* and *grk3*. HASM were treated with TGF-β1 (10 ng/ml, 18 hr), total RNA was isolated, and gene expression was assessed by TaqMan qPCR. Expression of *grk2* and *grk3* was normalized to endogenous *β-actin.* Data is representative of n=5-6 different donors as mean ± SEM, *p<0.05 by Student's t-test.

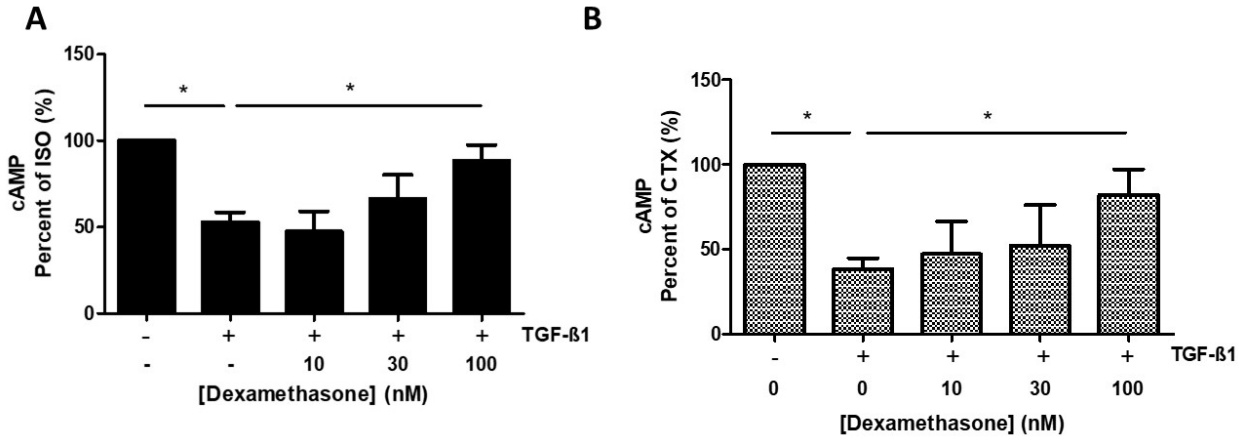

A
150
100
50
0
cAMP
Percent of ISO (%)
*
*
-
+
+
+
+
TGF-ß1
-
-
10
30
100
[Dexamethasone] (nM)
B
150
100
50
0
cAMP
Percent of CTX (%)
*
*
-
+
+
+
+
TGF-ß1
0
0
10
30
100
[Dexamethasone] (nM)

Figure 3-4: 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 hr) 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.

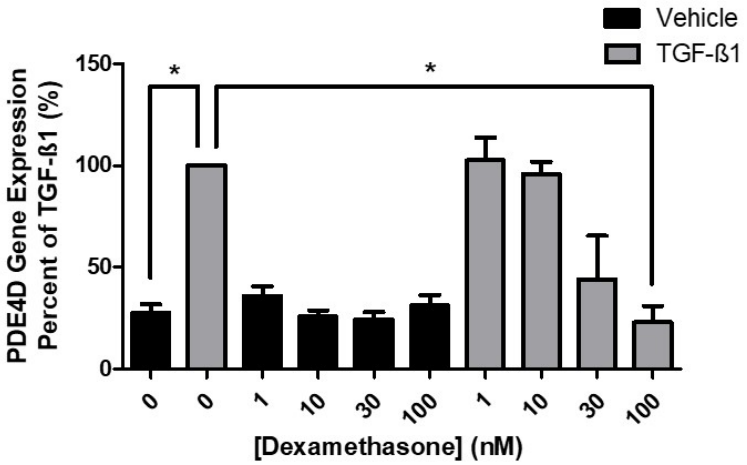

Vehicle
TGF-ß1
PDE4D Gene Expression
Percent of TGF-ß1 (%)
150
100
50
0
*
*
0
0
1
10
30
100
1
10
30
100
[Dexamethasone] (nM)

Figure 3-5: DEX inhibits TGF-β1-induced *pde4d* expression in HASM. HASM were pretreated with DEX (1-100 nM, 30 min) prior to stimulation with TGF-β1 (10 ng/ml, 18 hr). mRNA was extracted, reverse transcribed, and assessed for *pde4d* expression by TaqMan qPCR analysis. Data is represented as % of *pde4d* expression induced by TGF-β1. Data is representative of n=4-6 separate cell lines as mean ± SEM, *$p<0.05$ using a one-way ANOVA, and comparisons between two conditions assessed by Student's t-test.

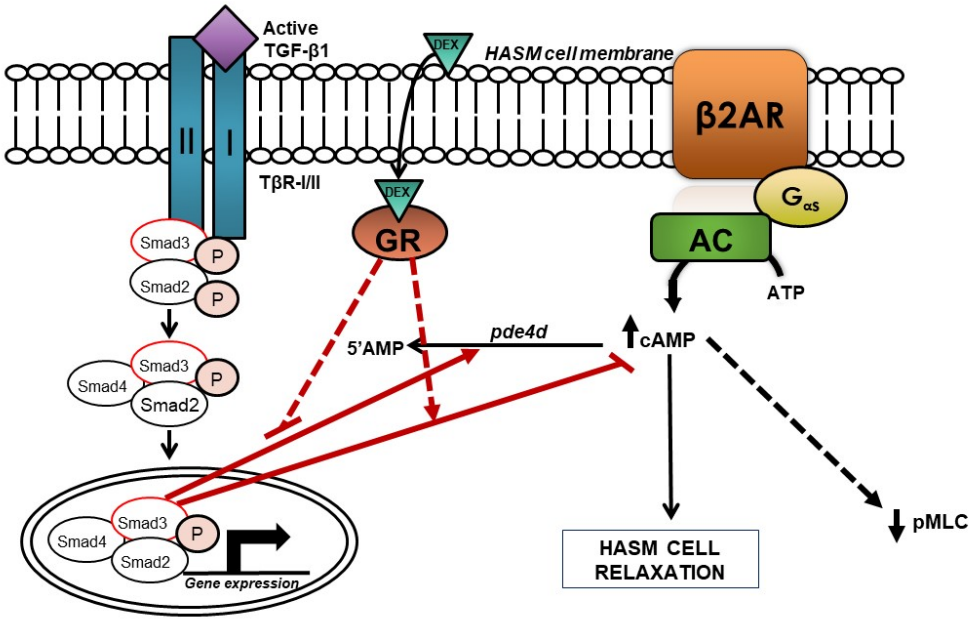

Active
TGF-β1
DEX
HASM cell membrane
β2AR
II
I
TβR-I/II
DEX
GR
$G_{\alpha s}$
AC
ATP
Smad3
P
Smad2
P
Smad3
Smad4
P
Smad2
5'AMP
pde4d
cAMP
Smad3
Smad4
P
Smad2
Gene expression
HASM CELL
RELAXATION
pMLC

Figure 3-6: A proposed model of GC-mediated rescue of TGF-β1-induced hyporesponsiveness to bronchodilators. We previously demonstrated that TGF-β1 induces Smad2/3 activation to increase *pde4d* expression, leading to increased cAMP hydrolysis and attenuating HASM cell relaxation responses. We posit that DEX binds to the glucocorticoid receptor (GR), inhibiting both increased *pde4d* expression and rescuing TGF-β1-induced attenuation of β_2AR and $G_{\alpha s}$-induced cAMP production. AC=Adenylyl Cyclase; β_2AR= β_2-adrenergic receptor; DEX=Dexamethasone; $G_{\alpha s}$= Stimulatory G_α protein; GR=Glucocorticoid Receptor; PDE4D=Phosphodiesterase 4D; TBR-I/II = TGF-β receptor I/II; pMLC= phosphorylated myosin light chain

DONOR CHARACTERISTICS*

Sex, M/F	27/14
Age, y/o	30.88 (14.07)
Race, C/B/H	28/7/6
BMI, kg/m^2	26.88 (6.74)

Data represent means (SD); n=41 donors. M, male; F, female; C, Caucasian; B, Black; H, Hispanic; BMI, body mass index
*Donors died of head trauma or cardiovascular incident, including stroke

Table 3-S1: Donor demographics for cAMP and *pde4d* expression studies. All cells were derived from subjects with no history of chronic disease.

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CHAPTER FOUR

Summary, Conclusions, and Future Directions

4.0. Summary

β2 adrenergic receptors (β2AR) are G protein coupled receptors essential for maintenance therapies for asthma and COPD as they are the targets for $G_{\alpha s}$/adenylyl cyclase (AC)/cAMP/protein kinase A (PKA) signaling. The profibrotic cytokine TGF-β1 has been shown to modulate contraction and dilation mechanisms of the airway smooth muscle (1, 2). These adverse effects by TGF-β1 may be mediated by β2AR desensitization, internalization, and phosphorylation as alterations in signaling have been shown to be induced by inflammatory mediator release following exposure to cytokines like IL-13 and TNFα (3–7). In addition, inflammatory mediators have been shown to alter signaling downstream of the receptor to blunt the effects of β2-agonists (4, 8, 9). Intracellular cAMP accumulation relies on the inhibition of phosphodiesterases (PDEs), playing a critical role in airway smooth muscle relaxation (10). Specifically, PDE4 represents a major subtype and PKA participates in the phosphorylation and the activation of PDE4 (11). Thus, we set out to quantify the contribution of TGF-β1 in altering each mechanism through modulation of gene expression and phosphorylation levels. Steroids have been shown to reverse the β2AR desensitization (12) and we posited that glucocorticoids may reverse the effects of TGF-β1.

In this framework, the present thesis addresses the following hypotheses:

(1) TGF-β1 attenuates β2-agonist-induced relaxation via alterations in cAMP/protein kinase A pathways.

(2) Steroids reverse TGF-β1-mediated β2AR desensitization and modulate β2AR-induced bronchodilation.

In Chapter 2 of this thesis, I worked in collaboration with Dr. Christie Ojiaku and the laboratories of Dr. Steven An and Dr. Rennolds Ostrom to uncover a mechanism for TGF-β1 in the β2AR. Dr. An's laboratory demonstrated that TGF-β1 augments contractility of HASM using Magnetic Twisting Cytometry (MTC), in a similar manner shown in IL-13-treated HASM (13). In addition, I have demonstrated that TGF-β1 decreases relaxant responses in HASM cells, suggesting the effects on cAMP depend on a mechanism by which TGF-β1 alters signaling both proximal and downstream of the β2AR. Forskolin, a pharmacological activator that bypasses the the β2AR, had little effect on agonist-induced cAMP production following TGF-β1 treatment. The differential response by forskolin may indicate desensitization and uncoupling of the β2AR. The Ostrom laboratory showed that TGF-β1 increases PDE4D gene expression, potentially leading to the inhibition TGF-β1 in relaxation responses. This suggests that PDE4D is the main player in TGF-β1-mediated attenuation of β-agonist-induced relaxation responses. The necessity of PDE4 in mediating the effects of TGF-β1 on agonist-induced relaxation was examined by inhibiting PDE activity with roflumilast, a selective PDE4D inhibitor. Our laboratory previously demonstrated that TGF-β1 induces MLC phosphorylation, and this thesis work shows that reversal of MLC phosphorylation is blunted by TGF- β1. We show that PDE4 inhibition rescues the ability of ISO to effectively reduce MLC phosphorylation in TGF-β1-treated cells. We observed that TGF-β1 augments PDE4D gene expression via a Smad2/3-dependent mechanism. Collectively, our results suggest that TGF-β1 is a novel therapeutic target in asthma as it blocks the effectiveness of β2-agonist bronchodilators.

The data in Chapter 3 of this thesis demonstrates that TGF-β1 alters β2AR signaling by affecting events proximal to the receptor, including $G_{\alpha s}$ activation. We found that TGF-β1 blocks attenuates relaxation induces by a β2-agonist (ISO) after TGF-β1-stimulation, that this attenuation also occurs in response to stimulation with a $G_{\alpha s}$ activator, cholera toxin (CTX) We also demonstrated that dexamethasone (DEX) reverses TGF-β1-induced PDE4D expression and rescues ISO and CTX-induced cAMP production.

With our experiments, we have been able to show that TGF-β1-induced signaling attenuates agonist-induced relaxation and posit that by targeting TGF-β1 in asthma responsiveness of the airways to bronchodilator therapy will be restored.

4.1. Future Directions

4.1.1. Investigating the Role of TGF-β1 Modulating Expression of $G_{\alpha i}$, *let-7f*, and Adenylyl Cyclase Activity

Our data demonstrates that TGF-β1 affects signaling β2-agonist-induced bronchodilation, however, it would be of interest to determine if TGF-β1 alters expression or the activity of $G_{\alpha i}$.

While specific microRNAs (miRNAs) like let-7f modulate β2AR expression (14), with prolonged β2-agonist activation attenuating let-7f levels, thus repressing downregulation of β2AR expression. We hypothesize that TGF-β1 modulates let-7f miRNA, thereby playing a role in downregulation of β2AR and promoting translational repression after treatment with a β2-agonist.

The data reported in Chapter 2 and 3 of thesis indicates that TGF-β1 mediates HASM cell hyporesponsiveness to β2AR and $G_{\alpha s}$ activation but has little effect on FSK-induced cAMP generation. TGF-β1 inhibition of cAMP generation is Smad3 dependent. It would be fruitful to explore the effects of TGF-β1 on AC activity and on its isoforms, which can offer a different therapeutic approach for asthma treatment as the enzyme catalyzes the conversion of ATP to cAMP.

4.1.2. The Effect of Budesonide on TGF-β1-mediated Hyporesponsiveness to Bronchodilators

Interestingly, another GC, budesonide (BUD), did not rescue TGF-β1-induced hyposensitivity to ISO or CTX (Figure 4-1), and had little effect on attenuating *pde4d* expression (Figure 4-2). Interestingly, RNAseq analysis of BUD-stimulated HASM (Figure 4-3A), showed a substantial increase in *pde4d* expression ($p=8.93\times10^{-39}$), whereas DEX pretreatment had little effect on *pde4d* expression p=0.13495 (Figure 4-3B).

The differential effects of these steroids may be structurally-related (15, 16). Studies have shown varying effectiveness of different glucocorticoids in patients (17, 18), which may in part contribute to the differential effects on reversing TGF-β1-induced effects on bronchodilation that we've observed. It would be interesting to further delve into a possible mechanism underlying the lack of reversal observed with BUD treatment in the presence of TGF-β1.

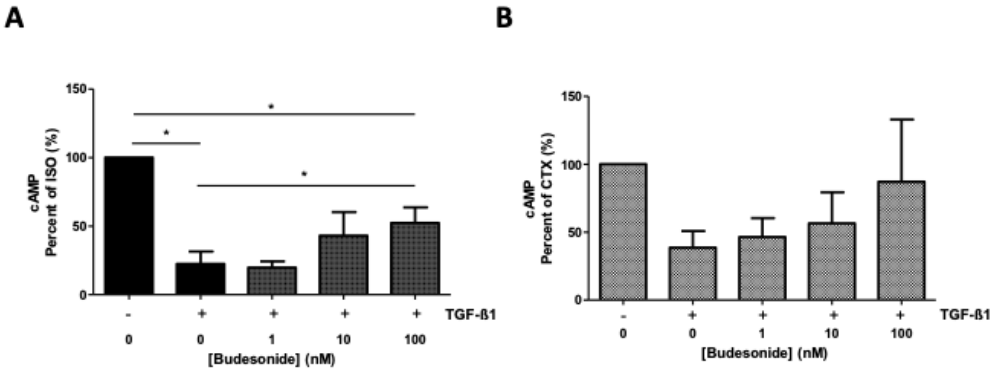
A
B
cAMP
Percent of ISO (%)
cAMP
Percent of CTX (%)
150
100
50
0
*
*
*
-
+
+
+
+
TGF-ß1
0
0
1
10
100
[Budesonide] (nM)

Figure 4-1: Budesonide (BUD) has little effect on TGF-β1-mediated attenuation of ISO- and CTX-induced cAMP production in HASM. HASM were pretreated with BUD (1-100 nM, 30 min) prior to TGF-β1 (10 ng/ml, 18 hr) stimulation. HASM were subsequently stimulated with (A) ISO or 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 (A) ISO or (B) CTX. Data is representative of n=6 separate cell lines as mean ± SEM, *$p<0.05$ compared to TGF-β1 and ISO treatments.

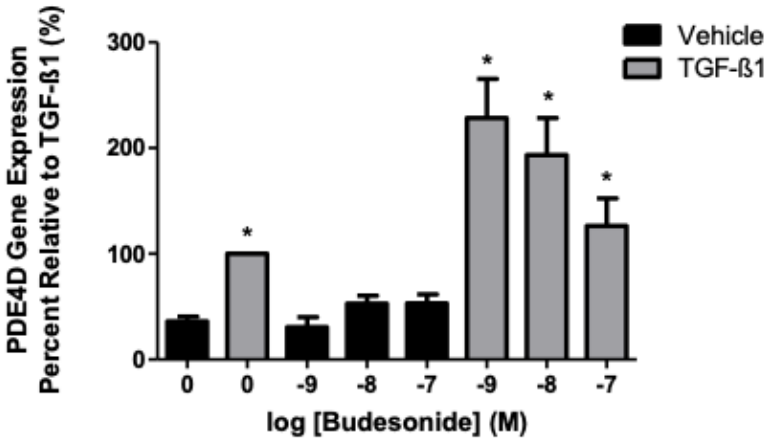
PDE4D Gene Expression
Percent Relative to TGF-ß1 (%)
300
200
100
0
Vehicle
TGF-ß1
*
*
*
*
0
0
-9
-8
-7
-9
-8
-7
log [Budesonide] (M)

Figure 4-2: Budesonide (BUD) does not decrease TGF-β1-induced *pde4d* expression in HASM. HASM were pretreated with BUD (1-100 nM, 30 min) prior to stimulation with TGF-β1 (10 ng/ml, 18 hr). mRNA was extracted, reverse transcribed, and assessed for *pde4d* expression by TaqMan qPCR analysis. Data is represented as % of *pde4d* expression induced by TGF-β1. Data is representative of n=4-6 separate cell lines as mean ± SEM, *$p<0.05$.

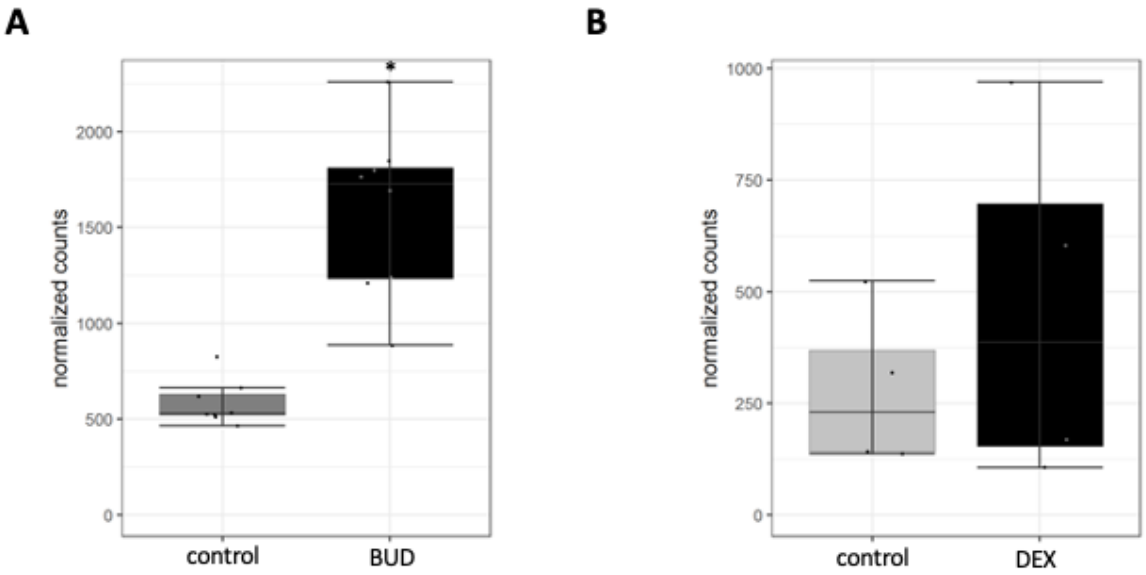
A
normalized counts
2000
1500
1000
500
0
control
BUD
B
normalized counts
1000
750
500
250
0
control
DEX

Figure 4-3: BUD, but not DEX, induces *pde4d* expression in HASM. HASM derived from non-diseased subjects were stimulated ± (A) BUD (100 nM, 24 hr) or (B) DEX (1 μM, 24 hr), mRNA prepared, and samples subjected to RNAseq analysis (as described in (19)). Data is representative of (A) n=8, p=8.93E-39 and (B) n=4, p=0.135 separate cell lines and expressed as normalized counts. Donor demographics are as previously described (Table 3-S1).

4.1.2. Albuterol-induced Desensitization of E-type Prostanoid (EP) Receptors

We found that TGF-β1 blunts not only cAMP levels to isoproterenol (ISO), but also to other agonists that signal through G-coupled protein receptors termed the E-type prostanoid (EP) receptors. The prostaglandin E_2 receptor (P2) is also $G_{\alpha s}$-coupled. Our lab previously demonstrated homologous desensitization of the β2AR in human precision cut lung slices (12). I demonstrate, using phosphorylation of MLC as a readout, this process in HASM, and examine whether heterologous desensitization of EP2R occurs with β2-agonist pretreatment.

Contractile agonist-induced MLC phosphorylation is decreased with increased PKA activity, a critical signaling event in airway smooth muscle relaxation (20, 21). To investigate whether the desensitization of the β_2AR is mediated through $G_{\alpha s}$ activation, HASM cells were treated with albuterol overnight and then stimulated with carbachol (Cch, a contractile agonist) and CTX. We observed that CTX exhibited similar effects as β2-agonist, ISO, showing significantly increased MLC phosphorylation (Figure 4-4) following overnight desensitization to albuterol and Cch stimulation.

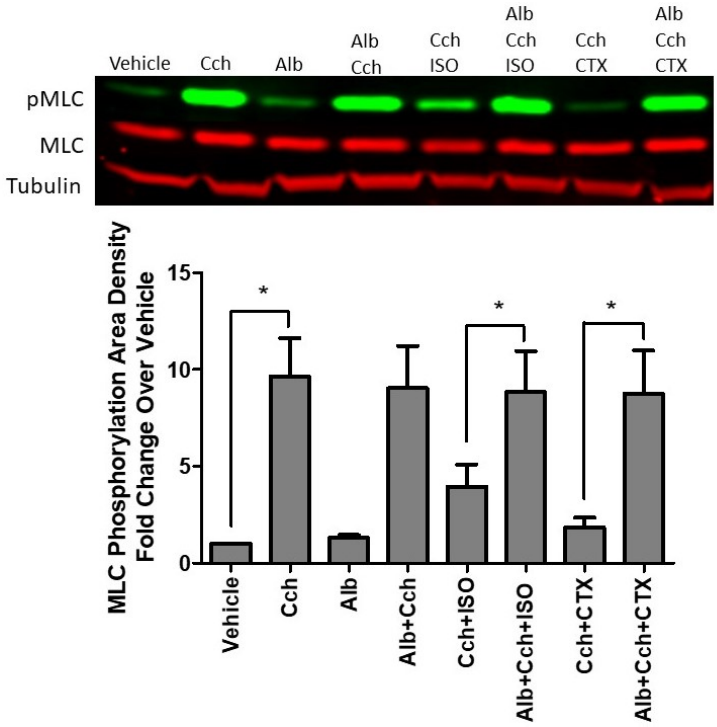
Vehicle
Cch
Alb
Alb Cch
Cch ISO
Alb Cch ISO
Cch CTX
Alb Cch CTX
pMLC
MLC
Tubulin
MLC Phosphorylation Area Density Fold Change Over Vehicle
15
10
5
0
*
*
*
Vehicle
Cch
Alb
Alb+Cch
Cch+ISO
Alb+Cch+ISO
Cch+CTX
Alb+Cch+CTX

Figure 4-4. **Albuterol-induced desensitization of agonist-induced β2AR and $G_{\alpha s}$ activation in HASM cells.** HASM cells were serum starved and treated with albuterol (10 μM; 24 hr). The media was removed, and cells were stimulated with carbachol (Cch) (20 μM; 13 min) ± ISO (1 μM; 10 min), or CTX (0.25 μg/mL; 1 hr) prior to collection. Cells were lysed and probed for phosphorylation of MLC and tubulin. Data represents mean + SEM of seven independent normal donors with the phosphorylation normalized to baseline levels. *$p<0.05$

To further examine whether heterologous desensitization of the EP2 receptor occurred following overnight albuterol treatment, PGE2-induced relaxation was measured with or without overnight albuterol then HASM cells were stimulated with contractile agonist, Cch. Opposite to what was observed with CTX or ISO treatment, PGE_2 decreased MLC phosphorylation in a dose-dependent manner despite albuterol pretreatment (Figure 4-5). Figure 4-5 illustrates that β2AR tolerance has little effect on EP receptor-induced relaxation of HASM. Like the β2AR, the EP receptor is coupled to $G_{\alpha s}$ leading to increased cAMP production. These data demonstrate that desensitization of the β2AR does not induce heterologous desensitization, suggesting that PGE_2 can still stimulate PKA activity in airway smooth muscle cells to induce relaxation. This also may suggest that the EP receptor may be selective when activating $G_{\alpha s}$.

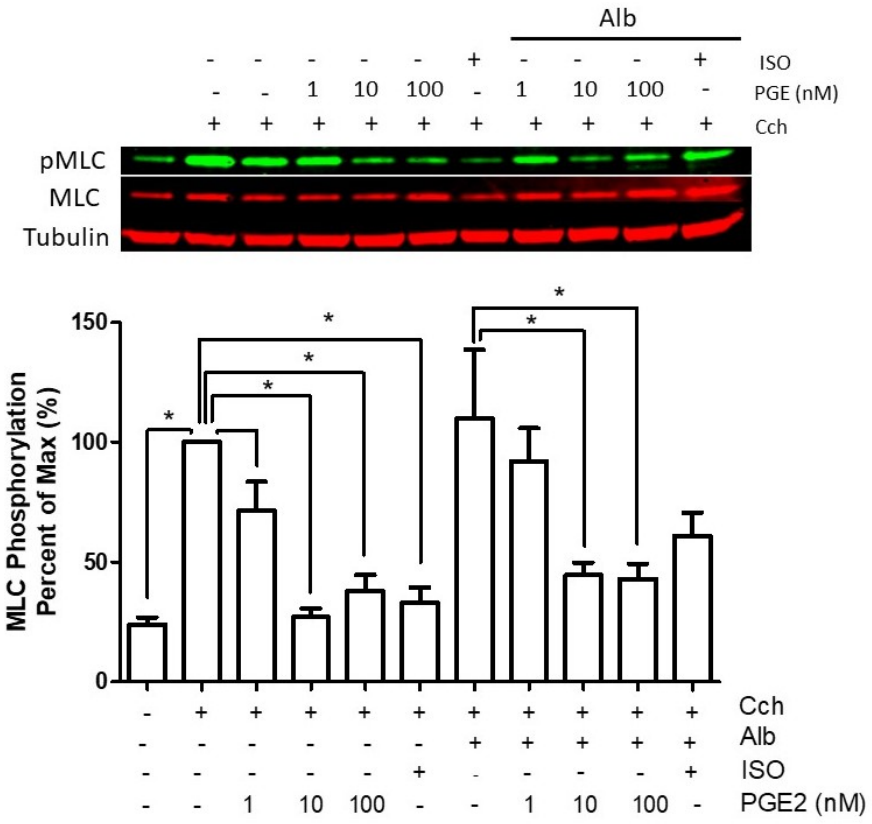
Alb
ISO
PGE (nM)
Cch
pMLC
MLC
Tubulin
MLC Phosphorylation
Percent of Max (%)
150
100
50
0
Cch
Alb
ISO
PGE2 (nM)

Figure 4-5. Albuterol-induced desensitization has minimal effect on signaling through the EP2/EP4 receptor. HASM cells were preincubated with albuterol (10 μM; 24 hr) after serum deprivation. HASM cells were stimulated with Cch (20 μM; 13 min) ± PGE2 (1-100 nM; 10 min) or ISO (1 μM; 10 min), then lysed and subjected to immunoblot. Data represents mean ± SEM of 8-10 different donors. *$p<0.05$, one-way ANOVA, comparing Cch versus Alb and Cch treatment.

In summary, the work described in this thesis indicates that TGF-β1 signaling through Smad2/3 contributes to β2AR dysfunction, and that dexamethasone restores TGF-β1-induced dysfunction in HASM cells. Additionally, we demonstrate that β2AR desensitization does not induce heterologous desensitization of another $G_{\alpha s}$-coupled GPCR.

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