ESTROGEN RECEPTORS SIGNALING IN AIRWAY SMOOTH MUSCLES: ROLE IN INTRACELLULAR CALCIUM REGULATION AND CONTRACTILITY

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Title

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

Epidemiological evidence suggests higher incidence and severity of asthma in pre-menopausal women and aging men, suggesting a role of sex steroids, especially estrogen. In this regard, it is not clear whether specific estrogen receptors (ERα and ERβ) play differential roles or whether there is any imbalance in their normal signaling during asthma. Airway smooth muscle (ASM) cell is of contractile phenotype which is involved in contractions and airway hyperresponsiveness. Therefore, this research focused to understand ER signaling in the context of airway hyperresponsiveness and bridge the gaps in knowledge about the role of ERs in human ASM cells.

The first aim demonstrate the long-term differential signaling of ERs in the regulation of $[Ca^{2+}]_i$ handling in the human ASM. It was found that ERα activation increases the $[Ca^{2+}]_i$ response in baseline conditions, while ERβ activation has neutral effect. Moreover, the differential signaling of ERs is more evident in asthma or inflammation where ERβ activation decreases the $[Ca^{2+}]_i$ response in the presence of inflammation while ERα increases it. Further elucidation of the mechanisms of their signaling on $[Ca^{2+}]_i$ suggests that ERβ results in decreased $[Ca^{2+}]_i$ response through increased SERCA2 expression and function, inhibition of pathways involved in activating the voltage gated LTCC and maintenance of the morphology of mitochondria.

In the next aim, it was found that ERβ causes a potentiation of the activity of β_2-AR which leads to an increase in cAMP. Also, ERβ is found to be involved in dephosphorylation of contractile apparatus ultimately leading to bronchodilation. Presenting a contrasting picture, ERα causes an increase in pro-contractile machinery such as RhoA activity and phospho-MYPT leading to increased overall contractility in ASM cells.

To confirm these *in vitro* findings in the presence of other structural and immune cells involved in inflammation, I have further evaluated the *ex vivo* and *in vivo* roles of ER signaling in airways. Interestingly, ERβ was found to be protective for the airways while ERα further aggravated the contractility of the airways. These novel findings of ER signaling in the context of contractile mechanisms of the airways can be utilized in designing novel therapeutics for bronchodilation.

ACKNOWLEDGMENTS

I came to NDSU for my PhD program, in the Department of Pharmaceutical Sciences in September 2016 and it had been a wonderful experience. First and foremost, I would like to express my heartfelt gratitude to Dr. Sathish Venkatachalem, my advisor, whose continuous guidance and support allowed me to complete this research and dissertation successfully. Throughout these years, he was constantly involved in polishing my research and writing skills that helped me to receive several accolades and shape my career as a scientist.

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My gratitude towards Dr. Jagdish Singh, Chair in the Department of Pharmaceutical Sciences for his inspirational words and providing excellent research facilities in the department. On similar note, I also appreciate all faculty, staff and graduate students from College of Health Professions for their valuable advice and motivation.

My parents have been the motivation behind all the things I do in my life. I cannot thank them enough for their selfless love, support and sacrifices. I am extremely grateful to my brother and sister-in-law who have backed me for all important decisions in life and whose support made me strong even through the worst phase. I have also been very blessed to find a source of everlasting motivation, courage, and strength in Karthik, my husband. I want to thank him from the bottom of my heart for deciding to shift to Fargo from Canada and enduring all my frustrations and stress with tremendous patience throughout my PhD. My mother-in-law, father-in-law, and

brother-in-law as well had been exceptionally cooperative and understood all my commitments towards my PhD.

I have been fortunate to be working with many post-doctoral fellows. All of them have been constant source of guidance and have helped in learning many research skills. Interactions with graduate and undergraduate fellows in the lab and in the department have helped me to learn science and I have gained some life -long friends through this lab.

I would like to give a special thanks to Janet Krom, Diana Kowalski and Tiffani for smooth handling of the administrative processes and the animal core facility staff which has helped in animal breeding and maintenance.

Finally, I would like to thank the support from various funding agencies and our collaborators at Mayo Clinic.

DEDICATION

I dedicate this work to my family.

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LIST OF ABBREVIATIONS

Abam …… Antibiotic antimycotic

AC..... Adenylyl cyclase

Ach……. Acetylcholine

AHR……..... Airway Hyperresponsiveness

cAMP……..... 3'-5'-cyclic adenosine monophosphate

ASM…….....Airway smooth muscle

β_2-AR…….……. β_2-adrenergic receptor

BSA…… Bovine Serum Albumin

$[Ca^{2+}]_i$ ……..……..Intracellular calcium concentration

CAM…….……Calmodulin

Cch……...Carbachol

CICR……Calcium induced calcium release

COPD..... Chronic obstructive pulmonary disease

DAG…… Diacylglycerol

DBD……. DNA binding domain

DMEM…….…….……... Dulbecco's Modified Eagle's Medium

DPN…….…….……Diaryl-propionitrile

Drp1…….…….Dynamin-related Protein 1

E_2…….…….17β-estradiol

ER……..…Estrogen receptor

ECM……. Extracellular Matrix

ERE…….…… Estrogen Response Elements

EDTA…….……... Ethylene Diamine Tetra Amino Acetate

ELISA……..... Enzyme linked immunosorbent assay

FBS…….…….. Fetal Bovine Serum

FOT……..…… Forced Oscillatory Technique

GPCR……..…….. G-Protein coupled Receptor

GPER……..…….. G-Protein Estrogen Receptor

.

GFP……..……. Green Fluorescent Protein

GTPase……..…….…... Guanosine Triphosphate

HBSS…….……... Hank's balanced salt solution

HDM ……..... House Dust Mite

H and E…….……...…..... Hematoxylin and Eosin

IgE……..…..…… Immunoglobulin – E

IL……....…….……..... Interleukin

INF…….…….…….. Interferon gamma

IP_3…….…….……... Inositol triphosphate

ISO……..…….…….. Isoproterenol

IR…….…..……... InfraRed

IC……..…….…… Inhibitory Concentration

IBMX…….…….. 3-isobutyl-1-methylxanthine

KCl……..... Potassium Chloride

KO…… Knock-out

LBD……..…… Ligand binding domain

LTCC…… L-Type Calcium Channel

$LaCl_3$ …… Lanthanum Chloride

MA…….……..... Mixed Allergen

MPP……..…….1,3-*Bis*(4-hydroxyphenyl)-4-methyl-5-[4-(2-piperidinyl ethoxy) phenol] – 1*H*-pyrazole dihydrochloride

MLC_{20}……..……..Myosin light chain (regulatory 20)

MLCK…….……...Myosin light chain kinase

MLCP……Myosin light chain phosphatase

MYPT…….....Myosin Phosphatase Target Subunit

Mfn……Mitofusin

Mch…….…….....Methacholine

NCX……..……..Sodium-calcium exchanger

OVA…….…….……Ovalbumin

PKA…….…….…….Protein kinase A

PKC…….……..Protein kinase C

PLC…….....Phospholipase C

PDE…….…….……Phosphodiesterase enzyme

PHTPP……..……4-[2-Phenyl-5, 7-*bis* (trifluoromethyl) pyrazolo[1,5-*a*]pyrimidin-3-yl]phenol

PMCA…….……..Plasma membrane calcium ATPase

PPT……(4,4′,4″-(4-propyl-[^{1}H]-pyrazole-1,3,5-triyl) tris-phenol

PBS…….....……Phosphate Buffered Saline

PVDF……..……Polyvinylidene fluoride

PDGF……..……Platelet Derived Growth Factor

PCLS……...Precision Cut Lung Slices

qRT-PCR……Quantitative Real Time Polymerase Chain Reaction

ROCC……..Receptor operated calcium channel

RYR……Ryanodine receptor

ROCK…….....……..Rho Kinase

Rrs …….……....…..Resistance

SERCA……Sarco-endoplasmic reticulum calcium ATPase

SOCC……..Store operated calcium channel

SOCE……...Store operated calcium entry

SR……Sarcoplasmic reticulum

siRNA……..Small Interference RNA

TBS……..Tris-buffered saline

TBST……Tris-buffered saline tween

THC…….(R,R)-5,11-Diethyl-5,6,11,12-tetrahydro-2,8-chrysenediol

Th cell……...……..T-Helper Cell

TNFα……....Tumor necrosis factor alpha

VDCC……..Voltage-dependent calcium channel

VASP……Vasodilator-stimulated phosphoprotein

WT……...Wild type

CHAPTER 1. GENERAL INTRODUCTION

1.1. Asthma

Asthma is one of the most prevalent non-communicable diseases and affects almost 20 million people in the U.S and more than 330 million people globally. It is also estimated that severe asthma accounts for about 250000 deaths per year worldwide. It is at present, the fourth leading cause of death globally, and is estimated to become the third leading cause by 2030. Currently available drugs such as corticosteroids and bronchodilators have provided symptomatic relief to several patients despite having several side effects. However, these drugs generally do not reverse the changes in airways and hence their effects rapidly vanish as drugs are discontinued. Thus, prolonged asthma treatments results in~$50 billion per year cost to society and calls for better therapeutic strategies [1].

1.2. Pathogenesis of Asthma

Asthma is a chronic disease of the airways in which inflammatory milieu is established in the airways, mediated by pathogens and mediators. Due to various triggers, T lymphocyte cells generate cytokines and chemokines involved in the recruitment and activation of mast cells, basophils, and eosinophils. Depending on the inflammatory profiles, T-helper cell mediated inflammatory processes are classified into two broad categories- Th2 high and Th2 low. The Th2 high or allergic subtype is characterized by significant increase in eosinophilic infiltration of the airways and is associated with predominance of Th2 cytokines (for e.g., IL-13). Conversely, the Th2 low or non-allergic subtype is characterized by neutrophilic infiltration and include either Th1 (for e.g., IFN-γ, TNFα) or Th17 (for e.g., IL-17, IL-22) responses [2, 3].

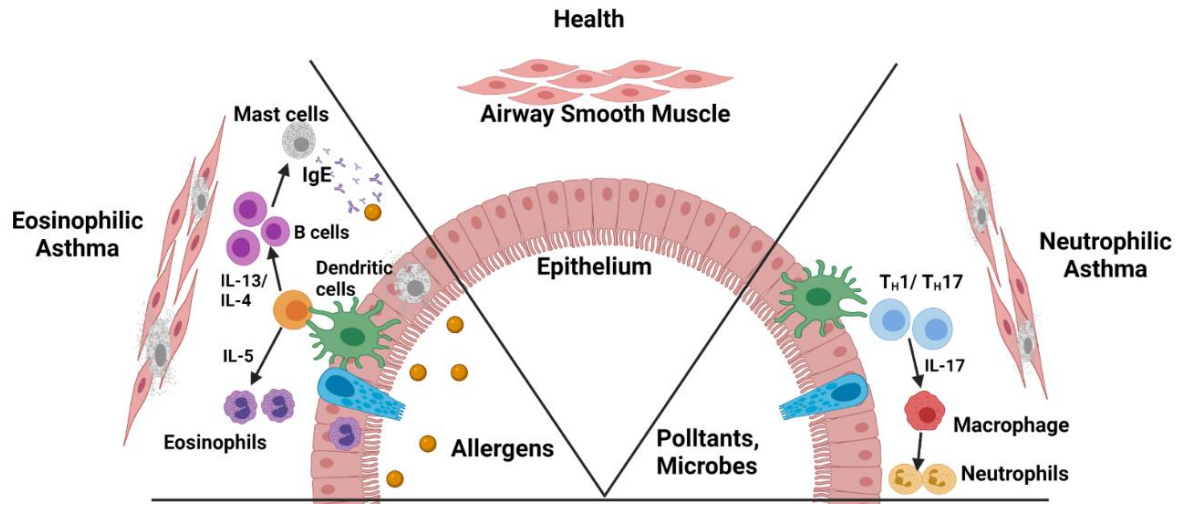

Figure 1. Mechanisms of asthma pathophysiology.
The Th2-high asthma subtype portrays a high level of eosinophils in the airways, and the Th2-low subtype is characterized by neutrophilic inflammation. IL=interleukin TH=T helper (Adapted from [2, 3]).

Because of trafficking of a myriad of immune cell types and chemokines, structural layers of airways are inflamed leading to variable airflow obstruction. In susceptible individuals these inflammatory events cause repeated episodes of wheezing, breathlessness, chest tightness, and recurrent coughing.

1.3. Airway Smooth Muscle

The two integral features of asthma are airway hyperresponsiveness (AHR) and airway remodeling. Amongst the three structural layers, fibroblasts, ASM and epithelial layer, it is the ASM layer which plays a principal role in both features [4-6]. Structurally, ASM cells increase in mass and size and produces a more extracellular matrix (ECM) resulting in reduced airway lumen; this phenomenon causes airway remodeling. Functionally, there is an uncontrolled contractile response of ASM resulting in inordinate bronchoconstriction in response to relatively subtle stimuli; this phenomenon is referred as AHR [6].

Several therapies aimed at various signaling molecules in ASM have shown success to some extent for the treatment of remodeling and hyperresponsiveness in asthma. However, a crucial barrier in our ability to effectively treat individuals with asthma is the gap of knowledge in various predisposing factors, phenotypes, and epidemiological patterns in the prevalence and severity of asthma. Hence, in the recent years, the research is focused on in-depth knowledge of asthma prevalence for personalized targeted treatment of patients suffering from asthma. Although corticosteroids have become a standard therapy for managing asthma symptoms in many cases, they represent a broad-spectrum empiric approach. Such approach is flawed since its strategy is based on one-size-fits all concept, whilst asthma being a heterogeneous disease is characterized by various endotypes. Hence, specific therapies targeting pathological mechanisms, particularly found in asthma in different population groups are still to be developed and a deeper understanding of the pathogenesis and demographics of asthma is a prerequisite for such specific therapies.

1.4. Gender Disparity in Asthma Prevalence

There is inherent sex driven changes in the anatomy of the lung at all stages of life right from the embryonic lung development through adulthood. Although, the female lungs are smaller compared to that of men, the ratio of airways to lung parenchyma of females grow proportionally, as opposed to that in males where airways grow slower resulting in inordinately fewer airways. Due to such structural variations, flow rates in the airways are higher in adult women than that in men. Thus, overall, the airways of females are innately superior to that of the males [7, 8]. Paradoxically, the clinical surveys suggest that these favorable anatomical traits are completely nullified by the greater incidence of asthma in boys as compared to girls and more in women as opposed to men [9, 10]. Such stark transition in asthma prevalence based on gender conforms with the changes in sex hormones and suggest an implication of sex hormones in asthma pathogenesis.

Although higher asthma prevalence in women hints towards the deleterious role of female sex hormone estrogen, a scrutinization into the fluctuations of asthma exacerbations during different phases of menstrual cycle suggests symptom severity in asthma during lower plasmatic estrogen levels [11, 12]. This complexity in the pattern of asthma prevalence in women during various stages of life calls for the need to study the receptors involved in estrogen signaling.

1.5. Estrogen Receptors

The role of estrogen is observed in a diverse range of body organs in both males and females. Estrogen's mechanism of action primarily occurs through two estrogen receptor isoforms (ERs), ERα and ERβ. These full-length ER receptors comprises of many functional domains which are denoted as A/B, C, D, and E/F. Amino-terminal A/B region leads to the gene transcription. The C region is the DNA binding domain, which contributes to estrogen receptor dimerization and contributes to binding to specific sequences in the DNA. These sequences on DNA are referred as estrogen response elements (ERE). The D domain is a hinge region that connects the C and E domains. This region also signals for the nuclear localization, and thus it causes the translocation of receptor-ligand complexes to the nucleus. In E/F region, which is also known as the ligand binding domain, estrogen binding region is located. The ligand binding domains of ERα and ERβ share a 55% homology and majority of the endogenous estrogenic compounds have similar affinities towards both ERα and ERβ [12, 13].

Alternative processing of a precursor mRNA results in formation of a variety of splice variants and truncated isoforms, many of which are translated into proteins. Hence, multiple splice variants and truncated isoforms of estrogen receptors are found, and their expression and functions are highly cell specific and tissue specific. ERα-36 and ERα-46, the truncated isoforms of ERα, are characterized by the lack of activation factor (AF-1) domain and confers complex effects on

the full length ERα. There are three ERα splice variants - ERαΔ3, ERαΔ5 and ERαΔ7 and five ERβ splice variants termed as ERβ1 -ERβ5 which are experimentally validated [14, 15]. ERα splice variants undergo exon deletions, for instance, ERαΔ3 lacks a DNA binding domain and ERαΔ5 is truncated, missing the entire ligand binding domain. Specific alternative slicing of exon 8 results in ERβ splice variants, causing variations at the E and F regions and hence the LBD of the protein is the main functional component that is altered in these splice variants[15].

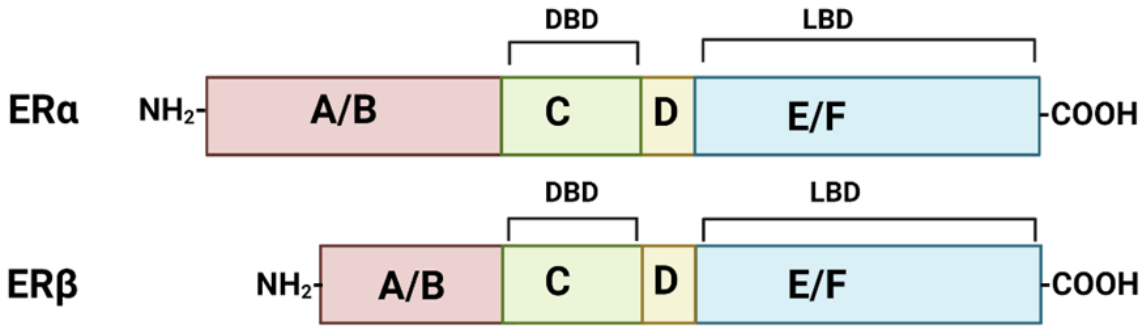

Figure 2. Structure of estrogen receptors.
Six domains of estrogen receptors are A-F. Functionally these are N-terminal domain (NTD, A/B domains), DNA binding domain (DBD, C domain), the hinge (D domain), and ligand binding domain (LBD, E/F domains) with (Adapted from [12, 16]**).**

1.6. Estrogen Receptor Signaling

ERα and ERβ belong to the steroid hormone superfamily of nuclear receptors and primarily function via activation of target genes through a genomic pathway causing transcription of genes, translation of mRNAs into proteins, and their integration into functional pathways and all these processes are reported to take hours or days to develop [17-19]. Apart from the genomic signaling, they also act by rapid activation of signaling pathways through a non-genomic signaling which occurs within seconds to minutes and involves simple biological events such as activation of kinases and phosphatases and fluctuations in calcium ions across membranes [20]. Recently, a membrane associated G-protein-coupled estrogen receptor known as GPR30 or GPER has been

discovered through which estrogen acts less predominantly. All these receptors are present and exert their actions in a wide variety of tissues/cells, such as vascular endothelial, cardiomyocytes, neuron, and smooth muscle. Although ER signaling is established in other cell types and is highly cell and context-dependent, knowledge of their mechanisms, especially in disease states, such as asthma, seems limited.

1.7. Research Gaps

Previous studies have demonstrated that these specific estrogen receptors (ERs) show differentiation in expressions with a multi-fold increase in the expression of ERβ as compared to ERα in asthmatic ASM and that might suggest a greater role of ERβ than ERα in asthma. Also, these studies have reported minuscule or undetectable expression and function of GPR30 in non-asthmatic or asthmatic human ASM [21]. It has also been reported that acute (possibly non-genomic) activation of ERs reduces intracellular calcium ($[Ca^{2+}]_i$) response and increases cAMP in normal ASM cells aiding bronchodilation [22, 23]. While the non-genomic E_2 effects on ASM contractility governed by $[Ca^{2+}]_i$ and cAMP are interesting, it does not give a complete picture of what happens during inflammation. Also, asthma as well as variations in hormonal levels in men or women occurs over prolonged time periods, hence it is likely that the complex and conflicting data on estrogen and asthma presumably reflect genomic ER effects in the presence of persistent inflammation. **Thus, the central hypothesis of this proposed research is that inflammation leads to a differential and greater role for ERβ in ASM as compared to ERα, and that ERβ can blunt contractility.** The clinical significance of the proposed work will be to demonstrate how the long-term (supposedly genomic) ER signaling differentially regulates airway tone and hyperresponsiveness in normal and inflamed conditions, setting the stage for future exploration of specific ERs in immune cells vs. structural cells of the airway, and helping to explain the overall

paradox of estrogens in asthma. I laid the following specific aims to explore my hypothesis and accomplish the overall objective of this research.

- **Specific Aim 1: To determine the differential role of ERα vs. ERβ in intracellular calcium regulation in human ASM cells with inflammation/asthma.** Previous data demonstrated that acute (possibly non-genomic) activation of ERs reduces intracellular calcium ($[Ca^{2+}]_i$) response in non-asthmatic ASM cells. Hence, this aim is to evaluate long-term (possibly genomic) effects of ERα vs. ERβ signaling on $[Ca^{2+}]_i$ handling in non-asthmatic and asthmatic human ASM cells and in the presence of cytokines**.**
- **Specific Aim 2: To determine the differential role of ERα vs.ERβ in the human ASM contractility.** Previous data demonstrated that acute estrogen exposure increases cAMP accumulation and potentiates β-adrenoreceptor in non-asthmatic ASM cells. Hence, this aim is to evaluate long-term effects of ERα vs. ERβ signaling on various mediators of contractility.
- **Specific Aim 3: In a mouse model of allergic asthma, to determine the effect of ERα vs. ERβ on structural and functional airway changes**. The aim is to apply a mixed allergen model to WT vs. ERα vs. ERβ KO mice and to explore the relative roles of ERβ vs. ERα, respectively in the context of airway hyperresponsiveness.

This research will have a significant impact on several ongoing research on sex differences in lung diseases. It will present a comprehensive understanding about differential role of estrogen receptors in airway contractility, which will help in designing estrogen receptor based personalized and targeted therapies with higher beneficial effects and lower risks. Estrogen and its receptor have multifaceted roles in various other diseases and therefore, this research will also have broader

significance and will advance our understanding about the overall role of estrogens in health and disease.

CHAPTER 2. ESTROGEN RECEPTORS DIFFERENTIALLY REGULATE INTRACELLULAR CALCIUM HANDLING IN HUMAN NONASTHMATIC AND ASTHMATIC AIRWAY SMOOTH MUSCLE CELLS[1]

2.1. Introduction

The rise of the cytosolic Ca^{2+} concentration is a key event in the contraction of ASM cells. During asthma, it is observed that there is alteration in the baseline Ca^{2+} and agonist induced Ca^{2+} increase in ASM [24]. Calcium is a second messenger not only in contractility, but also plays indispensable role in many other physiological events such as the activity of enzymes and ion channels, secretion, differentiation, cell proliferation, and death. Therefore, an exploration of the mechanisms underlying calcium handling in ASM during airway hyperresponsiveness are of paramount importance.

2.1.1. Calcium Influx and Efflux

Basal $[Ca^{2+}]_i$ is maintained at ~80 to ~100 nM, which is necessary to sustain the airway tone and shape. When triggered to an excited state, an ASM cell has a capacity to hold calcium in the micromolar range (10^{-6} M), which is 10 times higher than the levels at resting condition[25]. To compensate the high amount of extracellular calcium influx, the ASM cells also possess mechanisms by which accumulated cytosolic calcium is expelled out of the cell to maintain the approximately 10^4 - fold transmembrane gradient for calcium [26].

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[1]The material in this chapter was co-authored by Bhallamudi Sangeeta, Jennifer Connell, Dr. Christina Pabelick, Dr. YS Prakash and Dr. Venkatachalem Sathish. Bhallamudi Sangeeta and Dr. Venkatachalem Sathish conceived the idea. Bhallamudi Sangeeta developed the techniques and performed all the experiments. Jennifer Connell assisted in compilation and calculation of calcium imaging data. Dr. YS Prakash, Dr. Christina Pabelick provided the human ASM cells and reviewed the manuscript. Dr. Venkatachalem Sathish served as proofreader and checked the math in the statistical analysis conducted by Bhallamudi Sangeeta [27].

ASM cells contain several pumps and channels proteins which carries out the influx and efflux of calcium in a synchronized fashion. These are the voltage-dependent calcium channels (VDCC), store operated calcium channels (SOCC) receptor-operated calcium channels (ROCCs). VDCCs perceive the voltage gradient across the membrane and open when the membrane becomes depolarized beyond a certain limit. However, the membrane depolarization triggers only a transient and limited calcium influx through VDCCs. On the other hand, the opening of SOCC and ROCC does not depend on membrane depolarization, and they are activated by a G protein coupled receptor agonists such as acetylcholine and they are thought to be calcium independent. Calcium efflux is carried out majorly by Plasma membrane calcium ATPase (PMCA) and sodium-calcium ATPase (NCX) pump [26].

2.1.2. Internal Calcium: Uptake and Release

Intracellular organelles such as the sarcoplasmic reticulum and mitochondria stores enormous amount of calcium (in millimolar range) [28]. These stores act as a physiological source of calcium through which contractile agonists exert their actions in muscle cells. Most of these GPCR agonists act through receptors that are coupled to phospholipase C, and this enzyme is responsible to metabolize phosphatidyl inositol phosphate leading to the generation of the second messenger molecules diacylglycerol (DAG) and inositol trisphosphate (IP_3). The latter opens various channels and pumps on the sarcoplasmic reticulum, thereby causing a transient elevation of the cytosolic calcium concentration [26, 29].

Once the calcium is released due to various triggers, the pool of calcium in the sarcoplasmic reticulum must be refilled and this is accomplished by an ATP driven calcium pump on the sarcoplasmic reticulum membranes – the sarco-endoplasmic reticulum calcium ATPase (SERCA). Calcium released from the sarcoplasmic reticulum triggers several nonselective channels to open,

ultimately leading to membrane depolarization and initiation of voltage-dependent calcium influx [26].

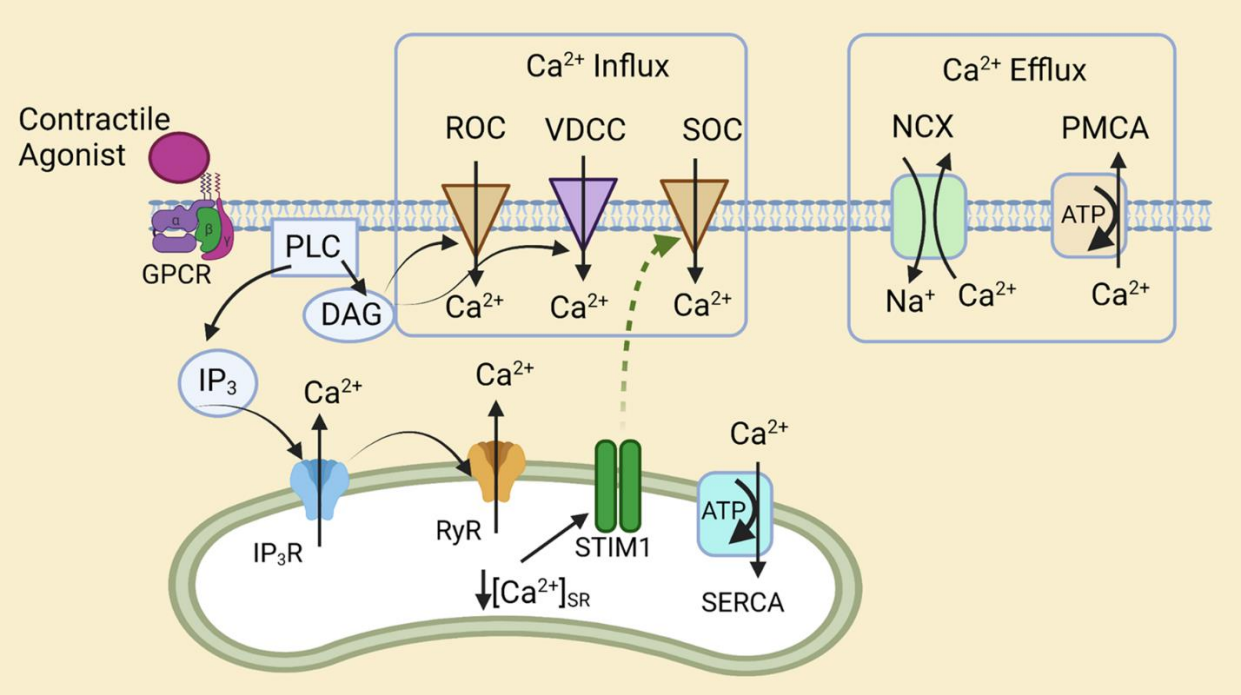

Figure 3. Influx and efflux of calcium ions through various channels and pumps involved in ASM cells.
PLC- Phospholipase, ROC-Receptor Operated Calcium Entry; STIM1 – Stromal Interacting Molecule 1; SOC- Store Operated Calcium Entry; NCX – Sodium Calcium Exchanger; PMCA- Plasma membrane Calcium ATPase; RyR- Ryanodine Receptor, DAG- Diacylglycerol; IP3- inositol trisphosphate [Adapted from [26, 28].

Inflammatory cells in the proximity of ASM and mitochondria release reactive oxygen species and free radicals into the airways (e.g., superoxide) and these free-radicals alter the calcium-handling [30]. Also, inflammatory mediators such as cytokines alter the properties of ASM through effects on calcium signaling. For e.g., IL-13 by itself elicits a transient calcium response in murine ASM cells and results in their contraction [31]. However, human ASM cells do not show this calcium spike with IL-13 treatment but exhibits increased calcium responses due to histamine [5, 32, 33]. TNFα also enhances the calcium response by similar mechanisms [34]. IL-13 and TNFα additionally increases the sensitivity of the contractile molecules to calcium by

stimulating Rho kinase [5, 35]. IL-13 also reduces the β-adrenergic mediated relaxation in ASM cells. Th2 cytokine is the major cytokine category that has been primarily linked to AHR and demonstrates the characteristics of eosinophilic asthma [5, 32].

Several human studies have demonstrated that female sex hormones improve contractile performance in postmenopausal women [9, 10, 36]. Previous studies have shown the constitutive expression of ERs in the lungs [21], and the expression levels seem to be comparable in both men and women. Studies have suggested that physiologically relevant concentrations of estrogens acutely reduce $[Ca^{2+}]_i$ responses to bronchoconstrictor agonists in human ASM cells and that this rapid estrogen effect on the reductions in $[Ca^{2+}]_i$ are largely mediated via ER[23]. Here, the full-length expression of ERα and ERβ in ASM was also confirmed with no notable expression of GPR30 in human ASM. In addition, recent studies suggested the noticeable difference in ER isoform-specific signaling in human ASM proliferation, especially during inflammation or in asthma. However, it is not clear whether and how genomic ERs specific signaling impacts the ASM $[Ca^{2+}]_i$ and airway contractility in asthma. In this study, I hypothesized that the conflict regarding estrogen's effects on the airway involves differential roles of ERs in ASM. Using a molecular approach and specific agonists/antagonists for ERα and ERβ, I examined $[Ca^{2+}]_i$ handling in ASM cells of non-asthmatic and asthmatic patients in the presence of pro-inflammatory cytokines.

2.2. Materials and Methods

2.2.1. Chemicals and Reagents

Cell culture media and reagents such as Dulbecco's Modified Eagle's Medium/F12 (DMEM/F12), trypsin and AbAm were purchased from Gibco (Carlsbad, CA). Charcoal Stripped Fetal Bovine Serum (FBS) was used throughout the studies to avoid hormonal contamination in

the media and was purchased from Sigma (St. Louis, MO). Pro-inflammatory cytokines tumor necrosis factor alpha (TNFα) and interleukin-13 (IL-13) were purchased from Invitrogen (Carlsbad, CA). Chemicals and supplies were purchased from Sigma (St. Louis, MO) unless otherwise specified. Pharmacological modulators for ERs, 17-β Estradiol (E_2), ERα agonists ERα-agonist (PPT, Propyl pyrazole triol; THC, Tetra Hydro Chrysenediol) and ERβ agonists (WAY-200070; FERB-033; DPN, Diaryl-Propio-Nitrile), ERα and ERβ antagonists (MPP and PHTPP respectively) were obtained from Tocris (Minneapolis, MN). B-actin antibody was obtained from Applied Biological Sciences (Cat#G043), SERCA2 antibody was obtained from Abcam (Cat#ab2861) and Mfn2 antibody was obtained from Santa-Cruz biotechnology (sc-515647). IRDye at goat anti- mouse secondary antibody was used followed by scanning in Li-COR Odyssey imaging system (Li-COR Systems, Omaha, NE).

ESR1 silencer select pre-designed siRNA (Ambion, Austin, TX, USA, Catalog# 4392420), ON-TARGET plus human ESR2 (2100) siRNA-Individual (Dharmacon, Lafayette, CO, USA, Cat # J-003402-13-0005) were used to knockdown ERα and ERβ, respectively. For negative controls, the Silencer Negative Control #1 (Cat# AM4611) from Ambion was used. For overexpression studies, ERα and ERβ plasmids tagged with GFP were created and gifted by Dr. Ratna Valdamudi (obtained via AddGene, Cat # 65211 and 65212). Zymopure Plasmid Midiprep Isolation kit (Zymoresearch, Cat # D4200 and D4201) was used for isolating the plasmids. Imaging experiments were performed in HBSS obtained from Gibco (Carlsbad, CA). Calcium sensitive dye, Fura-2 AM was obtained from Invitrogen and calibration was done using a calibration kit from Invitrogen (Cat # C3008MP).

2.2.2. Isolation of Human ASM Cells

Briefly, third to sixth generation human bronchi were obtained from lung specimens from patient's incidental to thoracic surgeries at Mayo Clinic for focal, non-infectious causes. Mayo clinic's Institutional Review Board approved protocols allowed for initial review of patient histories, with complete de-identification of samples for storage and subsequent usage. This study was limited to samples from 25 male and female non-smoker adults of ages from 21 to 65 years from both non-asthmatic and asthmatic (mild to moderate) patients**.** For each study five to seven non-asthmatic and asthmatic cells, each were chosen randomly and utilized independently. After removing the debris, airway samples were denuded of epithelium and ASM tissue was enzymatically dissociated according to previously described procedures following manufacturer's instructions (Worthington Biochemical, Lakewood, NJ) to generate ASM cells. Cultures (<5th passage) were maintained under optimal conditions of 37°C (5% CO_2, 95% air) in DMEM/F12 growth media. Periodically, ASM phenotype was verified by visualizing the hill and valley pattern of cells under microscopy. Western analyses for smooth muscle markers such as α- smooth muscle actin, transgelin and caldesmon are also carried out periodically (Figure 4). Cells were serum starved for 24h prior to treatment with agonists or antagonists.

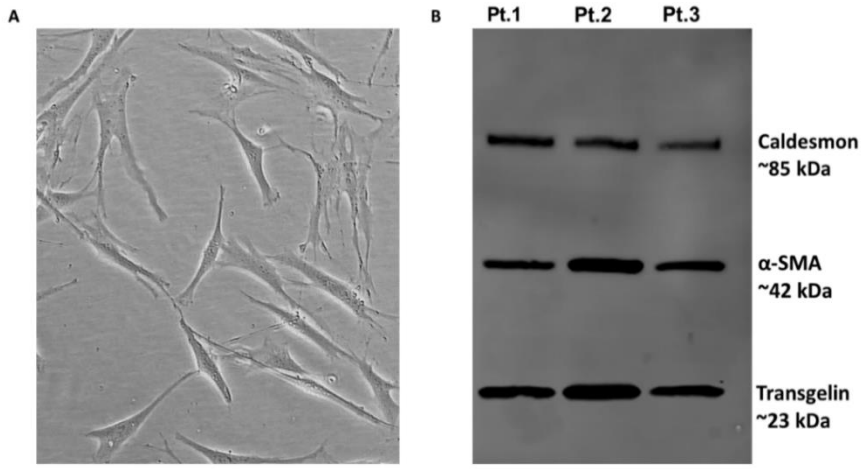

Figure 4. Characterization of human ASM cells.
The phase-contrast microscopy of confluent cells shows a typical hill-and-valley pattern. Confirmation of ASM cells obtained from different patients is done by western analyses of smooth muscle markers- caldesmon, α-smooth muscle actin and transgelin.

2.2.3. Cell Culture and Treatment

Fully confluent T-75 flasks of ASM cells were trypsinized mixed in 10% FBS (Charcoal Stripped) growth medium (DMEM/F12 with 1% AbAm), counted and seeded ~10,000 cells/well into Biotek 8-well chamber plates. Cells were allowed to adhere, grown up to 60-70% confluence and incubated in serum free medium (DMEM/F12 without FBS) for 24h to mature. ASM cells were treated with pro-inflammatory cytokines in the presence and absence of ER specific agonists such as PPT and THC (10nM), WAY, DPN and FERB-033 (10nM) and E_2 (1nM). Briefly, After 2h of pre-incubation with respective agonist or antagonist treatment groups, cells were then exposed to TNFα (20 ng/ml) or IL-13 (50 ng/ml) for 24h. Serum free media alone served as a vehicle. ASM cells were grown on 60 mm petri plates and treated similarly to collect lysates for mRNA used in qPCR techniques.

2.2.4. Small Interfering RNA Transfection and Overexpression

Human ASM cells were grown in 8-well plates to approximately 50% confluence. Transfection was achieved using 20 nM siRNA and Lipofectamine 3000 transfection reagent (Invitrogen) in DMEM/F-12 media without FBS and antibiotics. 6h after transfection, growth medium was added and maintained for 24h. Subsequently, cells were serum starved for another 24h before being subjected to treatments followed by live-cell imaging. Efficiency of knockdown and specificity was verified by mRNA analysis by performing transfection of ASM in 60 mm plates.

Overexpression studies were performed with ER specific plasmids tagged with GFP obtained in bacterial stabs from Addgene (Watertown, MA). These plasmids had bacterial resistance to kanamycin. The respective bacteria were streaked onto agar plate and single colonies were obtained. Using the LB broth, single colonies were inoculated overnight, plasmids were isolated using Zymopure plasmid Midiprep isolation kit and verified using gel electrophoresis. Prior to plasmid transfection, ASM cells were grown in 8-well plates to approximately 50% confluence. Transfection was achieved using Lipofectamine 3000 transfection reagent (Invitrogen) in reduced DMEM/F-12 (5% FBS and no antibiotics) with 0.5μg of plasmids. Cells were incubated as such for 24h in reduced media and then withdrawn to serum-free media for 24h for further imaging studies. More than 70% transfection efficiency was achieved and the efficiency of the transfection was confirmed using Olympus microscope with FITC filter. The effect of siRNA and overexpression on the $[Ca^{2+}]_i$ regulation in the presence of E_2, WAY or PPT were analyzed by real time $[Ca^{2+}]_i$ imaging.

2.2.5. Western Analysis

ASM cells were washed, harvested in lysis buffer (Cell Signaling Technologies, Beverly, MA, USA) containing protease inhibitors, and resultant supernatants were assayed for total protein content using the DC Protein Assay kit (Bio-Rad, Hercules, CA, USA). 30 μg equivalent protein from each treatment group lysate were loaded on 4-15% gradient gels (Criterion Gel System; Bio-Rad, Hercules, CA, USA) and transferred into 0.22 μm PVDF membranes using a Bio-Rad Trans-Blot Turbo rapid transfer system (Bio-Rad, Hercules, CA, USA). Non-specific binding was blocked using 5.0% bovine serum albumin (BSA) in Tris-buffered saline (TBS) for 1 h at room temperature and the membranes were probed overnight at 4°C with specific primary antibodies of interest. Following three washes with Tris-buffered saline with 0.1% tween (TBST), blots were then incubated with Li-COR near-infrared conjugated secondary antibody. Protein expression was detected by imaging the membrane on a Li-COR Odyssey XL system. β-actin was used as a loading control. The normalized values were obtained by dividing the raw values of proteins of interest with the raw values of β-Actin. The obtained values were then normalized to vehicle.

2.2.6. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR) Analysis

Cells were washed with RNA-grade DPBS, trypsinized and centrifuged. Total RNA was extracted using Quick-RNA™ MiniPrep kit (Zymo Research, Irvine, CA) following manufacturer's protocol and cDNA was synthesized using One Script cDNA Synthesis Kit (Applied Biological Materials Inc, Richmond, BC, Canada) using 500 ng of quantified RNA (Take3™ Micro volume plate, Synergy HTX, Biotek, USA) for each sample. BrightGreen 2X qPCR Master Mix (Applied Biological Materials catalog #MasterMix-S-XL) protocol was followed using QuantStudio 3 qPCR system as per the manufacturer's instructions. The following primers were used for qPCR analysis: ERα (forward 5′-GCC TGA ATG GCG AAT GGA-3′;

reverse 5′-GAA GGG AAG AAA GCG AAA GGA-3′), ERβ (forward 5′-GCTTACTCCGAC CATGATTTC T-3′; reverse 5′-GCC GAT GCTTGCAATAGTTTAG-3′), Drp1 (Forward 5′-CACTTGTGGATTTGCCAGGAATGACC-3′; reverse 5′-TGCGACCATCTGGATCTACCT CTCTT-3′) Mfn2 (forward 5′-CCTGCTCTTCTCTCGATGCAACTCTA-3′; reverse 5′-CTGCATTCCTGTACGTGTCTTCAA GG-3′). The fold changes in mRNA expression were calculated by normalization of cycle threshold [C(t)] value of target genes to reference gene s16 using the ΔΔCt method as previously described.

2.2.7. Real-Time $[Ca^{2+}]_i$ Imaging

The technique for $[Ca^{2+}]_i$ imaging of human ASM cells using Fura-2 have been previously described[22]. After 24h of incubation with the respective treatments, solutions containing various treatments were aspirated and human ASM cells were washed with freshly prepared HBSS (1X) buffer solution having 2.0 mM calcium and 1 mM magnesium ions (from HBSS 10X Gibco). After the wash, ASM cells were incubated with 4 μM Fura-2, AM (prepared in HBSS solution) for 1h at room temperature. Following 1h, the dye was aspirated, and cells were washed thrice with a 1X HBSS buffer solution. The ASM cells were imaged at 20X magnification on ratio metric Fura-2 filters (alternatively excited at 340 and 380 nm and emissions at 510 nm were collected in the Olympus Fluorescence Microscope (Fluoview FV300) equipped with Hamamatsu camera and Cool-LED lamp. A custom-built fluid level controller allowed cell perfusion with a rapid exchange of perfusate with the help of Gilson mini-pump and vacuum. $[Ca^{2+}]_i$ responses of at least 10 cells per chamber were obtained. Results were obtained in the ratio of 340/380 nm wavelengths and quantification of $[Ca^{2+}]_i$ was performed using previously described calibration procedures. The change in fluorescence intensities in the individual software facilitated-selected regions of interests and the corresponding $[Ca^{2+}]_i$ responses were recorded in response to 10 μM histamine, 1 μM

acetylcholine (Ach) and 10 nM bradykinin. For the overexpression studies, regions of interest were selected in those areas of the cells showing high green fluorescence (GFP) with the help of FITC filter and the then real time live-cell imaging were performed with Fura-2 filter.

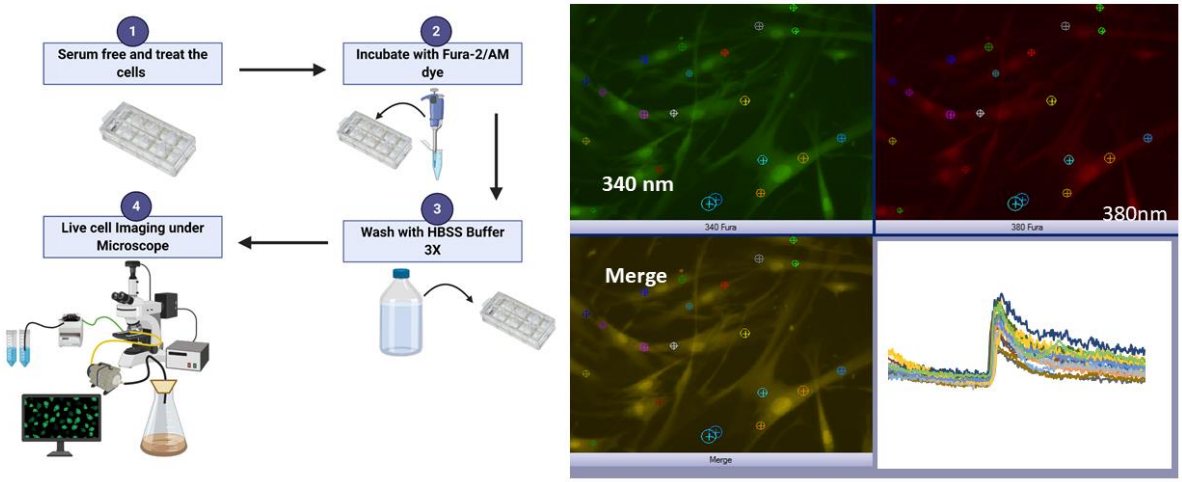

Figure 5. Schematic representation of a real-time calcium imaging using Fura-2 AM.
The cells are serum deprived, treated and incubated with Fura-2AM. These then washed and then placed under microscope for imaging.

To isolate the cells from the calcium environment, following initial incubation with Fura-2, AM containing HBSS with calcium, cells were perfused in zero calcium containing HBSS and the Ca^{2+} channels were non-specifically blocked with 1 μM $LaCl_3$ in HBSS for the "0" Ca^{2+} experiments. The rate of decline of the $[Ca^{2+}]_i$ was calculated in terms of time to decay (s). For the purpose of studying the L-type calcium channels (LTCC), the cells were perfused in calcium containing HBSS and prior to histamine, 1 μM nifedipine in HBSS was exposed to the cells for 15min resulting in blockage of LTCC. Further confirmatory studies on LTCC were performed using 100 mM KCl instead of histamine, which effectively depolarizes the membrane through voltage-gated channels.

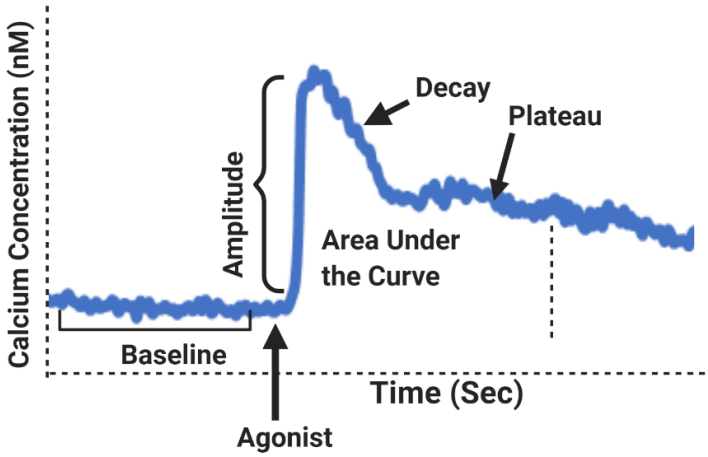

Figure 6. Calcium response.
A typical $[Ca^{2+}]_i$ response obtained from contractile agonists (Histamine, Bradykinin, Acetylcholine) induced $[Ca^{2+}]_i$ response in Fura-2,AM loaded cells.

2.2.8. Mitochondrial Fission-Fusion Morphology

ASM cells in eight-well Labteks were washed with HBSS buffer, and then loaded with 0.5 µM MitoTracker Green (for 1 min), and visualized under Bio-Tek Lionheart FX Live Cell Imager. Images were processed with MATLAB analysis software using NIH Image J software. MATLAB program was used to identify mitochondria. Average of mitochondrial form factor (an index of mitochondrial branching) and aspect ratio (an index of length of mitochondria) for each treatment group were calculated [30, 37, 38].

2.2.9. Statistical Analysis

Up to twenty ASM cells from different non-asthmatic and asthmatic patients were used. Each set of $[Ca^{2+}]_i$ experiments were performed in at least 5 randomly selected different patient cells, although not all protocols were performed in each sample. All the experiments were repeated at least three times for each patient sample. Statistical analysis was performed using GraphPad Prism version 8.0.0 for Windows, San Diego, California USA. Statistical differences between the experimental groups were analyzed using student's *t*-test or 1-way ANOVA followed by Dunnet

or Tukey's post-hoc test for multiple comparisons where appropriate. Statistical significance was established minimum at a minimum of $p \leq 0.05$. All values are expressed as mean ± SEM.

2.3. Results

2.3.1. Effect of ER Specific Activation on $[Ca^{2+}]_i$ Response to Agonist

To determine the role of long-term ER signaling (likely genomic) in $[Ca^{2+}]_i$ regulation, ASM cells were exposed to ERα (PPT, 10nM), ERβ (WAY, 10nM) agonists, E_2 (1nM) for 24h and $[Ca^{2+}]_i$ response were evaluated using Fura-2 AM (Figure 6). $[Ca^{2+}]_i$ response was measured in both asthmatic and non-asthmatic ASM cells using three different agonists induced elicitation: histamine (10µM), Ach (1µM) and bradykinin (10nM). ERα agonist PPT treated cells showed a significant increase in $[Ca^{2+}]_i$ response to histamine as compared to the vehicle in non-asthmatic ASM cells (Figure 7A, $p \leq 0.05$). This effect of PPT was much more pronounced in asthmatic ASM cells (Figure 7D, $p \leq 0.01$). Non-selective ER agonist E_2 did not show any significant changes in the $[Ca^{2+}]_i$ compared to vehicle. In response to bradykinin, the effect of PPT in increasing the $[Ca^{2+}]_i$ response was similar for both asthmatic and non-asthmatic ASM cells (Figure. 7B, E, $p \leq 0.01$). However, in Ach elicited $[Ca^{2+}]_i$ response, PPT showed a much more pronounced increase in $[Ca^{2+}]_i$ response in non-asthmatic ASM cells (Fig 7C, $p \leq 0.001$) compared to asthmatics (Fig 7E, $p \leq 0.05$). Notably, there were no differences observed in baseline calcium levels.

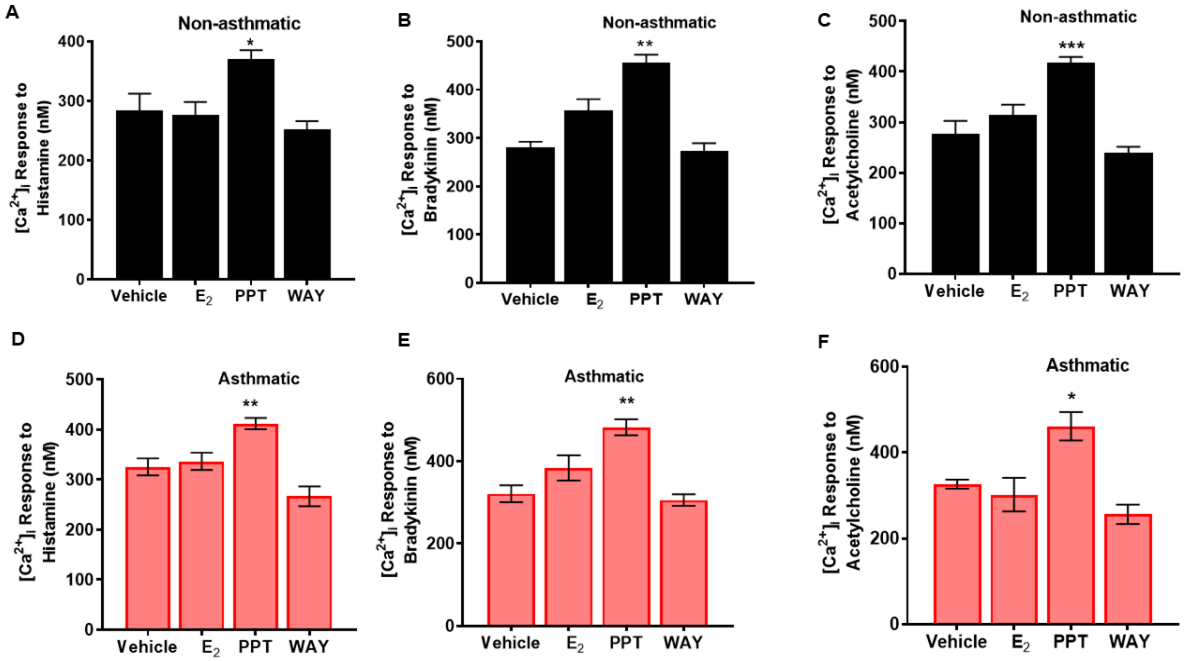

Figure 7. Effect of long-term exposure of estrogen receptor (ER)-specific agonists on intracellular Ca^{2+} ($[Ca^{2+}]_i$)response in non-asthmatic and asthmatic human airway smooth muscle (ASM) cells at baseline.
In both non-asthmatic and asthmatic ASM cells treated with ER specific agonists, the $[Ca^{2+}]_i$ responses to 10μM histamine (A,D), 1μM bradykinin (B,E) and 1μM Ach (C,F) were measured. *p<0.05, **p<0.01, ***p<0.001 vs. vehicle. Data represented as mean ± SEM from N of 5 patients each.

2.3.2. Effect of Various ER Selective Agonists and Antagonists on $[Ca^{2+}]_i$ Response

Considering our previous studies where acute ERα activation (likely non-genomic) decreased ASM $[Ca^{2+}]_i$ response, our results from 3.1 were distinct and hence additional validation of the long-term role of differential ER signaling in $[Ca^{2+}]_i$ response in ASM was needed. Here, different ERα (PPT and THC), ERβ (WAY, FERB-033 and DPN) agonists were utilized to study the histamine elicited $[Ca^{2+}]_i$ response in non-asthmatic human ASM (Figure 8A). In addition, ER specific antagonists, ERα (MPP) and ERβ (PHTPP) were also employed in the presence of E_2 to study the specific ER effects on ASM $[Ca^{2+}]_i$ response (Figure 8B). 10nM of ERα agonist THC, which has a dual role as ERα agonist as well as ERβ antagonist, significantly increased the $[Ca^{2+}]_i$

response to histamine ($p \leq 0.001$) compared to vehicle. Similarly, the effect of PPT on $[Ca^{2+}]_i$ response was found to be significantly higher ($p \leq 0.01$) albeit lower than that of THC. There were no significant changes observed in $[Ca^{2+}]_i$ response in cells treated with ERβ agonists (10nM: WAY, FERB-033 and DPN) compared to vehicle. In a separate set of experiments, cells pre-treated with ERα antagonist MPP and followed by 24h exposure to E_2 showed lower $[Ca^{2+}]_i$ response to histamine indirectly indicating the ERβ effect. Similarly, cells pre-treated with ERβ antagonist prior to treatment with E_2 for 24h showed a significantly higher $[Ca^{2+}]_i$ response as compared to vehicle pointing indirectly towards an ERα effect (Figure 7B, $p \leq 0.001$). All these data indicates ERα activation (genomic) leads to increased $[Ca^{2+}]_i$ response.

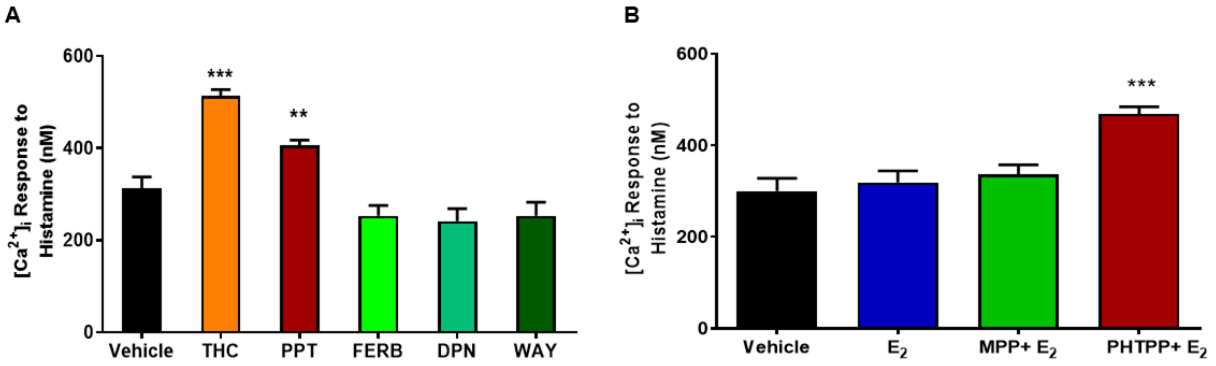

Figure 8. Effect of various ER agonists and antagonists on $[Ca^{2+}]_i$ response in non-asthmatic human ASM cells.
The long-term effects of various ERα agonists (THC and PPT), ERβ agonists (WAY, DPN and FERB-033) on $[Ca^{2+}]_i$ response to histamine were evaluated in non-asthmatic human ASM cells (A). In the presence of ERα antagonist (MPP) or ERβ antagonist (PHTPP), the effect of E_2 on the $[Ca^{2+}]_i$ response to histamine was measured (B). **p<0.01, ***p<0.001 vs. vehicle. Data represented as mean ± SEM from N of 5 patients each.

2.3.3. Effect of Specific ER SiRNA and Overexpression on $[Ca^{2+}]_i$ Response

To further confirm the observed pharmacological based results on the ER specific $[Ca^{2+}]_i$ response, molecular level experiments were performed using ER overexpression or ER specific siRNA on human ASM cells (Figure 9). The ER specific siRNA transfection was performed using

standard protocols in non-asthmatic human ASM cells and the efficiency of ER specific siRNA (~70%) was evaluated using mRNA analysis (Figure 9B). In ERα siRNA transfected ASM cells, treatment with E_2 (24h) as well as with ERα agonist PPT ($p \leq 0.01$) showed a significantly reduced $[Ca^{2+}]_i$ response to histamine as compared to the negative siRNA treated cells (Figure 9A). E_2 exposure on ERβ siRNA transfected cells showed significantly higher $[Ca^{2+}]_i$ response compared to negative siRNA ($p \leq 0.05$), whereas no changes observed with WAY exposure. For overexpression studies, GFP tagged ER specific plasmids were used and their transfection efficiency was confirmed through fluorescence imaging (Figure 9E). ASM cells overexpressed with ERα-GFP plasmid and subsequently treated with E_2 (24h), showed a higher $[Ca^{2+}]_i$ response ($p \leq 0.01$) as compared to lipofectamine, whereas ERβ-GFP transfected cells treated with E_2 showed a reduced $[Ca^{2+}]_i$ response ($p \leq 0.001$) compared to lipofectamine treated cells (Figure 9C). Representative traces of $[Ca^{2+}]_i$ response to histamine regulated through specific overexpressed ERs in the presence of E_2 are shown in the Figure 9D.

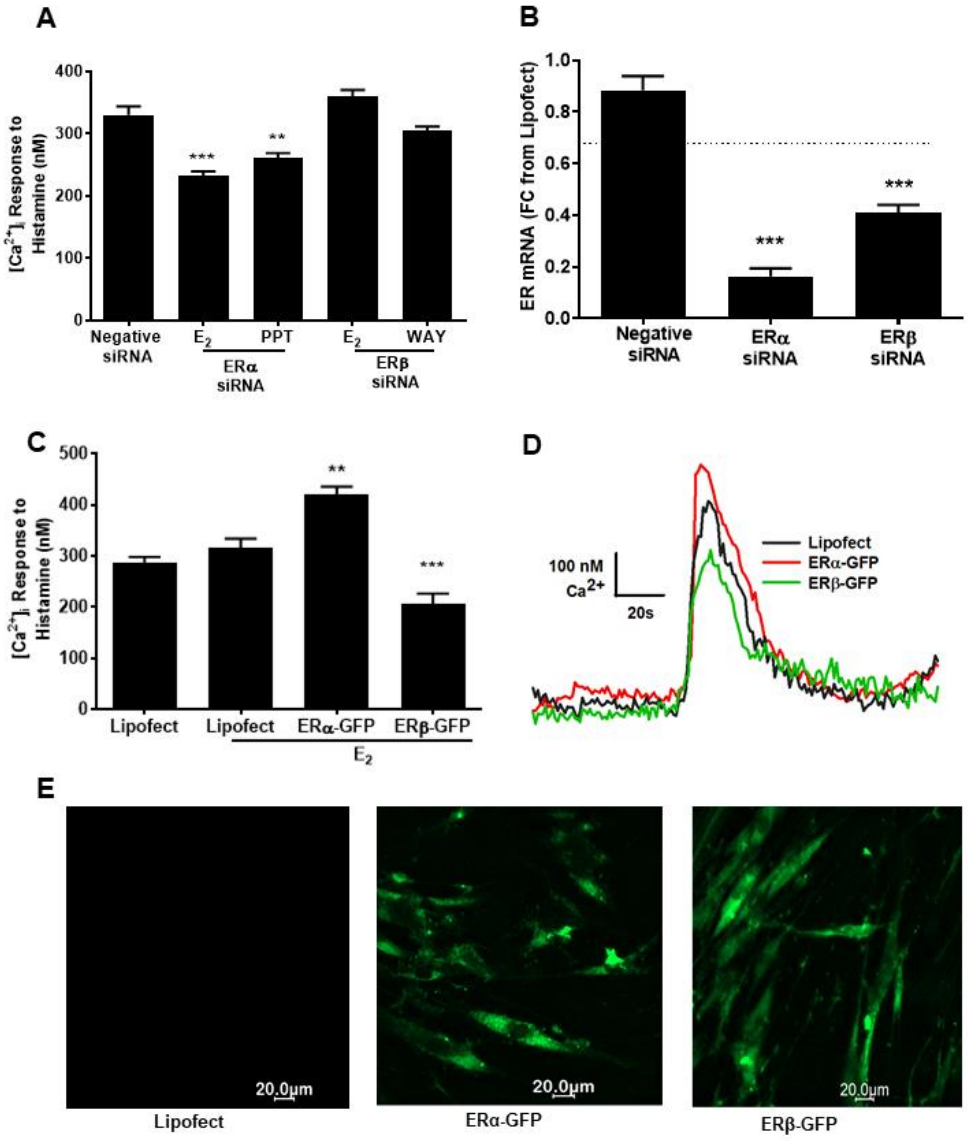

Figure 9. Effect of specific ER small interference RNA (siRNA) and overexpression on $[Ca^{2+}]_i$ response in non-asthmatic human ASM cells.

The effects of E_2, PPT and WAY in ER specific siRNA-transfected cells on the $[Ca^{2+}]_i$ response to histamine were evaluated in no-asthmatic human ASM cells (A). Transfection efficiency of siRNA was evaluated using mRNA expression studies (B). ERα and ERβ overexpressed ASM cells were treated with non-selective agonist E_2 and their $[Ca^{2+}]_i$ response to histamine were evaluated (C). Representative traces showing effect of overexpression of specific ERs on the $[Ca^{2+}]_i$ (D). The representative images of ERα-GFP and ERβ-GFP transfections on non-asthmatic ASM cells (E) **p<0.01, ***p<0.001 vs. Negative siRNA or Lipofectamine. Data represented as mean ± SEM from N of 5-7 patients.

2.3.4. Effect of Specific ER Signaling on TNFα Mediated $[Ca^{2+}]_i$ Response

Non-asthmatic and asthmatic ASM cells were treated with E_2, PPT or WAY for 2h and then treated with TNFα (20ng/ml) for 24h in these experimental conditions. TNFα alone produced a marked increase in $[Ca^{2+}]_i$ responses to Ach, bradykinin and histamine as compared to the vehicle ($p \leq 0.001$) in both asthmatic and non-asthmatic ASM cells. In non-asthmatic and asthmatic ASM cells, ERβ agonist WAY but not E_2 or PPT showed a reduction in the enhancing effect of TNFα on $[Ca^{2+}]_i$ response to histamine ($p \leq 0.001$, Figure. 8A-F). In bradykinin and Ach-induced $[Ca^{2+}]_i$ responses in the ASM cells (Figure. 8B, C, E, F), WAY in the presence of TNFα restored back the $[Ca^{2+}]_i$ response comparable to vehicle ($p \leq 0.001$) in both non-asthmatic and asthmatic cells. Here, the effect of E_2 in reducing the increased $[Ca^{2+}]_i$ response was varied depending on the conditions and showed a moderate reduction in TNFα induced effect on $[Ca^{2+}]_i$ response with bradykinin and Ach in asthmatic cells ($p \leq 0.05$, Figure. 10 E, F). In non-asthmatic ASM cells, E_2 and ERα agonist PPT did not alter the effect of TNFα in increasing the $[Ca^{2+}]_i$ response to all the three agonists used.

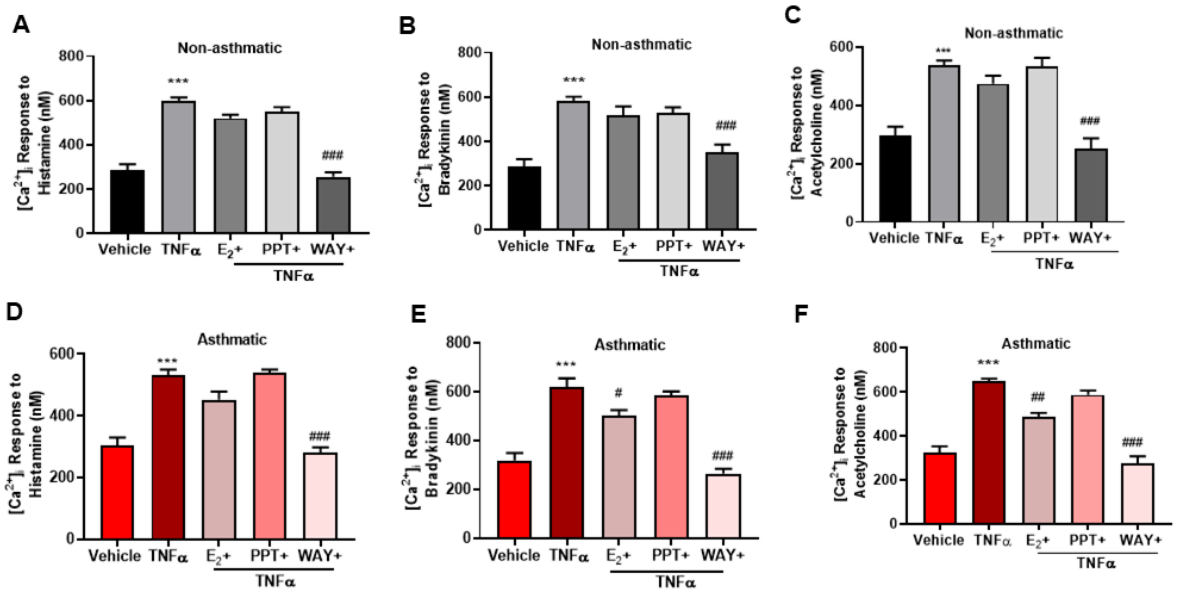

Figure 10. Effect of differential ER signaling on the TNFα enhancement of $[Ca^{2+}]_i$ response in human ASM cells.
Human non-asthmatic and asthmatic cells were treated with E_2, PPT or WAY for 2h prior to treatment with TNFα (20ng/ml) for 24h and their effects on the $[Ca^{2+}]_i$ response to histamine (A, D), bradykinin (B, E) and Ach (C, F) were evaluated. ***p<0.001 vs. vehicle. #p≤0.05, ##p<0.01, ###p<0.001 vs. TNFα. Data represented as mean ± SEM from N of 5 patients each.

2.3.5. Effect of Specific ER Signaling on IL-13 Mediated $[Ca^{2+}]_i$ Response

Similar to the effect of TNFα above, exposure of IL-13 (50ng/ml) for 24h also produced a significant increase in $[Ca^{2+}]_i$ response to Ach, bradykinin and histamine as compared to the vehicle (p≤0.001) in both non-asthmatic and asthmatic cells (Figure. 11), demonstrating Th2 effect in increasing $[Ca^{2+}]_i$ in human ASM cells, which is comparable to our previous findings. In non-asthmatic ASM cells, significant reduction in the $[Ca^{2+}]_i$ response to histamine was observed in IL-13 exposed cells treated with WAY (p≤0.001, Figure. 11A). In asthmatic ASM cells (Figure. 11D), E_2 showed a reduction in IL-13 induced $[Ca^{2+}]_i$ response to histamine (p≤0.01). Here more pronounced reduction of $[Ca^{2+}]_i$ response was observed with WAY (p≤0.001). No significant effect were observed in PPT and IL-13 treated groups. With respect to bradykinin and Ach-elicited

$[Ca^{2+}]_i$ response, WAY in the presence of IL-13 significantly reduced the $[Ca^{2+}]_i$ response in both non-asthmatic and asthmatic ASM cells (Figure. 11 B-F, $p \leq 0.01$). Notably, E_2 moderately decreased the IL-13 induced increase in $[Ca^{2+}]_i$ response only in non-asthmatic ASM cells ($p \leq 0.01$, Figure 11 B,C). Overall, WAY invariably showed a decreased $[Ca^{2+}]_i$ response in IL-13 treated cells in both non-asthmatic and asthmatic cells ($p \leq 0.001$) whereas PPT did not show any significant changes in $[Ca^{2+}]_i$ response.

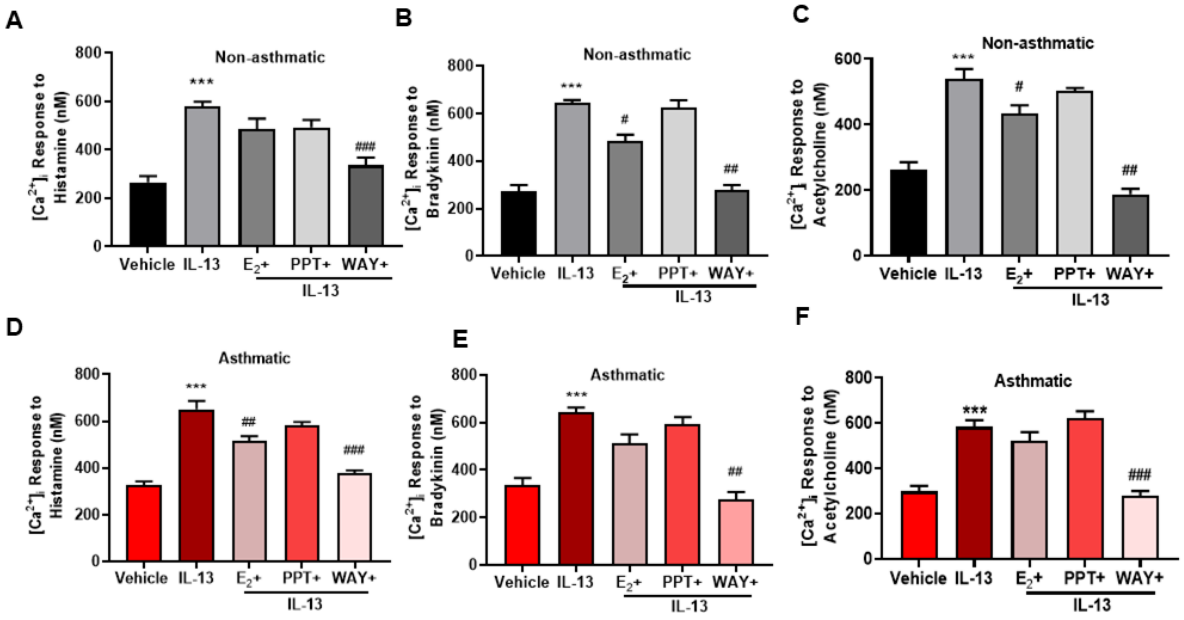

Figure 11. Effect of differential ER signaling on the IL-13 enhancement of $[Ca^{2+}]_i$ response in ASM cells.
Human non-asthmatic and asthmatic cells were treated with E_2, PPT or WAY for 2h prior to treatment with IL-13 (50ng/ml) for 24h and their effects on the $[Ca^{2+}]_i$ response to histamine (A,D) , bradykinin (B,E) and Ach (C,F) were evaluated. ***$p<0.001$ vs. vehicle, #$p \leq 0.05$, ##$p<0.01$, ###$p<0.001$ vs. IL-13. Data represented as mean ± SEM from N of 5 patients each.

2.3.6. Effect of Differential ER Signaling on $[Ca^{2+}]_i$ Reuptake

To confirm the $[Ca^{2+}]_i$ regulation of ER through intracellular stores, cells were isolated from the calcium containing environment ("0" Ca^{2+} HBSS) and their $[Ca^{2+}]_i$ response to histamine was studied in non-asthmatic human ASM cells. Cells were treated with ER agonists for 2h, then exposed to TNFα or IL-13 for 24h and the $[Ca^{2+}]_i$ response was evaluated in the absence of

extracellular Ca^{2+} and blocked plasma membrane Ca^{2+} fluxes. Pro-inflammatory cytokines, TNFα or IL-13 both showed significant increase in the $[Ca^{2+}]_i$ response to histamine as compared to vehicle ($p \leq 0.001$, Fig 12 A,B). As expected, the $[Ca^{2+}]_i$ responses in the environment devoid of Ca^{2+} was lower, indicating the effect only on the intracellular stores. In the presence of either of these pro-inflammatory cytokines, ERβ agonist WAY treated ASM cells significantly reduced the $[Ca^{2+}]_i$ response ($p \leq 0.001$), whereas PPT or E_2 treated cells showed negligible effects on the $[Ca^{2+}]_i$ response in the "0" Ca^{2+} HBSS environment. In these experiments, the effects of ERs on sarco (endo) plasmic reticulum Ca^{2+}-ATPase (SERCA2) reuptake were evaluated by calculating time to decay (Fig. 12 C, D). Time to decay was significantly higher in TNFα or IL-13 alone treated groups ($p \leq 0.001$) and was significantly lower in cells treated with WAY ($p \leq 0.001$). E_2 and PPT did not effectively change the TNFα or IL-13 induced increase in time to decay. Furthermore, SERCA2 protein expressions were significantly reduced in TNFα or IL-13 treated cells compared to vehicle (Fig 13). ERβ agonist WAY treatment reversed this trend in TNFα ($p \leq 0.01$) or IL-13 ($p \leq 0.001$) exposed ASM cells and showed increased SERCA2 expression, but there were no differences observed in PPT or E_2 treated cells. Overall, these support the concept that ERβ activation can blunt $[Ca^{2+}]_i$ responses by increasing Ca^{2+} sequestration at intracellular level.

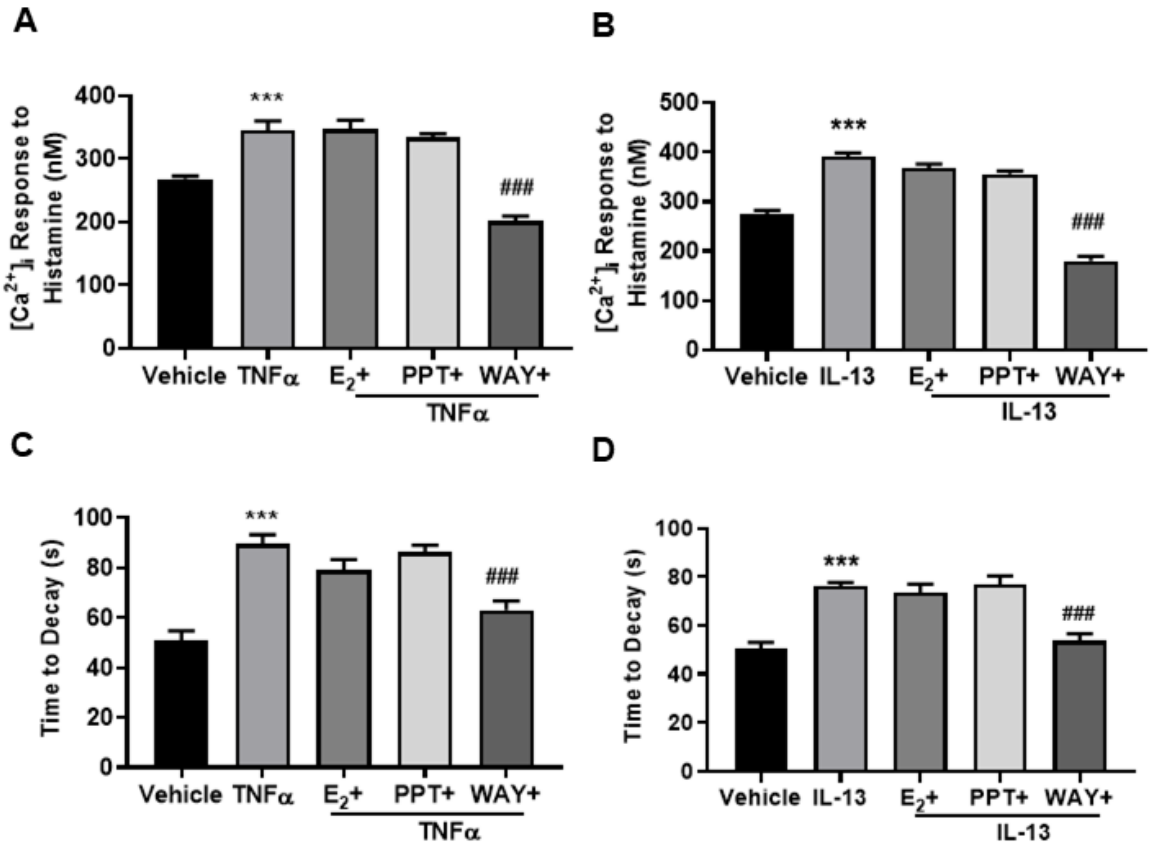

Figure 12. Effect of differential ER signaling on sarcoplasmic reticulum (SR) $[Ca^{2+}]_i$ reuptake activity.

In zero calcium environment, effect on the $[Ca^{2+}]_i$ response due to pro-inflammatory cytokines TNFα (A) and IL-13 (B) in the presence of specific ER agonists we were evaluated in non-asthmatic human ASM cells. The rate of fall of $[Ca^{2+}]_i$ response representing $[Ca^{2+}]_i$ reuptake by SR was measured in TNFα (C) or IL-13 (D) exposed ASM cells. ***$p<0.001$ vs. vehicle, ###$p<0.001$ vs. cytokines. Data represented as mean ± SEM from N of 5-6 patients.

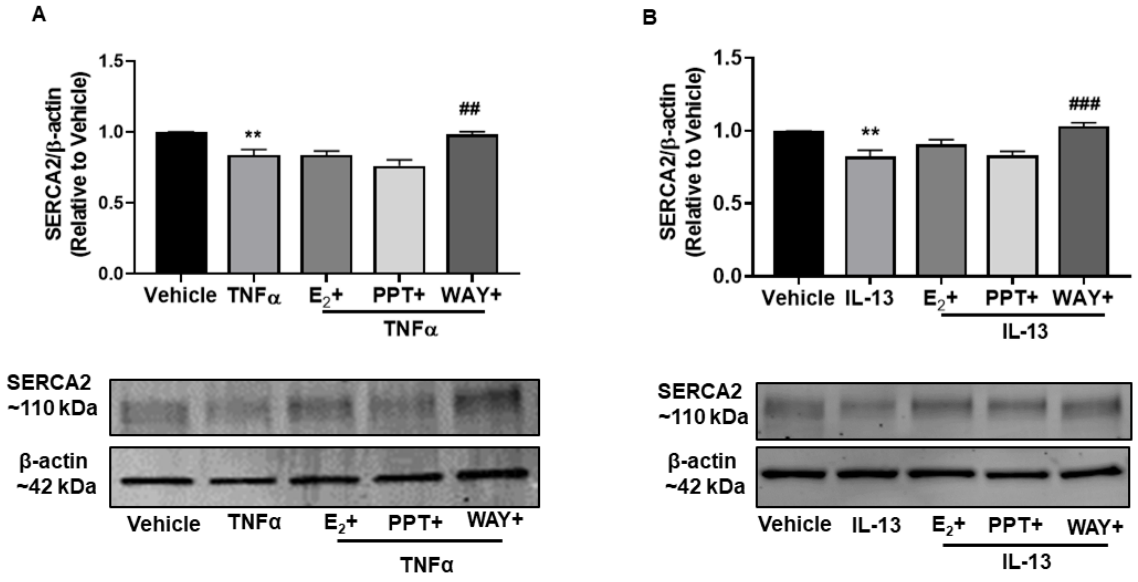

Figure 13. Effect of differential ER signaling on sarcoplasmic reticulum (SR) $[Ca^{2+}]_i$ reuptake expression.
SERCA2 protein expression analysis of ER agonists treated with TNFα (A) or IL-13 (B) exposed non-asthmatic human ASM cells. **p<0.001 vs. vehicle, ##p<0.01, ###p<0.001 vs. cytokines. Data represented as mean ± SEM from N of 5-6 patients.

2.3.7. Effect of ER Signaling on L-Type Calcium Channel Influx

Previous studies showed decreased Ca^{2+} influx in human ASM due to acute action of estrogen. To identify the specific contribution of ERβ signaling in ASM $[Ca^{2+}]_i$ regulation, additional possible mechanism through LTCC was also tested (Figure 14). After the 24h treatment (with WAY and cytokines), ASM cells were exposed to LTCC inhibitor nifedipine (1μM) for 20 min before being subjected to histamine in calcium containing HBSS. Acute nifedipine exposure significantly decreased the $[Ca^{2+}]_i$ response ($p \leq 0.001$) compared to vehicle. WAY treated ASM cells in the presence of nifedipine showed similar effects to that of nifedipine exposed cells in decreasing the $[Ca^{2+}]_i$, indicating the role of WAY effect partially through LTCC. TNFα or IL-13 induced increases in $[Ca^{2+}]_i$ response was significantly reduced by acute nifedipine exposure.

Furthermore, WAY treatment substantially and significantly reduced $[Ca^{2+}]_i$ response in TNFα or IL-13 exposed ASM cells in the presence of nifedipine ($p \leq 0.001$) and this was comparable to nifedipine or WAY alone treated groups (Figure 14A). To further confirm the involvement of ERβ in Ca^{2+} influx mechanisms, subsequent set of studies were performed in similar experimental conditions, where KCl (100 mM) instead of histamine was used as a depolarizing agent. It was found that $[Ca^{2+}]_i$ response to KCl was drastically reduced ($p \leq 0.001$) in the presence of nifedipine as compared to vehicle, suggesting that KCl depolarizes the ASM cell membrane primarily through LTCC. WAY treated cells showed a $[Ca^{2+}]_i$ response significantly lower than vehicle ($p \leq 0.001$), indicating an inhibitory effect primarily via LTCC. Furthermore, TNFα or IL-13 increased KCl induced $[Ca^{2+}]_i$ response in ASM cells ($p \leq 0.001$) and WAY treated cells showed decreased $[Ca^{2+}]_i$ response ($p \leq 0.001$) in the presence of either of the cytokines (Figure 14B).

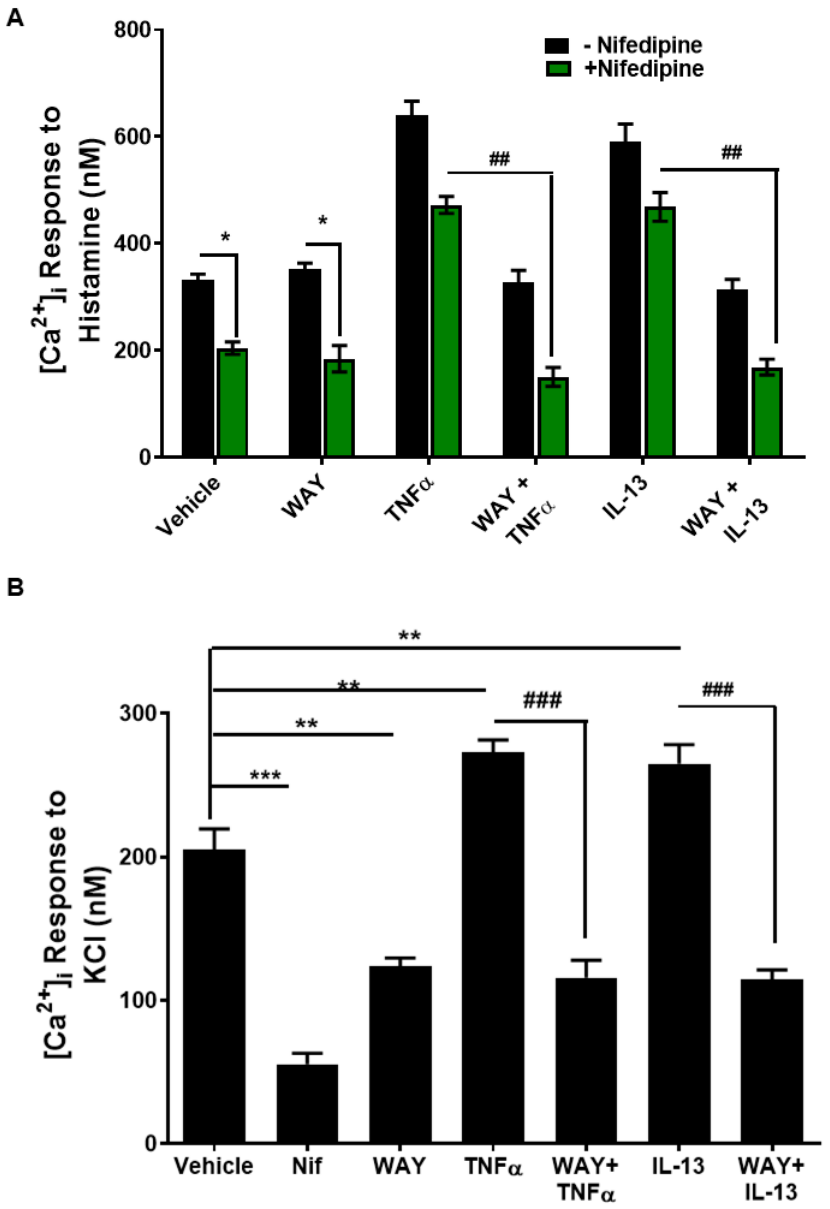

Figure 14. Effect of ERβ signaling on $[Ca^{2+}]_i$ response through L-type calcium channel (LTCC) inhibition.

Human non-asthmatic ASM cells treated with TNFα or IL-13 in the presence of WAY, were subjected to $[Ca^{2+}]_i$ response and comparisons were made with blunting effect of LTCC inhibitor nifedipine (1μM, 20min, A). $[Ca^{2+}]_i$ response to KCl - induced depolarization was evaluated and compared with nifedipine (B). *p≤0.05, **p≤0.01, ***p<0.001 vs. vehicle or WAY, ##p<0.01, ###p<0.001 vs. cytokines. Data represented as mean ± SEM from N of 5-7 patients.

2.3.8. Effect of ER Signaling on Mitochondrial Morphology

Increased ROS formation and $[Ca^{2+}]_i$ causes mitochondrial fragmentation and impairs the mitochondria's ability to uptake of cytosolic calcium leading to further increase in $[Ca^{2+}]_i$ [39, 40]. To identify the role of ER signaling in modulating the morphology of mitochondria, ASM cells were treated with E_2, PPT or WAY for 2h and then treated with TNFα (20ng/ml) for 24h in these experimental conditions. Cells were treated with Mito-tracker green and visualized under 40X (Figure 15 A). Form factor (degree of branching) and aspect ratio (mitochondrial branch length) were calculated after the raw images were taken and higher values of each of these denoted lower fragmentations.

It was found that, TNFα alone treated cells showed a marked increase in mitochondrial fragmentation as compared to the vehicle. ERβ agonist WAY showed a significant reduction in the TNFα induced fragmentation of mitochondria as signified by increased form factor and aspect ratio ($p \leq 0.05$ Figure 15 B, C). E_2 showed significant increase in form factor ($p \leq 0.05$) and no significant change in the aspect ratio. Here, the PPT did not show any significant changes in TNFα induced fragmentation. mRNA expression of fission protein Drp1 significantly increased with TNFα treatment ($p \leq 0.05$, Figure 15 D) as compared to vehicle group. WAY showed a marked reduction in the TNFα induced increase in the mRNA expression of Drp1 ($p \leq 0.001$). mRNA and protein expression of fusion protein Mfn2 (Figure 15, E, F) significantly dropped as compared to vehicle group. mRNA expression was increased in groups treated with TNFα in the presence of E_2 ($p \leq 0.05$) and more prominently with WAY treatment ($p \leq 0.001$). Also, protein expression of Mfn2 was restored in cells treated with WAY along with TNFα ($p \leq 0.05$).

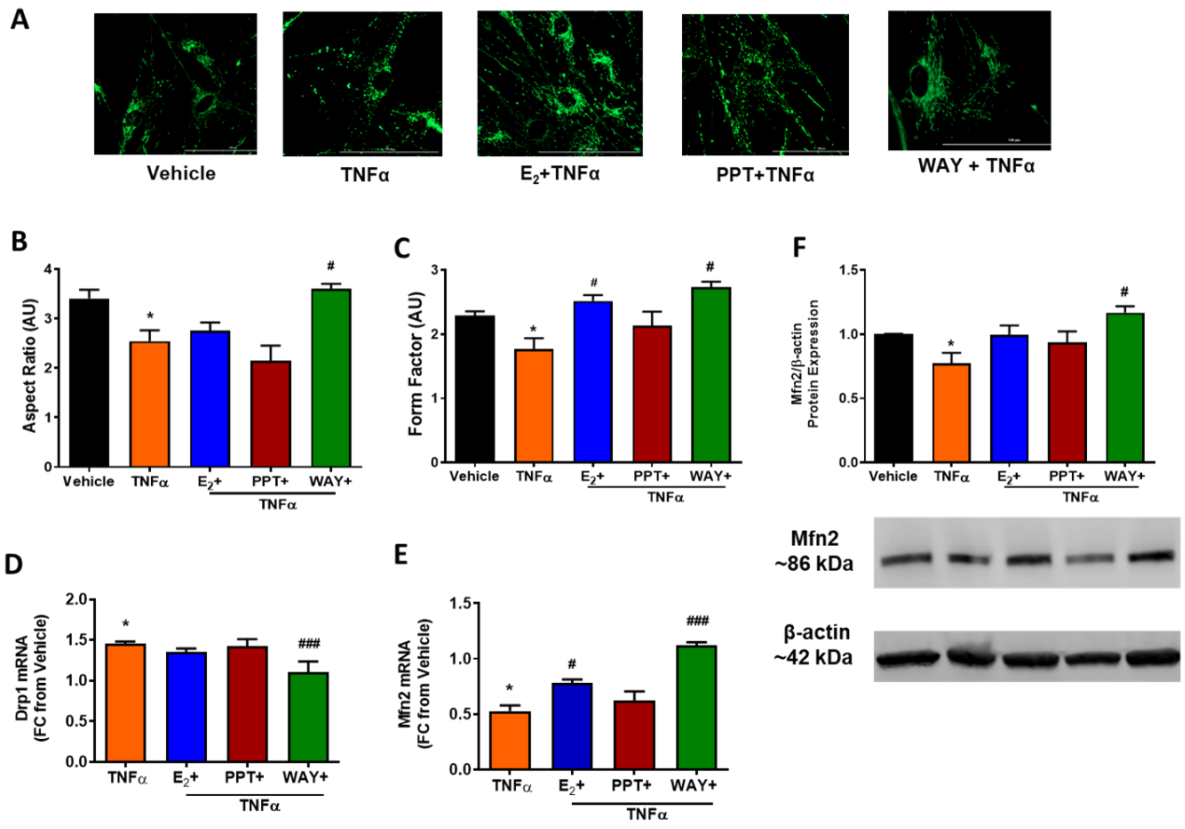

Figure 15. Effect of ERβ signaling on mitochondrial morphology.
Human non-asthmatic ASM cells treated with TNFα in the presence ER specific agonists, were analyzed for their mitochondrial morphology Inset panels shows representative images of mitochondria in cells having different treatment groups (A). Aspect ratio signifies mitochondrial branch length (B) and form factor signifies the degree of mitochondrial branching (C). mRNA expression studies were carried out for fission and fusion proteins - Drp1 (D) and Mfn2 (E). Protein expression studies were carried out for Mfn2 protein and normalized with β-actin as a loading control (F). *p≤0.05, vs. vehicle, ##p<0.01, ###p<0.001 vs. TNFα. Data represented as mean ± SEM from N of 5 patients.

2.4. Discussion

In asthma, various inflammatory pathways increase AHR and airway obstruction leading to recurrent episodes of coughing and wheezing [41, 42]. These hallmark features of asthma are majorly mediated through ASM layer, which balances between bronchodilation and bronchoconstriction to maintain the tone of airways [43]. $[Ca^{2+}]_i$ in ASM cells is one of the major factor for the overall constriction of airways. A bronchoconstrictor stimulation (muscarinic or

histaminergic) causes transient increase in $[Ca^{2+}]_i$, due to influx of extracellular calcium and release of calcium from the intracellular stores [44]. As such, there are several channels and pumps which affects the $[Ca^{2+}]_i$. Therefore, it is imperative to study various signaling mechanisms, which affects the $[Ca^{2+}]_i$ regulation in ASM cells.

Several clinical findings have stressed on the gender disparity existing in asthma prevalence and severity. Due to increased evidences of asthma prevalence in women, focus has been diverted towards the role of estrogen on ASM cells. In the present study, I have attempted to establish the link between long-term activation of ERs on the $[Ca^{2+}]_i$ regulation in both non-asthmatic and asthmatic ASM cells in the presence of inflammation.

The first study on the modulatory role of estrogen in the airway dates back to 1983 where it was found that these sex steroids causes potentiation of isoproterenol-mediated relaxation. More recent studies have shown the expression of both ERα and ERβ in human ASM. Here, acute exposure of physiological concentration of E_2, via ERα, inhibits plasma membrane influx, thereby reducing ASM $[Ca^{2+}]_i$. However, the long-term (possibly genomic) effect of ERs on the $[Ca^{2+}]_i$ levels in healthy and inflamed ASM cells was not addressed owing to the complexities of signaling mechanisms of ER isoforms.

In recent studies, it was found that during asthma, expression of ERs is found to be increased, which laid the foundation for examining the specific role of ERs in asthmatics. Interestingly, ERβ shows a marked increase in expression as compared to ERα[45, 46]. However, the significance of a transmembrane estrogen receptor GPR30, remains unclear owing to its non-expression by Western analysis and ineffective G1 (GPR30 agonist) role in ASM proliferation [45, 46]. The results of the present study will establish the differential contribution of ER isoforms in the maintenance of $[Ca^{2+}]_i$ and overall airway tone in normal and inflamed conditions.

My first set of studies demonstrated that the long-term ERα activation led to increased $[Ca^{2+}]_i$ response in both asthmatic and non-asthmatic ASM cells. Here, a physiological concentration of E_2 (1nM) has no significant effect on the $[Ca^{2+}]_i$ response to histamine, bradykinin, or ACh with 24h treatment. These results were interesting, owing our previous studies, which showed acute exposure of E_2 decreased the $[Ca^{2+}]_i$ response, indicating the differences in the ER signaling depends on the duration of action. In our studies, we used three different contractile agonists to confirm our results, due to Gq-coupled receptor activation and involvement of voltage gated, receptor-operated, store-operated $[Ca^{2+}]_i$ changes in varying degrees. Notably, ERβ activation showed a significant reduction in $[Ca^{2+}]_i$ in the asthmatic cells under normal conditions with histamine exposure.

It is important to note that the baseline $[Ca^{2+}]_i$ levels were observed to be higher in all the asthmatic cells compared with the non-asthmatic cells (data not shown) and not changed with ER-specific agonist treatment. Here, the asthmatic samples were derived from mild to severe asthmatic patients and had differences in the amplitude response as well as baseline levels of $[Ca^{2+}]_i$.

Modulation of receptors pharmacologically may not be precise, owing to cross-reactivity of the chemical compounds with a variety of other receptor types. Therefore, to confirm our results, various ER-specific agonists and antagonists were randomly screened, and an ERα activation-mediated increase in ASM $[Ca^{2+}]_i$ was validated. Particularly, ERα agonist THC, which also shows antagonism toward ERβ receptor exhibited highest $[Ca^{2+}]_i$ response, suggesting divergent $[Ca^{2+}]_i$ regulation by ERα versus ERβ [47-52]. The selection of estrogen receptor-selective agonists/antagonists and concentrations used were based on several previous studies. For example, WAY200070 has an IC50 of 2.3 nM for ERα and 155 nM for ERβ with 410 times more selectivity to ERβ over ERα. Similarly, PPT acts as an ERα -selective agonist, as it is inactive through ERβ

[48]. All other pharmacological ligands were also selected on the basis of their IC50 and binding affinity reported, as well as from our previous studies done by our group [48-51, 53]. Hence, on the basis of their IC50 values, it is safe to consider that the agonist concentrations used in our studies (10 nM) have high selectivity toward specific ER activation with minimal crossover. Furthermore, the differential role of ERα and ERβ activation in the regulation of $[Ca^{2+}]_i$, was confirmed by molecular biology techniques, where effects of ERs were prominently observed as a result of their specific overexpression, using plasmids or knockdown through siRNA. Here, an interesting point to note was that the effectiveness of ERβ in reducing the $[Ca^{2+}]_i$ was much more pronounced in ERβ-overexpressed cells, suggesting a regulatory role of ERβ isoform under higher expression.

The important role of TNFα and IL-13 in airway inflammation and hyperreactivity is well established. These cytokines affect several regulatory pathways through Th1 and Th2 responses, thereby increasing the overall $[Ca^{2+}]_i$ levels. Previous studies have demonstrated an increased expression of both ERα and ERβ in the TNFα or IL-13-treated human ASM cells, hinting toward possible modulation of inflammation due to differential signaling mechanisms of ER isoforms. In our present functional studies, in the context of inflammation, intriguingly, downregulation of $[Ca^{2+}]_i$ response in the presence of cytokines was particularly highly pronounced with ERβ activation than that with ERα activation, indicating the possible bronchodilatory effects the ERβ isoform could exert on the inflamed or asthmatic airways.

The present study is the first to demonstrate the role of long-term ER signaling on the mechanisms of $[Ca^{2+}]_i$ regulation. Notably, the regulatory role of ERβ in reducing $[Ca^{2+}]_i$ during inflammation was more prominent in the zero $[Ca^{2+}]_i$ environment. We have previously shown that the human ASM does not express the regulatory phospholamban and that inflammation or

asthma leads to decreased SERCA expression [54]. Interestingly, in previous acute exposure studies, there was no observable effect of estrogen on the SR $[Ca^{2+}]_i$ reuptake. Whereas long-term ERβ activation reverses the trend of cytokine effect with increased SERCA2 expression and function, leading to increased $[Ca^{2+}]_i$ sequestration. Additional possible mechanisms of ERβ also appear to be inhibition of LTCC. Here, KCl was used as a depolarizing agent to elicit the transient $[Ca^{2+}]_i$ response because it effectively causes depolarization of the membrane primarily through voltage-gated channels [34, 55-57]. These results corroborate other reported studies on the LTCC inhibitory effect of E_2. The $[Ca^{2+}]_i$ homeostasis in ASM cells is due to the interplay between the vast majority of protein channels and pumps, among which LTCC and SERCA have a major contribution.

Mitochondria undergoes more fission under the conditions of inflammation, increased $[Ca^{2+}]_i$, stress and increased ROS production. Several reports suggest that during asthmatic conditions, there is an increased fragmentation of mitochondria [58]. Increased fragmentation, further lead to leakage of mitochondrial calcium and reduction in mitochondrial calcium buffering capacity. This leads to a detrimental cycle of $[Ca^{2+}]_i$ generation [37, 59]. Therefore, it is important to observe whether ER signaling plays a role in modulating these mitochondrial events. Interestingly, ERβ activation restores the balance of fission-fusion of mitochondria in the inflammatory conditions.

The $[Ca^{2+}]_i$ homeostasis in ASM cells is due to the interplay between the vast majority of protein channels and pumps among which LTCC and SERCA have a major contribution. If ER signaling affects the SR function (as evidenced by the $LaCl_3$ experiments), it may also modulate regulatory proteins such as STIM1 and Orai1 that are critical to store-operated calcium entry (SOCE) which needs to be evaluated [4, 25, 26, 29, 54, 60, 61]. As transient receptor potential

(TRP) channels and sodium-calcium exchange (NCX) pumps modulate the SOCE [62-64], involvement of these mechanisms of genomic ER signaling remain to be examined. The differential signaling of ERs in the mitochondrial morphology needs to be further confirmed in the mitochondrial function of calcium buffering capacity as well. Here, it is important to note that there may be a differential signaling of ERs on various calcium regulators and the differences in may $[Ca^{2+}]_i$ handling may arise due to the possible signaling of ERs in the expression of muscarinic, histaminergic and bradykinin receptors as well.

Overall, the present study demonstrates the differential role of ERs in regulating $[Ca^{2+}]_i$ in baseline and inflamed conditions. ERα activation at baseline shows increased $[Ca^{2+}]_i$ response and is non-effective in regulating the cytokines induced increase in $[Ca^{2+}]_i$ responses. Whereas, ERβ activation tends to decrease $[Ca^{2+}]_i$ response slightly at baseline and significantly in the inflamed conditions. ERβ contributes to decreased $[Ca^{2+}]_i$ response through increased SERCA2 function as evident by the increased rate of sequestration of Ca^{2+}. ERβ exerts signaling through inhibition of pathways involved in activating the voltage gated LTCC. ERβ is also involved in the maintenance of morphology of mitochondria and thus most likely play a role in regulating mitochondria calcium. Overall, these results suggest that estrogen signaling is maintained in the presence of inflammation, and indeed more enhanced via ERβ activation, providing a potential avenue to blunt effects of inflammation on $[Ca^{2+}]_i$ in ASM.

CHAPTER 3. ESTROGEN RECEPTORS DIFFERENTIALLY REGULATE THE OVERALL CONTRACTILITY OF THE HUMAN AIRWAY SMOOTH MUSCLE[1]

3.1 Introduction

Prolonged inflammation causes structural and physiologic changes in the cells in the airway, including immune cells, epithelial cells, and ASM [4-6, 65]. These changes include ASM migration, proliferation, extracellular matrix deposition (ECM) and secretion in ASM which contribute to airway remodeling and lead to a hypercontractility. Here, mechanisms regulating ASM contraction, and the differential role of estrogen receptors discussed briefly.

3.1.1. Ca^{2+} Dependent Contraction of ASM Cells

Like any other smooth muscle, ASM cells are composed of two main proteins: actin and myosin. ASM cells use the second messenger system to open various calcium channels and pumps that release the calcium ions which leads to regulation of contractile apparatus. ASM can undergo phasic or tonic (sustained) contractions and both types of contraction depends on an increase in cytosolic calcium concentration. Increased cytosolic calcium in ASM cells binds to the calmodulin (CAM) and further activates the contractile apparatus via activation of myosin light chain kinase (MLCK) enzyme. Upon activation, MLCK phosphorylates myosin light chain (MLC) at S19, which forms cross-bridges with actin, forming actomyosin complex which leads to contraction. Thus, in ASM cells, MLC acts as the ultimate target for triggering contraction.

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[1]Publication forthcoming.

As the intracellular calcium decreases, the calcium dissociates from the CAM, and this leads to termination of contraction process. However, phosphorylation of MLC is maintained at a low level in the absence of any external stimuli or activation to maintain the tone of airways and this is termed as tonic contraction. In asthmatic ASM cells, there is an increased expression of myosin heavy chain and MLCK, which explains the hypercontractility in these patient [66].

3.1.2. Ca^{2+} Sensitization Mechanism and Contraction of ASM

Apart from Ca^{2+} dependent activation of MLC kinase, the state of myosin light chain phosphorylation is additionally regulated by MLC phosphatase (MLCP), which removes the phosphate from the light chain of myosin to promote smooth muscle relaxation. MLCP consists of a myosin binding subunit, known as myosin phosphatase target (MYPT) which, when phosphorylated, inhibits the activity of MLCP, allowing the MLC to remain phosphorylated, thereby sustaining contraction [29, 35]. RhoA and its downstream target Rho kinase play an important role in the regulation of MLCP activity. Rho kinase phosphorylates the MYPT subunit, inhibiting the activity of MLCP and thus promoting the phosphorylated state of the MLC_{20}. Factors which decreases MLCP activity have a net result of increased ASM contraction. Acetylcholine activates M2 muscarinic receptors, leading to RhoA to translocate from the cytosol to the membrane [56, 67].

3.1.3. ASM Relaxation

The relaxation process involves a decreased $[Ca^{2+}]_i$ concentration and increased MLC phosphatase activity. Also, second messengers such as cAMP promotes muscle relaxation. Bronchodilators which target β-adrenergic Gs coupled receptor (β_2-AR) acts through this mechanism to cause relaxation [68, 69]. Adenylyl cyclase (AC) exists downstream of β_2-AR, which mediates the hydrolysis of ATP into cAMP. cAMP is a short-lived molecule and is degraded

by the activity of phosphodiesterase enzyme (PDE). Increased cAMP acts via the effector protein kinase A (PKA) on the contractile process in three ways: 1) It phosphorylates the calcium pumps to remove the Ca^{2+} ions 2) It antagonizes MLCK 3) It causes a reduction in the sensitivity of the contractile machinery by partially inhibiting the GTPase RhoA. This increases MLCP activity and causes MLC dephosphorylation and muscle relaxation [67, 70]. Hence, the activity of PKA is important for relaxation and is assessed by measuring the phosphorylation of its substrate-Vasodilator-stimulated phosphoprotein (VASP) [71-73].

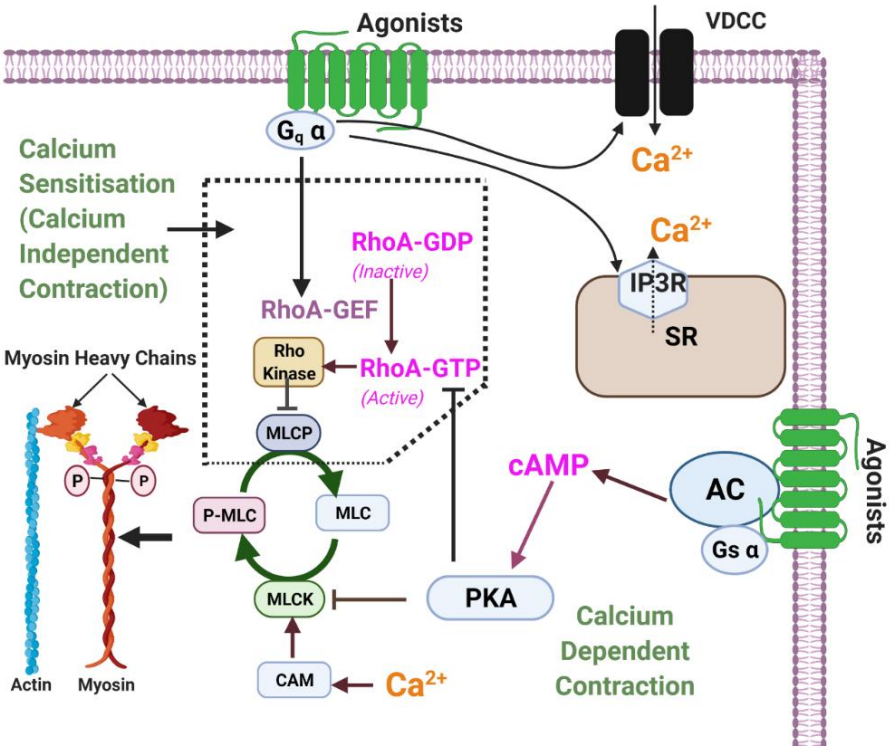

Figure 16. Regulation of smooth muscle contraction in ASM cells.
When agonists for G-protein coupled receptor binds, the Phospholipase C produces inositol 1,4, 5-trisphosphate (IP3) causing transient release of calcium $[Ca^{2+}]_i$ which binds to calmodulin(CAM), subsequently leading to activation of myosin light chain kinase (MLC kinase). This kinase transiently phosphorylates the light chain of myosin. The contractile response is also sustained by a $[Ca^{2+}]_i$ sensitizing mechanism through the inhibition of myosin phosphatase (MLCP)activity by Rho kinase.

β_2-AR agonists are the most effective and quick acting bronchodilators for treatment for acute asthma exacerbations. Although β_2-agonists are generally effective, tachyphylaxis with repeated use as well as reduced efficacy are well-recognized limitations. Accordingly, the focus of ongoing research is in development of adjunct and novel approaches to enhance bronchodilation.

It is known that estrogens regulate of contraction in many different types of non-airway tissues. For e.g., an increase in estrogen levels causes contraction of the pregnant uterus and contribute to the parturition process [69]. Estrogenic hormones are also shown to exert their action on breast cancer cells by increasing cAMP levels [20, 74]. There is a precedent for studies showing estrogen-induced relaxation in vascular smooth muscle and in ventricular myocytes [75, 76]. Also, several studies have shown that there exists a crosstalk between β_2-AR of vascular endothelium and cardiac cells which explains the sex specific differences in cardiovascular diseases[77-79]. Although these studies point towards the effect of estrogen on the regulation of muscle tone, most of these studies are focused on the non-genomic signaling pathways of GPR30 and ERs especially ERα [80-83]. Previous studies done on human ASM cells also demonstrated increased cAMP due to estrogen, colocalization of ERs with β_2-AR and its potentiation through non-genomic signaling of estrogen [84]. These *in vitro* studies with non-genomic signaling of estrogens may not give a complete explanation of the discrepancies observed in clinical findings that have also shown the detrimental effects of estrogen. These inconsistencies in effects of estrogen in the women may arise due to imbalance between non-genomic and genomic signaling mechanisms at different life stages and menstrual cycle suggesting a need for the exploration of long-term genomic ER signaling.

Through our recent findings, ERα and ERβ are found to be expressed in the human ASM and their increased during inflammation (specifically ERβ). In my previous aim, the $[Ca^{2+}]_i$ response to various agonists were found to be regulated through differential signaling of ERα and ERβ which may lead to differences in contractile mechanisms through these receptors. I also showed that ERβ exerts its action partly due to LTCC and SERCA2, which are one of the targets of β_2-AR activation[85]. Hence, in the present study, I tested the hypothesis that genomic ER signaling in normal human ASM and regulates various signaling mechanisms of contraction during agonist stimulation, and that their signaling overlaps with β_2-AR signaling in human ASM cells. This will set the foundation for examining the role of ERs in asthmatic ASM contractility.

3.2. Materials and Methods

3.2.1. Isolation of Human ASM Cells

Briefly, third to sixth generation human bronchi were obtained from lung tissues of patients who underwent to lung surgeries at Mayo Clinic for focal, non-infectious reasons. Mayo clinic's Institutional Review Board approved protocols allowed for initial review of patient histories, with complete de-identification of samples for storage and subsequent usage. This study was limited to samples from 25 male and female non-smoker adults of ages from 21 to 65 years non-asthmatic patients. For each study 5 to 15 non-asthmatic cells each were chosen randomly and utilized independently. After removing the debris, airway samples were denuded of epithelium and ASM tissue was enzymatically dissociated according to previously described procedures following manufacturer's instructions (Worthington Biochemical, Lakewood, NJ) to generate ASM cells. Cultures (<5th passage) were maintained under optimal conditions of 37°C (5% CO_2, 95% air) in DMEM/F12 growth media [86]. Periodic assessment of ASM phenotype was performed by

verifying stable expression of smooth muscle actin, and agonist receptors. Cells were serum starved for 24h prior to treatment with agonists or antagonists.

3.2.2. Cell Treatment

Fully confluent T-75 flasks of ASM cells were trypsinized, mixed in 10% FBS (Charcoal Stripped) growth medium (DMEM/F12 with 1% AbAm), and seeded onto 100 mm petri plates. Cells were allowed to adhere, grown up to 80% confluence and incubated in serum free medium (DMEM/F12 without FBS) for 24h to mature. ASM cells were treated with pro-inflammatory cytokines in the presence and absence of ER specific agonists such as PPT, WAY, (10nM) and E_2 (1nM). Serum free media alone served as a vehicle.

3.2.3. Traction Force Microscopy (TFM)

Human ASM cells in serum free media were seeded onto a hydrogel plate (From Matrigel) of known stiffness (Young's moduli of gel substrates (12 kPa)) containing GFP beads. Cells were treated for 24 h with ER specific agonists and then kept under the microscope incubated with HBSS. Contractility was measured in a single cell at a time under the microscope at 20X using histamine (10μM) induced elicitation. GFP and phase-contrast images of fluorescent beads and human ASM cells were taken at baseline and following histamine exposure for 10 minutes. Cells were detached using trypsin, and bead images was acquired. For each cell, traction force were calculated from phase-contrast and bead images before vs. after cell removal, utilizing 2D cross-correlation algorithm. All the analysis was carried out using Traction for all software designed at Mayo Clinic using MATLAB script [87].

3.2.4. cAMP Assay

Human ASM cells were serum-starved for 24 h, and then treated with ER specific agonists for 24 hr. Cells were then incubated with 1 μM PDE inhibitor -IBMX (3-isobutyl-1-

methylxanthine) for 30 min followed by stimulation with 5 μM forskolin, or 1 μM isoproterenol (ISO) for 15 min. After treatment, cells were lysed in cAMP lysis buffer and protein quantification of lysates was carried out. Lysates were equalized and intracellular cAMP levels were measured by competitive cAMP ELISA kit (R and D Systems) as per the manufacturer's protocol. The concentrations obtained (pmol/ml) were then normalized to the total protein of the lysates to obtain results in terms of pmol/mg.

3.2.5. RhoA Activity

RhoA activity was determined by measuring levels of RhoA-GTP in cultured human ASM cells under agonist treatments. Briefly, human ASM cells were seeded in 100-mm dishes. After 80% confluence, cells were serum-deprived for 24 h and then treated with ER specific agonists. Before collecting lists, cells were activated using 1 μM Histamine [56, 67, 88]. Cell lysates were collected, snap-frozen immediately within 10 minutes of lysate collection and aliquots were made. Protein concentrations were estimated, and lysates were adjusted for equal protein concentrations. RhoA activity was assessed using G-LISA RhoA activation assay Biochem kit (Cytoskeleton) according to the manufacturer's instructions by detecting absorbance at 490 nm.

3.2.6. Western Blotting

For pVASP (from SCBT) protein expression studies, Human ASM cells were serum-starved for 24 h, and then treated with ER specific agonists for 24 hr. Cells were then incubated with 1 μM IBMX for 30 min followed by stimulation with 100 μM forskolin, or 1 μM ISO for 15 min. Cells were washed with ice cold PBS and lysed by adding lysis buffer supplemented with protease and phosphatase inhibitor along with PMSF. Lysates collected were briefly sonicated and centrifuged to remove the debris and resultant supernatants were assayed for total protein content using the DC Protein Assay kit.

For pMLC and pMYPT proteins (Cell Signaling Ab), owing to the fragile nature of phospho-proteins, the process of cell lysate collection was modified to avoid the breakdown of phospho-proteins. Human ASM cells were treated with either PPT, WAY (10nM) or E_2 (1nM) for 24 h followed by exposure to 1 µM Histamine for 5 minutes (Vehicle +/- Histamine). Thereafter, protein samples were prepared by treating the cells with stop buffer (0.3 N Perchloric acid) for 5 min on ice. Cells were rinsed with ice-cold PBS for three times and lysed in cell lysis buffer supplemented with protease and phosphatase cocktail inhibitor at 4°C for 30 min. Lysates collected were briefly sonicated and centrifuged to remove the debris and resultant supernatants were assayed for total protein content using the DC Protein Assay kit [89].

40 µg equivalent protein from each treatment group lysate were loaded on 4-15% gradient gels (Criterion Gel System; Bio-Rad, Hercules, CA, USA) and transferred onto 0.22 µm PVDF membranes using a Bio-Rad Trans-Blot Turbo rapid transfer system. Non-specific binding was blocked using 5.0% bovine serum albumin (BSA) in Tris-buffered saline (TBS) for 1 h at room temperature and the membranes were probed overnight at 4°C with tVASP, pMLC (S19) or pMYPT (Thr507) primary antibodies in 5% BSA in TBST. Following three washes with Tris-buffered saline with 0.1% tween (TBST), blots were then incubated with LI-COR near-infrared conjugated secondary antibody. Protein expression was detected by imaging the membrane on a LI-COR Odyssey CLx system. β-actin was used as a loading control. The normalized values were obtained by dividing the raw values of proteins of interest with the raw values of β-Actin. In case of VASP, ratio of p-VASP to tVASP were calculated. The obtained values were then normalized to vehicle to get relative protein expression of pMLC or pMYPT.

3.2.7. Statistical Analysis

Up to twenty ASM cells from different non-asthmatic and asthmatic patients were used. Each set of $[Ca^{2+}]_i$ experiments were performed in at least 5 randomly selected different patient cells, although not all protocols were performed in each ASM sample. Statistical analysis was performed using GraphPad Prism version 8.0.0 for Windows, San Diego, California USA. Statistical differences between the experimental groups were analyzed using student's *t*-test or 1-way ANOVA followed by Dunnet or Tukey's post-hoc test for multiple comparisons where appropriate. Statistical significance was established minimum at a minimum of $p \leq 0.05$. All values are expressed as mean ± SEM.

3.3. Results

3.3.1. Effect of ER Specific Signaling on Overall Contractility of ASM Cells

To determine the role of long-term ER signaling (likely genomic) in contractility, ASM cells were exposed to ERα (PPT, 10nM), ERβ (WAY, 10nM) agonists, E_2 (1nM) for 24h. Contractility was measured using TFM in single cell at a time under microscope at 20X using histamine (10μM) induced elicitation. ERα agonist PPT treated cells showed a significant increase in traction force as compared to the vehicle. Non-selective ER agonist E_2 and ERβ agonist WAY did not show any change in the traction force (Figure 17).

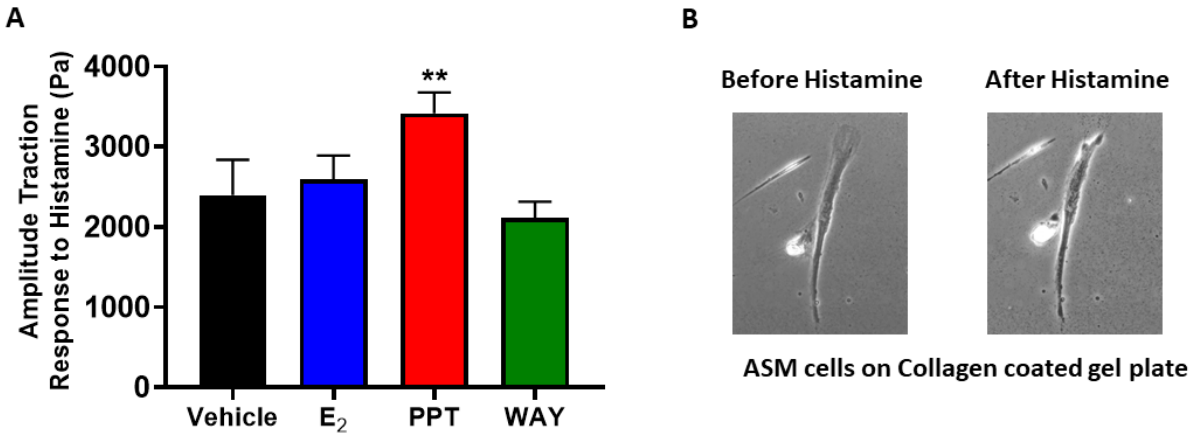

Figure 17. Effect of long-term ER signaling on overall contractility in ASM cell.
(A) In ASM cells treated for 24 h with ERα specific agonist PPT, ERβ specific agonist WAY, and non-specific E_2, the cellular contractility was assessed by measuring the traction force following exposure to histamine (10 μM). (B) Representative images took before addition of Histamine and after obtaining the maximum contraction within 10 minutes. **$p<0.01$, vs. vehicle. Data represented as mean ± SEM from N of 5 patients each.

3.3.2. Effect of ER Specific Signaling on β_2-AR Sensitization in ASM Cells

To investigate ER signaling effect on β_2-AR sensitization, cells incubated with ER specific agonists for 24 hours were treated PDE inhibitor IBMX followed by β_2-AR agonist- ISO for 15 minutes. IBMX alone treated vehicle group served as control. cAMP turnover in cells unstimulated with ISO remained at low levels. ISO alone treated groups significantly increased the cAMP accumulation in the cells (Figure 18, $p \leq 0.05$). ERβ agonist WAY treatment for 24 h significantly potentiated ISO-mediated increase in the cAMP accumulation ($p \leq 0.01$). Non-selective ER agonist E_2 and ERα agonist PPT did not show any significant changes in the cAMP accumulation compared to ISO alone (Figure 18).

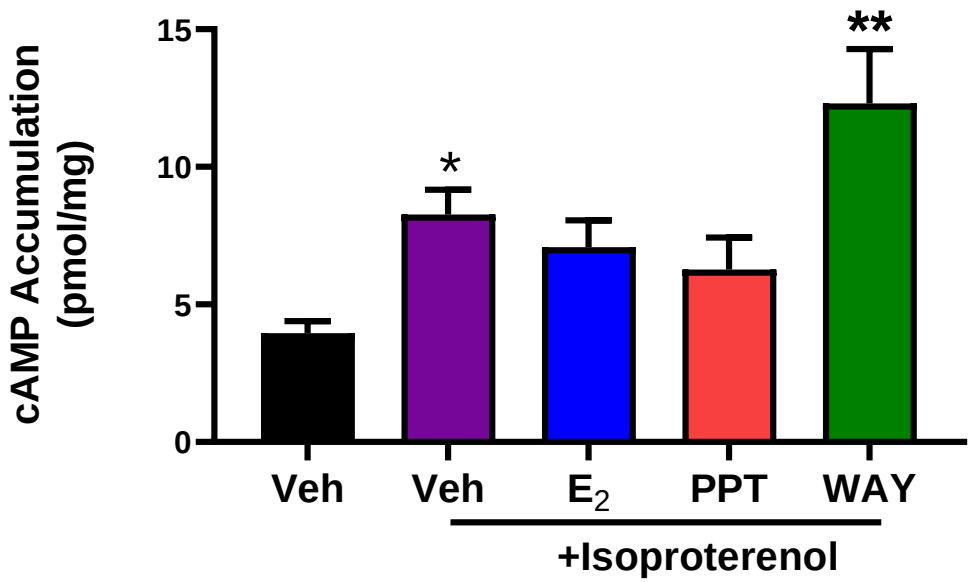

Figure 18. Effect of long-term ER signaling on β_2-AR sensitization in ASM cell.
In ASM cells treated for 24 h with ERα specific agonist PPT, ERβ specific agonist WAY, and non-specific E_2, the cAMP accumulations due to β_2-AR agonist ISO (1 μM) in the presence of IBMX were measured. *p<0.05, **p<0.01, vs. vehicle. Data represented as mean ± SEM from N of 5 patients each.

3.3.3. Effect of ER Specific Signaling on β_2-AR Mediated PKA Activity

To further confirm the effects of ER specific signaling on Isoproterenol induced relaxation, PKA activity was confirmed by immunoblotting for total VASP, which undergoes phosphorylation by PKA at Ser-157. The shift in VASP protein band by Western blot from 46 to 50 kDa is an indicative of PKA activity (Figure 19).

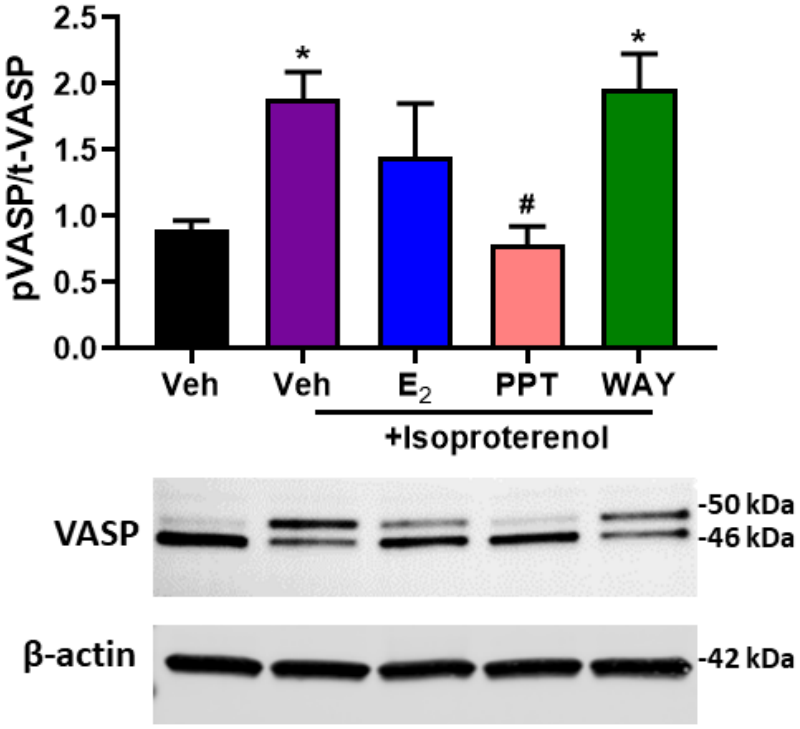

Figure 19. Effect of long-term ER signaling on β_2-AR mediated PKA activity in ASM cell. In ASM cells treated for 24 h with ERα specific agonist PPT, ERβ specific agonist WAY, and non-specific E_2, cells were treated with ISO for 15 minutes in the presence of IBMX. PKA activity was measured through the phosphorylation state of substrate VASP. Phospho- VASP was normalized to the total VASP. **p<0.01, ***p<0.01, vs. vehicle. Data represented as mean ± SEM from N of 10 patients each.

Cells treated as in (3.2) were subjected to western analyses for VASP proteins which is a Substrate of PKA mediated phosphorylation (Figure 19). ISO alone treated groups increased the phosphorylation of VASP in the cells ($p \leq 0.05$) as compared to the vehicle. Non-selective ER agonist E_2 did not show any significant changes in the ISO mediated VASP phosphorylation. In cells pre-treated with ERα agonist PPT, there was a significant reduction in the ISO mediated VASP phosphorylation ($p \leq 0.05$) as compared to ISO alone treated cells. ERβ agonist WAY treatment for 24 h retained the PKA activity of ISO as shown by the significant increase in VASP phosphorylation.

3.3.4. Effect of ER Specific Signaling on Adenylyl Cyclase (AC) Sensitization in ASM Cells

To investigate ER signaling effects on AC sensitization, cells incubated with ER specific agonists for 24 h were treated PDE inhibitor IBMX followed by AC activator, forskolin for 15 minutes. IBMX alone treated vehicle group served as control (Figure 20). Forskolin alone treated groups significantly increased the cAMP accumulation in the cells ($p \leq 0.001$). Pretreatment with ERα agonist PPT, ERβ agonist WAY, and non-selective ER agonist E_2 for 24 h did not show any significant changes in the cAMP accumulation compared to forskolin alone.

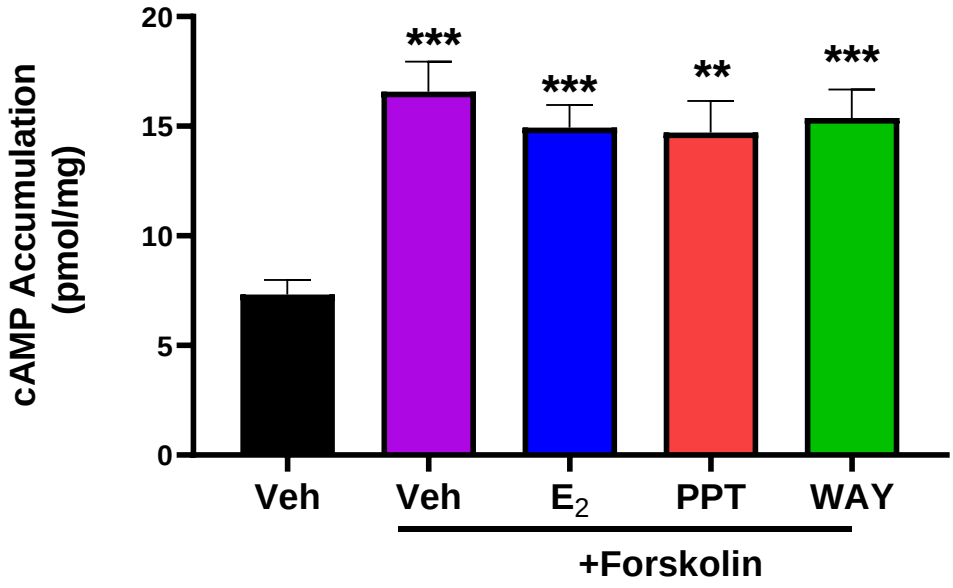

Figure 20. Effect of long-term ER signaling on adenylyl cyclase sensitization in ASM cell.
In ASM cells treated for 24 h with ERα specific agonist PPT, ERβ specific agonist WAY, and non-specific E_2, the cAMP accumulations due to AC activator forskolin (5 μM) in the presence of IBMX were measured. **p<0.01, ***p<0.01, vs. vehicle. Data represented as mean ± SEM from N of 10 patients each.

3.3.5. Effect of ER Specific Signaling on AC Mediated PKA Activity

To further confirm ER signaling on AC sensitization, forskolin mediated PKA activity (via VASP) in the presence of ER specific agonists was evaluated. Cells treated as in (3.4) were subjected to Western analyses for VASP proteins (Figure 21). Forskolin alone treated groups

increased the phosphorylation of VASP in the cells ($p \leq 0.05$) as compared to the vehicle. Pretreatment with ERα agonist PPT, ERβ agonist WAY, and non-selective ER agonist E_2 for 24 h did not show any significant changes in the cAMP accumulation compared to forskolin alone (vehicle treatment).

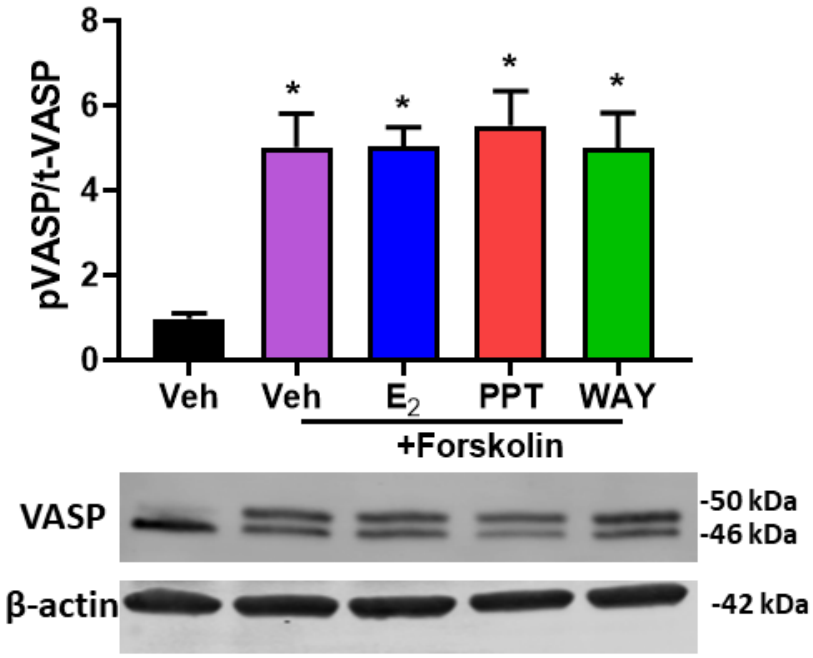

Figure 21. Effect of long-term ER signaling on AC mediated PKA activity in ASM cell.
In ASM cells treated for 24 h with ERα specific agonist PPT, ERβ specific agonist WAY, and non-specific E_2, cells were treated with Forskolin for 15 minutes in the presence of IBMX. PKA activity was measured through the phosphorylation state of substrate VASP. Phospho- VASP was normalized to the total VASP. **p<0.01, ***p<0.01, vs. vehicle. Data represented as mean ± SEM from N of 5 patients each.

3.3.6. Effect of ER Specific Signaling on Histamine Induced Phosphorylation of MLC

Human ASM tone and contractility depends on the phosphorylation state of the smooth muscle MLC, which is regulated through combined activities of $[Ca^{2+}]_i$, PKA and MLCP. Hence phosphorylation of MLC protein ultimate end contraction of ASM and was analyzed using our agonist treatments (Figure 22). Cells treated with ER specific agonists were incubated with 1 µM

histamine for 5 minutes and lysates were collected. Vehicle without histamine treatment served as control. Western analysis for pMLC protein was performed. At baseline, the expression of pMLC is negligible. Histamine treatment significantly increased the phosphorylation state of MLC ($p \leq 0.001$) as compared to the vehicle. Pretreatment with WAY showed a marked decrease in the histamine induced phosphorylation of MLC ($p \leq 0.05$). Non-selective ER agonist E_2 reduced the histamine induced phosphorylation of MLC, although its effects were less prominent than that of the WAY treated cells. ERα agonist PPT did not show any changes in the histamine induced phosphorylation of MLC.

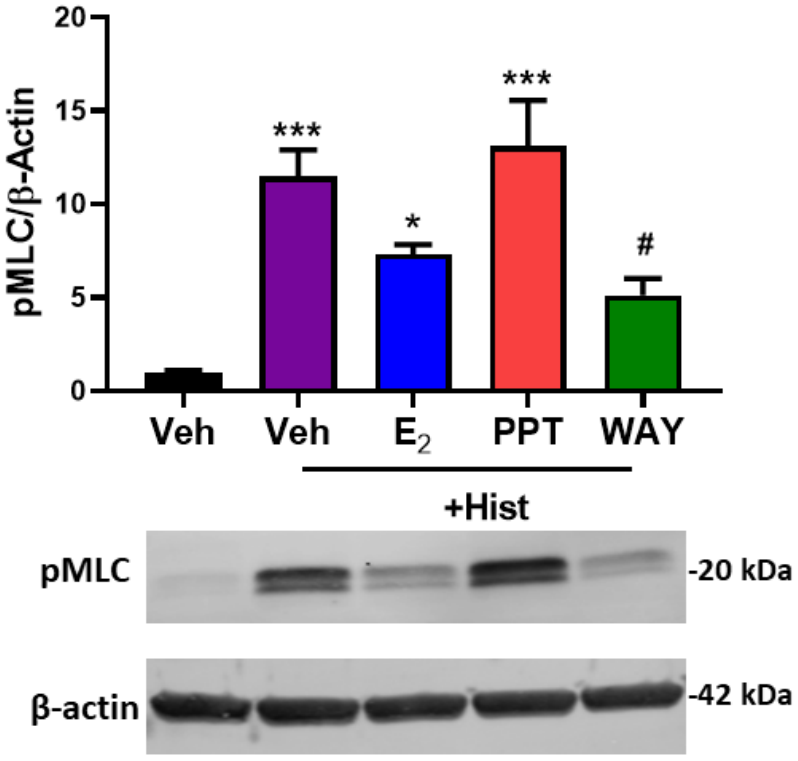

Figure 22. Effect of long-term ER signaling on the histamine induced phosphorylation of MLC in ASM cell.
Human ASM cells were pre-treated for 24 h with ERα specific agonist PPT, ERβ specific agonist WAY, and non-specific E_2, followed by stimulation with 1 μM histamine (Hist) for 5 min. Cell lysates were immunoblotted for pMLC and β-actin was used as loading control. Data represented as mean ± SEM from N of 4 patients each. *$p<0.05$, ***$p<0.001$ vs. vehicle (No Hist). $^{\#}p<0.05$ vs. vehicle (+Hist).

3.3.7. Effect of ER Specific Signaling on Phosphorylation of MYPT

Cells treated with ER specific agonists for 24 hours were treated with 1 µM histamine for 5 minutes same as in (3.3.6) and lysates were collected. Vehicle without histamine treatment served as control. Western analyses for pMYPT (Thr507) protein was performed (Figure 23). Histamine treatment increased the phosphorylation state of MYPT as compared to the vehicle. Pretreatment with WAY or E_2 did not cause any significant change in histamine induced phosphorylation of MYPT ($p \leq 0.05$). However, cells pre-treated with PPT showed marked increase in the phosphorylation of MYPT as compared to vehicle ($p \leq 0.001$) and this phosphorylation was significantly higher as compared to histamine alone treated cells ($p \leq 0.05$).

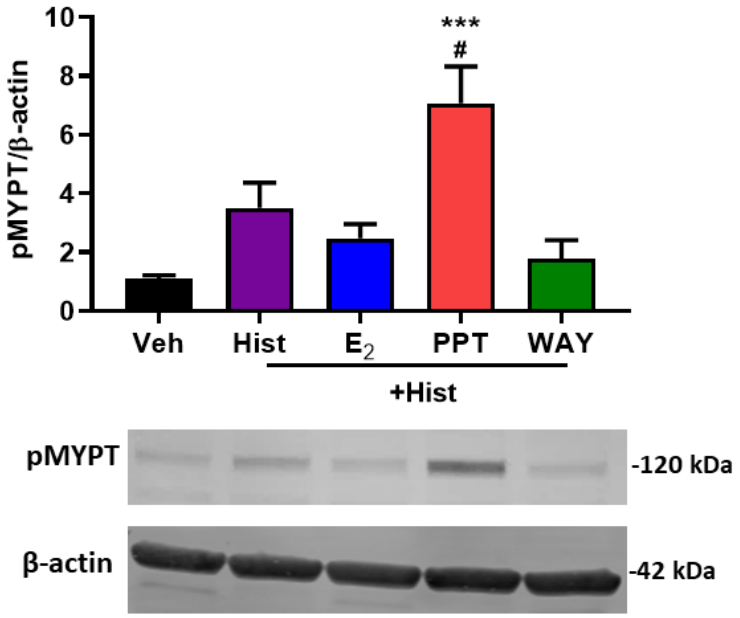

Figure 23. Effect of long-term ER signaling on phosphorylation of MYPT in ASM cell.
Human ASM cells were pre-treated for 24 h with ERα specific agonist PPT, ERβ specific agonist WAY, and non-specific E_2, followed by stimulation with 1 µM Hist for 5 min. Cell lysates were and immunoblotted for pMYPT and β-actin was used as loading control. Data represented as mean ± SEM from N of 4 patients each. *$p<0.05$, ***$p<0.001$ vs. vehicle (No Hist). #$p<0.05$ vs. vehicle (+Hist).

3.3.8. Effect of ER Specific Signaling on RhoA Activity

The phosphorylation at Thr507 of MYPT-1 is an outcome of Rho kinase activity [90]. Therefore, the activity of its upstream protein-RhoA needed to be measured (Figure 24). Cells were treated for 24 h with ER specific agonists and then stimulated with histamine for 5 min before the lysates were collected for the analysis of RhoA-GTP levels which signifies the RhoA activity. Treatment with PPT significantly increased the RhoA -GTP as compared to the vehicle. On the contrary, E_2 and PPT treatment did not show any significant changes as compared to the vehicle.

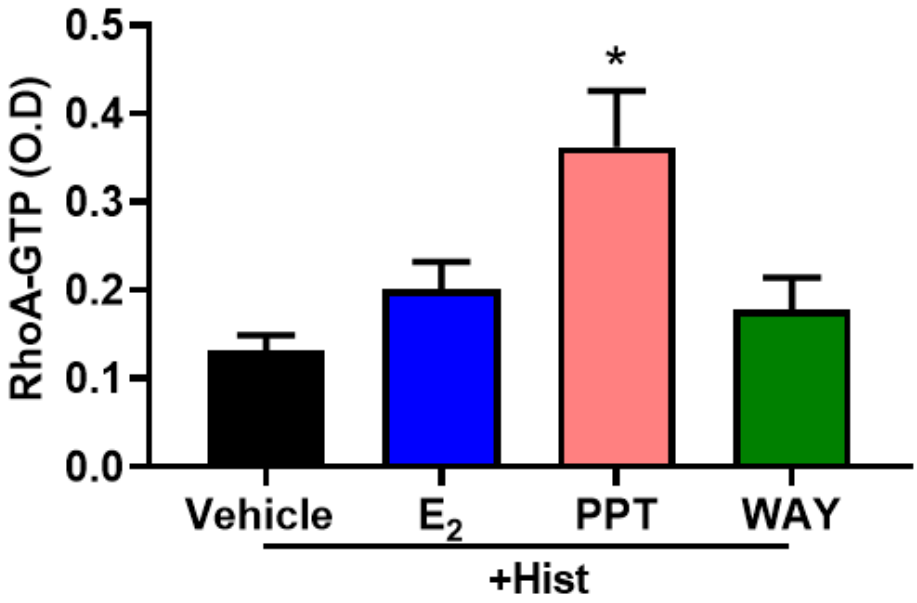

Figure 24. Effect of long-term ER signaling on RhoA activity in ASM cell.
Human ASM cells were pre-treated for 24 h with ERα specific agonist PPT, ERβ specific agonist WAY, and non-specific E_2, followed by stimulation with 1 µM histamine (Hist) for 5 min. Cells were lysed and snap-frozen immediately. Analyses of RhoA-GTP levels were done using ELISA. Data represented as mean ± SEM from N of 4 patients each. *$p<0.05$, ***$p<0.001$ vs. vehicle (No Histamine). $^{\#}p<0.05$ vs. vehicle (+Hist).

3.4. Discussion

Convincing evidence has shown that non-genomic ER signaling exerts bronchodilatory and Broncho protective effects. However, knowledge of ER genomic signaling in various events

of airway hyperresponsiveness is still lacking. In the first aim, I have demonstrated that $[Ca^{2+}]_i$ handling in asthmatic and non-asthmatic ASM cells is differentially regulated by ERα and ERβ. The current study demonstrates for the first time that differential ER activation regulates cAMP production, $[Ca^{2+}]_i$ sensitization. Signaling through ERs modulates PKA and RhoA activity both of which mediate their actions on MLC phosphorylation and thus overall contractility in human ASM cells is regulated.

The present study further demonstrates that differential ER signaling modulates the overall contractility of normal human ASM cells. To assess the contractility of single cell treated with ER specific agonists, I have used TFM technique. TFM allows to measure space distribution of the contraction forces in response to contractile or relaxing agonist of ASMC at the single-cell level. There was no significant change in the basal traction force in groups treated with ER specific agonists for prolonged duration. However, long-term ERα activation increased the traction force in response to contractile agonist histamine. This suggests that ERα activation leads to a hypercontractile state of the cell at baseline conditions.

Since the overall contractility was affected due to differential signaling of ERs, ER signaling could have a direct effect on cAMP production. ISO is a potent bronchodilator which mediates its action acutely through β_2-AR and brings quick relief in hypercontractile response of asthma. However, repeated dose of β_2 agonists leads to desensitization of the receptor and reduced activity. Moreover, cAMP is a short-lived molecule which is degraded by PDE. There are several subtypes of PDE found in the ASM cells, with PDE4, the most found and active subtype in ASM cells of humans. There are several inhibitors available to date, which stops the activity of this enzyme, and they serve as indirect bronchodilators however their efficacy is lesser than the

activation of β_2-AR. Therefore, such drugs which may serve to potentiate the activity of β_2-AR could be added as an adjuvant medicine to the already existing bronchodilator therapy.

ISO-induced cAMP is found to be increased due to short-term effect of estrogen through previous studies. My goal of the present study was to check the prolonged (possibly genomic) effect of ER signaling on the ISO-induced bronchodilation. In this study, non-selective PDE inhibitor IBMX was used to stop the degradation of cAMP. Here, physiological concentrations of E_2 (1 nM) given for prolonged duration to the cells had no significant potentiation effect on β_2-AR. These results were interesting, owing to previous studies, which showed acute exposure of E_2 increased the cAMP levels and caused potentiation of ISO-induced relaxation. ERβ activation led to significant potentiation of β_2-AR. Cells pre-treated with ERα agonist PPT, tend to reduce cAMP accumulation. Furthermore, cAMP acts through effector molecule PKA to bring about relaxation by its inhibitory activity on MLCK. To further confirm the effects of ER signaling on ISO-induced relaxation, PKA activity was assessed through VASP phosphorylation. Notably, ERβ activation retained the ISO-mediated PKA activity, whereas ERα activation significantly diminished the VASP phosphorylation, indicating the differential and contrasting broncho modulatory effects of ER signaling.

To further investigate the downstream effects of ERs on the cAMP signaling pathway, Forskolin, an AC activator was used in this study [68, 83, 91]. This experiment helped to isolate β_2AR effect and measure the direct activation of AC in human ASM. It was found that none of the ERs significantly alter the forskolin-stimulated cAMP levels in human ASM cells. Furthermore, there was no significant change in the forskolin mediated VASP phosphorylation. Since, only ISO-induced cAMP was affected by differential ER activation, these data suggests that ER acts exclusively on β_2-AR cascade mechanism, not via AC.

The previous aim (Chapter 2) has shown differential $[Ca^{2+}]_i$ regulation due to ER signaling. Coupled with several other key modulators of contraction, $[Ca^{2+}]_i$ leads to the phosphorylation of MLC causing overall contraction of ASM cells. Hence, MLC is the target contractile apparatus for all the second messengers and enzymes to cause contraction or relaxation. In normal tonic contraction without any external stimuli, the phospho-MLC is present is low amount. Various Gq agonists trigger the phosphorylation by activating various pumps and channels. To understand the role of ER signaling on the overall contraction, it is important to study the phosphorylation state of MLC. The present study for the first time have successfully established the differential effect of ERs on the histamine induced phosphorylation of MLC linking the role of ERs with overall contractility.

Studies suggest that cAMP/PKA signaling pathway may be involved in the partial inactivation of RhoA by phosphorylating RhoA at Ser188[92]. This results in a decrease in the inhibitory effect of RhoA on MLCP, which causes MLCP to dephosphorylate MLC and relax airway smooth muscle independent of $[Ca^{2+}]_i$ level. RhoA inhibits MLCP activity through phosphorylation of its regulatory subunit MYPT-1 at Thr507, Thr696 and Thr853 [90, 93] (more specifically on Thr507 and Thr853). When any of these MYPT sites are dephosphorylated, MLCP gets activated to dephosphorylate MLC and induce relaxation. Most of the Gq agonists increase the RhoA activity leading to phosphorylated MYPT and consequently contraction. In case of the present study, specifically ERα activation significantly increased the histamine induced phosphorylation of MYPT suggesting its pro-contractile mechanism.

This study has focused on the differential signaling of ERs on the overall contractile machinery of ASM cells without particular focus on inflammatory condition. However, the results obtained in this study will set the foundation for examining the role of ERs in asthmatic ASM

contractility. Although the concentration of forskolin was used based on previous literatures, the lack of differential signaling of ERs on adenylate cyclase may arise due to the possible ceiling effect of forskolin. The overall study involved a fixed concentration of Isoproterenol and forskolin, and the differential role of ERs at different concentrations of these agonists is yet to be explored.

In conclusion, in this study, I have shown for the first time the genomic ER signaling on cAMP/PKA cascade as well as RhoA/ROCK pathway. This will explain some of the clinical discrepancies in the effect of estrogens and could be interpreted in the context hyperresponsiveness. Also, these findings can provide a molecular basis for developing new broncho modulatory compounds based on estrogen receptors.

CHAPTER 4. IN A MOUSE MODEL OF ALLERGIC ASTHMA, TO DETERMINE THE EFFECT OF ERα VS. ERβ ON STRUCTURAL AND FUNCTIONAL AIRWAY CHANGES.[1]

4.1. Introduction

The effect of estrogen in asthma is controversial due to the contradictory results from various groups of researchers [36]. Some groups have suggested that long-term and/or high doses of estrogen therapy increases risk of asthma while ovariectomy is shown to reduce asthma [94]. In contrast, another study reported that supplemental estrogens could be beneficial in women with asthma and also reduce AHR in murine models [95-97]. Changes in pulmonary function and airway hyperresponsiveness according to the menstrual cycle fluctuations have also been well documented, suggesting a role of estrogen hormone [9, 12, 36, 98, 99]. However, the correlation between estrogen levels and asthma is complex, and the mechanism for the association remains obscure. Limited studies on the role of estrogen in AHR has been mainly focused on ERα, while completely neglecting role of ERβ. Overall, these data on airway responses in females highlight the need for further research into the mechanisms by which estrogens affect the airway.

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[1]The material in this chapter was co-authored by Dr. Rama Satyanarayana Raju Kalidhindi, Dr. Nilesh Sudhakar Ambhore, Bhallamudi Sangeeta, Dr. Jagadish Loganathan and Dr. Venkatachalem Sathish. Dr. Rama Satyanarayana Raju Kalidhindi, Dr. Nilesh Sudhakar Ambhore, and Dr. Jagadish Loganathan performed the Flexivent analysis. Bhallamudi Sangeeta had primary responsibility for breeding of mice, genotyping, treatment of mice with mixed allergen, collecting samples for BALF and histology studies [100]. PCLS studies were designed and performed by Bhallamudi Sangeeta exclusively, and results of these studies will be published in future. Bhallamudi Sangeeta also drafted and revised all versions of this chapter. Dr. Venkatachalem Sathish served as proofreader and checked the math in the statistical analysis conducted by Bhallamudi Sangeeta for this chapter.

Asthma is a multifaceted and intricate disease involving diverse pathologies and many cells which make it challenging to identify and address the core mechanisms involved. Hence, in *vivo* studies which can replicate the complexity of airway disease in the presence of complex tissue components greatly validates the investigation of the underlying pathophysiology of AHR. The most used animal to model allergic airway inflammation is the mouse, due to relatively low handling costs, short gestation period, litter size and the feasibility to manipulate mice genetically by switch on or off certain genes and thus study specific pathways.

4.1.1. FOT and PCLS

Forced Oscillatory technique (FOT) is an advanced technique to assess the lung function. It facilitates measurements of lung mechanics through the analysis of wavelengths acquired in reaction to oscillatory airflow waves which are applied at the subject's airway opening [101]. Apart from *in vivo* lung function studies, ex vivo studies are also used for assessing AHR. For intact ASM structure, tracheal pieces in organ baths have been traditionally utilized for estimating smooth muscle function and constriction *in vivo*. However, for more mechanistic understanding, precision cut lung slices (PCLS) are utilized which has the capabilities to investigate multiple regions of the lung as well as distal airways. The terminal airways of the lung located beyond the 7th or 8th generation of the tracheobronchial tree accounts for approximately >98% of the cross-sectional area of the lung [102]. These small airways are responsible for majority of the resistance offered by the airways. PCLS and FOT both are powerful tools to assess the lung function in the smaller distal airways.

4.1.2. Murine Model of Asthma

It is important to note that murine model used for studying asthma do not spontaneously develop the disease. Hence, these murine models of asthma are artificially developed models of

allergic airway inflammation based on an initial sensitization phase to an antigen followed by local challenge to induce airway inflammation. The antigen generally used in the allergic asthma models is ovalbumin. However, continuous exposure to inhaled OVA leads to a state of immunological tolerance [103]. More recently, murine asthma models are developed by utilizing naturally occurring allergens [104]. The two most common environmental aeroallergens are house dust mites (HDM) and fungi such as Aspergillus and Alternaria. These environmental allergens replicate the natural conditions of human allergic asthma in murine models and is administered intranasally. I have utilized newly developed mixed allergen (MA) induced mouse model of asthma in my experiments. The rationale behind using the MA model is 1) Simpler sensitization regimen with early persistent inflammation, structural and functional airway changes as early as two weeks; 2) An eosinophilic and Th_2-weighted inflammatory response resembling human asthma; 3) We have verified that the MA model produces significant inflammation and remodeling in C57BL/6J mice.

With the advent of recent advancements of receptor-specific knockout mice, the present study aimed to confirm our hypothesis using ERα and ERβ specific knockout (KO) mice [105]. An interesting fact to note here is that ERβ expression is increased multifold during asthma when compared at baseline, justifying the need for *ex vivo* and *in vivo* study in the context of asthma. Considering these facts, the present study was focused to identify the role of ER specific signaling of endogenous estrogen during asthma in an MA induced murine model of asthma in ER specific KO mice (ERα and ERβ).

4.2. Materials and methods

4.2.1. Animals

Animal study protocol in this study was approved by the Institutional Animal Care and Use Committee at North Dakota State University and conducted in accordance with guidelines derived from the National Institutes of Health's Guide for the Care and Use of Laboratory Animals. ERα (Stock No: 004744, B6.129P2-Esr1^{tm1Ksk}/J) and ERβ (Stock No: 004745, B6.129P2-Esr2^{tm1Unc}/J) knock out heterozygous breeding pairs of C57BL/6J background were procured from Jackson Labs (Bar Harbor, ME). All the mice used in this study were homozygous obtained from in-house breeding using ERα or ERβ knock out of heterozygous breeding pairs. Obtained litters were separated based on genotyping and the resultant wild type mice and knockout mice were used for the study. Mice were always housed under constant temperature and 12-hour light and dark cycles provided with food and water *ad libitum*. Mice from either gender were used in this study with a minimum of 5-6 mice in each group.

4.2.2. Genotyping

The pups obtained from the breeding process were subjected to genotyping after 7 weeks using a tail biopsy method following instructions provided by Jackson laboratories (Bar Harbour, ME). The genomic DNA from mouse-tails were isolated by a hotshot method. Briefly, the collected tail snips were homogenized in alkaline buffer (75 µl of 25mM NaOH and 0.2mM EDTA pH 8 solution) followed by heating at 95^{o}C for 30 minutes and immediately cooling at 4^{o}C for 15 minutes. Later Neutralizing buffer (75µl of 40mM Tris HCL) was added and the resultant DNA was used for PCR using following primer sequences; for ERα (WT 5'-GTAGAAGGCGGGAGGGCCGGTGTC-3', Common 5'-TACGGCCAGTCGGGC ATC-3', Mutant 5'-GCTACTTCCATTTGTCACGTCC-3') and ERβ (WT 5'-GTTGTGCCAGCCCT

GTTACT-3', Common 5'- TCACAGGACCAGACACCGTA-3', Mutant 5'-GCAGCCTCTGTTC CACATACAC-3'). The obtained cDNA then subjected to agarose gel electrophoresis in a 2% gel and viewed in a LICOR gel imaging station. Mice DNA samples showing 2 bands (300 bp and 234 bp for ERα and 160 bp and 106 bp for ERβ) were designated as heterozygous, samples showing a single band at 160 bp for ERβ and 300 bp for ERα were designated as Knockout and DNA samples showing 234 bp and 106 bp were designated as wild type.

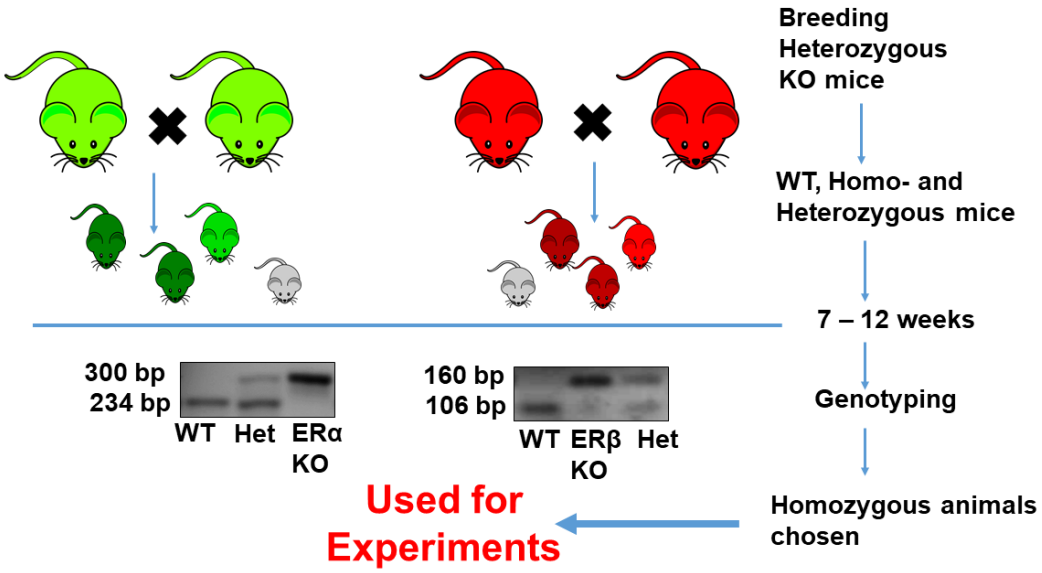

Figure 25. Schematic for breeding plan and genotyping of ERα KO and ERβ KO animals.

4.2.3. Mixed Allergen (MA) Exposure

Mice allotted to mixed allergen (MA) group were administered intranasally with a mixture of equal amounts (10 µg) of ovalbumin (Sigma Aldrich, USA), and extracts from *Alternaria alternata*, *Aspergillus fumigatus* and *Dermatophagoides farinae* (Greer labs, USA) for 4 weeks in phosphate-buffered saline (PBS), while PBS alone was administered as a vehicle for 28 days on every alternate day [103, 104, 106, 107].

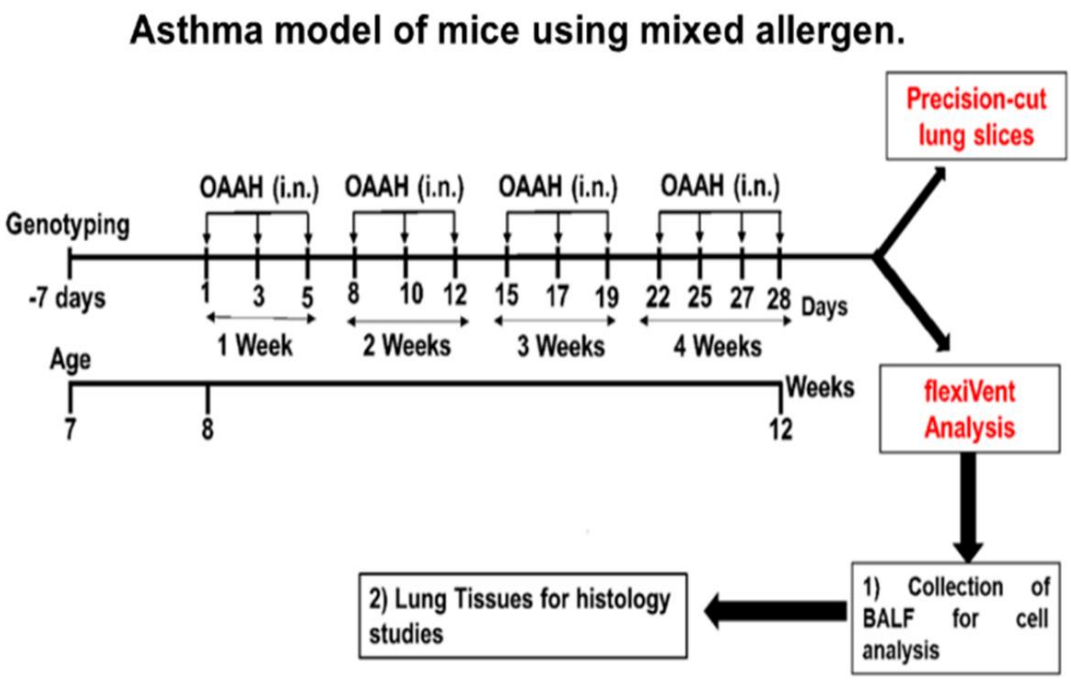

Figure 26. Experimental design of the mixed allergen model.
Genotyping was performed at the age of 7 weeks and mice were grouped according to their genotype. Mixed allergen (OAAH: 10 µg each of Ovalbumin, Alternaria Alternata, Aspergillus Fumigatus and Dermatophagoides Farina (house dust mite; i.n., intranasal) was administered on alternate days for 28 days while control mice received phosphate buffer solution as vehicle.

4.2.4. Precision-Cut Lung Slicing

Briefly, both male and female mice between 8 and 10 weeks were euthanized by intraperitoneal injection of pentobarbital. After performing tracheostomy, the lungs were inflated with 1.2 ml of warm 2% agarose-HBSS via a cannula inserted into the trachea. 0.5 ml of air was injected to quickly flush the agarose out of the trachea into the lobes. Post this, the agarose solution within the lungs were solidified by cooling to 4°C. The swollen lungs were excised, and a lobe was molded in gelatin with the help of specimen block and was sectioned into lung slices of 120 µm thick with a vibratome. The slices were collected immediately as they were slicing into the buffer tank using thin brushes and placed in an order into 24 well plates (each well contained

maximum 2 slices) and maintained in DMEM at 37°C for maximum up to 2 days. Trauma caused by tissue slicing releases mediators and causes modulation of airway tone. Hence, media was changed every 6 hours during the remainder of day 1 and day 2 to minimize trauma and reduce airway tone, as well as to remove any remaining agarose in the tissue. Estradiol treatment were given on 2nd day and experiments for contractility were carried out on the third day. During treatment slices from all the areas of lobe were randomized to avoid any deviation due to different generations of the airways [108].

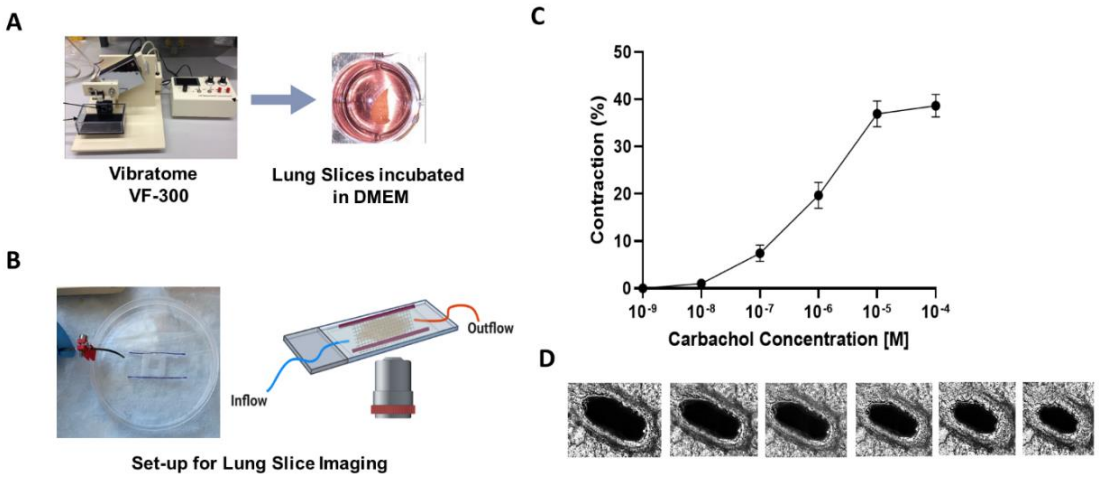

Figure 27. Experimental design of precision-cut lung slicing technique (PCLS).
Lung Slices were cut using a vibratome (A). A home-made set up was designed using coverslip, nylon mesh and grease for imaging the live tissue slices under the microscope (B). Airway luminal area narrowing in response to different concentrations of carbachol (CCh) was evaluated. A typical contraction of an airway to carbachol and a mean concentration-response curve is shown (C). Representative inset panel shows the narrowing of lumen with each dose of CCh (D).

4.2.5. Measurement of Airway Contraction

Lung slices with few microliters of HBSS buffer were placed onto a cover glass attached to a petri-plate with grease on the sides. A nylon mesh was placed on the surrounding of the slice and a cover glass was added slowly to the top. The airway was located with the Biotek Lionheart Fx microscope and live recordings were done. An image at baseline was taken (at 0% contraction)

followed by the addition of the increasing concentration of carbachol (10^{-8} to 10^{-4} M). Images were collected 10 minutes after each dose. The airway lumen area was measured. The minimum lumen area after each contraction was normalized to baseline, and percentage (%) of the contraction were measured. In a pilot study, PCLS obtained from wild type animals were incubated with E_2 at a concentration ranging from 1 nM to 100 µM for 24 h. There was no significant difference in contractility at 1 nM to 100 nM concentrations. However, beyond 100 nM concentration, E_2 significantly reduced the carbachol induced contraction suggesting some tissue damage due to high concentration. Hence, 100 nM of E_2 was chosen as an optimum concentration.

4.2.6. Lung Function Using FlexiVent

All mice were subjected to flexiVent (Scireq, Montreal, Canada) analysis to determine respiratory resistance (Rrs). The flexiVent based lung function analysis in murine models works similar to spirometry used to analyze the lung function in humans, except for the fact that it is an invasive method [109]. Male and female (WT, ERα KO and ERβ KO) mice were anesthetized using ketamine and xylazine (100 mg/kg and 10 mg/kg *i.p.* respectively) and immediately ventilated mechanically using flexiVent system. Respiratory resistance (Rrs) was measured and recorded at baseline (0 mg/mL Methacholine, MCh) followed by increasing doses of nebulized MCh (6.25, 12.5, 25.0, 50.0 mg/ml, respectively) delivered at 5 min intervals. The body temperature of mice was consistently maintained at 37°C with a heating pad placed underneath the mice and a bulb placed above at a 45° angle. Electrocardiogram (ECG) was monitored throughout the procedure. Mice were euthanized with an overdose of pentobarbital at the end of the experiment followed by a collection of broncho-alveolar lavage fluid (BALF). Following this, lungs were inflated with Carnoy's solution (100 % Ethanol, Chloroform, Glacial acetic acid in a ratio of 6:3:1 with added ferric chloride) and used for histology studies.

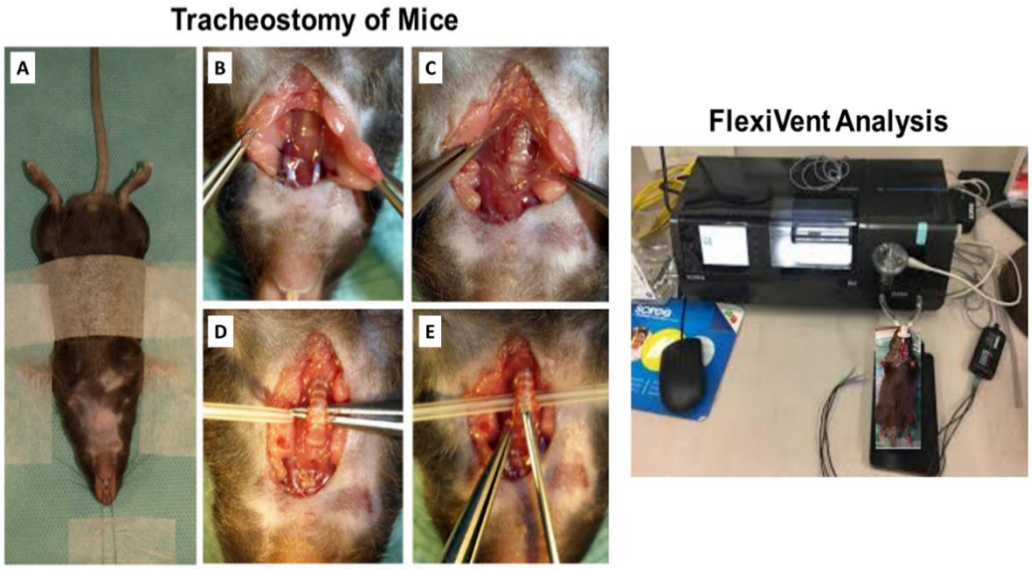

Figure 28. Step-by step-experimental design of flexiVent analysis.
Mice is anaesthetized, tracheostomy is performed, trachea is cut opened and a cannula is inserted. This cannula is then fitted to the flexiVent system and lung function is assessed by adding increasing dose of methacholine provided in the system.

4.2.7. Histopathology Using Hematoxylin and Eosin (H&E) Stains

Standard techniques were employed for histopathological studies using H&E for morphological analysis. Stained sections were scanned using Motic Easy Scan (Motic, Canada). Regions of interest were captured on the acquired H&E-stained images (20X) using Motic DS Assistant Lite software (Motic, Canada) followed by analyzing them for ASM thickness using image J macros (Image j, NIH, USA).

4.2.8. Total and Differential Leukocyte Count in BALF

Estimation of total and differential leukocyte count (DLC) in BALF was performed following previously published methods [110-113]. BALF was centrifuged at 2000 rpm for 5 min at 4^{o}C and the supernatant was discarded. The resultant cell pellet was re-suspended in 100 µl of PBS and total leukocyte count was performed using Countess-II FL cell counter. Following this, a smear was prepared using cytospin and the air-dried smeared slide was stained with Differential

Quick Staining Kit (Modified Giemsa, EMS) and washed with distilled water for 8 min. The differential cell count was carried out using a digital light microscope (Olympus, USA) at 100x magnification by oil immersion technique. At least 200 cells were differentiated on each slide.

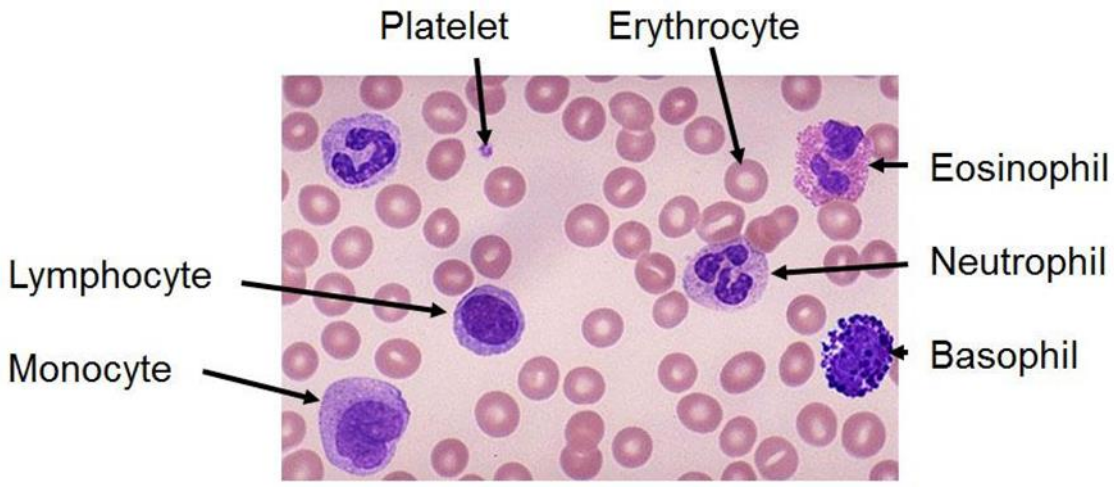

Figure 29. Overview of different inflammatory cells observed in the BALF analysis (Adapted from [110, 114]).

4.2.9. Statistical Analysis

All groups consisted of minimum six mice each of males and females (WT, ERα KO and ERβ KO). "n" values represent number of animals. For PCLS, slices were randomized in all wells before treating them with E_2. Lumen sizes were calculated using NIH Image J software. Percentage of contractions were measured as the % of difference between lumen size at baseline and at lumen size after each dose. Statistical analysis was performed using one way or two-way ANOVA followed by Dunnet's or Tukey post-hoc multiple comparisons using GraphPad Prism version 8.1.0 for Windows (GraphPad Software, San Diego, California USA, www.graphpad.com). All data are expressed as mean ± SEM. Statistical significance was tested at the minimum of $p<0.05$ level.

4.3. Results

4.3.1. Role of Differential ER Signaling on Airway Contractility at Baseline

To examine the effect of ER specific signaling on carbachol induced overall airway contractility, lung slices from WT, ERα KO and ERβ KO mice (from both male and female) were exposed to 100 nM of E_2 for 24 h and carbachol-induced narrowing was determined (Figure 30). Carbachol concentrations were added in every 10 minutes or till no further contractions in an increasing order from 10^{-4} to 10^{-9} M. It was found that all the groups had no significant difference in contractility till 1µM of CCh was added. WT slices treated E_2 showed a no significant changes in contractility as compared to WT without any treatment. In ERα KO mice slices (Figure 30A), there was a significant decrease in contractility in response to 10 µM CCh ($p<0.01$) and 100 µM CCh ($p<0.01$) as compared to WT mice slices treated with E_2 suggesting a possible role for ERβ mediated effect. There were no significant changes observed with ERβ KO mice lung slices. Representative slice images from each group are shown at baseline and highest contraction (Figure 30, B-I).

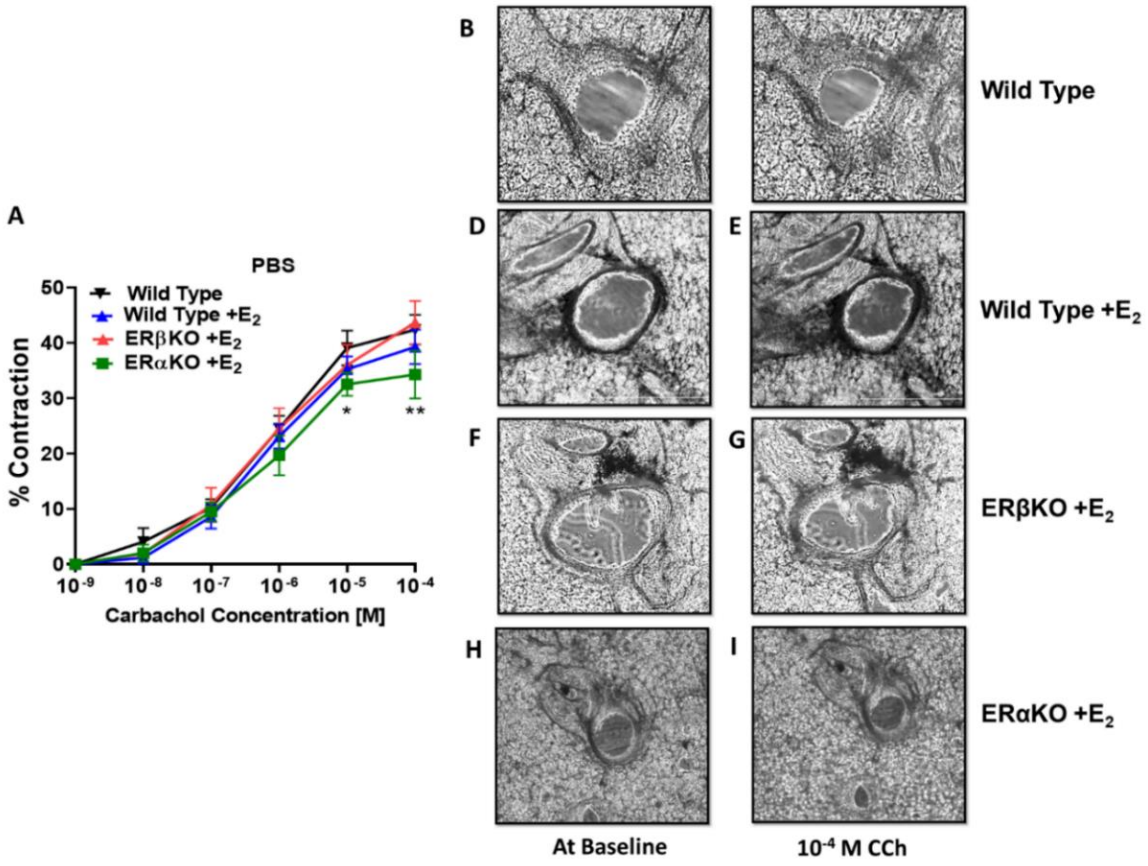

Figure 30. Effect of ER signaling on airway contractility of mice at baseline.
Lumen sizes were measured after 10 minutes of each dose of Carbachol (CCh) added to the slices of WT, ERα KO and ERβ KO. (A) % Contraction were measured as the % of difference between lumen size at baseline and at lumen size after each dose. Representative images show slices at baseline and after maximum contraction for WT (B, C), WT+ E_2 (D, E), ERβKO+ E_2 (F,G) and ERαKO+E_2 (H, I). *p<0.05 vs WT+ E_2. **p<0.01 vs WT+ E_2. Data represented as mean ± SEM of at least 5 mice per group.

4.3.2. Role of Differential ER Signaling on Airway Resistance (Rrs) at Baseline

Role of differential ER signaling in mouse lung *in vivo* at baseline were determined using the flexiVent FX1 module with an in-line nebulizer (SciReq, Montreal, Canada). Mice from all three control-study populations (WT, ERα and ERβ KO) showed a dose-dependent effect in lung function parameters after MCh challenge (Figure 31). Rrs indicates the dynamic resistance of the airways and quantitatively assesses the level of constriction in the lungs. ERβ KO male ($p<0.01$) and female ($p<0.001$) mice showed a significant increase in Rrs compared to WT mice at baseline

(Figure 31,B, D). Rrs in ERβ KO male ($p<0.05$) and female ($p<0.001$) mice were also significant as compared to ERα KO mice of respective gender. ERα KO mice (male and female) did not show any changes in the Rrs at baseline.

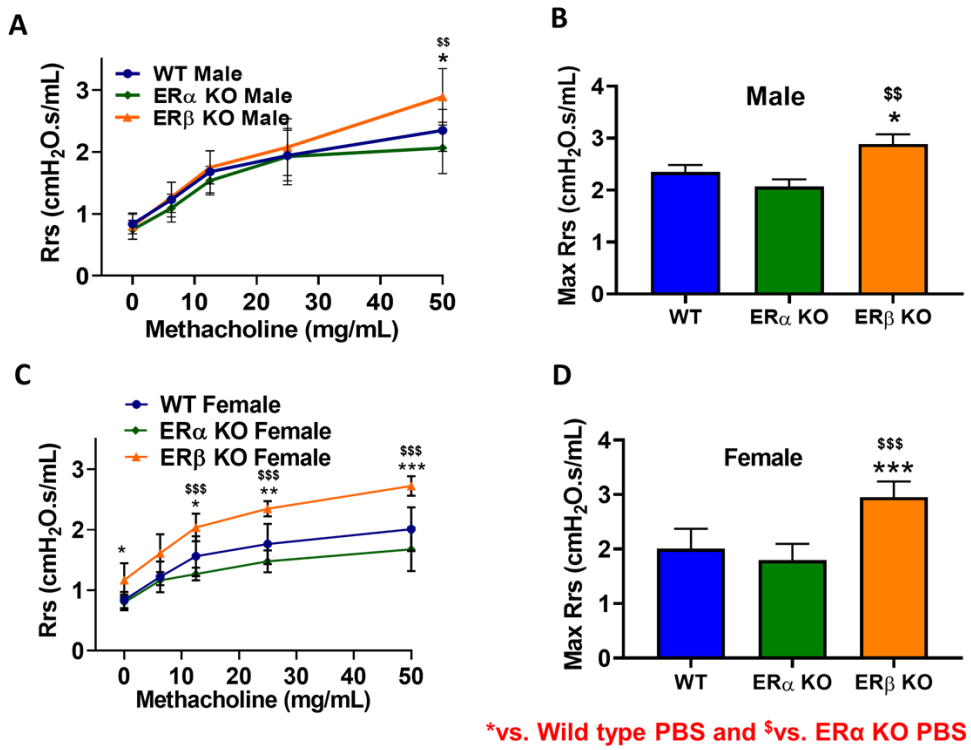

Figure 31. Effect of estrogen receptor (ER) signaling on airway resistance (Rrs) in the lungs of (a) male and (b) female mice at baseline.
Max Rrs (at 50 mg/mL) was used to compare WT, ERα KO and ERβ KO in (B) males and (D) female mice. Data represented as mean ± SEM of at least 5-6 mice per group; *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs. WT of PBS (ER specific KO effect) and $$p<0.01$, $$$p<0.001$ vs. ERα KO of PBS (ERβ effect).

4.3.3. Role of Differential ER Signaling on Airway Compliance (Crs) at Baseline

Crs depicts the ease with which respiratory system can be extended and provides insights into the overall elastic properties of the respiratory system that is needed to overcome during tidal breathing and is inversely proportional to resistance [109, 115-118]. ERβ KO male and female ($p<0.05$) mice showed a significant decrease in maximum compliance at baseline compared to WT

mice. Further, ERβ KO male mice showed significant reduction in compliance compared to ERα KO male mice (Figure 32 B, D).

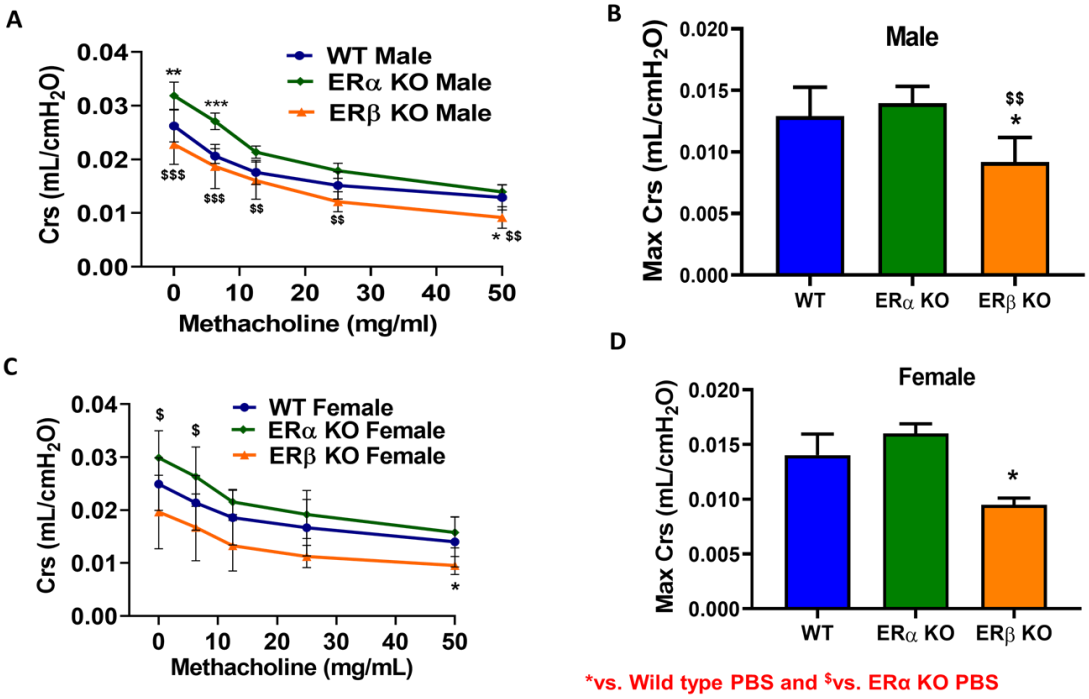

Figure 32. Effect of ER signaling on compliance (Crs) in the lungs of (a) male and (b) female mice at baseline.
Max Crs was used to compare WT, ERα KO and ERβ KO in (c) male and (d) female mice. $$p<0.01 vs. ERα KO (ERβ vs. ERα effect). *p<0.05 vs Wild type. Data represented as mean ± SEM of at least 5 mice per group. *p<0.05, **p<0.01, ***p<0.001 vs. WT of PBS (ER specific KO effect) and $p<0.05, $$p<0.01, $$$p<0.001 vs. ERα KO of PBS (ERβ effect).

4.3.4. Effect of ER Signaling on Lung Histology at Baseline

H&E staining showed increased thickness of the airway epithelium and ASM layer in of ERβ KO male and female mice compared to respective WT mice, indicating the endogenous estrogen signaling via ERβ at baseline (Figure 33). This was absent in the case of ERα KO mice. There were no observed inflammatory cells present in WT, ERα KO and ERβ KO mice (both males and females) at baseline conditions.

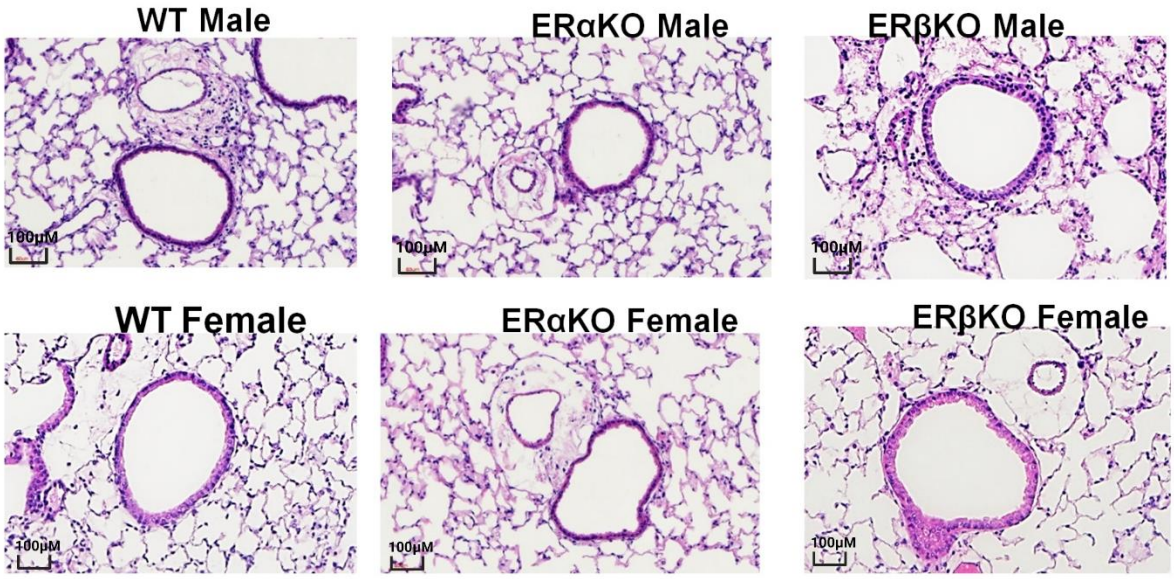

Figure 33. Effect of ER signaling on the structure of airways.
H&E-stained mice lung sections show increased thickness of the airway epithelium and ASM layer in ERβ KO male and female mice, while ERα KO mice did not show any prominent changes. The images are representative of those obtained from each group of animals. Scale bar, 100 μm

All these data hints towards protective role of ERβ receptor in airways. Since, expression of ERα and ERβ is significantly increased in inflammation and during asthma, as shown in previous studies, my goal was to observe the role of ERα and ERβ receptor in the presence of asthma. Hence, a murine model of asthma was created using the mixed allergen regimen as described in 4.2.3.

4.3.5. Role of Differential ER Signaling on Airway Contractility during Inflammation

To examine the effect of ER specific signaling on carbachol induced overall airway contractility, MA treated WT, ERα KO and ERβ KO mice were used for generating lung slices (as in Figure 34). Carbachol concentrations were added in every 10 minutes or till no further contractions in an increasing order from 10^{-4} to 10^{-9} M and contractions were measured. Contractility was relatively higher than the baseline data observed (Figure 30), where no MA treatment was given to mice. It was found that all the groups had no significant difference in

contractility till 10 µM of CCh was added. WT slices treated E_2 showed no significant changes in contractility as compared to WT without any treatment. In ERαKO mice slices (Figure 34A), there was a significant decrease in contractility in response to 10 µM CCh ($p<0.001$) and 100 µM CCh ($p<0.001$) as compared to WT mice slices treated with E_2 suggesting a protective role of ERβ. This reduction in contractility in ERαKO mice is more pronounced than in the baseline condition, suggesting an increased protective role of ERβ receptor during asthmatic or inflamed conditions. Representative slice images from each group are shown at baseline and highest contraction (Figure 34, B-I).

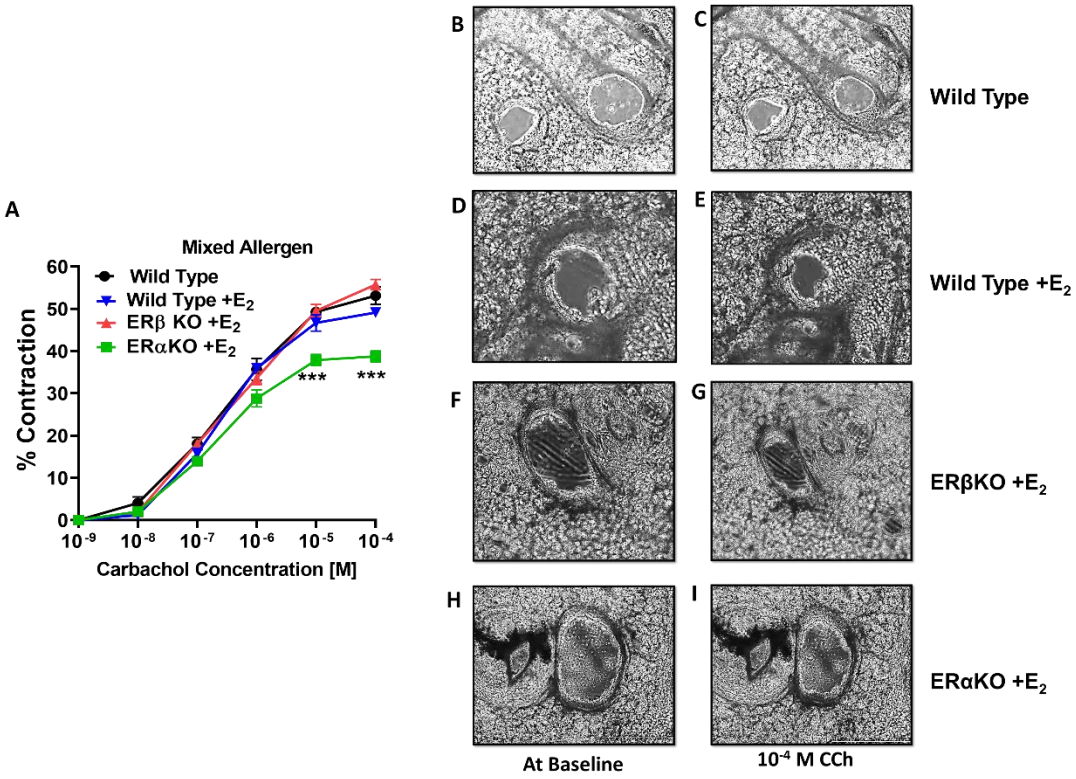

Figure 34. Effect of ER signaling on airway contractility of MA treated mice.
Lumen sizes and % contraction were measured same as in Figure 20. Representative images show slices at baseline and after maximum contraction for WT (B, C), WT+ E_2 (D, E), ERβKO+ E_2 (F, G) and ERαKO+E_2 (H, I). ***$p<0.001$ vs WT+ E_2. Data represented as mean ± SEM of at least 5 mice per group.

4.3.6. Role of Differential ER Signaling on Airway Resistance (Rrs) during Inflammation

Similar to baseline study, mice from all three-study populations (WT, ERα and ERβ KO) showed a dose-dependent effect in lung function parameters after MCh challenges. Male and female mice from all three-study populations (WT, ERα KO and ERβ KO) showed a significant increase in Rrs after MCh challenge in MA exposed groups compared to PBS (Figure 35 B, D). MA challenged mice from all three study populations showed a significant increase in Rrs in males ($p<0.05$ for WT; $p<0.001$ for ERα KO and ERβ KO) and in females ($p<0.001$ for WT, ERα KO and ERβ KO) compared to PBS challenged mice of respective populations with maximum changes observed in ERβ KO mice (both males and females; Figure 35 B, D). Interestingly, ERβ KO male and female mice showed a significant increase in Rrs compared to ERα KO mice in the presence of MA ($p<0.001$; Figure 35 B, D).

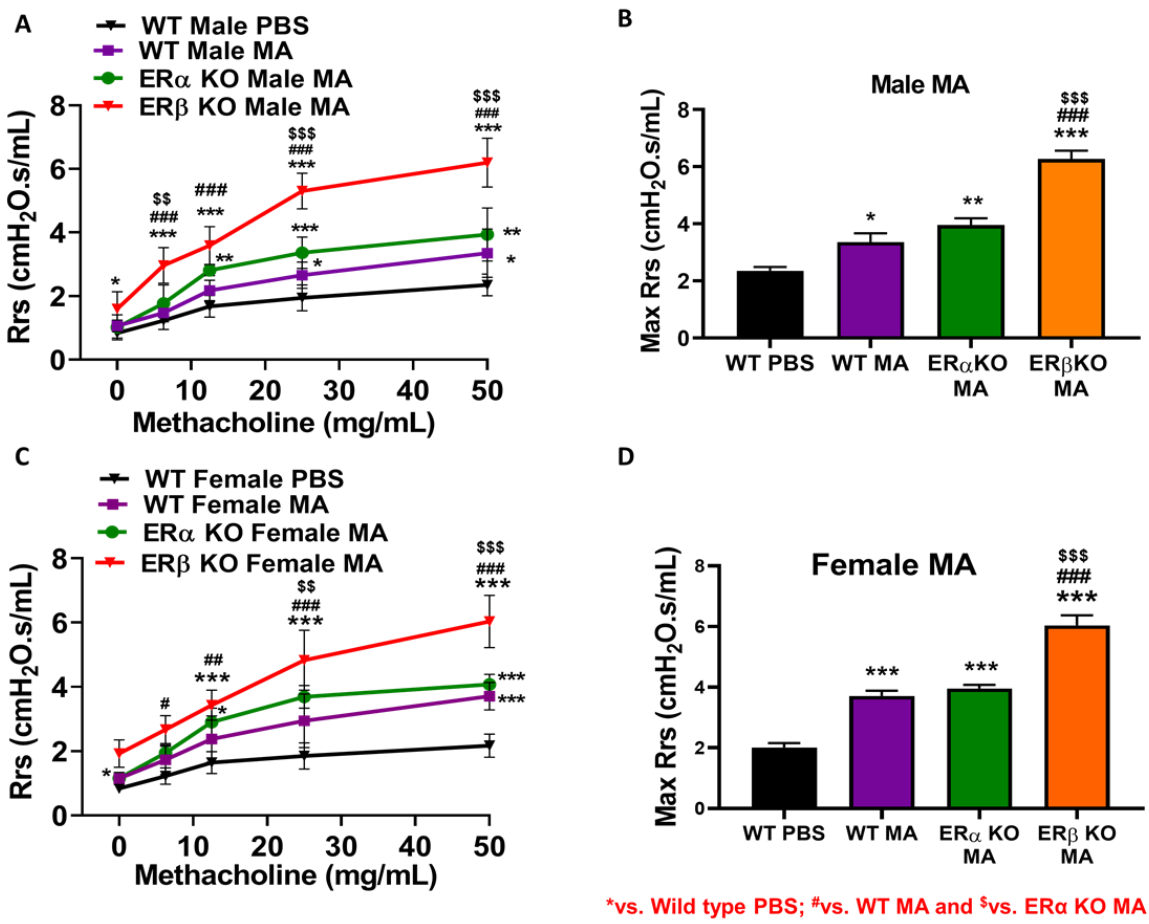

Figure 35. Effect of ER signaling on Rrs in the lungs of male and female mice exposed to MA. PBS treated mice in each group served as control. Max Rrs was used to compare WT, ERα KO and ERβ KO in (B) male and and (D) female mice exposed to MA. Data represented as mean ± SEM of at 5-6 mice per group; *p<0.05, **p<0.01, ***p<0.001 vs. WT of PBS, #p<0.05, ##p<0.01, ###p<0.001 vs. WT MA, and $$p<0.01, $$$p<0.001 vs. ERα KO MA.

4.3.7. Role of Differential ER Signaling on Airway Compliance (Crs) during Inflammation

Similar to the baseline study on the airway compliance, mice from all three-study populations (WT, ERα and ERβ KO) showed a dose-dependent effect in lung function parameters after MCh challenge (Figure 36 B, D). Male and female mice from all three-study populations (WT, ERα KO and ERβ KO) showed a significant increase in Max Crs after MCh challenge in MA exposed groups compared to PBS. Interestingly, ERβ KO male and female mice showed a much prominent decrease in Crs compared to WT or ERα KO mice in the presence of MA ($p<0.01$ Vs. Male WT, $p<0.01$ Vs. Female WT (Figure 36 B, D).

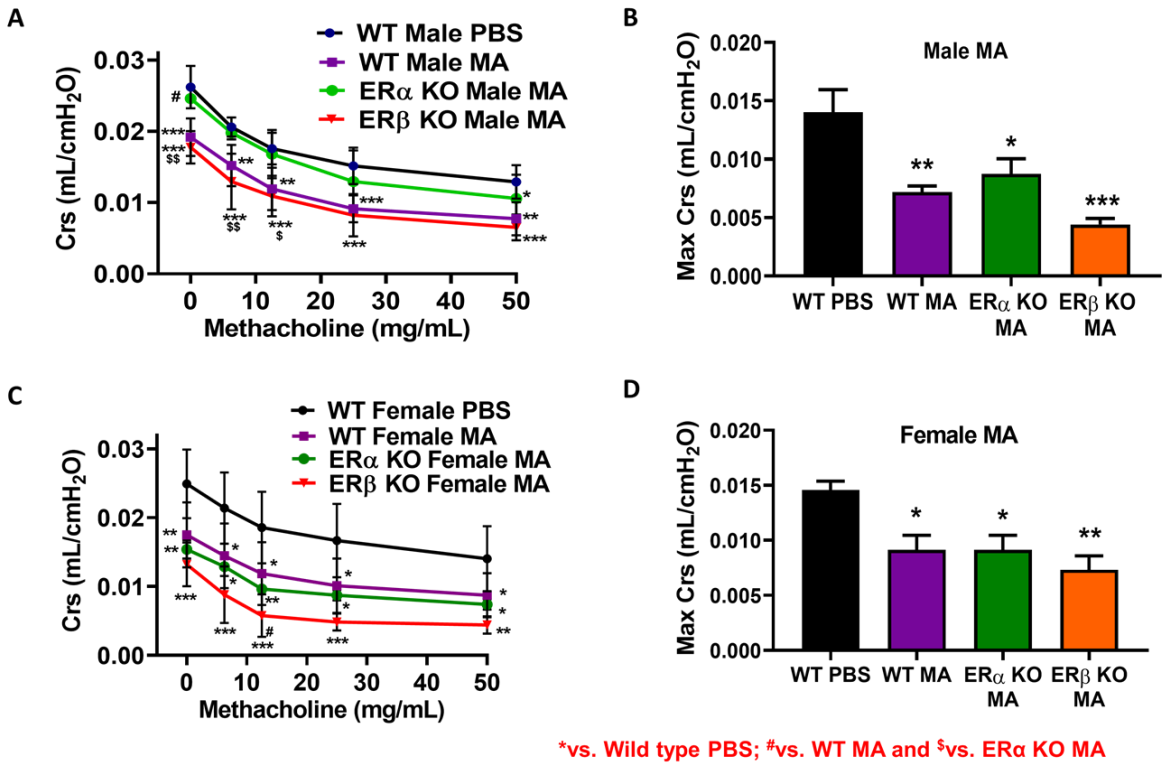

Figure 36. Effect of ER signaling on Crs in the lungs of male and female mice exposed to MA. PBS treated mice in each group served as control. Max Crs was used to compare WT, ERα KO and ERβ KO in (B) males and (D) female mice exposed to MA. Data represented as mean ± SEM of at 5-6 mice per group; *p<0.05, **p<0.01, ***p<0.001 vs. WT of PBS, #p<0.05, ##p<0.01, ###p<0.001 vs. WT MA, and $$p<0.01, $$$p<0.001 vs. ERα KO MA.

4.3.8. Effect of ER Signaling on Lung Histology during Inflammation

H&E staining showed increased thickness of the airway epithelium and ASM layer in MA challenged WT, ERα KO and ERβ KO male and female mice compared to respective PBS challenged mice, with higher thickness of ASM observed in ERβ KO female mice (Figure 37). Furthermore, it was observed that the infiltration of inflammatory cells was increased in the airways of MA challenged WT, ERα KO and ERβ KO mice (both males and females), with a robust increase in inflammatory cells was observed in ERβ KO mice.

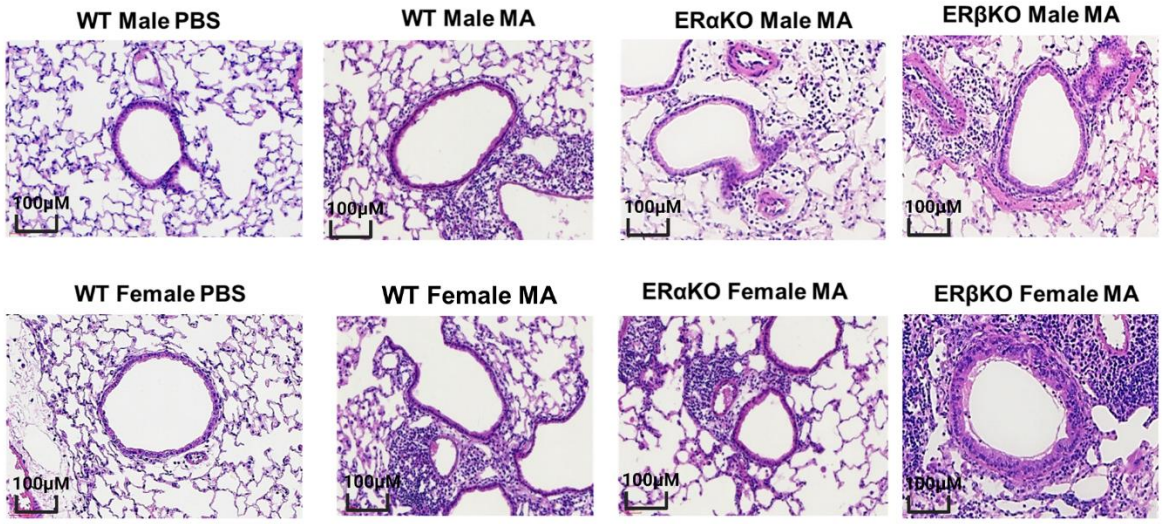

Figure 37. Effect of ER signaling on the structure of airways in MA treated mice.
H&E-stained mice lung sections show increased thickness of the airway epithelium and ASM layer in MA challenged WT and ERβ KO male and female mice, while ERα KO mice did not show any prominent changes. In addition, the infiltration of inflammatory cells was increased in the airways of MA challenged WT, ERα KO and ERβ KO mice (both male and female), with a robust increase observed in ERβ KO mice. The images are representative of those obtained from each group of animals. Scale bar, 100 μm

4.3.9. Effect of ER Signaling on Differential Leukocyte Count during Inflammation

Mice from all three study populations showed a significant increase in the total cell count upon MA challenge when compared to respective PBS challenged mice (Figure 38). Lymphocyte count was significantly increased upon MA challenge ($p<0.001$ for WT, ERβ KO and ERα KO males; $p<0.01$ for WT females and $p<0.001$ for ERα KO and ERβ KO females) compared to respective PBS challenged mice (Figure 38 Blue bars). Macrophage count in the BALF was significantly increased upon MA challenge ($p<0.001$ for WT males, $p<0.01$ for ERα KO and ERβ KO males; $p<0.01$ for WT and ERβ KO females, $p<0.05$ for ERα KO females) compared to respective PBS challenged mice (Figure 38, red bars). Furthermore, a significant increase in lymphocyte count was observed in MA challenged ERβ KO mice compared to MA challenged WT mice ($p<0.001$) and MA challenged ERα KO mice ($p<0.01$). Recruitment of neutrophils was significantly increased upon MA challenged mice ($p<0.001$ for WT and ERα KO males, $p<0.01$ for ERβ KO males; $p<0.001$ WT, ERα KO and ERβ KO females) compared to respective PBS challenged mice (Figure 38, Green Bars). Eosinophilic infiltration was significantly increased in MA challenged mice ($p<0.001$ for WT, ERα KO and ERβ KO males and females) compared to respective PBS challenged mice (Figure 38, Purple bars). Notably, MA challenged ERβ KO mice showed a significant increase in eosinophil count compared to MA challenged WT mice ($p<0.05$ for males and $p<0.01$ for females) as well as MA challenged ERα KO mice ($p<0.05$ for males and $p<0.001$ for females).

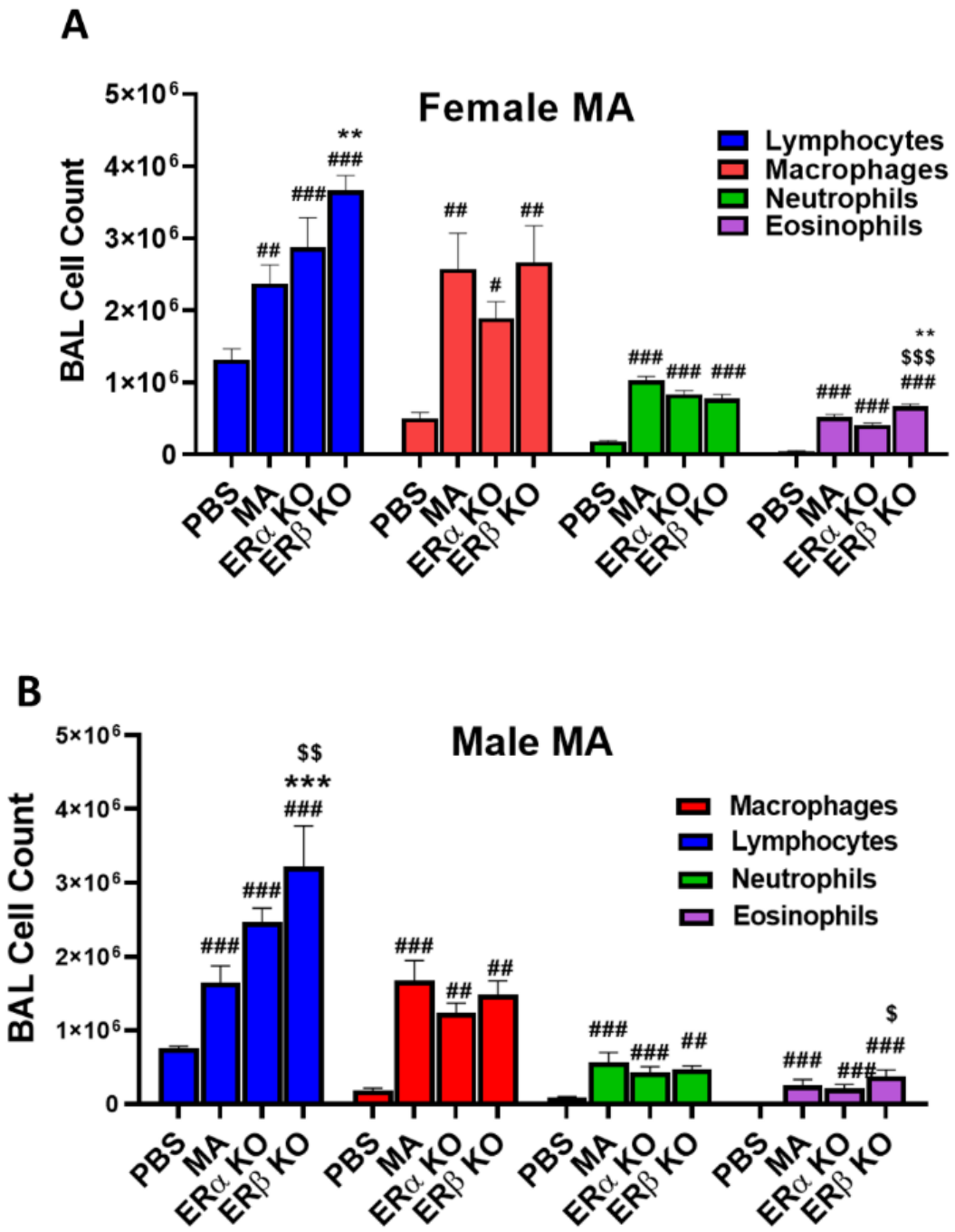

Figure 38. Effect of ER signaling on the differential leukocyte count during inflammation. Macrophage count (Red bars); Lymphocyte count (Blue bars); Neutrophils count (Green bars) and Eosinophils count (Purple bars) in (A) males and (B) females. Data represented as mean ± SEM of at least 5-6 mice per group; #p<0.05, ##p<0.01, ###p<0.001 vs. PBS (MA effect) and *p<0.05, **p<0.01 vs. WT MA (ER specific KO effect ON MA) and $p<0.05, $$p<0.01, $$$p<0.001 vs. ERα KO MA (ERβ vs. ERα effect).

4.4. Discussion

Airway inflammation, remodeling and AHR are considered as cardinal features of asthma leading to obstruction of the airways. Akin to clinical data, sex differences and sex steroids play a crucial role in the incidence and severity of asthma. Because women are more prone to the occurrence of asthma than men, identifying the role of sex steroids, especially estrogen in airways might shed some light on the pathology of asthma.

Estrogen has a systemic role beyond the reproductive system and the evidence suggests a wide array of roles for estrogen in both males and females in regulating cell growth and differentiation, intracellular calcium regulation and inflammation. Given the facts about estrogen and the lack of consensus whether it is pro-inflammatory vs. anti- inflammatory and to define the consequences in structural cells of the airways, it is important to understand the mechanisms involved in estrogen signaling. To identify the role of estrogen signaling in asthma *in vivo,* multiple studies have been performed in the past; however, none of them have been able to provide a complete picture. Most of these studies have largely focused on the role of estrogen *per se,* but did not focus on the receptor specific effects involved. Moreover, very limited data is available on the receptor-based mechanisms of estrogen *in vivo*, which or focused on ERα, completely disregarding ERβ. In connection to this, in order to establish the comprehensive role of physiological estrogen in the airways and to avoid the cross-reactivity of the pharmacological receptor agonists, ERα KO and ERβ KO mice were employed, which will provide valuable insights into the receptor-based effects of endogenous estrogen on AHR and airway remodeling.

MA induced model of asthma was utilised, as it is robust and the most effective model of mimicking human asthma in murine models [104]. Airway contractility were determined using precision cut lung slicing technique. The present study shows that MA treatment increased the

level of contractility in all the groups. Interestingly, ERαKO mice slices showing a significant decrease in contractility as compared to WT mice lung slices treated with E_2 at baseline and much pronounced in inflammatory condition, suggesting a protective role of ERβ and a pro-hyperreactivity role of ERα. This pronounced effect during inflammation could be attributed to the increased expression of ERβ in our previous studies.

Airway mechanics were determined using the forced oscillation technique (FOT) of the flexiVent Fx1 module, which is an invasive endpoint technique that delivers parameters like airway resistance (Rrs) and compliance (Crs) along with other remodeling and airway elasticity parameters. The present study shows that ERβ KO mice show deteriorated lung function compared to WT and ERα KO in both the genders at baseline, with prominent changes observed in females compared to males, which correlates with earlier clinical findings suggesting females are susceptible to asthma. Interestingly, ERα KO mice of either sex showed no changes in lung function compared to WT mice at baseline, which can be attributed to the protective role of ERβ or detrimental role of ERα in the airways, especially in ASM. In addition, MA challenged mice of all three populations (WT, ERα KO and ERβ KO) showed a significant decline in lung function compared to respective PBS treated mice. Here, female mice exposed to MA in all three-study populations showed prominent decline compared to males, which corroborates with clinical data suggesting increased severity of asthma in females [98, 119-122]. In addition, the severity of MA induced AHR was found to be more pronounced in ERβ KO mice compared to WT and ERα KO.

Histology studies using H&E stain show no prominent changes across all three-study populations at baseline; however, upon MA challenge significant changes in the thickness of the epithelium and ASM were observed in all three study populations (especially females), with the maximum changes observed in ERβ KO mice.

One of the cardinal features of asthma is infiltration of inflammatory cells like lymphocytes, monocytes/macrophages, neutrophils and eosinophils into the airways, especially eosinophilic infiltration, which is associated with the development and aggravation of AHR [123-125]. Lymphocytes, both T and B lymphocytes play a crucial role in regulating the inflammatory response in asthma. T-lymphocytes are responsible for recruiting various cytokines, especially Th2 cytokines, which contribute to remodeling and AHR; whereas B-lymphocytes secrete IgE and the factors regulating IgE secretion, which result in recruiting inflammatory cells into the airways, eventually contributing to airway inflammation [125, 126]. Neutrophils are not a predominant cell type observed in the airways of patients with mild-to-moderate chronic asthma, whereas they appear to be a more prominent cell type in the airways and induced sputum of patients with more severe asthma [127, 128]. Evidence suggests experimentally activated eosinophils induce airway epithelial damage [107, 123, 128]. In this study, it was found that MA exposure significantly increased the differential leukocyte count in the airways of mice, especially in females with prominent changes in ERβ KO mice, which corroborates with clinical evidence indicating females are more susceptible to asthma and that ERβ plays a protective role in regulating inflammatory cell infiltration. Very little information is available on the ER specific effects on inflammatory cells in the lung during asthma, which warrants more in-depth immune cell-based studies in the future [125, 129].

The present study was focused on global specific ER Knock-out mice and so the effects due to other tissues in lungs cannot be ruled out. However, these results laid the foundation for future studies in smooth muscle specific ER knockout mice.

In conclusion, the results from this study suggest the importance of ER's and their signaling in the lungs and their role in regulating the overall lung function. In addition, this study implicates the differential role of ERα and ERβ in airway physiology during asthma, especially in the context of AHR. Considering the "protective role of ERβ" during asthma, it is noteworthy to identify ERβ as a potential target to develop novel lead molecules that can be used as alternative therapies to treat asthma.

CHAPTER 5. SUMMARY AND CONCLUSIONS, LIMITATIONS AND FUTURE DIRECTIONS

Targeting sex steroids for airway hyperresponsiveness opens several avenues in respiratory research. Several epidemiological data have presented with confusing and conflicting evidence of estrogen's role in lungs. On one hand, there is a higher incidence and severity of asthma in women, on the other hand, in pre-menstrual asthma, symptoms and exacerbations are greatest when estrogen levels are lowest. Even in vivo and in vitro studies present a contrasting picture, where some studies suggest a pro-inflammatory role of estrogen hormone while others report protective role of estrogen in asthma. What is clear from these discrepancies is that E_2 effects are complex, and likely cell type- and context-dependent, and furthermore species-dependent. Moreover, estrogen mediates its action through estrogen receptors (ERs -ERα and ERβ) and acts through genomic and non-genomic signaling which leads to a barrage of different combinations of events. Hence, elucidating these complex estrogen effects in each cell type of asthma would provide fresh insights into asthma with a real prospect of novel gender- based therapies.

ER signaling is currently being evaluated by multiple researchers for its therapeutic potential in various immune and structural cells involved in asthma pathophysiology. Moreover, novel estrogen receptor specific ligands are being synthesized to help understand the estrogen signaling not only in the context of asthma but in several gender based physiological disorders. However, we still lack a complete understanding about the role of estrogen receptor signaling in airway hyperresponsiveness and asthma. Also, most of the studies done on ER signaling have focused on non-genomic short term signaling. Since, asthma as well as variations in hormonal levels in men or women occurs over prolonged time periods, it is possible that the complex and conflicting data on estrogen and asthma reflect genomic ER effects more than rapid effects. Thus,

the present study was focused to determine the long-term estrogen receptor signaling in contractility of airway smooth muscle (ASM) which is the main structural cell causing hyperresponsiveness of airways.

The results of the present study for the first time demonstrate the long-term differential signaling of ERs in the regulation of $[Ca^{2+}]_i$ handling in the human airway smooth muscle. It was found that ERα activation increases the $[Ca^{2+}]_i$ response in baseline conditions, while ERβ activation has neutral effect. Moreover, the differential signaling of ERs is more evident in asthma or inflammation where ERβ activation decreases the $[Ca^{2+}]_i$ response in the presence of inflammation while ERα increases it. The effect of estradiol acting through both these receptors seems to be intermediary between these two receptors. Further elucidation of the mechanisms of their signaling on $[Ca^{2+}]_i$ suggest that ERβ contributes to decreased $[Ca^{2+}]_i$ response through increased SERCA2, inhibition of pathways involved in activating the voltage gated LTCC and maintenance of the morphology of mitochondria.

Further, I evaluated ER signaling in overall airway contractility. Interestingly, it was found that ERβ causes a potentiation of the activity of β_2-AR which leads an increase in cAMP. Also, ERβ is found to be involved in dephosphorylation of contractile apparatus ultimately leading to bronchodilation. Presenting a contrasting picture, ERα causes an increase in pro-contractile machinery such as RhoA activity and phospho- MYPT leading to increased overall contractility in normal human ASM cells.

To confirm these novel *in vitro* findings in the presence of other structural and immune cells involved in inflammation, I have further evaluated the *ex vivo* and *in vivo* roles of ER signaling in airway contractility. Interestingly, ERβ was found to be protective for the airways while ERα have shown detrimental effects and further aggravated the contractility of airways.

These novel findings of ER signaling in the context of contractile mechanisms of airways can be utilized in designing novel therapeutics for bronchodilation.

To conclude my thesis, the results of my study suggest the importance of ER's and their differential signaling in the regulation of $[Ca^{2+}]_i$, contractile machinery and in overall contractility of the airways. In addition, this study implicates the differential role of ERα and ERβ in airway physiology during asthma, especially in the context of AHR. All these observations point towards the protective role of ERβ, and hence it can be identified as a potential target to develop novel lead molecules that can be used as alternative therapies to treat asthma.

5.1. Limitations

This study resulted in several novel findings but is not free from limitations. The result of the present study demonstrates the differential effect of estrogen receptors on influx channel LTCC and SERCA reuptake. However, to know their complete role in intracellular calcium handling, it is signaling through several other pumps and channels involved in sustained calcium entry, store-operated and receptor operated calcium entry (SOCE and ROCE) needs to be evaluated. Similarly, the effect of ER signaling on morphology of mitochondria was evaluated which gives a hint towards the protective role of ERβ signaling. However, the direct effect of ER signaling in calcium buffering capacity of mitochondria remains to be studied.

In addition, although well-known gender and age-based differences are established in asthma, the present study had not provided analyses based on puberty, age, menopause, or pregnancy. It would be interesting to find the effect of ER signaling in different subtype classifications to see if ER signaling is modified due to underlying factors.

The present study was focused on global specific ER Knock-out mice and so the effects due to other tissues in lungs cannot be ruled out. However, these results laid the foundation for future studies in smooth muscle specific ER Knockout mice.

5.2. Future Directions

The present research provides several new challenges and future directions. The present study not only generate new adjunct medications for bronchodilators but also answer the relevant question of discrepancies revolving around asthma occurrence. A logical extension of this study would be to understand the effects of ER signaling through various metabolites of estrogen present in the body. As the different metabolites of estrogen may have different levels of expression during various phases of menstrual events, this may give a clarity about the contrasting effects of estrogen hormones in women. Moreover, there are several truncated forms and splice variants of estrogen receptors as well. It would be interesting to see the signaling and cross-talk through those variants as well. The results of these comprehensive studies can be used to discover novel biomarkers in case of asthma exacerbations during increased estrogen levels (such as in estrogen replacement therapy) which will lead to omics-based precision medicines for the treatment of asthma.

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