Biased Signaling by Beta-2 Adrenergic Receptor Ligands in Murine Models of Asthma

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A Dissertation Presentation to

The Department of Pharmacological and Pharmaceutical Sciences

University of Houston

In Partial Fulfillment of

The Requirement for the Degree

Doctor of Philosophy

By

Vaidehi Jatin Thanawala

August 2014

Mom, Dad,
Raghu and Maithili

For always believing in me

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-Vaidehi Jatin Thanawala

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Abstract

Asthma is a chronic inflammatory disorder of the airways that affects over 300 million people worldwide. Inhaled corticosteroids (ICS) and β_2 adrenergic receptor (β_2AR) agonists are the mainstay of asthma therapy. However, chronic use of ICS and β_2AR agonists has been associated with adverse effects and loss of control of asthma symptoms. Clinical studies have shown the beneficial effects of chronic administration of the beta-blocker nadolol in attenuating forced expiratory volume (FEV1) in mild-asthmatics. However, not all beta-blockers are beneficial in the therapy of asthma. A clinical study published by Short and colleagues showed that chronic administration of the beta-blocker propranolol in a subset of moderate asthmatics did not improve FEV1.

Similar to human studies, such discrepancy in the beneficial effects of beta-blockers has also been seen in murine models of asthma. Our previous studies have shown that certain beta-blockers with inverse agonist activity such as nadolol, ICI-118,551 and high-dose metoprolol are beneficial in attenuating the inflammation and AHR associated with the murine asthma phenotype. However, other beta-blockers that are not inverse agonists, such as alprenolol, were not as effective in attenuating the murine asthma phenotype.

We have also reported that, β_2AR knock out (β_2AR KO) mice have an attenuated asthma phenotype, indicating the requirement of the β_2AR for development of the murine asthma phenotype. Moreover, nadolol does not further attenuate the asthma phenotype in the β_2AR KO mice, indicating that the beneficial effects of nadolol in the murine asthma phenotype are through its activity at the β_2AR.

The current project investigated the β_2AR activation and the signaling pathways mediating the asthma phenotype in antigen-driven murine models. Using pharmacological and genetic models to we show that constitutive activation of the β_2AR is not enough and ligand-activation of the receptor is required for development of the asthma phenotype. We used six β_2AR ligands with varying signaling profiles to show the role of the non-canonical extracellular signal-regulated kinases (ERK1/2) activation pathway in development of the asthma phenotype; and suggest the subset of beta-blockers capable of shutting down β_2AR-ERK1/2 signaling may have a role in the chronic management of asthma.

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List of abbreviations

7-TMR	7-transmembrane receptor
AC	Adenylyl cyclase
ACE inhibitors	Angiotensin converting enzyme inhibitors
AHR	Airway hyperresponsiveness
AKAP	A-kinase anchoring protein
ATP	Adenosine triphosphate
BAL	Broncho-alveolar lavage
BALF	Broncho-alveolar lavage fluid
cAMP	Cyclic adenosine monophosphate
CHF	Congestive heart failure
COMT	Catechol O-methyltransferase
COPD	Chronic obstructive pulmonary disorder
CysLT1	cysteinyl-leukotriene receptors
Epi-KO	Epinephrine null mice
ERK1/2	Extracellular signal-regulated kinases
FEV1	Forced expiratory volume
GDP	Guanosine diphosphate
GFP	Green fluorescent protein

GPCR	G protein-coupled receptor
GRK	G protein-coupled receptor kinase
GTP	Guanosine triphosphate
HDAC2	Histone deacetylase-2
HPLC	High performance liquid chromatography
i.p.	Intra-peritoneal
IACUC	Institutional animal care and use committee
ICS	Inhaled corticosteroids
IgE	Immunoglobulin E
IL	interleukin
Il-13	Interleukin-13
IL-4	Interleukin-4
IL-5	Interleukin-15
JNK	c-Jun N-terminal kinases
K	Airway reactivity
LABA	Long acting beta-2 adrenoceptor agonists
LAMA	Long acting muscarinic antagonists
LTD4	Leukotriene D4
MAO	Monoamine oxidases

MAPK	Mitogen-activated protein kinases
MAPKK	Mitogen-activated protein kinase kinases
MAPKKK	Mitogen-activated protein kinase kinase kinases
MKP7	Mitogen-activated protein kinase phosphatase 7
OCS	Oral corticosteroids
Ova S/C	Ovalbumin sensitization and challenge
PAFS	Periodic acid fluorescent Schiff's
PAR	Protease-activated receptors
PAR2	Protease-activated receptor-2
PAS	Periodic acid Schiff's
PC100	Airway sensitivity
PEEP	Positive end-expiratory pressure
PGD2	Prostaglandin D2
PI3K	Phospho-inositol kinase-3
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PNMT	Phenylethanolamine N-methyltransferase
PP2A	Protein phosphatase 2A

Rrs	Total respiratory resistance
SABA	Short acting beta-2 adrenoceptor agonists
SAMA	Short acting muscarinic antagonists
TH	Tyrosine hydroxylase
T_H17	T-helper cells 17
T_H2	T-helper cells 2
TNF- α	Tumor necrosis factor α
Ultra-LABA	Ultra long acting beta-2 adrenoceptor agonists
VMAT	Vesicular monoamine transporter
WT	Wild-type
β_2AR	β_2 adrenergic receptor
β_2AR KO	β_2 adrenergic receptor knock out
βAR	β adrenergic receptor
βARK	β adrenergic receptor kinase

1. Introduction and statement of problem

Asthma is a chronic inflammation of the airways and affects over 300 million people worldwide. It is more prevalent in children than in adults and is the leading cause of childhood disability (Foundation, 2014). The current mainstays of asthma therapy are inhaled corticosteroids and β2AR agonists. ICS prevent the inflammation associated with asthma. However, with chronic administration of ICS there is an increased risk of systemic absorption that has been associated with growth impairment in children and adolescents (Kelly et al., 2008; Fuhlbrigge et al., 2014). Moreover, some patients are resistant to the anti-inflammatory action of ICS, which makes their treatment difficult.

β2AR agonists are potent bronchodilators and reverse the bronchoconstriction of the airway smooth muscle in asthma. When used acutely, β2AR agonists are the most effective bronchodilators ever known, chronic administration of β2AR agonists is associated with worsening and loss of control of asthma symptoms (Theron et al., 2013). In spite of the high prevalence of asthma, there has been little advance in development of newer and better therapeutic options for asthma (Foundation, 2014). Our original hypothesis for a potential novel asthma therapy was based on the analogy of the paradoxical success of a subset of beta-blockers in congestive heart failure (CHF) (Bond et al., 2007).

CHF is a chronic condition of a failing heart characterized by fluid accumulation that leads to cardiac insufficiency. Consequently, βAR agonists were used for the therapy of CHF. When used acutely, βAR agonists are beneficial in increasing the rate and force of cardiac contraction, chronic administration of βAR agonists led to an increase in mortality (Weber et al., 1982). Beta-blockers were contraindicated for the therapy of heart failure because they inactivate the βAR and can reduce cardiac contractility and rate of contraction. However, in clinical studies done using chronic administration of beta-blockers such as metoprolol and carvedilol, patients showed an increase in the force of cardiac contraction (Waagstein et al., 1975; Hall et al., 1995; Bristow et al., 1996). A small subset of beta-blockers is now first line of therapy drugs for CHF. This was the first time certain members of a class of drugs went from being contraindicated in a disease to becoming drugs of choice for that disease.

Similarly, beta-blockers have been contraindicated in the therapy of asthma, because acute treatment with beta-blockers can cause bronchoconstriction. While, acute treatment with β2AR agonists is beneficial, the chronic administration of β2AR agonists has been associated with a loss of control of asthma symptoms. Analogous to the use of beta-blockers in CHF, in our murine models of asthma, we have shown that chronic administration of certain beta-blockers with inverse agonist activity such as nadolol, ICI-118,551

and high-dose metoprolol were beneficial in attenuating the inflammation and AHR of the murine asthma phenotype (Callaerts-Vegh et al., 2004; Lin et al., 2008; Nguyen et al., 2008). However, other beta-blockers such as alprenolol were not as effective in attenuating the murine asthma phenotype (Callaerts-Vegh et al., 2004; Nguyen et al., 2009).

Clinical studies have shown that chronic administration of a beta-blocker-nadolol can result in significant improvement in the forced expiratory volume (FEV1) of mild-asthmatics (Hanania et al., 2008; Hanania et al., 2010). However, a recent clinical study showed that another beta-blocker, propranolol, did not improve FEV1 with chronic administration in a subset of mild-asthmatics (Short *et al.*, 2013b). These clinical studies highlight a discrepancy- that not all beta-blockers are effective in asthma. A similar discrepancy was observed in the use of beta-blockers for the therapy of CHF. While chronic administration of carvedilol, metoprolol and bisoprolol is effective in improving the contractility of the heart, bucindolol, nebivulol and celiprolol have failed as potential therapies for CHF.

The differences in the efficacy of various beta-blockers in asthma and CHF may arise from the differences in the regulation of the βAR by the beta-blockers. However, the discrepancy in the efficacy of different beta-blockers indicates that even though beta-blockers are classified as a single group, there is

no ‘class effect’ and there may be significant differences in their therapeutic activities (Thanawala et al., 2014).

The purpose of the current study was to understand the reason for the therapeutic variability of different beta-blockers in asthma therapy. We used murine models of asthma to study the activation and signaling of the β2AR and the effect of beta-blockers on the murine asthma phenotype.

2. Review of literature

2.1 Asthma

2.1.1 Disease and prevalence

Asthma is a chronic inflammatory airway disorder characterized by inflammatory infiltration of the airways, mucous metaplasia and hypersecretion, and airway hyperresponsiveness (AHR). Over 300 million people of all age groups worldwide, and about 25 million in the U.S suffer from asthma. It is more common in children than adults and is the most prevalent cause of childhood disability. One out of every 12 adults and one out of nine children suffer from asthma. The cost impact of asthma is about $50 billion a year in the U.S. alone, including healthcare costs involving emergency room visits and hospitalizations in particular, expenses from 10 million missed work days, 13 million missed school days, and early mortality (Foundation, 2014).

Although the etiology of asthma is not completely understood, many factors are known to contribute to the propensity to develop asthma. The different subtypes of asthma based on varying disease etiology further complicate the understanding of the causes of asthma. Asthma can be allergic (e.g., fungi-driven), aspirin-sensitive, exercise-induced, and non-atopic mechanism-driven (Ishmael, 2011; Lotvall *et al.*, 2011). Genetic predisposition to develop allergic reactions to common aeroallergens is considered one of the most important

factors of asthma etiology. However it is the confluence of genetic-environmental factors such as exposure to allergens and genetic propensity that is considered most likely to lead to the manifestation of the disease. In addition, pre-natal (maternal smoking) and post-natal factors (e.g., type of child-birth, diet, stress and the use of antibiotics) can also act as risk factors (Subbarao *et al.*, 2009).

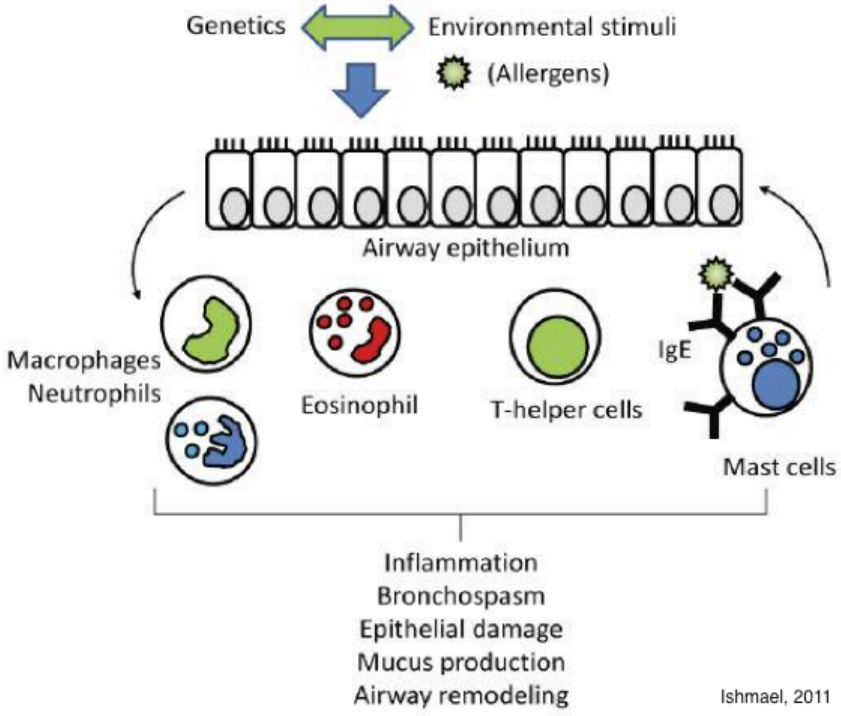

Figure 1. Chronic inflammation in allergic asthma: Inflammatory cells (leukocytes) and the airway epithelium contribute to the development of allergic asthma. Inflammatory stimuli cause the airway epithelium to release inflammatory mediators. Leukocytes are recruited to the airways by the inflammatory mediators released by the airway epithelium. The inflammatory cells infiltrate the lungs and release mediators that further increase the inflammatory damage on the airway epithelium, thus forming a vicious inflammatory cycle. IgE- immunoglobulin E. Adapted from (Ishmael, 2011)

Asthma occurs as a result of many factors that contribute to inflammation. These inflammatory responses further damage the airway-epithelial integrity and worsen the disease condition, resulting in a vicious cycle. Figure 1 shows the cycle of asthma and its 'cause and effect' mediators.

2.1.2 Therapy

Despite the extensive prevalence of asthma and severity of disease, patients often have limited therapeutic options. The currently available therapeutic options for asthma only partially control symptoms in most patients with some patients being therapy-resistant. Considering the prevalence and high impact of asthma and its related disabilities, there has been very little therapeutic advance in asthma treatment (Foundation, 2014). Figure 2 shows the stepwise treatment plan for asthma based on severity of the condition. The current therapeutic options for asthma include:

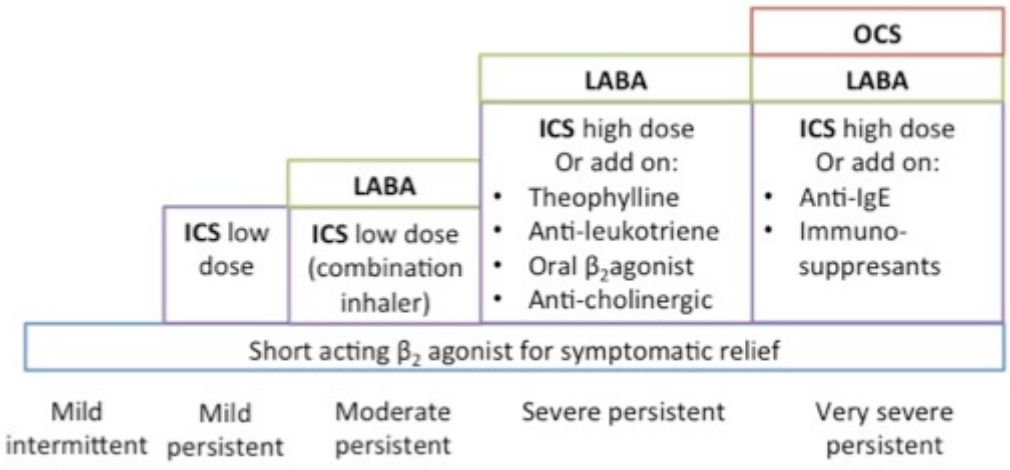

Figure 2. Current asthma therapies: The figure depicts the current stepwise asthma therapy regimen. This regimen is based on the severity of symptoms. For mild intermittent asthma, short acting β_2 agonists are used. Inhaled corticosteroids (ICS) remain the mainstay of asthma therapy. Long-acting β_2 agonists (LABA) like formoterol and salmeterol are added to the regimen with ICS for moderate persistent asthma. For further severe asthma, additional therapies are added to the mainstays of asthma. In very severe asthma, oral corticosteroids (OCS) are added to the treatment protocol. IgE-Immunoglobulin E. Modified from (Barnes, 2010)

2.1.2.1 Inhaled corticosteroids

Inhaled corticosteroids are currently the mainstay of asthma therapy. As seen in figure 2, corticosteroids form an integral part of the current asthma therapy. Anti-inflammatory ICS therapy is effective for the symptomatic treatment of most asthma patients. Figure 3 depicts the various targets of corticosteroids leading to the beneficial therapeutic effects of ICS. ICS exert their anti-inflammatory actions by inducing apoptosis of eosinophils, preventing activation of mast cells, reducing

the number of T-lymphocytes, and by inhibiting the release of cytokines. The effect of switching off inflammatory genes and recruiting the nuclear enzyme, histone deacetylase-2 (HDAC2), contributes to the anti-inflammatory effect of ICS (Barnes, 2012b). The indirect effect of reduction in inflammatory mediators and infiltration can be seen on the structural cells of the airways:

- Prevent differentiation of epithelial cells into mucin producing goblet cells,
- Reduce mucin production by mucin producing cells,
- Act on endothelial cell integrity and reduce the leakage of fluid and inflammatory mediators from the blood and
- Act on airway smooth muscles and reduce bronchoconstrictor effects of the cytokines and increase expression of the β_2AR to facilitate bronchodilation (Adcock *et al.*, 2008; Barnes, 2010; Barnes, 2012b; Barnes, 2012a; Kandeel *et al.*, 2013).

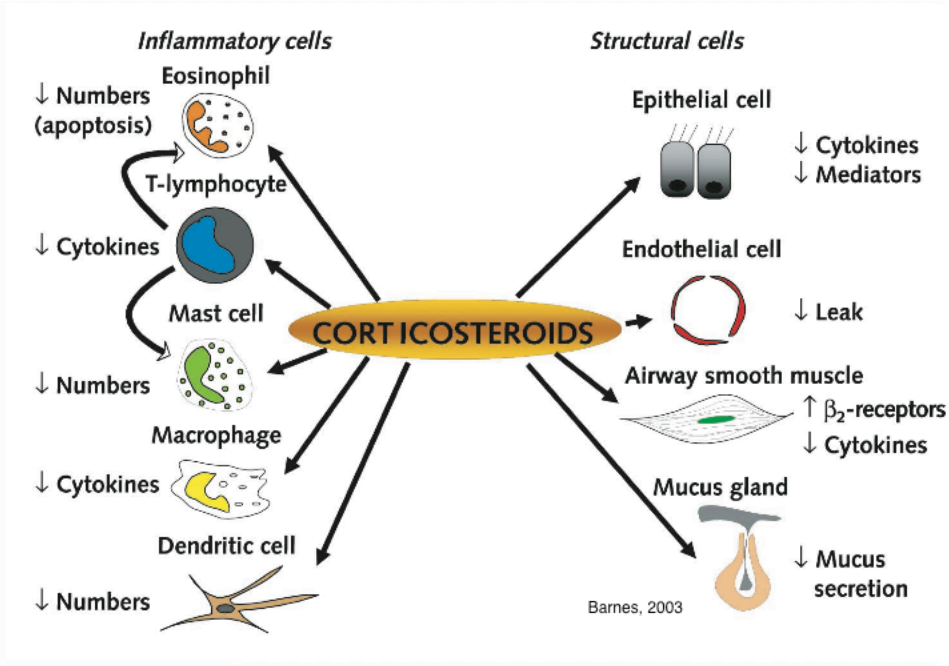

Figure 3. Anti-inflammatory effects of corticosteroids on cells: Corticosteroids remain the mainstay of asthma therapy for their potent inflammatory effects. Corticosteroids reduce the inflammation by decreasing the number of inflammatory cells, and thereby reduce release of cytokines. Apart from the anti-inflammatory effect on leukocytes, corticosteroids also affect the structural cells in the airways like the epithelium, smooth muscle, endothelium and mucin producing cells. Adapted from (Barnes *et al.*, 2003)

Although ICS therapy offers beneficial effects in most patients, it has certain drawbacks that are associated with chronic ICS use. Chronic therapy may increase systemic absorption of corticosteroids and lead to various complications and adverse effects. Concerns over the chronic use of ICS in children and

adolescents have been a primary issue. Recent studies indicate that chronic inhaled budesonide and beclomethasone dipropionate therapy affects the linear growth (height) of children that persists into adulthood. Notably, reduced bone mineral density was observed in these subjects (Kelly *et al.*, 2008; Fuhlbrigge *et al.*, 2014). Even though the effects of ICS on growth and bone density were significantly lower than with oral corticosteroids (OCS), the adverse effects did persist, suggesting systemic absorption after inhalation (Kelly *et al.*, 2008).

A small percentage of patients are resistant to corticosteroid therapy. This group of patients poses a therapeutic challenge. There are several proposed mechanisms for resistance to corticosteroids. The cytokines, interleukin-2 (IL-2), interleukin-4 (IL-4), and interleukin-13 (IL-13) play a role in asthma pathophysiology. Increased concentrations of these cytokines have been observed in the bronchial biopsy of patients with steroid resistance. This increase in concentration results in reduced affinity of the glucocorticoid receptor for the corticosteroid, which in turn causes a reduction in anti-inflammatory action, ultimately leading to corticosteroid resistance. Moreover, increased expression of the glucocorticoid receptor β also can contribute to steroid resistance by binding to the glucocorticoid response element, thereby preventing the binding of the glucocorticoid receptor α.

Human asthma can be either eosinophilic or neutrophilic. Glucocorticoids cannot suppress neutrophilic inflammation, which may be another cause for the steroid resistance (Nightingale *et al.*, 2000; Barnes *et al.*, 2003; Barnes, 2010; Barnes, 2012b). While ICS have several beneficial effects and are the mainstay for anti-inflammatory therapy in asthma, drawbacks associated with their use warrants the need for alternative therapeutic options.

2.1.2.2 Bronchodilators

β_2AR agonists

The most potent bronchodilatory drugs used for asthma are β_2AR agonists. β_2AR agonists remain the best drugs for acute asthma attacks and have saved countless lives. Short-acting β_2AR agonists (SABAs) like albuterol (or salbutamol) find extensive use as 'rescue' medication in the event of asthma attacks. They provide rapid-onset bronchodilation that is required in an acute asthma attack situation. However, their duration of action is short, and for a more sustained bronchodilation, long-acting β_2AR agonists (LABAs) are now available. LABAs currently used in the therapy of asthma include formoterol and salmeterol. Both formoterol and salmeterol have a long duration of action ~12 hours. The long duration of action allows for twice a day dosing. While LABAs possess strong bronchodilatory properties, they have been associated with loss of asthma

control, masking the inflammation associated with asthma, and a small yet significant increase in mortality (Theron *et al.*, 2013). The increase in mortality has forced the FDA to ban the use of LABAs as monotherapy in asthma and to put a 'black-box' warning on the packaging (Aaronson, 2006). LABAs can now be used only in combination with ICS. Moreover, studies have shown that LABAs may increase the sensitivity to ICS (Kobayashi *et al.*, 2012; Rossios *et al.*, 2012).

In order to increase patient compliance by reducing frequency of dosing, the recently developed ultra-long acting β_2AR agonists (ultra-LABAs) are currently being tested clinically. Ultra-LABAs such as indacaterol, abediterol, carmoterol, milveterol, olodaterol and vilanterol have a half-life of ~24 hours and require only once-a-day dosing. While, indacaterol and vilanterol have received FDA approval for use in chronic obstructive pulmonary disorder (COPD), they are still in clinical trials for asthma (Theron *et al.*, 2013). However, LABAs (formoterol and salmeterol) are being used as monotherapy in COPD without any reported problems (Theron *et al.*, 2013).

Anti-muscarinic

Muscarinic antagonists have been proposed for use in the therapy of asthma as bronchodilatory agents. Short-acting muscarinic antagonist (SAMAs) like ipratropium bromide and long acting muscarinic antagonists (LAMAs) such

as tiotropium have been used for asthma therapy. The mechanism of action of muscarinic antagonists is functionally opposite to the mechanism of action of β_2AR agonists. Muscarinic antagonists act by blocking the cholinergic arm of bronchoconstriction. However, β_2AR agonists can, not only *reverse* bronchoconstriction caused by all factors including the direct effects of histamine, prostaglandin D_2 (PGD_2) and leukotriene D4 (LTD_4), but also cause bronchodilation. Muscarinic antagonists therefore, are weaker than β_2AR agonists as bronchodilatory agents in asthma (Barnes, 2012a; Theron *et al*., 2013).

2.1.2.3 Other currently available therapeutic options

Corticosteroids and bronchodilators (especially β_2AR agonists) remain the mainstay of asthma therapy. There are other therapeutic options available in market, however these are used mainly as add-on therapy. Drugs that act on leukotrienes by targeting the cysteinyl-leukotriene receptors (eg $CysLT_1$ receptors) are used for their anti-inflammatory properties. Commerically available anti-leukotrienes include montelukast, zafirlukast and pranlukast. Montelukast is used primarily for exercise-induced asthma (Theron *et al*., 2013). However, the efficacy of anti-leukotrienes to reduce inflammation is lower than ICS and they may not have much therapeutic benefit in severe asthma unless used in

combination with ICS (Riccioni *et al.*, 2004; Mastalerz *et al.*, 2010; Mathew *et al.*, 2012).

Another anti-inflammatory therapy option available is to use an anti-immunoglobulin E (IgE) antibody (eg: Omalizumab) to block the IgE response. In the majority of cases, asthmatic reactions are caused by allergic IgE-mediated responses; using omalizumab provides an alternative to counteract inflammation. Omalizumab is a monoclonal antibody that prevents activation of the IgE receptor by binding to the Fc of IgE (Barnes, 2012a). While, ICS work by reducing the inflammation, anti-IgE prevents the formation of inflammation. Using anti-IgE as add-on therapy reduces the dosage of ICS required for therapy of asthma (Buhl, 2005; Nowak, 2006; Morjaria *et al.*, 2007).

2.1.3 Limitations and need for newer therapeutic options

In spite of the availability of a wide variety of therapies for asthma, there is an unmet clinical need for better therapeutic avenues. The first line of therapy, corticosteroids, has inherent problems of systemic effects and steroid-resistant asthma. β_2AR agonists remain the drugs of choice in acute asthma conditions but have little utility in long-term control of the disorder. Other therapeutic options including muscarinic antagonists, leukotriene inhibitors and anti-IgE therapy lack required efficacy and need to be co-administered with ICS. While limited

progress has been achieved in approved therapeutic options for asthma, it is encouraging to see a variety of mechanistically variable options being tested and developed. Tables 1, 2 and 3 enlist the therapeutic options in asthma that are in development, are potentially viable or have failed, respectively. Using combination therapy is the current treatment regimen for asthma; however, better options beyond symptomatic treatment are needed.

Table 1. Asthma therapies currently under development

Class	Drug or group	MOA	Expected potency compared to prototype	Expected potency compared to standard therapy
Bronchodilators	Ultra-LABAs	Activate β_2AR	Similar	Similar (Cazzola *et al.*, 2011)
Bronchodilators	Glycopyrolate, GSK573719 and aclidinium bromide	Anti-muscarinic (LAMAs)	Better	Lesser than LABAs (Cazzola *et al.*, 2013; Gras, 2014) (Approved for COPD)
Bronchodilator	Ro 25-1553	Vasoactive intestinal peptide analog	N/A	Lesser than formoterol (Linden *et al.*, 2003)
Anti-inflammatory	GSK2190915	5'-LO and 5'-LO activating protein inhibitors	May be useful in exercise-induced asthma	(Grant *et al.*, 2009; Kent *et al.*, 2014)
Anti-inflammatory	Recombinant soluble IL-4 receptor	IL-4 receptor antagonist	N/A	Lesser than ICS (Borish *et al.*, 2001)
Anti-inflammatory	Benralizumab (MEDI-563)	IL-5 receptor α blocker	N/A	Lesser than ICS (Laviolette *et al.*, 2013)

MOA-mechanism of action, GCR-glucocorticoid receptor, CS-corticosteroids, β_2AR- β_2 adrenergic receptor, LABAs-Long acting β_2AR agonists, N/A- not available, 5'LO- 5' lipooxygenase, BLT_1- leukotriene B_4 receptor 1, CRTH2- chemoattractant homologous receptor on T_H2 cells, IL-4- interleukin 4, IL-5- interleukin 5

Table 2. Potential therapies for asthma

Class	Example	MOA	Potential problem	Potential benefit
Bronchodilators	N/A	Rho Kinase inhibitor	Toxicity	N/A (Barnes, 2012b)
Bronchodilators	Quinine, chloroquine, saccharine	Bitter taste receptor (TAS2R) agonists	Unknown	Hyperpolarization of ASM (Deshpande *et al.*, 2010)
Bronchodilators	Cilostazol, milrinone	PDE3 inhibitors	Cardio-vascular mortality	Relax ASM (Banner *et al.*, 2009)
Bronchodilators	N/A	EP_4 agonists	Unknown	Prevent coughing (Buckley *et al.*, 2011)
Bronchodilators	N/A	Potassium-channel activators	Unknown	Relax ASM by activating K^+ channels (Barnes, 2012b)
Bronchodilators	Rosiglitazone	PPAR-Y agonist	Unknown	Improved lung function (Spears *et al.*, 2009; Richards *et al.*, 2010)
Anti-inflammatory	Roflumilast (Approved for COPD)	PDE4 inhibitor	Nausea, headaches, diarrhea	Inhibits allergen-induced responses (Bousquet *et al.*, 2006; Baye, 2012)
Anti-inflammatory	N/A	Phospholipase A_2 inhibitors	Difficult to design selective inhibitors	Inhibit generation of all lipid mediators (Magrioti *et al.*, 2010)
Anti-inflammatory	N/A	DP_1-DP_2 dual antagonist	Unknown	Prevent actions of PGD_2 (Barnes, 2012a)
Anti-inflammatory	N/A	PGD synthase inhibitor	Unknown	Inhibit synthesis of PGD_2 (Barnes, 2012a)
Anti-inflammatory	N/A	IL-13 inhibitor	Unknown	Inhibition of IL-13 regulation of IgE (Spahn *et al.*, 1996; Matthews *et al.*, 2004)

Table 2 (continued) Potential therapies for asthma

Class	Example	MOA	Potential problem	Potential benefit
Anti-inflammatory	AS1517499	STAT6 inhibitor	Unknown	Block downstream signaling of IL-4 and IL-13 (Chiba *et al.*, 2009)
Anti-inflammatory	MEDI-528	IL-9 antibody	Unknown	Reduced inflammation and mucin hypersecretion (Parker *et al.*, 2011)
Anti-inflammatory	N/A	TSLP inhibitor/antibody	Unknown	Unknown (Shikotra *et al.*, 2012)
Anti-inflammatory	N/A	IL-17, IL-25, IL-33, GM-CSF	Unknown	Unknown (Hansbro *et al.*, 2011)
Anti-inflammatory	Mogalmulizumab (KW-0761, AMG-761)	Defucosylated antibody CCR4	Unknown	Depletion of T_H2 cells (Antoniu, 2010)
Anti-inflammatory	Navarixin (SCH-527123)	CXCR1/CXCR2 antagonist	Unknown	Blocks ozone-induced neutrophilia (Holz *et al.*, 2010)
Anti-inflammatory	Mapracorat	Non-steroidal GCR activator	Unknown	Lesser than CS (De Bosscher, 2010)
Anti-inflammatory	N/A	Inhibitor of κB kinase	Unknown	Block inflammation (Karin *et al.*, 2004)

N/A-not available, PDE3-phosphodiesterase 3, ASM-airway smooth muscle, EP_4-prostanoid receptor (one of four receptors for prostaglandin E_2), PDE4-phosphodiestease 4, DP_1-DP_2- Prostaglandin D2 receptor, PGD_2-prostaglandin D_2, (IL) interleukin, STAT6-signal transducer and activator of transcription 6, TSLP-Thymic stromal lymphopoietin, GM-CSF-granulocyte macrophage colony-stimulating factor, CCR/CXCR-chemokine receptor, GCR-glucocorticoid receptor, CS-corticosteroids,

Table 3. Failed asthma therapies

Class	Example	MOA	Reason for failure
Anti-inflammatory	N/A	LTB_4 receptor (BLT_1) antagonist	No clinical benefit (Ohnishi *et al.*, 2008)
Anti-inflammatory	AMG317	mAb for IL-4 receptor α	Not beneficial in across overall group of patients (Corren *et al.*, 2010)
Anti-inflammatory	Mepolizumab, reslizumab	mAb for Il-5	Reduced eosinophilia but no effect on AHR, exacerbation or lung function (Leckie *et al.*, 2000; Flood-Page *et al.*, 2007; Castro *et al.*, 2011; Prazma *et al.*, 2014)
Anti-inflammatory	TPI-ASM8	Antisense oligonucleotide for IL-5, GM-CSF and CCR3	Little beneficial effect (Gauvreau *et al.*, 2008; Gauvreau *et al.*, 2011)
Anti-inflammatory	Infliximab Golimumab	Antibodies for TNF-α	No beneficial effect but exacerbated pneumonia and cancer (Howarth *et al.*, 2005; Berry *et al.*, 2006)
Anti-inflammatory	AMG-853,	CRTH2 antagonists	No beneficial effects (Busse *et al.*, 2013)
Anti-inflammatory	N/A	Small molecular inhibitors of CCR3	Toxicology issues (Barnes, 2012a)

LTB_4- leukotriene B_4, BLT_1- leukotriene B_4 receptor 1, IL-4- interleukin 4, mAb- monoclonal antibody, IL-5- interleukin 5, AHR- airway hyperresponsiveness, GM-CSF- granulocyte macrophage colony stimulating factor, CCR3- chemokine receptor 3, TNF-α-Tumor necrosis factor α

2.2 Murine models of asthma

In the search for better therapeutic options for asthma treatment, it is necessary to have a good model to study the pathophysiology and the implications of therapeutic interventions. Humans, cats, and horses are the only known animals that can spontaneously develop asthma. However, testing different therapies in feline or equine models of asthma is not feasible. Moreover, human asthma is multi-faceted and has multiple varying etiological factors. Mice provide a valuable tool to study asthma with the flexibility of genetically modified strains and a number of asthma models that 'mimic' different human asthma symptoms. However, mice do not develop asthma, and we can only mimic the symptoms of human asthma by using different methods.

Murine models of asthma can be allergen-driven which include any allergens from fungi, house dust mites, epitopes of insect (cockroach) to completely unrelated proteins like chicken egg albumin (ovalbumin). Murine asthma models can also be induced by directly stimulating the airways using inflammatory cytokines like interleukin-13 (IL-13) that have been known to play a role in asthma. The challenge in using murine models of asthma is to find the balanced model that can best represent human asthma. While there exists an argument that mice are not the ideal model for human asthma, they do form one of the best possible models to study asthma given the limitations of using larger animals and

the advantage of a wide variety of genetically modified available and ones that can be easily generated (Mullane *et al.*, 2014).

2.2.1 Model 1: Ovalbumin sensitization and challenge

One of the most widely used and accepted murine models of asthma is the ovalbumin sensitization and challenge (Ova S/C). The Ova S/C model is an allergen-induced model of asthma. The mice are exposed (sensitized) to a foreign protein ovalbumin using a T-Helper cells-2 (T_H2) skewing adjuvant, alum (Conrad *et al.*, 2009). Once the mice develop 'sensitivity' to ovalbumin, their airways are exposed to ovalbumin. Usual protocols for Ova S/C vary, but the overall outline remains same. Mice are sensitized with ovalbumin and alum with 2-4 intra-peritoneal injections, followed by multiple intra-nasal, intra-tracheal or inhaled (nebulized) exposures to ovalbumin (Kips *et al.*, 2003; Callaerts-Vegh *et al.*, 2004; Nguyen *et al.*, 2008; Nials *et al.*, 2008; Nguyen *et al.*, 2009; Thanawala *et al.*, 2013). This results in development of the asthma 'phenotype', since the mice do not develop asthma but merely mimic the symptoms or phenotype of asthma.

Asthma is chronic inflammation of airways and is characterized by inflammation (inflammatory cell infiltration into the airways, mucus hypersecretion) and airway hyperresponsiveness. The Ova S/C model results in

development of these characteristics and hence forms a good murine model to study asthma. Some debate that ovalbumin is a completely unrelated protein and there is a remote possibility of the mouse or human ever getting 'allergic' to ovalbumin to develop asthma and that using fungi or house dust mites may form a better model for human asthma (Nials *et al.*, 2008). However, this works to the advantage of Ova S/C model. Since there is no possibility of accidental exposure of mice to ovalbumin except through the experimental protocol, the chances of confounding experimental factors is limited.

2.2.2 Model 2: Protease-activated receptor type-2

Another model of asthma is a modification of the Ova S/C model, by addition of a protease (or proteinase)-activated receptor type 2 (PAR2) ligand. PAR2 is a member of the G protein-coupled receptors (GPCRs) super family belonging to the protease-activated receptors class. PARs have a tethered ligand on their N-terminal extracellular motif that is usually inactive. As shown in figure 4A, specific proteases can cleave the N-terminal sequence and allow the tethered ligand to activate the receptor. The tethered ligand is a protein sequence that binds to the ligand-binding domain of the PAR2. For PAR2, the protein ligand sequence is SLIGRL. The binding of the ligand to the receptor can result in activation of downstream signaling cascades.

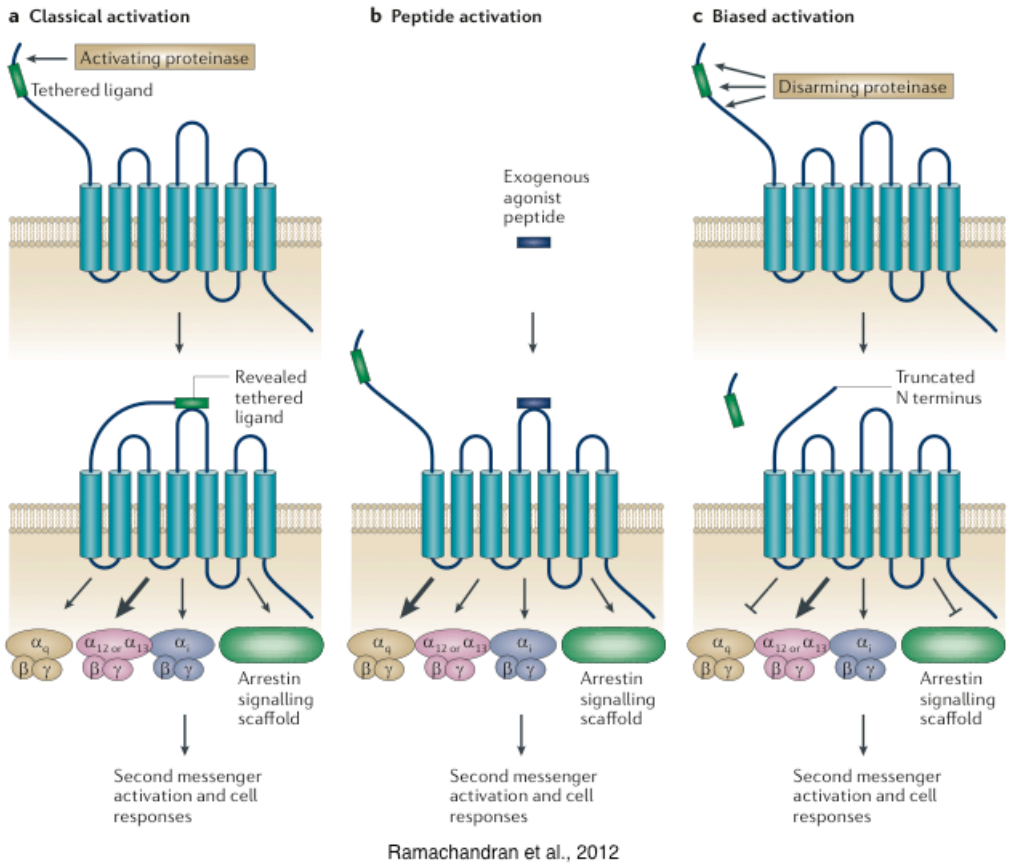

Figure 4. Protease-activated receptor activation: The figure shows the different mechanisms of activation of the protease-activated receptor (PAR). A) The classical activation of PAR is by activating proteases, these proteases are found in allergens that cause asthma. The PAR receptor has a tethered ligand in its protein sequence. The activating protease cleaves the tethered ligand, allowing it to bind to the receptor and activate downstream signaling cascades through G proteins and arrestin. B) Alternatively, the receptor can be activated by an exogenous peptide that can activate the receptor resulting in downstream signaling. C) In a third scenario, the PAR can be activated by disarming proteases that can cleave the tethered ligand and truncate it such that it can activate the receptor in a preferential manner. In this case, the receptor will only activate some pathways over others (biased signaling). In addition, the 'disarmed' receptors can be further targets for exogenous peptide activation. Adapted from (Ramachandran *et al.*, 2012)

The signaling of GPCRs will be discussed in detail in the following sections. Briefly, PAR2 activates $G\alpha_q$ and/or $G\alpha_i$ G proteins (Ramachandran *et al.*, 2012). In addition, it also has an arrestin-dependent signaling pathway that leads to activation of ERK1/2 (DeFea *et al.*, 2000; Kumar *et al.*, 2007; Ramachandran *et al.*, 2012). The activation of multiple pathways also leads to the possibility of 'biased signaling' by the PAR2 that can be brought about by using different protein ligand sequences to activate the receptor (Figure 4C). These pathways will be discussed in detail in the following section on GPCR signaling. The activation of PAR2 leads to the increase in the inflammatory cytokines IL-13 and tumor necrosis factor-α (TNF-α). These cytokines have been implicated in and result in development of the asthma phenotype (Ebeling *et al.*, 2005)

In addition to being activated by its tethered ligand, PAR2 can also be activated by an external ligand as shown in figure 4B. In the absence of a protease to cleave and 'unmask' the tethered ligand, an external ligand can be used. This property of PAR2 is utilized in the PAR2 model of murine asthma. Mice are sensitized to ovalbumin similar to the Ova S/C model but in the challenge phase, mice are exposed to both ovalbumin and the PAR2 ligand SLIGRL-NH_2. The PAR2 model has been shown to present a worsening of the asthma phenotype in Balb/c mice in comparison to Ova S/C model alone.

However, the PAR2 model only induced a worsening of the asthma phenotype when the challenge phase included both the PAR2 ligand and ovalbumin. While the PAR2 ligand alone initiated an inflammatory response, it needed an allergen to induce a worse asthma phenotype (Ebeling *et al.*, 2005).

The PAR2 model of asthma also simulates human asthma conditions where certain allergens (responsible for allergic asthma) possess protease activity and result in activation of the PAR2 in addition to the allergic responses (Ebeling *et al.*, 2005). The details of this model have been discussed in the methods section.

2.2.3 Characterization of asthma phenotype:

The murine model of asthma can be characterized by analyzing many different parameters. Here, the three cardinal features of asthma: inflammatory cell infiltration, mucous metaplasia and AHR are discussed.

2.2.3.1 Inflammatory cell infiltration:

One of the hallmarks of asthma is infiltration of the airways by inflammatory cells. These cells are recruited to the airways upon exposure to an antigen in sensitized mice. In Ova S/C model of asthma, ovalbumin exposure to sensitized mice results in recruitment of lymphocytes, on ovalbumin exposure. As

described previously, Ova S/C model of murine asthma is skewed for a T_H2 response using alum, hence an increase in T_H2 lymphocytes is seen in this model. The exposure to allergen results in the endocytosis of the allergen by antigen presenting cells and in turn to naïve T cells. Dendritic cells induced by the allergen response result in differentiation of T cells into T_H2 helper cells and T_H17 cells. The T_H2 cells induce IgE production as a result of stimulation by IL-4 and IL-13. IgE causes bronchoconstriction by inducing release of inflammatory mediators. In addition, T_H2 cells release IL-5, which increase eosinophil recruitment. The inflammatory mediators released from the eosinophils cause further damage to the airway epithelium (Ishmael, 2011).

In the murine models of asthma based on Ova S/C, the increase in total number of cells and the number of eosinophils in airways are analyzed as a measure of inflammatory infiltration. Figure 5 shows the pathways involved in the increase in inflammatory (eosinophilic) infiltration into the airways.

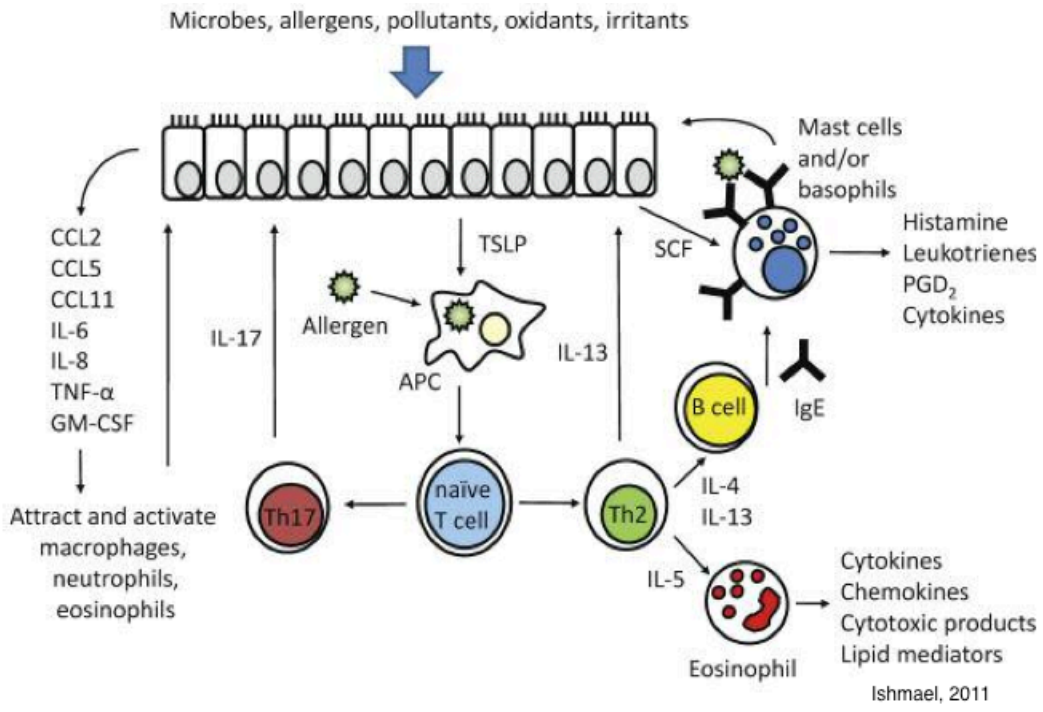

Figure 5. Allergic asthma pathogenesis: Different allergens are endocytosed by antigen-presenting cells and are presented to the naïve T-cells. Th2 cells induce IgE, via B cells, release of IL-13, Il-4, IL-5 that result in recruitment of eosinophils. Adapted from (Ishmael, 2011)

2.2.3.2 Mucous metaplasia

Mucus hypersecretion is the leading cause of death in asthma patients. Mucus plugs can restrict airflow and even block airways with hypersecretion (Evans *et al.*, 2009b). 'Mucus' refers to the secreted extra-cellular mucins. Mucins are large, heavily glycosylated proteins which are synthesized by specific MUC (human) or Muc (murine) genes (Williams *et al.*, 2006; Evans *et al.*, 2009a). Under normal conditions, airway epithelium is covered with a thin layer of mucus

(approximately 5-50 μm thick) and it has a protective function to trap inhaled particles or allergens (Knowles *et al.*, 2002). The epithelial cilia beat and move the mucus towards the pharynx and allow it to be swallowed. In asthma, the mucus secretion and production increases. An up-regulation of the mucus/mucin producing genes occurs that results in mucus hyper-production and hyper-secretion. Figure 6 shows the transcriptional regulation of mucin production that result from of a variety of inflammatory signals (Evans *et al.*, 2009a).

Mucous metaplasia results from differentiation of the epithelial cells into mucus producing goblet cells. Metaplasia refers to change in the type of cells. The goblet cells that secrete mucus increase in numbers and lead to increased mucus secretion into airway lumen. In asthma, there is not only an increase in the amount of mucus produced and secreted, but also an increase in number of cells producing and secreting mucus (Evans *et al.*, 2009a).

Visualizing and quantifying the amount of mucus produced is vital to the characterization of asthma. One of the most commonly used stains to visualize mucus is periodic acid Schiff's stain (PAS). The PAS stain stains different polysaccharides including glycoproteins like mucins. A modification of the PAS stain is known as the periodic acid fluorescent Schiff's (PAFS) stain. PAFS similar to PAS stains all mucin glycoproteins, however it has an advantage over PAS, in that it can be used to visualize the epithelium and the mucus globules

separately. PAFS involves oxidation of the alcohols on the mucin glycoproteins to aldehydes or ketones such that mucin fluoresces red with TritC excitation and the epithelium fluoresces green with GFP excitation (Kim *et al.*, 2008; Piccotti *et al.*, 2012). Details of the staining procedure have been discussed in the methods section.

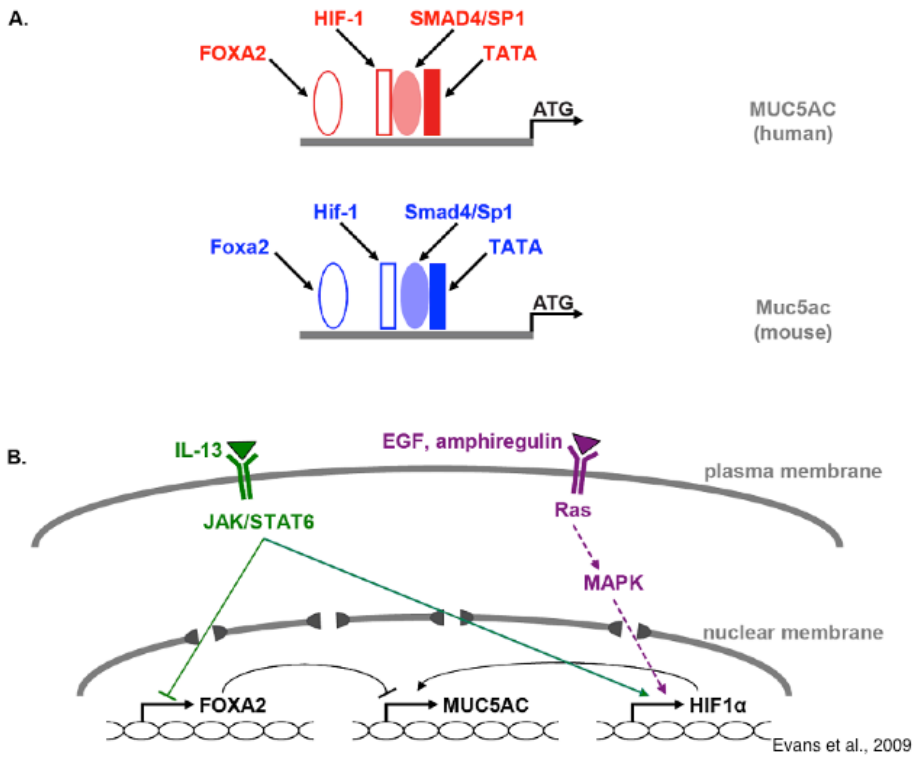

Figure 6. Transcriptional regulation of mucin production: The figure depicts the transcriptional regulation of mucin production, A) Activation of MUC5AC and Muc5ac the human and murine mucin producing genes respectively. B) Activation of MUC5AC (mucin producing gene) by inflammatory mediators like IL-13. Adapted from (Evans *et al.*, 2009a)

2.2.3.3 Airway hyperresponsiveness

Asthma is characterized by hypersensitive airways. In comparison to a normal person, an asthmatic's airways are more responsive to allergens and bronchoconstrictors. At the same dose of a bronchoconstrictor such as methacholine, an asthmatic airway demonstrates a more pronounced narrowing of airways and resultant increase in resistance to airflow compared to a normal person at the same dose of the bronchoconstrictor (Figure 7). This characteristic of an asthmatic airway is known as airway hyperresponsiveness (Doeing *et al*., 2013). AHR is measured in humans to diagnose asthma using methacholine and measuring the FEV1 of air. In murine models, the FEV1 cannot be measured so an equivalent measurement of the airway resistance is employed using an invasive forced oscillation technique. Mice are subjected to increasing doses of methacholine and the resultant airway resistance is measured using FlexiVent® (Scireq, Montreal, Canada).

The invasive forced oscillation technique using a mechanical ventilator is widely accepted and has advantages over the use of whole-body plethysmography. The invasive method of measuring airway resistance involves the use of a tracheal cannula. This avoids interference from the upper respiratory tract and allows for direct measurement of airway resistance. Mice are kept under general anesthesia and mechanically ventilated so any external effects resulting

in changes in air pressure with the whole-body plethysmograph are avoided. Another advantage of the mechanical ventilation is that the tidal volume and respiratory rate are controlled and constant (Drazen *et al.*, 1999). However, it also has its disadvantages. The procedure is invasive and usually terminal, preventing the use of the same animal for further studies.

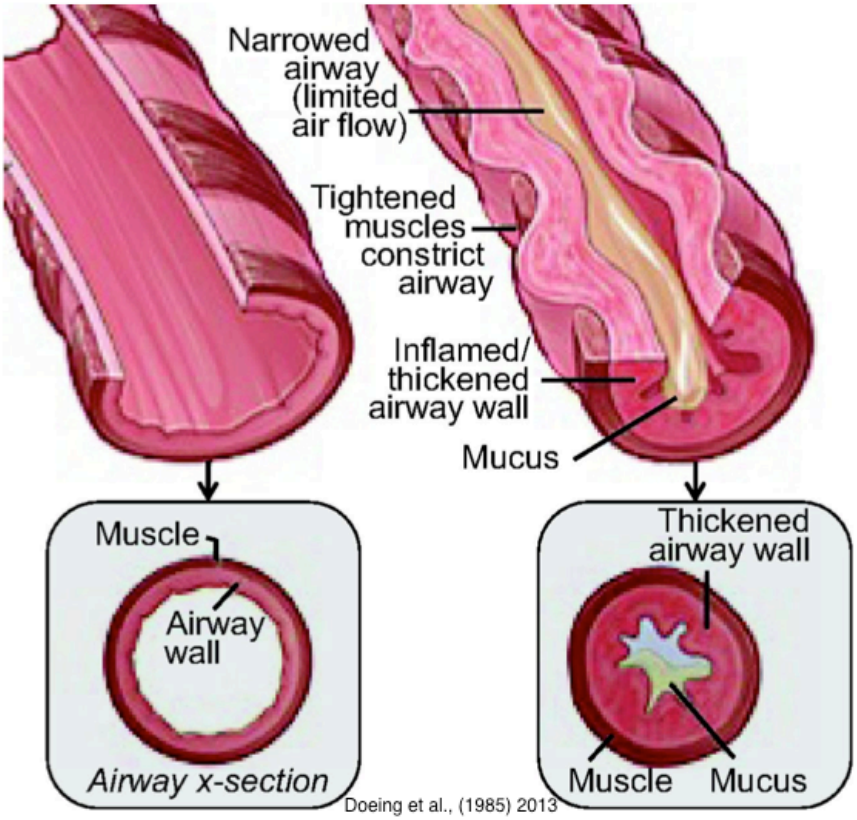

Figure 7. Airway narrowing and bronchoconstriction in asthma: The figure depicts the airway smooth muscles of normal (left) and asthmatic (right) airways, with the cross-sectional view in the inserts. The asthmatic airway has non-uniform airway narrowing and bronchoconstriction that is much more pronounced resulting in airway hyperresponsiveness. The airway smooth muscle also thickens in asthmatics. Mucin overproduction leads to further blockade of the airways. Adapted from (Doeing *et al.*, 2013)

It has been proposed that AHR is a result of airway remodeling that occurs in asthma conditions. Airway remodeling may include the structural changes such as thickening of the epithelium by collagen, goblet cell metaplasia, angiogenesis, increase in extracellular matrix and airway smooth muscle mass increases (Al-Muhsen *et al.*, 2011). This increase in airway smooth muscle mass has been implicated in the increased responsiveness or increased contraction in response to bronchoconstrictors (Pelaia *et al.*, 2008). For years, the airway smooth muscle has been a therapeutic target for designing newer therapies (Camoretti-Mercado, 2009). In these studies we have used AHR as one of the measures of the asthma phenotype that is a function of changes in the airway smooth muscle.

2.3 β_2 adrenergic receptor

β_2AR agonists remain the mainstay of asthma therapy with (LABAs) or without (short-acting β_2 agonists) corticosteroids. β_2AR belong to the super-family of GPCRs or 7-transmembrane receptors (7-TMRs). GPCRs are named because they couple to intracellular guanine nucleotide binding proteins (G proteins) that transduce the extracellular stimuli to the receptor into intracellular signaling cascades (Gilman, 1987). GPCRs are also called 7-TMRs because they are made up of 7 membrane-spanning helices. The β_2AR is one of the most widely studied GPCRs and is considered the prototype receptor for most GPCR studies.

Consequently, the structure and signaling pathways of β_2AR have been studied extensively (Benovic, 2002).

2.3.1 Activation and regulation of the β_2AR

The β_2AR like all other GPCRs, are bound to the heterotrimeric G protein complex made up of α, β and γ subunits. The α subunit of the G protein is bound to guanosine diphosphate (GDP) in its inactive state along with the β and γ subunits. Upon activation, the conformation of the β_2AR changes and results in decreased affinity of the α subunit for GDP. The α subunit exchanges GDP for guanosine triphosphate (GTP). This exchange of nucleotide decreases affinity of the α subunit for the β and γ subunits and causes a dissociation of the G protein complex into GTP-bound α subunit and βγ complex (Gilman, 1987). There are at least 23 different types of known α subunits of the G protein, having different signaling cascades (Hermans, 2003). The β_2AR is predominantly known to couple to the $G\alpha_s$ type of subunit. The $G\alpha_s$ subunit stimulates the enzyme adenylyl cyclase (AC). Adenylyl cyclase catalyzes the conversion of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP) (Pierce *et al.*, 2002). cAMP in turn activates a downstream kinase protein kinase A (PKA) which phosphorylates numerous cellular substrates (Taylor *et al.*, 2008; Pidoux *et al.*, 2010). The α subunit hydrolyzes GTP to GDP resulting in the increase of its

affinity for the βγ complex. The re-association of G protein subunits allows their binding to the receptor and to revert to the original state of receptor-bound G proteins.

2.3.2 Desensitization of the β_2AR

Prior to re-association, the βγ complex of the G protein recruits another kinase called the G protein-coupled receptor kinase (GRK) (certain subtypes were previously known as βARK) (Benovic *et al.*, 1987). There are many subtypes of GRK and the β_2AR is primarily regulated by GRK2 (Seibold *et al.*, 1998). GRKs are serine/threonine kinases that phosphorylate the receptor and result in desensitization (Krylova, 1977; Ferguson *et al.*, 1996). The phosphorylated receptor cannot couple to its G proteins and recruits another intra-cellular protein called arrestin (certain subtypes were previously known as β-arrestins) (Lohse *et al.*, 1990). Apart from GRKs, other kinases like PKA and protein kinase C (PKC) also regulate the β_2AR by phosphorylation. The role and function of the phosphorylation of the β_2AR by PKA and PKC have been reviewed (Pitcher *et al.*, 1992; Penn, 1998; McGraw *et al.*, 2005).

Arrestins bind to the GRK-phosphorylated receptor and prevent further association with G proteins. Arrestins also act as adaptors for clathrin-mediated endocytosis of the receptor into intracellular vesicles. The receptor may then be

degraded or recycled back to the surface (Krylova, 1977; Ferguson *et al.*, 1996; DeWire *et al.*, 2007). It was recently reported that even an internalized receptor could signal from the endocytic vesicle after endocytosis (Irannejad *et al.*, 2013). The activation of the G-protein mediated signaling cascade by a receptor is known as the classical or canonical model of GPCR regulation by GRKs and arrestins and is summarized in figure 8. However, many other signaling pathways that are associated with the β_2AR exist (Benovic, 2002).

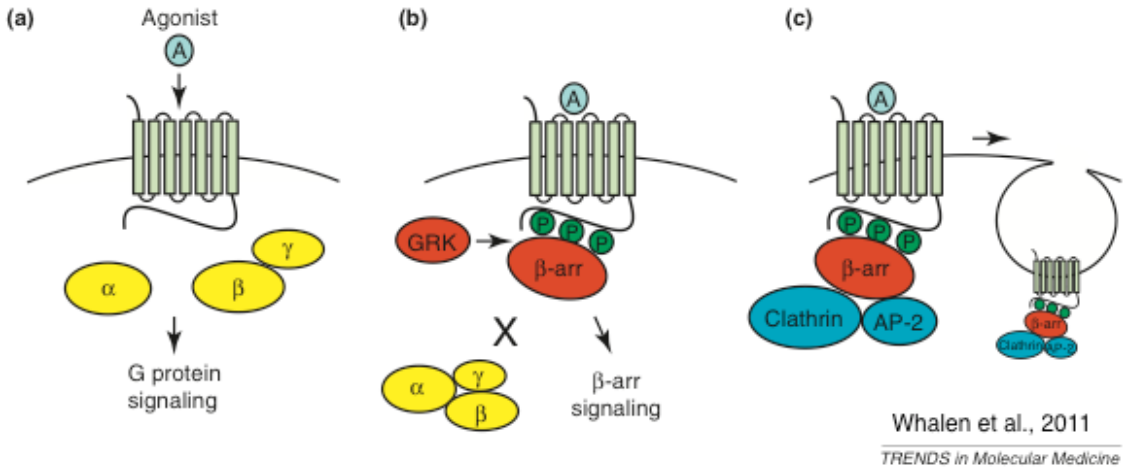

Figure 8. Canonical signaling pathways of G protein-coupled receptors (GPCRs): The figure shows the canonical pathway of GPCR signaling. A) When activated by an agonist, the GPCR results in signaling through its G proteins. B) An activated GPCR results in the recruitment of G protein-coupled receptor kinase (GRK) that phosphorylates the receptor. The GRK phosphorylation results in recruitment of arrestins. C) Arrestins bind to the receptor and prevent further association of G proteins with the receptor. Arrestin also scaffolds clathrin and AP-2 proteins to facilitate internalization of the receptor. Adapted from (Whalen *et al.*, 2011)

2.3.3 Alternate signaling pathways

2.3.3.1 PKA mediated $G\alpha_s$ to $G\alpha_i$ switch

We have discussed the classical model of β_2AR signaling that included activation of AC and cAMP-mediated activation of PKA. In addition, we discussed the phosphorylation of β_2AR by PKA leading to heterologous desensitization. However, PKA phosphorylation can also lead to a switch in the coupling preference of the β_2AR from $G\alpha_s$ to $G\alpha_i$ (Daaka *et al.*, 1997). This switching of preferred G protein subtype also has signaling implications. β_2AR leads to the downstream activation of mitogen-activated protein kinases (MAPK) like ERK1/2 by signaling via $G\alpha_i$. Another possible role of PKA phosphorylation involves A-kinase anchoring proteins (AKAPs). AKAPs are scaffolding proteins that compartmentalize PKA to specific cellular organelles (Michel *et al.*, 2002).

2.3.3.2 Regulation by tyrosine kinases

Apart from GRK, PKA and PKC, some studies have shown that tyrosine kinases like insulin-receptor and insulin-like growth factor receptor-1 can phosphorylate β_2AR. The role of this phosphorylation is not clear but appears to involve generation of Src homology-2 binding domains on the receptor (Fan *et al.*, 2001).

2.3.3.3 Arrestin-mediated signaling pathway

While various alternative signaling pathways and interactions of the β_2AR have been shown to exist, the most commonly studied alternative signaling pathway of the β_2AR involves arrestin. Previously, we have described the role of arrestin to desensitize the receptor and 'prepare' it for internalization. However, in the last decade it has been shown that arrestins play a much more elaborate role in β_2AR signaling. Arrestins can act as scaffolds for different proteins mainly MAPKs. MAPKs are a family of serine/threonine kinases that include:

- ERK1/2, also known as p44 or p24 MAPK
- p38 kinases
- c-Jun N-terminal kinases (JNK)

The MAPKs are involved in regulation of many cellular functions including cell cycle progression, transcriptional regulation and apoptosis. The activation of each MAPK involves a prototype-signaling cascade, for example, ERK1/2 is a MAPK activated by phosphorylation by MEK that is a MAPK kinase (MAPKK). MEK is phosphorylated by Raf protein isoforms that are triple MAPKs or MAPKKKs. Therefore each MAPK is phosphorylated by a MAPKK, which is in turn phosphorylated by a MAPKKK (DeWire *et al.*, 2007).

Arrestin acts as a scaffold for the different kinases of the MAPK cascade as shown in figure 9. Arrestin scaffolds MAPKs in close proximity to allow their

interaction with each other. In addition, there is data to show that arrestin also scaffolds other proteins like phosphatases that help negatively regulate MAPKs. As shown in figure 9, arrestin can scaffold all kinases of the JNK activation cascade and its inactivating phosphatase, MAPK phosphatase 7 (MKP7) (DeWire *et al.*, 2007). β_2AR can activate MAPKs via the cAMP pathway as well as described previously. Apart from MAPKs, arrestin can act as a scaffold for Akt or protein kinase B (PKB). Akt is a downstream kinase of phosphoinositide-3 kinase (PI3K) and can regulate cell survival and apoptosis. Arrestin scaffolds Akt with its phosphatase, protein phosphatase 2 (PP2A) (DeWire *et al.*, 2007).

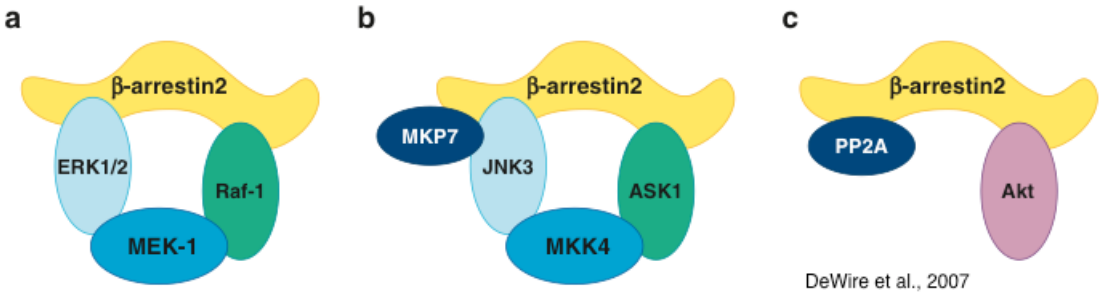

Figure 9. Arrestin scaffolds and signaling: The figure depicts the different kinases and phosphatases β-arrestin2 (or arrestin-3) scaffolds. A) Arrestin scaffolds extracellular signal-related kinase (ERK1/2) and Raf-1. Mitogen-activated protein kinase kinase (MEK-1) indirectly binds to arrestin. B) Arrestin scaffolds c-Jun N-terminal kinase (JNK) 3 and apoptosis signaling kinase 1 (ASK1) directly and MAP kinase kinase 4 (MKK4) indirectly. In addition, the negative regulator phosphatase MAP kinase phosphatase 7 (MKP7) is bound directly to arrestin. C) arrestin also scaffolds Akt and PP2A. Adapted from (DeWire *et al.*, 2007)

Figure 10 summarizes two major signaling pathways of the β_2AR, activation of cAMP and PKA via G proteins and arrestin-mediated activation of the MAPKs.

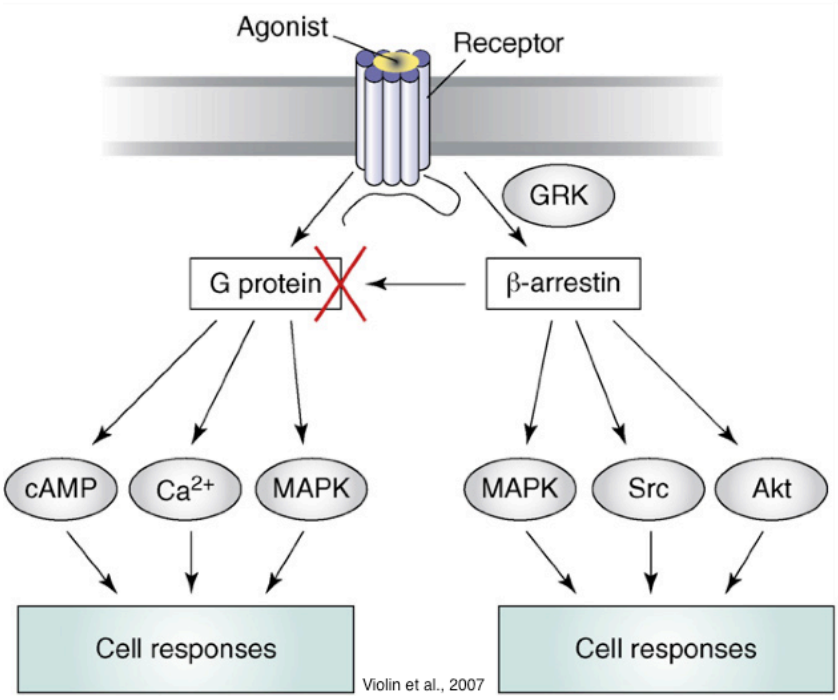

Figure 10. Multiple signaling pathways of the G protein coupled receptors (GPCRs): On activation, GPCRs can signal through the canonical G protein-dependent pathway via the heterotrimeric G proteins. G proteins can lead to the activation of second messenger systems like cyclic adenosine monophosphate (cAMP), calcium (Ca^{+2}) or activation of mitogen activated protein kinases (MAPK). The activated receptor is phosphorylated by G protein-coupled receptor kinase (GRK) and desensitized by β-arrestin (arrestin). Arrestins can further activate different downstream signaling kinases like MAPK, Src and Akt. Adapted from (Violin *et al.*, 2007)

2.3.4 Biased signaling

Many GPCRs activate more than one signaling pathway. For most GPCRs signaling through G proteins remains the canonical pathway. For β_2AR, the

arrestin-mediated pathway has been shown to be another major signaling pathway. It should be noted that an 'arrestin-biased' ligand refers to the activation of MAPK or ERK1/2 by the ligands and NOT the recruitment of arrestin. This pathway is of major interest not only does this pathway defies its namesake 'arrestin' (coined because it 'arrested' the GPCR's signaling) but it also helps differentiate seemingly similar ligands. Previously, ligands were classified based on their activity at the canonical pathway; they were either agonists or antagonists. With the discovery of constitutively active receptors, another class 'inverse agonists' was added. However, all these ligand classifications were solely based on the activity at the canonical G protein-mediated signaling pathway.

With the addition of alternate signaling pathway, the possibility of ligands not conforming to pre-set classes arose, and various class 'subsets' became possible. As shown in figure 11, ligands could now not only activate both pathways or inactivate both pathways but also affect pathways preferentially. The ligands could now exhibit a 'bias' or preference for one of the two pathways and preferentially activate that pathway while having no effect or even shutting down the other pathway. This behavior of ligands was termed as 'biased signaling'. Many other definitions for this behavior have been cited- 'ligand-directed trafficking of receptors' or 'functional selectivity'.

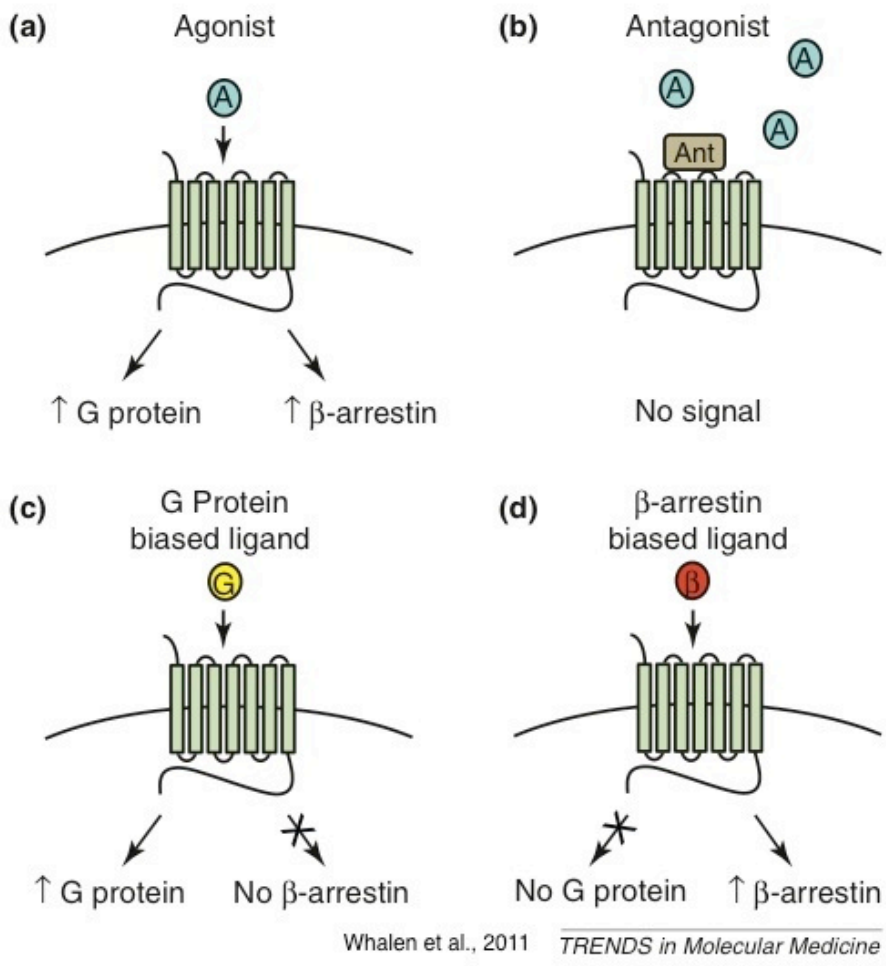

Figure 11. Biased signaling by β_2 adrenergic receptor (β_2AR): β_2AR can activate at least 2 major signaling pathways: G protein-dependent pathway and βarrestin (or arrestin)-dependent signaling pathway. A) A normal agonist can activate both the signaling pathways with equal efficacy (eg., epinephrine). B) An antagonist would inactivate both the signaling pathways (eg., nadolol). C) A G protein pathway biased ligand will preferentially activate the G protein-dependent pathway compared to the arrestin pathway (eg., salmeterol). It may also inactivate the arrestin pathway (eg, carvedilol). D) An arrestin biased ligand will activate the arrestin-dependent pathway preferentially compared to the G protein-dependent pathway. It may inactivate the G protein-pathway signaling, however such a ligand for β_2AR is not known yet. Adapted from (Whalen *et al.*, 2011)

Biased signaling is an only recently appreciated property of receptors that opens a number of possibilities to exploit these preferential signaling properties for therapeutic benefit. It is possible to design ligands that can activate a specific potentially therapeutic signaling pathway while inhibiting another signaling pathway that may result in unwanted effects. There are biased-ligands in clinical and pre-clinical testing for different receptors including angiotensin-1, β_2AR, parathyroid hormone receptor and opioid receptors (Rajagopal *et al.*, 2010; Whalen *et al.*, 2011).

For the β_2AR, two major signaling pathways include the G protein-mediated and arrestin-mediated pathways (Shenoy *et al.*, 2006). Both these pathways can be independently activated and this helps drug design for diseases that need biased signaling for therapeutic benefit. Different methods have been proposed to determine and quantify the bias of a ligand (Rajagopal *et al.*, 2011). A biased ligand is determined on an arbitrary scale where the endogenous ligand for a receptor is considered as the 'neutral' ligand that can activate both signaling pathways equally (Figure 12). In case of β_2AR, epinephrine, the endogenous ligand is set as the standard neutral ligand. All other ligands for β_2AR are compared to epinephrine for signaling at the two different pathways. For example, carvedilol is an arrestin-biased ligand that inhibits the G protein-dependent signaling pathway via β_2AR (Wisler *et al.*, 2007). Similarly, formoterol

and salmeterol are 'arrestin-biased' β_2AR ligands, they can activate ERK1/2 (Rajagopal *et al.*, 2011). Biased ligands can induce different receptor conformations that result in their differential signaling profiles and many studies are currently pursuing different molecular structures induced by β_2AR ligands (Rajagopal *et al.*, 2011).

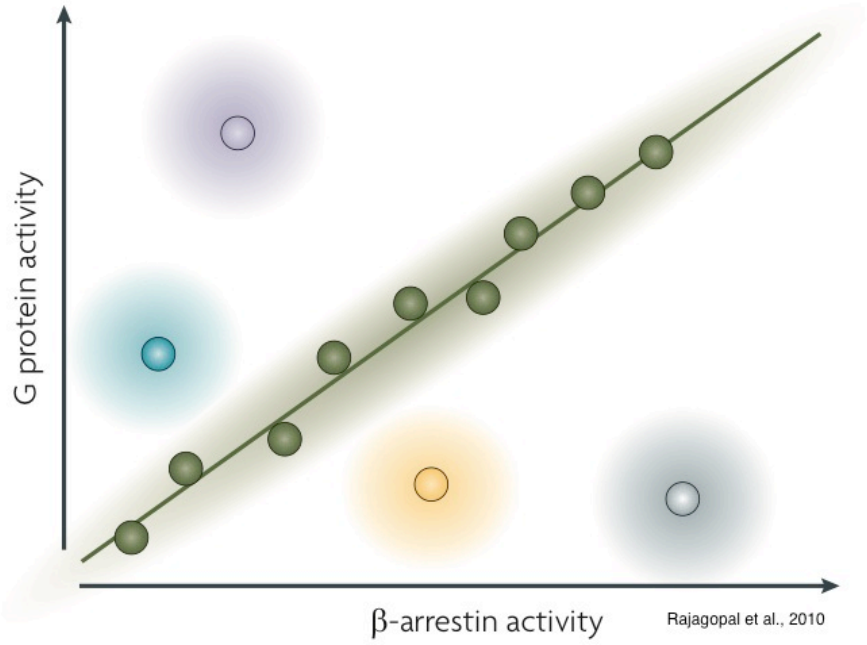

Figure 12. Quantifying ligand bias at G protein-coupled receptors (GPCRs): Biased signaling can be quantified by plotting the signaling of different ligands at the two signaling pathways. As shown in the graph, the G protein-activity versus the arrestin activity of a ligand can be plotted on an X and Y plot. Balanced ligands or ligands (green dots) that can activate both signaling pathways with equal efficacy and lie on the line in the graph. Biased ligands can be of four different types: G protein-biased full agonists (purple), G protein-biased partial agonist (blue), arrestin-biased full agonist (grey) and arrestin-biased partial agonist (yellow). Adapted from (Rajagopal *et al.*, 2010)

Another type of bias that can exist is based on the receptor, where the receptor is biased (Sprang *et al.*, 2012). Neutral ligands acting through a biased receptor will activate downstream pathways with a bias (Figure 13). It could be possible that certain disease conditions transform a normal receptor into a biased receptor such that even the endogenous ligand (assigned as neutral), would now act as a biased ligand.

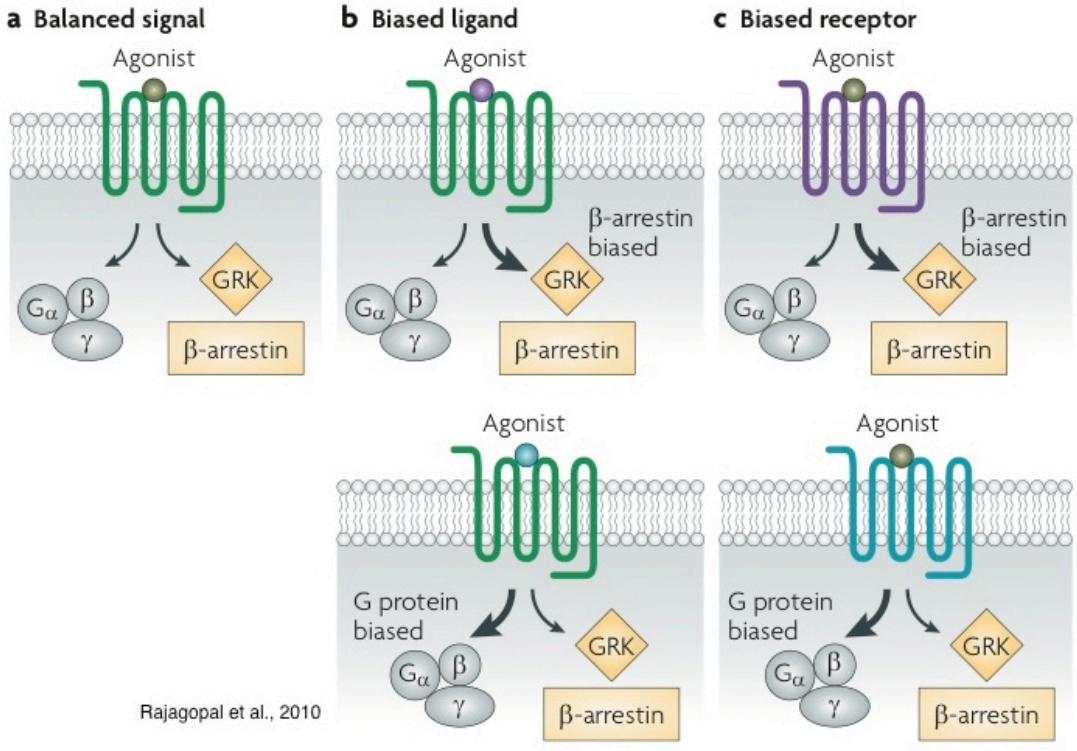

Figure 13. Biased signaling: Signaling through a G protein-coupled receptors (GPCRs) can be: A) balanced, such that it can activate the G protein-dependent and the arrestin-dependent signaling pathways equally, B) ligand-based bias towards either of the two signaling pathways depending, and C) receptor-based bias. The receptor-based bias involves a biased receptor that can make a balanced ligand signal with downstream signaling bias. Adapted from (Rajagopal *et al.*, 2010)

2.4 Receptor theory models

2.4.1 Classic receptor theory model

The classical model of receptor activation states that receptors exist in an inactive state R, which when bound to a ligand, L produces a binary complex LR. As shown in figure 14A, the binary complex may activate downstream effectors if the ligand L is an agonist and will not activate the downstream effectors if the ligand is an antagonist (Costa *et al.*, 1989).

2.4.2 Two-state model of receptor activation

With the discovery of constitutively active or spontaneously active receptors that could signal in the absence of a ligand, the classic receptor theory model needed to be expanded. In addition, the classic receptor theory model failed to accommodate inverse agonists. It became necessary to add another state (conformation) of the receptor, R* which was constitutively active. This led to the proposal of the two-state model of receptor activation (Figure 14B). The two-state model of receptor activation posits, that receptors can exist in two states or conformations at equilibrium, the active conformation R* and the inactive conformation R. The active state R* is able to constitutively signal in the absence of a ligand. This model allows us to explain three types of ligands that can act on the system: agonists, inverse agonists and antagonists.

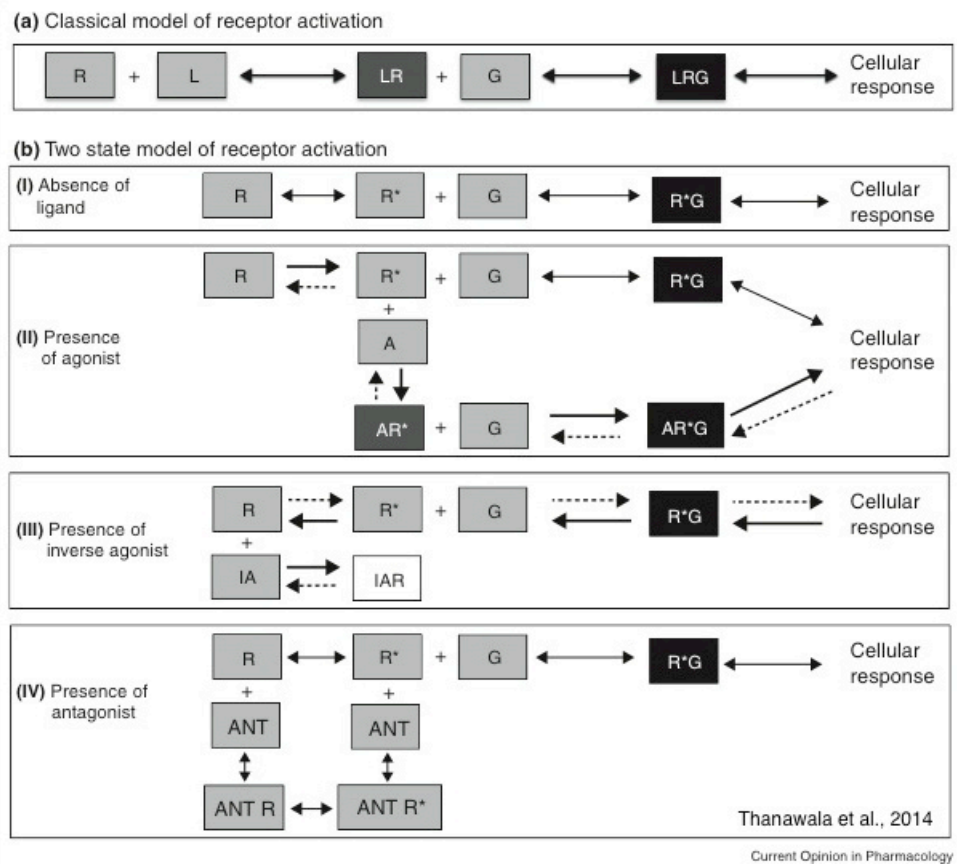

Figure 14: Classical and two-state models of receptor activation. A) This figure depicts that classical model of GPCR activation. Receptors exist in a single conformation 'R'. When R is activated by a ligand 'L' they form a binary complex 'LR'. If L is an agonist, then LR has high affinity for the downstream signaling component 'G' and leads to the formation of the 'LRG' complex. The LRG complex can stimulate downstream signaling pathways. If L is an antagonist, the LR complex does not have affinity for G and downstream signaling is stopped. B) According to the two-state model of receptor activation, receptors can exist in two conformations, 'R' (inactive) and R* (active) at equilibrium with each other. (I) R* has affinity for G and In the absence of a ligand R* can spontaneously or constitutively signal via the binary complex R*G. (II) An agonist 'A' has higher affinity for R* than R and binds to R* to form the binary complex 'AR*', shifting the equilibrium away from R and towards R*. AR* also has high affinity for G and results in the formation of AR*G to elicit downstream signaling responses. (III) An inverse agonist 'IA' has higher affinity for R as compared to R* and results in the shift in the equilibrium towards R and away from R*. This shift in equilibrium results in reduction of the constitutive activity of R*. (IV) An antagonist 'ANT' has similar affinities for both R and R* and maintains the equilibrium. ANT can inactivate or reverse agonist or inverse agonist activities but does not affect the constitutive activity of R*. Adapted from (Thanawala *et al.*, 2014)

When an agonist, A is presented to the system, it has a relatively higher affinity for the active conformation R* and results in the formation of the binary complex AR*. This binary complex can activate downstream signaling effectors like G proteins. As a consequence of formation of AR*, the equilibrium shifts towards formation of more R* and away from R. Similarly, when an inverse agonist, IA is presented to the system, it has a relatively higher affinity for the inactive conformation R and results in formation of the binary complex IAR. In this case, the binary complex is inactive and results in the shift in the equilibrium away from the active conformation R* and towards R. An antagonist is 'neutral', ANT has similar affinities for both the receptor conformations R and R* and it forms two binary complexes ANT-R and ANT-R* respectively. This maintains equilibrium between the two conformations.

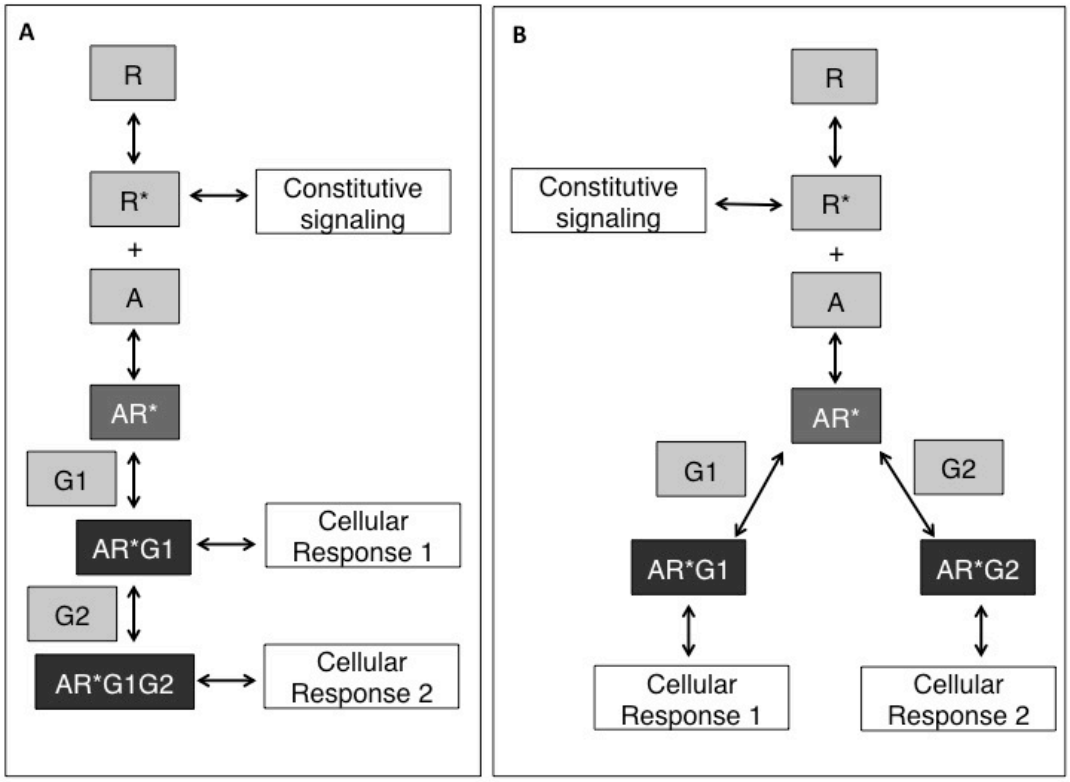

Figure 15. Multiple signaling pathways of a receptor explained using two-state model of receptor activation: If the receptor exists in two conformations R* (active) and R (inactive) at equilibrium, the addition of an alternative signaling pathway activated by the receptor can be explained by the two-state model of receptor activation under two conditions. A) Sequential activation: An agonist 'A' is added to the system and results in the formation of the binary complex 'AR*' with high affinity for a downstream signaling molecule 'G1'. AR* bind to G1 to form the complex 'AR*G1'. AR*G1 further has high affinity for another downstream signaling effector G2 such that they form the complex AR*G1G2. AR*G1 and AR*G1G2 activate distinct signaling pathways. B) Consequent activation An agonist added to the system forms the binary complex AR*, such that AR* has affinity for two distinct downstream signaling molecules G1 and G2. Binding of AR* to G1 results in the formation of the complex AR*G1 and binding to G2 forms the complex AR*G2. AR*G1 and AR*G2 activate distinct downstream signaling pathways. The affinity of AR* for G1 and G2 may vary but will remain in the same ratio even with a change in the system.

It is important to note that while an inverse agonist can inhibit activity of constitutively active receptors by binding to the inactive conformation and reducing amount of active receptor by a shift in the equilibrium, an antagonist cannot inhibit constitutively active receptors since it does not affect the equilibrium. However, an antagonist can inhibit activities of both an agonist and an inverse agonist (Barker *et al.*, 1994; Chidiac *et al.*, 1994; Bond *et al.*, 1995; Leff, 1995). According to the classical receptor theory model, the antagonist 'blocks' or inactivates the receptor and prevents association with the agonist. However according to the two-state model of receptor activation, an antagonist binds to R and R* to draw the receptor states away from their interactions with the agonist or inverse agonist.

With addition of a second state of a receptor, the theoretical possibility of an infinite number of receptor conformations exists. However, addition of another (third, fourth or multi-state) receptor conformation needs to be warranted by data or experimental observations. Without this data, there is no need for additional complexity in the receptor activation model.

2.4.3 Three-state model of receptor activation

Recently, it has been shown that certain GPCRs can activate more than one signaling pathway. This addition of another pathway does not warrant

addition of another receptor state unless certain criteria are met. The classical or two-state model of receptor activation can still explain addition of another signaling pathway. The additional pathway may be a result of sequential activation of downstream effectors as shown in figure 15A, where activation of R* or LR* leads to activation of G1 which leads to activation of G2. Alternatively, the ligand L may have very high efficacy and the excess active receptors may be sufficient to activate an additional activation pathway, such that LR* can lead to activation of both G1 and G2 (Figure 15B). Similarly, LR, the binary complex in classic receptor theory, can lead to activation of G1 and G2 either individually or sequentially. This explanation for 'strength of signal' by Kenakin however, could not explain change in rank order of agonist potencies (Kenakin, 1995). If it can be shown that two pathways G1 and G2 are independently activated by the receptor such that it is possible to observe potency reversals then an additional active receptor conformation is required to be able to explain the activation.

Potency reversal is a change in the potency order of activation of the two pathways activated by a ligand via a receptor. For example, if a ligand has higher potency for pathway G1 than for pathway G2 in a particular system when activated by a receptor but in another system has higher potency for G2 than for G1 then potency reversal has occurred and cannot be explained by classical or two-state receptor activation models. This is because if pathways G1 and G2

were either sequential or activated by the same receptor conformation, it would not be possible to observe this reversal of potencies even if the system conditions change because whatever factors would affect the activation of G1 will affect the activation of G2. However, if the two pathways are not sequential or activated by the same receptor conformation, then it will be possible to see potency reversal since some factor that affects G1 may not affect activation of G2 and vice versa. This would lead to the observation that while in system X, activation of G1>G2 but in system Y, G2>G1 and this could not be possible if G1 and G2 were activated sequentially or by the same receptor conformation.

In such an event, it becomes necessary to incorporate a modification (like addition of an active receptor conformation) to help explain the phenomena. This led to the postulation of the three-state model of receptor activation. This theory posits that receptors can exist in three distinct conformations, R (inactive), R* (one active) and R** (another active conformation). While, R is the inactive state, R* and R** can independently activate their own signaling pathways. However, another consideration needed to be addressed with addition of a receptor conformation. Activation of the pathway by R* could either affect activation of the pathway by R** or they could be independent of each other. This led to two types of systems that can be described with the three-state model of receptor activation: intact system (Figure 16) and isolated system (Leff *et al.*, 1997).

The intact system as the name suggests is a system where R, R* and R** all exist in equilibria with each other. A ligand that has affinity for and affects equilibrium between R and R* will also affect the equilibrium between R and R** and vice versa. It is important to note that while receptor states equilibrium will be affected, the downstream pathways still can be activated independently hence it is a three-state system (Leff *et al.*, 1997).

The isolated system on the other hand, distinguishes the two pathways to be independent of each other such that equilibrium between R and R* does not affect equilibrium between R and R** (Leff *et al.*, 1997). For this purpose, it may help to think of the isolated system of the three-state model as 2 individual two-state models of receptor activation. Even though they have a common denominator in the inactive conformation in R, they are independent of each other's equilibria. This can be theoretically explained by three possible explanations:

1) An infinite pool of R (Scaramellini *et al.*, 2002)
2) Two inactive states (with distinct but inactive conformations) of receptor
3) Compartmentalized pools of R with R* and R with R**

It is difficult to be able to eliminate or prove any of these probabilities. Since the rate-limiting step in activation of receptors is the concentration of downstream effectors, it may not be able possible to determine if there exists an infinite pool

of inactive receptors. Also, number of receptors in the inactive state cannot be determined because of the lack of readout; we cannot truly determine if there exist two inactive states. And it is difficult to distinguish if these receptor conformations can exist in the same cell type compartmentalized from each other.

As discussed previously, while the three-state model is sufficient to explain activation of two distinct signaling pathways, there exists a possibility of an infinite number of states that can exist. However, Occam's razor prevents us from adding more states unless it is warranted by experimental data that cannot be explained by classical, two-state or even three-state receptor activation models.

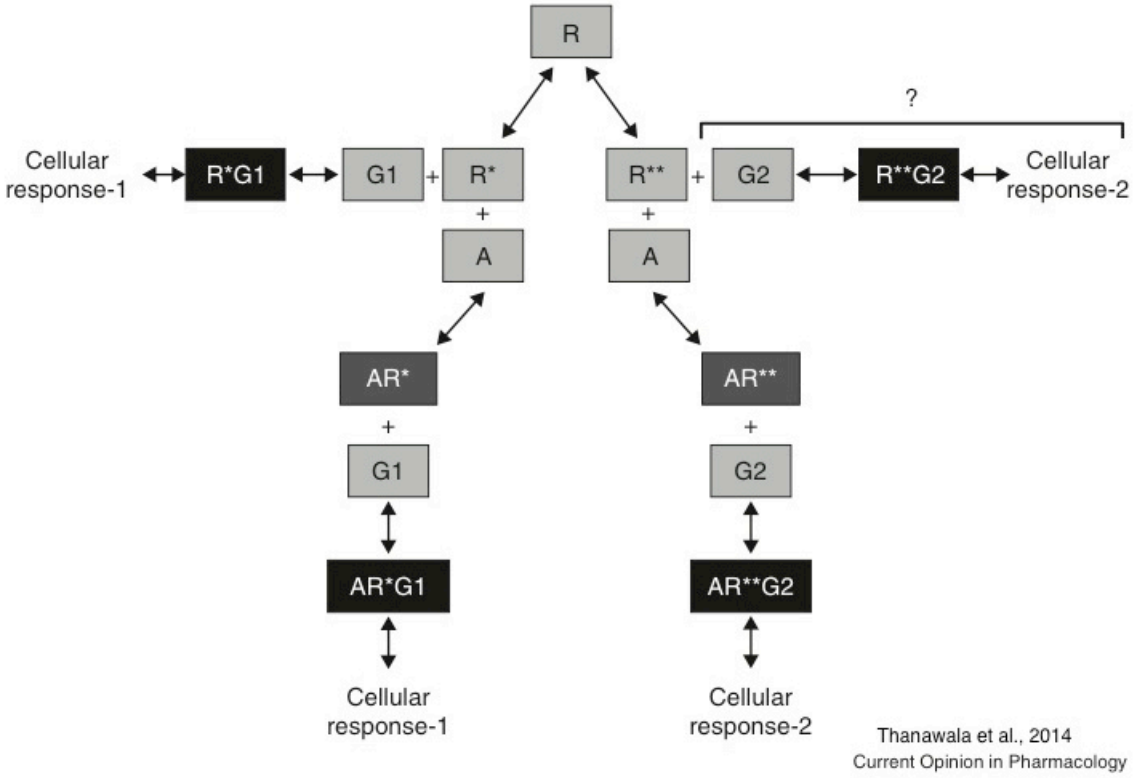

Figure 16. Three-state model of receptor activation (Intact system): Receptors can exist in three different conformations: R (inactive) and two active states (R and R**) such that R and R** can activate two different downstream cellular responses. R* activates cellular response-1 via G1 and R** activates cellular response-2 via G2. R* can activate cellular response-1 constitutively (left lateral arm of schematic). R** can also activate cellular response-2 (arrestin-mediated pathway) constitutively (right lateral arm of schematic), however, this has not been shown experimentally and has been denoted with '?'. When ligand 'A' binds to R* to form the binary complex AR* and activates cellular response-1 via G1 (left descending arm of schematic). 'A' can also bind to the other active conformation R** and form AR** that can activate cellular response-2 via binding to G2 (right descending arm of schematic). Note: This schematic is only a representation of the intact system in the three-state model of receptor activation. The three-state model has two modes of operation, the first where all equilibria are interconnected (intact) and the second where they are disconnected (isolated). The isolated system can be considered as two distinct two-state models. Also, the equilibria can change based on the ligands added to the system. Therefore, the schematic is not an accurate representation of the dynamic nature of the equilibria between the different states of the receptors and their binary or ternary complexes. Also, there is no experimental evidence of the existence of a direct equilibrium between R* and R** and is therefore excluded from this schematic. Adapted from (Thanawala *et al.*, 2014)

2.5 Background

2.5.1 Beta-blockers in heart failure

Beta-blockers had been contraindicated in therapy of CHF for decades. CHF is a condition that results from inability of the heart to meet the needs of the body. 'Congestive' refers to the increase in fluid accumulation or edema in peripheral tissues caused by insufficient circulation due to the failing heart. The contraindication for use of beta-blockers in CHF was rationalized because of the mechanism of action of beta-blockers. Beta-blockers inactivate the β adrenergic receptor and result in a reduction in inotropy and chronotropy of the heart. In a patient with CHF, the heart is already failing and administration of beta-blockers could further lead to a reduction in heart rate and force of contraction. The obvious rationale would be to use β adrenergic agonists to help increase heart rate and force of contraction. Acutely, as expected β adrenergic agonists were beneficial in increasing inotropy and chronotropy, however, with chronic use there was worsening of symptoms and increase in mortality (Weber *et al.*, 1982).

Alternative therapy for CHF included, digoxin, ACE inhibitors, diuretics etc. While, digoxin is beneficial in increasing cardiac contractility, its narrow therapeutic window poses a high risk of toxicity. ACE inhibitors and diuretics reduce blood volume subsequently reducing the stress on the heart but do not

affect the etiology of CHF. The therapy of CHF required innovative options to help increase inotropy and chronotropy without increasing mortality.

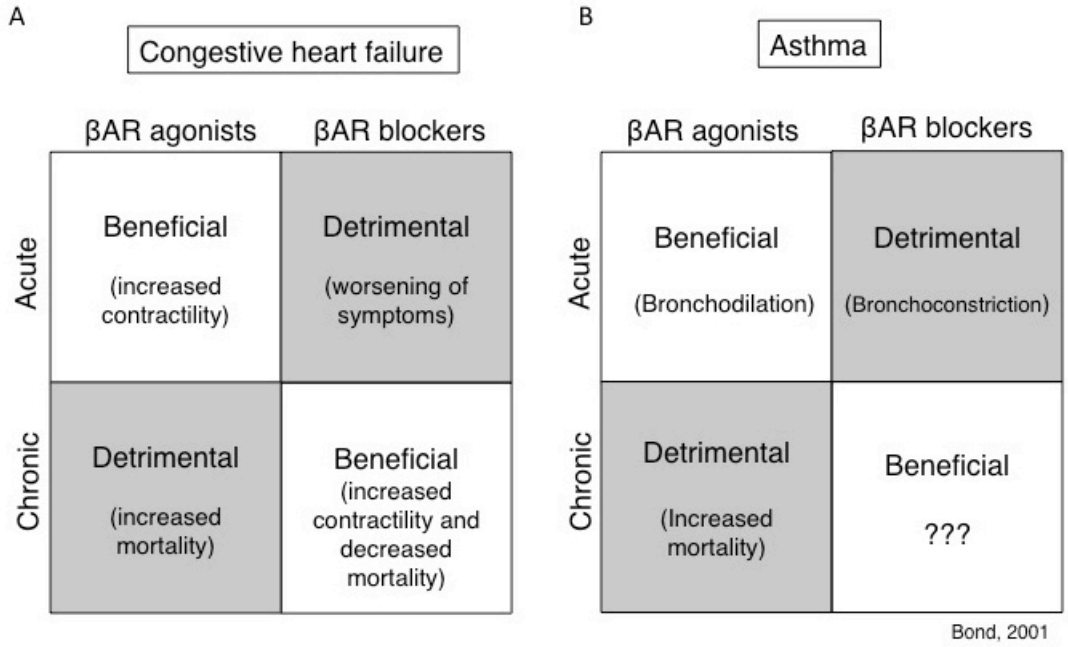

Figure 17. Relationship between acute and chronic treatment of congestive heart failure and asthma with β adrenoceptor agonists and β-blockers: A) In congestive heart failure (CHF), acute treatment with β adrenoceptor (βAR) agonists results in beneficial effects like increased cardiac contractility, however, chronic use of βAR agonists is detrimental and can lead to increased mortality. β-blockers were contraindicated in the therapy of CHF because acute treatment with β-blockers led to worsening of symptoms in CHF patients. However, chronic treatment with certain beta-blockers results in increased contractility and decreased mortality. B) Similarly, in asthma, βAR agonists acutely result in beneficial effects like bronchodilation, however, chronic use of βAR agonists is associated with increased mortality. Beta-blockers are currently contraindicated in asthma therapy because they can acutely result in bronchoconstriction. However, the effect of their chronic administration is still unknown. In comparison to CHF, βAR ligands respond similarly in three out of the four boxes, and there is murine and small clinical study data to show that the fourth box may also have similar results. Modified from (Bond, 2001)

While, acute treatment with beta-blockers led to a decrease in chronotropy and inotropy, studies showed that chronic administration of certain beta-blockers was beneficial in CHF (Figure 17A). Chronic carvedilol administration is now a first line of therapy for CHF. In addition, bisoprolol and metoprolol have also been approved for use in CHF (Packer *et al.*, 1996; 1999b; 1999a; Hjalmarson *et al.*, 2000). However, other beta-blockers like bucindolol, nebivolol and celiprolol have been ineffective in heart failure (Witchitz *et al.*, 2000; 2001; van Veldhuisen *et al.*, 2009; Mulder *et al.*, 2012). Patients were administered low doses of the beta-blocker during the start of the therapy and then doses are doubled weekly. Initial administration resulted in a reduction in the inotropy and chronotropy of the heart while, with chronic administration, the heart rate remained the same but the cardiac contractility increases (Hall *et al.*, 1995; Bristow *et al.*, 1996). The mechanism of action of the beta-blockers that results in their beneficial effect in CHF has not been elucidated but there are various theories that have been proposed (Wisler *et al.*, 2007).

2.5.2 Beta-blockers in asthma

Similar to beta-blockers in heart failure, beta-blockers have been contraindicated in the therapy of asthma. Asthma is a chronic inflammation of

airways and is characterized by bronchoconstriction. The rational therapeutic option is to administer β_2AR agonists. β_2AR agonists result in bronchodilation and help reverse the bronchoconstriction associated with asthma. Administration of beta-blockers would inactivate the β_2AR in the airways and result in worsening of the bronchoconstriction. Hence beta-blockers were contraindicated in therapy of asthma. While, β_2AR agonists were beneficial acutely, their chronic administration much like in CHF was associated with loss of control of asthma symptoms and an increase in mortality. As shown in figure 17B, we know the effects of acute administration of beta-blockers but what happens when beta-blockers are administered chronically? Does it resemble the fourth box in CHF (Figure 17B)?

Our previous studies using murine models of asthma showed the beneficial effects of chronic administration of certain beta-blockers in the therapy of asthma. We had tested acute and chronic administration of albuterol (salbutamol), alprenolol, carvedilol, and nadolol in murine models of asthma. Albuterol is a short acting β_2AR partial agonist and is used in 'rescue' inhalers. Acute administration of albuterol resulted in a reduction in the AHR, whereas with chronic administration, the beneficial effect was lost (Callaerts-Vegh *et al.*, 2004). We tested the beta-blockers, alprenolol, carvedilol and nadolol in this model as well. Similar to albuterol, alprenolol reduced AHR on acute treatment but resulted

in a loss of response with chronic treatment. Carvedilol resulted in worsening of AHR on acute treatment and on chronic treatment it resulted in a left ward shift in the curve. However, nadolol was the most promising, while it also worsened the AHR upon acute administration, chronic administration resulted in reduction in AHR (Callaerts-Vegh *et al.*, 2004). In addition, we have also shown that ICI-118, 551 and high dose of metoprolol on chronic administration resulted in a reduction in AHR (Lin *et al.*, 2008). Moreover, nadolol and ICI-118, 551 but not alprenolol result in a reduction in the inflammatory cell infiltration and mucus hypersecretion in a murine model of asthma (Nguyen *et al.*, 2009).

These studies led to successful small-center clinical trials using nadolol in mild-asthmatics. Hanania and colleagues have shown that in mild asthmatics, nadolol has a beneficial effect on the airway hyperresponsiveness (Hanania *et al.*, 2008). In addition, these patients also responded to short-acting β_2AR agonist: salbutamol that would be required in the event of an acute asthma attack. The bronchodilation by salbutamol alleviated concern that the beta-blocker nadolol would render β_2AR agonists ineffective during an asthma episode (Hanania *et al.*, 2010). This led to the approval of another multi-center clinical trial using nadolol in asthmatics that is currently underway (Clinical trail ID: NCT01804218). However, another clinical study done using a different beta-blocker, propranolol, failed to improve AHR in mild-asthmatics (Short *et al.*,

2013a; Short *et al.*, 2013b). This caused a controversy in the already controversial field of using beta-blockers in asthma (Bond, 2014; Kazani *et al.*, 2014; Lipworth *et al.*, 2014; Penn, 2014). The data from Short and colleagues showed that propranolol was ineffective in improving asthma in mild-asthmatics and drew an editorial (Kazani *et al.*, 2013). This study questioned the theory of using beta-blockers in asthma.

However, upon understanding the differences between nadolol and propranolol in terms of their signaling profiles via the β_2AR, it becomes clear that while, nadolol and propranolol are both classified as beta-blockers they are in fact different in terms of their signaling profiles. Comparison of the different beta-blockers as shown in figure 18, helps explain the discrepancy in their therapeutic efficacy. Nadolol, ICI-118,551 and metoprolol are inverse agonists at the G protein signaling pathway and blockers of ERK1/2 activation (Wisler *et al.*, 2007). Carvedilol and propranolol are inverse agonists at the G protein-signaling pathway but are partial agonists at ERK1/2 activation (Wisler *et al.*, 2007). Alprenolol on the other hand is a partial agonist at both the signaling pathways (Wisler *et al.*, 2007). Even though nadolol, propranolol, carvedilol, alprenolol, metoprolol and ICI-118,551 are grouped as beta-blockers there exist critical differences in their signaling profiles (Thanawala *et al.*, 2014).

The aim of this project is to unravel the role of G protein-mediated and arrestin-mediated signaling pathways and their effect on the asthma phenotype. Even with promising data from nadolol in small clinical trials there remain many unanswered questions about the efficacy of beta-blockers in asthma that this project aims to address.

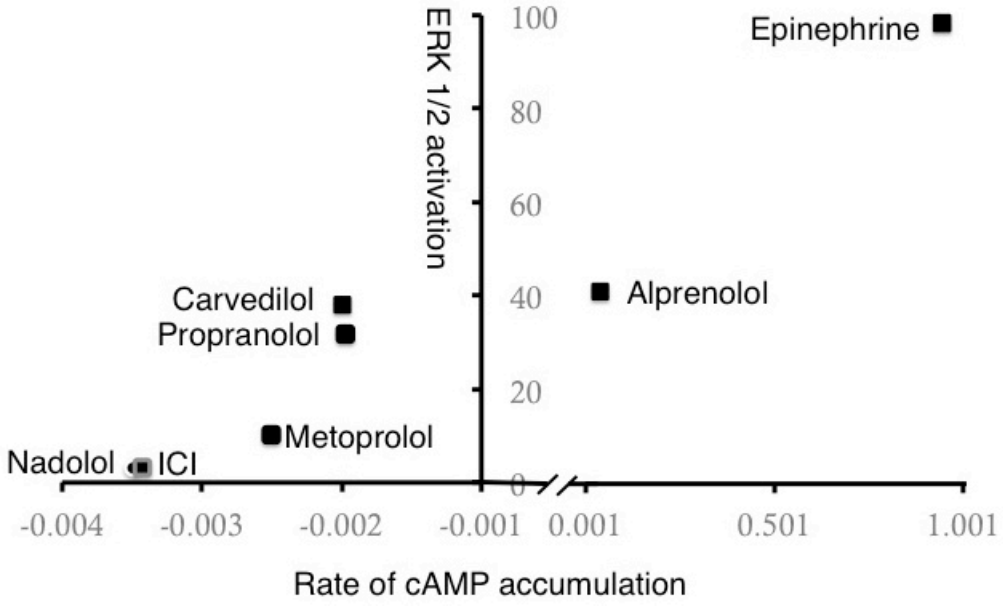

Figure 18. Signaling profiles of different βAR blockers at two signaling pathways: The figure is a representation of the data from the manuscript by Wisler and colleagues. It depicts the accumulation of cyclic adenosine monophosphate (cAMP) (representing the G protein signaling pathway) and activation of extracellular related-signal kinase (ERK1/2) (representing the arrestin-signaling pathway). Epinephrine is assigned as the balanced ligand with 100% activation of both signaling pathways at 100 (for ERK1/2 activation) and 1 (at cAMP accumulation). Alprenolol is a partial agonist at both pathways, Nadolol, ICI-118,551 and metoprolol are inverse agonists at cAMP accumulation and have little or no activation of the ERK1/2 pathway. Carvedilol and propranolol are also inverse agonists at the cAMP pathway but are partial agonists at ERK1/2 activation. Modified from the data by (Wisler *et al.*, 2007)

2.5.3 Requirement of β_2AR in asthma

Chronic treatment with certain beta-blockers has beneficial effects in murine models of asthma and in mild-asthmatic patients. However, we needed to understand the mechanism by which certain beta-blockers like nadolol were exerting their beneficial effects. Our previous study aimed to study if the effects of beta-blockers in asthma were indeed through β_2AR. In order to study the role of β_2AR, we used β_2AR knockout mice (β_2AR-KO). The β_2AR-KO mice exhibited significantly attenuated inflammatory responses and AHR compared to wild-type mice. These data indicated that the β_2AR was required in development of the asthma phenotype. Moreover, chronic administration of nadolol to the β_2AR-KO mice did not result in further alleviation of the asthma phenotype, indicating that the effect of nadolol was probably through its activity at β_2AR (Nguyen *et al.*, 2009).

However, it is important to note that while the β_2AR was required for development of the asthma phenotype, it was not required for the sensitization of the mice to ovalbumin in the Ova S/C model of asthma. We have previously shown that mice exhibited no difference in asthma phenotype when treated with beta-blockers during or after the sensitization phase of the Ova S/C model (Nguyen *et al.*, 2008).

3. Methods

3.1 Animals

Male and female mice aged 5-8 weeks were used in the studies. Stephen Ebert (University of Central Florida) generously gifted mating pairs of the epinephrine knock out (Epi-KO) mice. Epi-KO mice lack the enzyme phenylethanolamine N-methyltransferase (PNMT) to synthesize epinephrine from norepinephrine as shown in figure 19. These mice are referred to as PNMT-/- or Epi-KO mice. The Epi-KO mice used for the experiments were bred in-house. The Epi-KO mice were backcrossed with the Sv/129J strain (Ebert *et al.*, 2004). The wild type Sv/129J mice used in the studies were obtained from Jackson laboratories. The mice were housed under specific pathogen-free conditions and all the procedures and protocols were approved by the IACUC at the University of Houston.

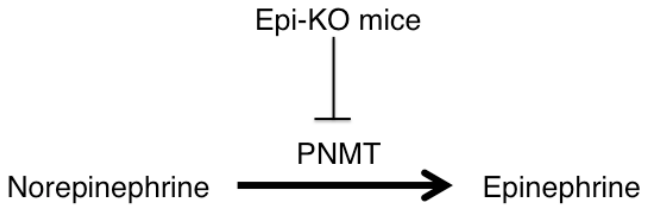

Figure 19. Epinephrine knock-out mice: Epinephrine is synthesized from norepinephrine by the enzyme phenylethanolamine N-methyltransferase (PNMT). The epinephrine knock-out (Epi-KO or PNMT-/-) mice have a disrupted gene for the enzyme PNMT, they cannot synthesize the enzyme PNMT. The lack of PNMT prevents the conversion of norepinephrine to epinephrine resulting the lack of epinephrine in these mice.

3.2 Genotyping

All mice used in the studies were genotyped to ensure disruption of the PNMT gene. In addition, all Epi-KO mice were phenotyped to ensure lack of circulating levels of epinephrine in their plasma. This measurement was done using high performance liquid chromatography (HPLC) as is described in the HPLC method below.

Genotyping was done by clipping ~ 3 mm of tail and digesting it using 300 μl of tail digestion buffer + 5 μl of proteinase K (Promega®) per tail at 55-60°C for ~16 hours, mixing intermittently. Protein was removed from the digested tails by addition of 100 μl protein precipitation solution. The DNA was isolated from the solution by addition of 300 μl isopropanol. The precipitated DNA was then washed with 70% ethanol and dissolved in PCR-grade water.

PCR was run with ~100 ng of DNA using the primers: forward 5' CAG GCG CCT CAT CCC TCA GCA GCC 3', reverse for wild type 5' CTG GCC AGC GTC GGA GTC AGG GTC 3' and reverse for knockout 5' GGT GTA CGG TCA GTA AAT TGG ACA CCG TCC TC 3' with an annealing temperature of 60°C and extension time of 1 min. DNA was run on a 1-1.5% agarose gel with a 1Kb ladder (Promega®) and control DNA in the presence of ethidium bromide. DNA was visualized using UV illumination. Knockout bands were observed at 100 bp and the wild-type bands were observed at 160 bp.

3.3 Murine asthma models

Two different models of asthma were used for the studies.

3.3.1 Ovalbumin sensitization and challenge

The primary model of asthma used in the studies was Ova S/C model of murine asthma (Figures 20A and 20B). Ovalbumin is chicken egg albumin and was obtained from Sigma Aldrich. The mice were sensitized to 2 mg/kg/day to ovalbumin with 2 mg of alum (Imject® alum, Thermo Scientific) in saline on days: 0, 7 and 14 via intra-peritoneal (i.p.) injections. At end of the study on days: 23-27 (Figure 20A) or 41 to 45 (Figure 20B), mice were subjected to an intra-nasal challenge with 1 mg/kg/day of ovalbumin in saline or saline alone for controls. At the end of the protocol, the mice were studied for three cardinal features of asthma; inflammatory cell infiltration, mucous metaplasia and airway hyperresponsiveness.

3.3.2 Protease-activated receptor ligand and ovalbumin sensitization and challenge

It has been previously shown that the PAR2 model of murine asthma has a worse asthma phenotype compared to the Ova S/C (Ebeling *et al*., 2005). This model is similar to the Ova S/C, with the difference in the challenge phase. The mice were sensitized to ovalbumin similar to the Ova S/C model, with three i.p.

injections of 2 mg/kg/day in 2 mg of alum on days: 0, 7 and 14. During the challenge phase on days: 41-45, the mice were challenged with 1 mg/kg/day of ovalbumin and 100 μM SLIGRL-NH_2 (Tocris Biosciences). At the end of the protocol, mice were characterized for inflammatory cell infiltration and mucous metaplasia (Figure 20C).

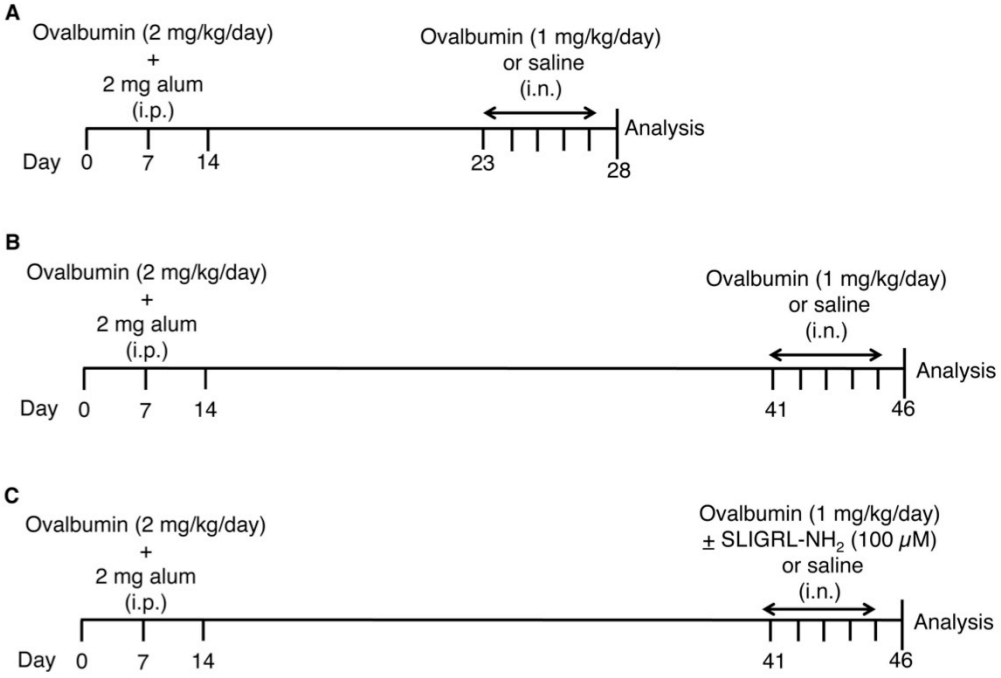

Figure 20. Schematic representation of the timeline of murine models of asthma: A & B) Ovalbumin sensitization and challenge protocols. Mice were sensitized intra-peritoneally on days: 0, 7 and 14 with 2 mg/kg/day ovalbumin and 2 mg alum. The mice were challenged on days: 23-27 (A) or days: 41-45 (B) with 1 mg/kg/day intranasal ovalbumin or saline (control). At the end of the protocol the mice were analyzed for the different asthma parameters. C) Protease-activated receptor type 2 (PAR2) model of asthma. The mice were sensitized to ovalbumin similar to A and B on days: 0, 7 and 14 but the challenged intranasally with 1 mg/kg/day ovalbumin with or without 100 μM PAR2 ligand SLIGRL-NH_2 or saline on days: 41-45. At the end of the study the mice were analyzed for the different asthma parameters.

3.4 Drugs treatments

3.4.1 Reserpine

Reserpine was used to deplete epinephrine from the mice. Reserpine acts by inhibiting the vesicular monoamine transporter (VMAT). VMAT is needed for the transport of catecholamines into the vesicles for storage and protection from degradation from monoamine oxidases. Reserpine inhibits VMAT, preventing the storage of catecholamines and leading to the degradation of the catecholamines by monoamine oxidases. Reserpine was dissolved in 4% ascorbic acid solution in saline and administered to wild type mice as shown in figure 21. A bolus dose of 5 mg/kg/day i.p. reserpine was administered to mice one day prior to the start of the challenge phase on day 22 followed by maintenance doses of 0.3 mg/kg/day i.p. on days 23-27 days. The plasma and adrenals of the mice were collected and catecholamine levels were measured using HPLC to ensure depletion of circulating epinephrine.

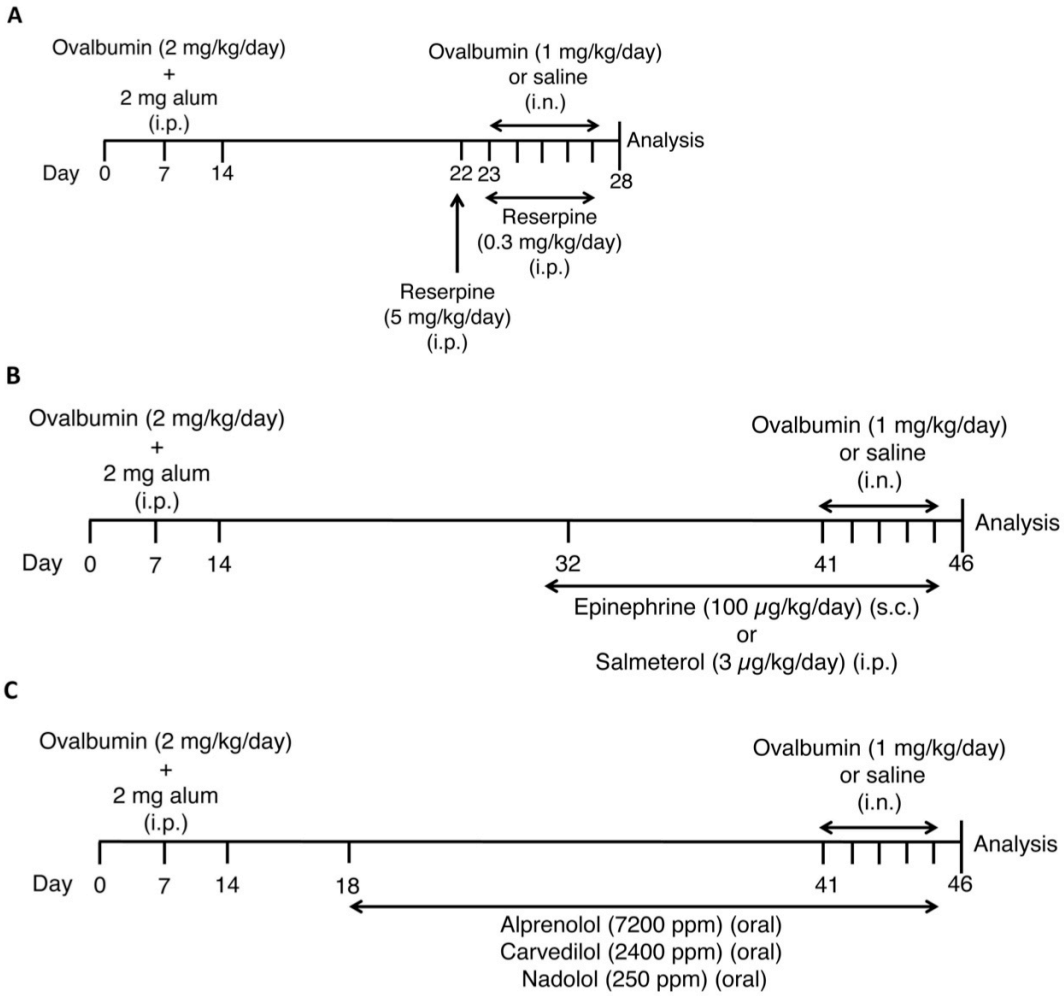

Figure 21. Schematic representation of treatment protocols in the murine asthma model: Mice are sensitized and challenged to ovalbumin as shown in figure 20. A) Reserpine administration. Mice received a bolus dose of 5 mg/kg/day of reserpine in 4% ascorbic acid on day 22 and 0.3 mg/kg/day of reserpine in 4% ascorbic acid on days: 23-27 intra-peritoneally. The mice were analyzed for the different asthma parameters on day 28. B) Administration of epinephrine and salmeterol. Mice received 100 μg/kg/day of epinephrine via osmotic pumps for 14 days. Salmeterol (3 μg/kg/day) was administered intra-peritoneally twice daily for 14 days. C) Administration of beta-blockers. Alprenolol (7200 ppm), carvedilol (2400 ppm) or nadolol (250 ppm) was administered to mice orally in powdered chow or propranolol (80-140 mg/ml) in water *ad libitum* for 28 days.

3.4.2 Epinephrine

In order to replace endogenous epinephrine in the Epi-KO mice, epinephrine was administered to Epi-KO mice using osmotic pumps (Alzet® 2004 pumps). Since epinephrine has a very short plasma half-life of 2-3 mins, sub-cutaneous infusion is a better route of administration than injecting epinephrine. Sub-cutaneous infusion of epinephrine allows the maintenance of a steady-state plasma level of epinephrine whereas injections every few minutes would still result in large fluctuations in plasma epinephrine concentrations.

The objective of administration of epinephrine was to replace the endogenous epinephrine in Epi-KO mice to the plasma level of epinephrine in wild-type mice. To obtain WT endogenous plasma levels of epinephrine, two different doses of epinephrine were titrated in Epi-KO mice. Epinephrine bitartarate (Sigma Aldrich) equivalent to 50 μg/kg/day or 100 μg/kg/day of epinephrine in 0.2% ascorbic acid was administered to Epi-KO mice. Epinephrine was dissolved in 0.2% ascorbic acid in saline to prevent oxidation. The solution was then bubbled with nitrogen for 15 minutes to reduce the amount of oxygen in solution, another measure to reduce degradation of epinephrine by oxidation. The solution was sterilized by filtration through a 0.45 μm filter. Any microbial contamination could contribute to oxidation and/or interfere with the osmotic

pump's function by plugging the pores of the osmotic membrane. The pumps were filled as per the manufacturer's instructions.

Even though extensive measures were taken to ensure minimal degradation of epinephrine, epinephrine is highly prone to degradation by oxidation and is also sensitive to light. At the end of 14 days, an average of 20% of epinephrine was degraded. Based on literature, and after measuring the amount of non-degraded epinephrine left in the osmotic pump after 14 days, epinephrine infusion was done for 14 days (Figure 21B) (Terres *et al.*, 1989; Khasar *et al.*, 2003; Khasar *et al.*, 2005).

The pumps were implanted in Epi-KO mice, under isoflurane anesthesia. The mice were prepared for surgery by inducing anesthesia using an isoflurane chamber. The surgical site was shaved, sterilized with 70% ethanol and swabbed with Betadine®. A small incision ~6-10 mm was made near the neck on the dorsal side of the mouse and the subcutaneous layer was separated from the skin by using tissue-separating scissors. The osmotic pump was inserted subcutaneously on one side of the spine. The incision was sutured using Autoclips® and mice were given analgesic and antiseptic treatment until the incision healed.

The plasma and adrenals from the mice were collected after euthanasia and the epinephrine and norepinephrine levels were determined using the HPLC method as described below.

3.4.3 Salmeterol

Salmeterol is a β_2AR agonist currently used in the therapy of asthma in combination with a corticosteroid. Salmeterol has a long duration of action of ~12 hours and was administered i.p. twice a day for 14 days (Figure 21B). The duration of treatment was based on the duration of treatment of epinephrine. Salmeterol xinafoate (Sigma Aldrich) was dissolved in 0.05% methanol and 99.95% saline. Mice were administered 1.5 μg/kg (approximately 100 μl) twice a day for 14 days. This dose of salmeterol was based on literature and to achieve only replacement of endogenous signaling at the β_2AR and not for therapeutic efficacy (Maris *et al.*, 2004; Singam *et al.*, 2006; Riesenfeld *et al.*, 2010; Qian *et al.*, 2011)

3.4.4 Alprenolol, Propranolol, Carvedilol and Nadolol

Alprenolol, propranolol, carvedilol and nadolol are classified as β-blockers. Alprenolol (Santa Cruz Biotechnology Inc.), propranolol (Sigma Aldrich), carvedilol (Glenmark Generics Inc.) and nadolol (Sigma Aldrich) were

administered for 28 days to achieve chronic administration in the mice. Mice had access to 7200 ppm alprenolol, 2400 ppm carvedilol or 250 ppm nadolol mixed in powdered chow *ad libitum* for 28 days (Figure 21C). The doses of alprenolol, carvedilol and nadolol were determined based upon doses used in mice and our previous studies (Callaerts-Vegh *et al.*, 2004; Nguyen *et al.*, 2009). Regular diet rodent powdered chow was triturated with the appropriate beta-blocker and then added to J-feeders in the mouse cages. The food intake was monitored daily through the duration of treatment to ensure ~5 g of food intake per mouse per day.

Propranolol was administered in water for 28 days based on 2-4 ml/day consumption of water. Mice had free access to 80-140 mg/ml of propranolol in water. The doses of propranolol were determined based on previous mouse studies (Knowles *et al.*, 2001)

3.5 Broncho-alveolar lavage

At the end of the treatment protocols (day 28 or 45), mice were euthanized with 100 mg/kg i.p. pentobarbital sodium. The pentobarbital sodium solution for euthanasia was prepared as per the UH-IACUC guidelines. After the mice were euthanized, the chest cavity was opened and the trachea was cannulated with an 18G luer stub adapter. Left lobe of the lung was isolated using a hemostat and

the right lung lobe was lavaged with 400-500 μl of sterile saline to obtain the broncho-alveolar lavage fluid (BALF). BALF was then used to count the total number of cells in the airways using a hemocytometer. 10 μl of BALF from each mouse was placed on the hemocytometer and standard procedure for counting cells from four chambers of the hemocytometer was followed. The cell count obtained was multiplied by a factor of 10^4 to obtain the total number of cells in the BALF per ml.

The remaining BALF was spun onto a charged slide using Cytospin®. BALF on the slides was then stained with Wright-Giemsa stain (Sigma) to allow visualization of the different granulocytes. Using light microscopy, the differential leukocyte count was obtained for all BALF samples. Since Ova S/C is an eosinophilic model of asthma, number of eosinophils was determined from 5 random fields at 40X as a percentage of the total cells in each field. Eosinophils can be identified by red staining of the cytoplasmic granules as opposed to blue granules in basophils and colorless granules in neutrophils. The total number of eosinophils was calculated using the percentage of the known total cell count for each sample and expressed as eosinophils/ml.

3.6 Mucous metaplasia

3.6.1 Perfusion of lungs

After obtaining BALF from the right lung lobe, the hemostat isolating the left lobe was removed. Both lobes of the lungs were then perfused with cold 4-10% formalin (Sigma Aldrich) via the cannula. The cannula was then removed and the trachea was tied up with a suture. The lungs and heart were then isolated from the thoracic cavity to maintain the perfused state of the lungs and kept in formalin for 24 hours. After 24 hours, a sharp blade was used to remove the top and bottom of the left lobe of the lung. The remaining lobe was sectioned transversely to obtain two halves. The two halves of the left lobe were then encased in plastic cassettes, dehydrated and perfused with paraffin. The sections were then embedded in paraffin blocks ready for sectioning.

3.6.2 Sectioning and staining

Each block was used to obtain three-5 μm sections of both sections of the lung using a microtome and the sections were then transferred onto a charged slide. The sections were stained with periodic acid fluorescent Schiff's (PAFS) stain.

The sections were first de-waxed with histoclear or xylene and then rehydrated using a series of ethanol dilutions, 100%, 95%, 80%, 70% and

phosphate-buffered saline. Once the slides were rehydrated, they were oxidized using 1% periodic acid to oxidize alcohols on the glycoproteins to aldehydes or ketones. Following oxidation, the slides were incubated in fluorescent Schiff's reagent for 20 mins at room temperature. After staining, the slides were washed with distilled water and rinsed in acid alcohol. The sections were then mounted with cover slips using a Canada balsam mounting medium (Piccotti *et al.*, 2012).

3.6.3 Imaging

PAFS stain stains the glycoproteins and parenchymal cells such that when excited using TritC or texas red filter (emission peak 628 nm), the glycoproteins emit red fluorescence and GFP excitation results in green emission (peak 531 nm) from parenchymal cells. This property of the PAFS stain can be used to perform a morphometric analysis of mucin content in the airways. Mucin is a glycoprotein as described previously and emits red fluorescence with PAFS staining. This mucin is qualitatively analyzed from 6 random images taken after TritC or texas red excitation (Piccotti *et al.*, 2012).

3.6.4 Quantification

The mucin volume density from the images was morphometrically determined by calculating the area of mucin/glycoprotein in the epithelium per

length of the basement membrane using ImageJ. The images were scaled based on the magnification as indicated by the scale bar during image acquisition (Kim *et al.*, 2008; Piccotti *et al.*, 2012). The mucin volume density was determined using the formula:

Mucin volume density = (MA) / (LBM)(4/π)

Where,

MA- mucin area

LBM- length of the basement membrane

3.7 Airway hyperresponsiveness

Airway hyperresponsiveness to increasing doses of methacholine was measured using the forced oscillation technique on the Flexivent® (Scireq, Montreal, Canada) with an in-line nebulizer. Airway hyperresponsiveness of an refers to the hyper-reactivity of an asthmatic airway to a bronchoconstrictor like methacholine when compared to normal airways. Mice were anesthetized with a mixture of ketamine (240 mg/kg) and xylazine (48 mg/kg) and booster doses administered as needed. The trachea was cannulated using an 18G cannula. Tube calibration was done before each subject using the respective cannula. The airway calibration for 0 mm and 300 mm of mercury was done using a sphygmomanometer attached to a Y-tube. The anesthetized mouse was

attached to the Y-tube via the cannula and artificially ventilated at 150 breaths per minute. The EKG leads were attached to the limbs of the mouse to monitor heart rate. If heart rate fell below 40 bpm, the mouse was considered dead for the purpose of the experiment. The mouse was maintained on a water-heated pad to avoid hypothermia due to anesthesia during the entire procedure.

The perturbations were started approximately after 4 mins of regular ventilation with saline nebulization as a baseline measurement followed by increasing doses of methacholine (1, 2.5, 10, 25 and 50 mg/ml). The positive end-expiratory pressure (PEEP) was set at 2-4 cm of Hg. Each dose was administered for 15 secs via the in-line ultrasonic nebulizer (Aeroneb®) and respiratory resistance was measured every 10 secs for the 3 mins. The dose was followed by two deep sighs to inflate the lungs and avoid any residual effects of the previous dose on the following dose.

The airway resistance was calculated by averaging the three peak resistance values for each dose. The airway sensitivity (PC100) and reactivity (K) were calculated by a non-linear regression analysis of the dose-response curve. PC100 refers to the provocative concentration that is required to double the baseline resistance, or a 100% increase in baseline and obtain the airway reactivity K.

3.8 High performance liquid chromatography

High performance liquid chromatography (HPLC) coupled with coulometric detection was used to determine amounts of catecholamines in the plasma and adrenals of the mice. Plasma and adrenals from the mice were collected post-mortem and stored at -80°C. Adrenals were collected, cleaned from fat tissue and homogenized in 0.2N perchloric acid. Plasma was separated from the blood collected from descending aorta of the mice. Both samples were adsorbed on alumina using an extraction buffer (1.5M Tris HCl, 0.1% EDTA, pH 8.6). The adsorbed catecholamines were eluted into 0.2N perchloric acid and filtered through 0.45 μm syringe filters prior to injection.

The binary 1525 HPLC system from Waters® (Milford, MA) was used with a 3μ C-18 LUNA® (Phenomenex®, Torrance, CA) column coupled with a Coulochem III detector (ESA, Thermo Scientific, Sunnyvale, CA). The software system Breeze® v3.30 (Waters) was used for the data collection and analysis. The catecholamines levels epinephrine and norepinephrine were measured by analyzing the peak area. Epinine was used as an internal standard to account for the recovery of the catecholamines. For the standard curves, epinephrine bitartarate (Sigma Aldrich), norepinephrine bitarate (Sigma Aldrich) and epinine hydrochloride (Pfaltz and Bauer Inc.) were used. The concentration range of the standard curve was between 50 pg/μl to 10 ng/μl.

3.9 Statistics

Statistical analysis was done using Graphpad Prism 4® (Graphpad, San Diego, CA). Data are expressed as mean $\pm$ S.E.M. Data were analyzed for normal distribution and Student's T-test was done to compare two groups and multiple groups were compared using one-way ANOVA with Dunnett's multicomparison test. For data that did not follow normal distribution, non-parametric statistics were done using Kruskal-Wallis test with Dunn's post-hoc analyses. Non-linear regression was done to obtain the PC100 and K values of AHR measurement. Statistical significance was considered at $p<0.05$.

4. Chapter 1

The requirement of epinephrine in development of the asthma phenotype in a murine model*

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4.1 Rationale

We have previously shown that the β_2AR is required for development of the asthma phenotype in murine models of asthma (Nguyen *et al.*, 2009). In addition, chronic β_2AR inverse agonists like nadolol but not antagonists like alprenolol attenuate the asthma phenotype in murine models of asthma (Callaerts-Vegh *et al.*, 2004; Nguyen *et al.*, 2009). According to the two-state model of receptor activation, while antagonists and inverse agonists can inactivate the agonist-activated receptor, only inverse agonists can inactivate constitutively active receptors (Bond *et al.*, 1995; Leff, 1995).

4.2 Hypothesis and objective

Using the knowledge of the two-state receptor activation model and based on the requirement of inverse agonism for therapeutic efficacy, we formed the

hypothesis that, 'Constitutive activity of the β_2AR is required for development of the asthma phenotype in murine models of asthma.'

4.3 Approach

To test this hypothesis, we required a method able to distinguish between the ligand-activated and constitutively active β_2AR signaling. While epinephrine activates all 9 adrenoceptors, norepinephrine activates only 8 of the 9 adrenergic receptors. The only adrenergic receptor that norepinephrine cannot activate at endogenous concentrations is the β_2AR. Epinephrine is the only endogenous ligand of the β_2AR. If epinephrine is removed, the role of constitutively active β_2ARs could be studied for their role in development of the murine asthma phenotype, as there would not be any ligand-activated β_2AR signaling. In other words, by eliminating the epinephrine, we could study the effect of constitutive signaling in isolation from any ligand activated signaling for the β_2AR.

Two approaches were used to eliminate epinephrine and test this hypothesis:

4.3.1 Genetic method of epinephrine depletion

Epinephrine is synthesized from norepinephrine by means of an enzyme, phenylethanolamine N-methyltransferase (PNMT) (Figure 19). The genetic

method utilized mice PNMT null mice that are unable to synthesize epinephrine (herein termed Epi-KO mice). These PNMT null (Epi-KO) mice have been described in detail the 'animals' section of the methods (Ebert *et al.*, 2004).

4.3.2 Pharmacological method of epinephrine depletion

The pharmacological method used reserpine to eliminate sympathetic signaling. Reserpine administration results in the depletion of all catecholamines including epinephrine from the synaptic terminals. Reserpine inhibits vesicular monoamine transporter (VMAT). VMAT is responsible for the incorporation of monoamines (or catecholamines) into neuronal vesicles. Within the vesicles, the catecholamines are stored and protected from degradation by monoamine oxidases (MAO) or catechol *O*-methyltransferases (COMT) (Goodman LS, 2011). Administration of reserpine prevents storage of these catecholamines into the vesicles resulting in their degradation in the cytosol by MAO (Figure 22).

These two methods were used to study the role of constitutive β_2AR signaling in development of the asthma phenotype. Figure 20A is a schematic of the treatment protocol using Epi-KO and WT mice treated with reserpine to eliminate epinephrine activation of the β_2AR and test the role of constitutive β_2AR signaling. Briefly, mice were sensitized by i.p. administration of 2 mg/kg/day of ovalbumin and 2 mg of alum on days: 0, 7, and 14. The mice were then

challenged by once-daily intranasal administration with either 1 mg/kg/day ovalbumin (or saline for controls). Pharmacological depletion of epinephrine in WT (Sv/129J) mice was achieved by i.p. administration of reserpine. A 5 mg/kg loading dose was given on day 22, followed by five maintenance doses of 0.3 mg/kg on days 23 – 27. The mice were then analyzed for mucous metaplasia, inflammation, and AHR.

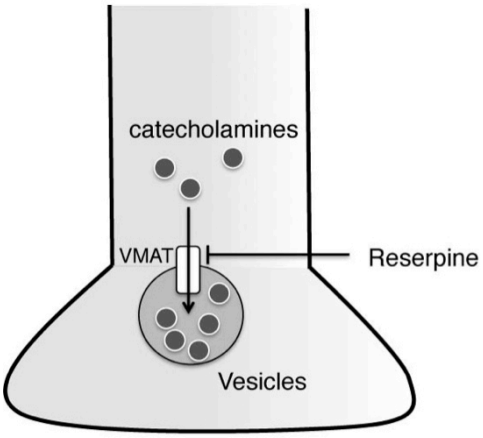

Figure 22. Mechanism of action of reserpine: Catecholamines are stored in neuronal vesicles to prevent degradation by cytosol monoamine oxidases. The vesicular monoamine transporter (VMAT) transports the catecholamines from the cytosol into the vesicles. Reserpine blocks the VMAT and prevents the transport of the catecholamines into the vesicles resulting in the degradation of the catecholamines.

4.4 Results

4.4.1 Catecholamine levels (HPLC)

The Epi-KO and reserpine-treated WT mice had no detectable levels of epinephrine (>50 pg/ μl) in their plasma (Table 4); and had very low levels of epinephrine in their adrenals compared to WT mice (Table 4). The norepinephrine levels in the plasma or adrenals of the Epi-KO and WT mice without reserpine administration were not significantly different from each other. However, reserpine administration in WT mice resulted in significant reduction in the norepinephrine levels in plasma and adrenals compared to WT control mice.

Table 4. Plasma and adrenal content of epinephrine and norepinephrine after genetic and pharmacological depletion of epinephrine

	Plasma (pg/μl)		Adrenals (ng/mg of tissue)	
	Norepinephrine	**Epinephrine**	**Norepinephrine**	**Epinephrine**
WT	166.94 $\pm$ 18.06	135.88 $\pm$ 18.87	233.64 $\pm$ 71.45	244.13 $\pm$ 50.86
WT Ova S/C	96.64 $\pm$ 25.80	107.93 $\pm$ 27.98	185.85 $\pm$ 59.33	217 $\pm$ 43.26
Epi-KO	153.11 $\pm$ 7.52	BLQ	326.97 $\pm$ 54.06	12.36 + 5.28
Epi-KO Ova S/C	86.91 $\pm$ 15.66	BLQ	374.12 $\pm$ 25.41	1.19 $\pm$ 0.37
WT + Reserpine	BLQ	BLQ	8.64 $\pm$ 1.55	6.48 + 2.31
WT + Reserpine Ova S/C	BLQ	BLQ	1.12 $\pm$ 4.85	5.86 $\pm$ 2.91

The table shows the norepinephrine and epinephrine levels in the mice measured using high performance liquid chromatography (HPLC) coupled with coulometric detection. WT- wild type mice (Sv/129J),Epi-KO- Epinephrine null mice, Ova S/C- ovalbumin sensitized and challenged mice and BLQ- below limits of quantification (50 pg/μl)

4.4.2 Inflammatory cell infiltration

The total number of cells and the number of eosinophils that infiltrated the airways were analyzed from the broncho-alveolar lavage (BAL) fluid of the right lung lobe of the mice. WT mice showed a significant increase in the total cells and eosinophils in the airways with Ova S/C compared to control mice (Figure 23A and 23B). Epi-KO and reserpine-administered WT mice on the other hand did not show any difference with or without Ova S/C in the total cell or eosinophil counts (Figure 23C and 23D).

4.4.3 Mucous metaplasia

Mucin production was morphometrically analyzed from sections of airway epithelium stained with periodic acid fluorescent schiff's stain. The epithelium and parenchymal cells emit green fluorescence (531 nm) and glycoproteins like mucin emit red fluorescence (628 nm) with TritC filters. PAFS is not specific for mucins alone as it can stain all glycoproteins. Epi-KO and reserpine-administered WT mice did not show any increase in mucin production in the airway epithelium with Ova S/C (Figure 24A-C). However, the WT mice showed an increase in mucin production with Ova S/C (Figure 24A-C).

4.4.4 Airway hyperresponsiveness

Airway hyperresponsiveness is a measure of hyper-reactivity of an asthmatic airway to methacholine compared to a normal airway. Here AHR was characterized by measuring three different parameters: peak airway resistance, airway sensitivity (PC100) and airway reactivity (K) using an invasive forced oscillation technique with FlexiVent® (Scireq, Montreal, Canada). An increase in peak resistance, increase in airway reactivity (high K value) and airway sensitivity (low PC100) indicates airway hyperresponsiveness. Epi-KO mice and reserpine-administered WT mice do not show any significant change in any of the three AHR parameters with Ova S/C (Figures 25A-F). However, WT mice show an increase in AHR measured by an increase in peak airway resistance, airway reactivity and a decrease in airway sensitivity with Ova S/C compared to control WT mice (Figures 25A-F).

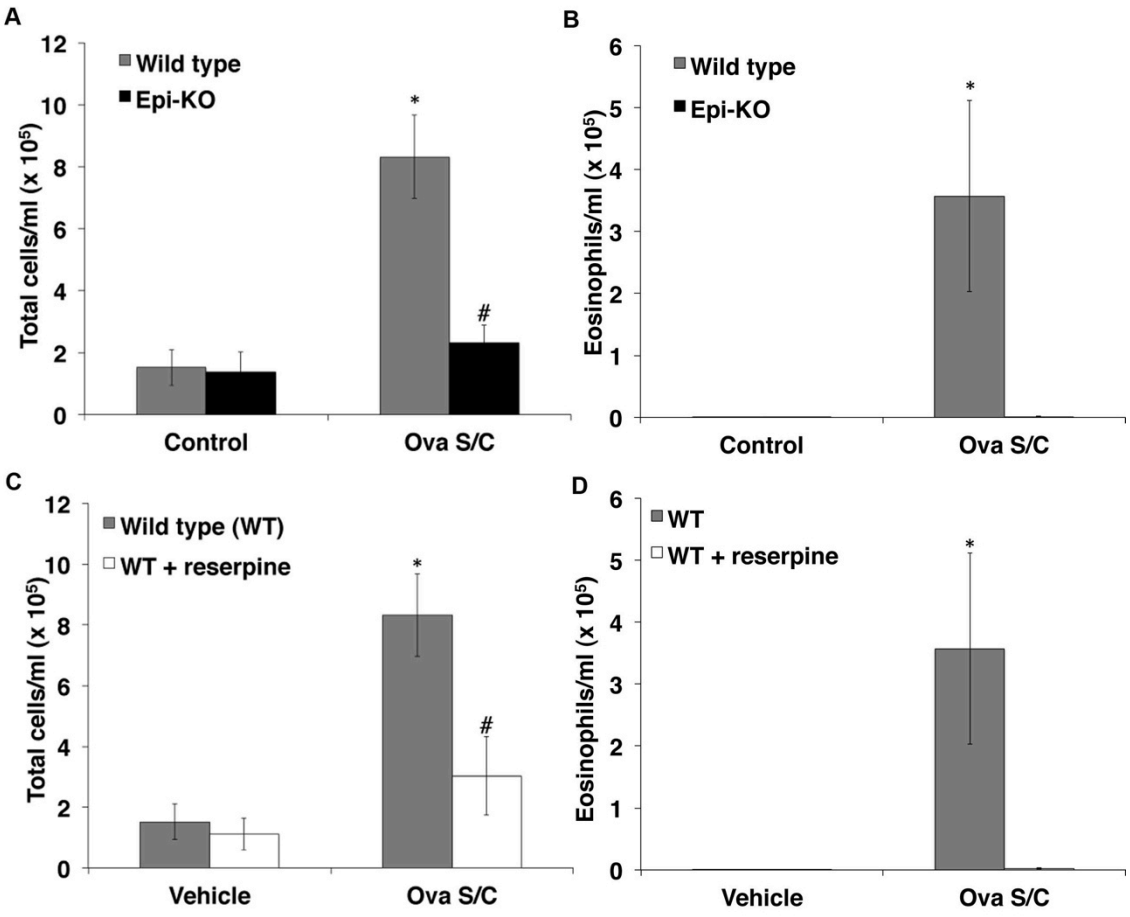

Figure 23. Epinephrine is required for the inflammatory cell infiltration in the ovalbumin sensitization and challenge (Ova S/C) model: The figure represents the total cells and eosinophilic infiltration into the murine airways measured from broncho-alveolar lavage fluid (BALF) after pharmacological or genetic depletion of epinephrine. Controls represent mice sensitized to ovalbumin and challenged with saline. A and B) The effect of genetic ablation. A) The total cell count in the BALF of Ova S/C wild type (WT) (grey) and epinephrine knock out (Epi-KO) (genetic ablation) (black) mice in comparison to control mice. B) The eosinophil count in the BALF of Ova S/C WT (grey) and Epi-KO (black) mice in comparison to control mice. C and D) The effect of pharmacological ablation. C) The total cell count in the BALF of Ova S/C WT (grey) and reserpine-treated WT (pharmacological ablation) (white) mice in comparison to control mice. B) The eosinophil count in the BALF of Ova S/C WT (grey) and reserpine-treated WT (white) mice in comparison to control mice. Data for each group represents mean $\pm$ SEM for n=3-7 .*$P<0.05$ compared to respective control mice. $^{\#}P<0.05$ compared to Ova S/C WT mice

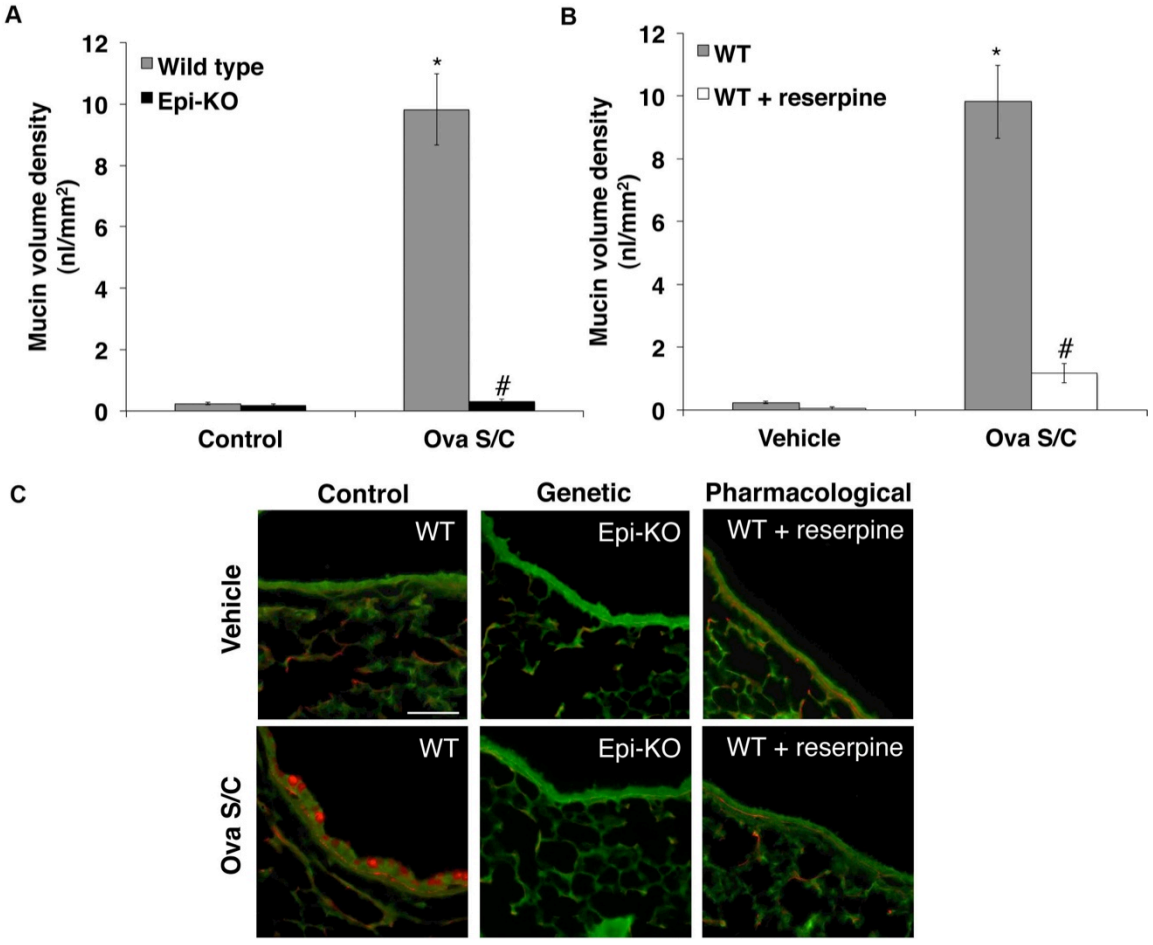

Figure 24. Epinephrine is required for mucin hypersecretion in the ovalbumin sensitization and challenge (Ova S/C) model: The figures represent mucin content (red) in the airway epithelium (green) measured after periodic acid fluorescent Schiff's (PAFS) staininng following pharmacological or genetic depletion of epinephrine. Controls represent mice sensitized to ovalbumin and challenged with saline. A) Morphometric quantification of mucin volume density for Ova S/C wild type (WT) (grey) and epinephrine knock out (Epi-KO) (black) mice in comparison to control mice. B) Morphometric quantification of mucin volume density for Ova S/C WT (grey) and reserpine-treated WT (white) mice in comparison to control mice. C) Mucin staining in the airway epithelium for Ova S/C and control WT mice with or without reserpine administration and Epi-KO. Scale bar (white) represents 100 μm. Data for each group represents mean $\pm$ SEM for n=3-7. *P<0.05 compared to respective control mice. $^{\#}$P<0.05 compared to Ova S/C WT mice

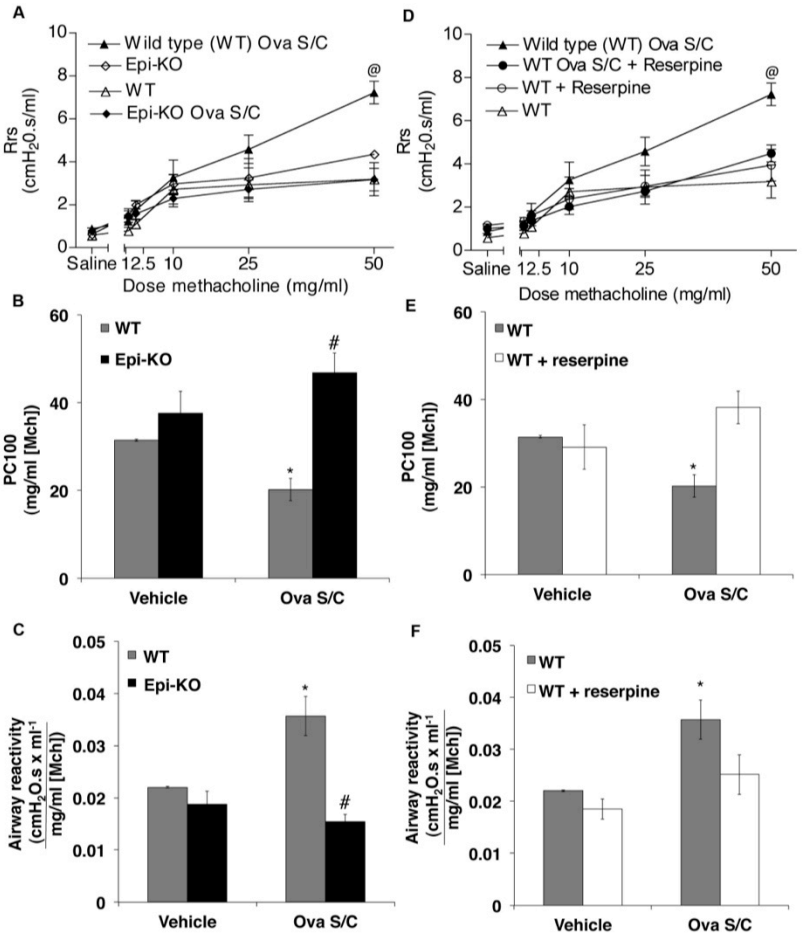

Figure 25. Epinephrine is required for airway hyperreponsiveness (AHR) in the ovalbumin sensitization and challenge (Ova S/C) model: Total respiratory system resistance (Rrs) to increasing doses of nebulized methacholine (Mch) (0–50 mg/ml) was measured using an invasive forced oscillation technique. Rrs was determined by averaging the three highest resistance responses for each mouse at each methacholine dose. A lower value for sensitivity (provocative concentration of methacholine that causes a doubling of baseline airway resistance [PC100]) and a higher value for reactivity to methacholine (K) indicates increased AHR. Controls mice were challenged with saline. A-C) Effect of genetic ablation. A) Dose-response curve for Rrs to increasing doses of MCh for Ova S/C wild type (WT) and epinephrine knock out (Epi-KO) mice in comparison to control WT and Epi-KO mice respectively. B and C) PC100 and K for Ova S/C WT and Epi-KO mice compared to controls. D-F) Effect of pharmacological ablation. D) Dose-response curve for Rrs to increasing doses of MCh for for Ova S/C WT and and reserpine-treated WT mice in comparison to control WT and reserpine-treated WT mice. E and F) PC100 and K for WT and reserpine-treated WT bars. Data for each group represents mean$\pm$SEM for n=3-7. @P<0.05 significant effect of Ova S/C on WT mice relative to other groups; *P<0.05 significance compared to respective control mice; #P < 0.05 significance of Epi-KO or reserpine-treated mice compared to respective WT mice.

Discussion

All of the long acting β_2AR agonists that have been used or are in use for asthma have been developed and designed using the bronchodilatory effects of epinephrine as a template. The data from this current set of experiments investigating the role of ligand-induced versus constitutive β_2AR signaling indicates that epinephrine is required for development of the asthma phenotype in our murine models. We have also shown that restoration of β_2AR signaling by administering the long-acting β_2AR agonist formoterol to Epi-KO mice also restored the asthma phenotype (Thanawala *et al.*, 2013). These data show the requirement for ligand-activated β_2AR signaling in development of the asthma phenotype. Formoterol was administered to the Epi-KO mice after the sensitization phase, indicating that β_2AR signaling played no role in the sensitization of the mice to ovalbumin.

To confirm that ablating PNMT or performing a pharmacological sympathectomy with reserpine depleted circulating epinephrine, HPLC analysis of the plasma and adrenal content of all mice was done (Table 4). Epinephrine can be synthesized from dopamine by conversion to norepinephrine and then methylated by PNMT as shown in figure 26. In addition, an alternate pathway for the synthesis of epinephrine has been shown *in vivo* involves the conversion of dopamine to epinine and then to epinephrine as shown in figure 26 (Laduron,

1972; Laduron *et al.*, 1974; Schumann *et al.*, 1976). The conversion of norepinephrine to epinephrine by PNMT is the major *in vivo* pathway for the synthesis of epinephrine. However, we wanted to ensure the disruption of the PNMT gene depleted circulating epinephrine. Plasma epinephrine levels were below detection in Epi-KO mice; and Epi-KO mice had significantly lower levels of epinephrine in the adrenals compared to WT mice. The epinephrine remaining in the adrenals of Epi-KO mice was possibly due to the contribution of the epinine pathway. However, the epinine pathway did not produce detectable circulating levels of epinephrine.

Norepinephrine levels in Epi-KO mice were not different from WT mice. We expected that the lack of epinephrine may result in an increase in the norepinephrine levels as a reflex mechanism to maintain overall sympathetic activity. However, there was no change in the levels of the norepinephrine. This could be explained by the feedback system in catecholamine synthesis. Tyrosine hydroxylase (TH) is the enzyme required for the synthesis of dopamine from tyrosine (Figure 26), and is the rate-limiting step in the catecholamine synthesis pathway. TH can be regulated by variety of factors ranging from genomic DNA, phosphorylation of the enzyme to catecholamine feedback. Discussing all these factors is beyond the scope of this thesis (Fujisawa *et al.*, 1989; Zigmond *et al.*, 1989; Kumer *et al.*, 1996; Dunkley *et al.*, 2004; Fujisawa *et al.*, 2005). However,

while catecholamine levels of dopamine help regulate the activity of TH, this is just one of several factors regulating TH activity. This could explain why there was no reflex increase in norepinephrine, in the absence of epinephrine. Moreover, the levels of dopamine are still able to regulate TH making the presence or absence of epinephrine inconsequential.

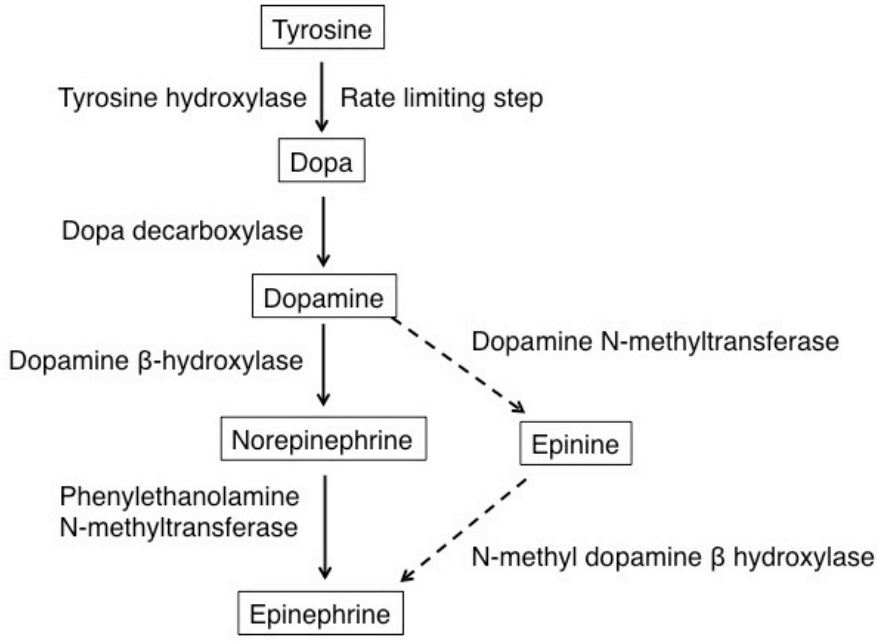

Figure 26. Major and alternate pathways for epinephrine synthesis: The flowchart illustrates the two pathways for the synthesis of epinephrine from tyrosine. Tyrosine is converted to dopa by the rate-liimiting enzyme tyrosine hydroxylase. Dopamine is synthesized from dopa by the enzyme dopa decarboxylase. Epinephrine can be synthesized from dopamine by two different pathways. The major pathway involves the synthesis of norepinephrine from dopamine by the enzyme dopamine β-hydroxylase followed by conversion of norepinephrine to epinephrine by phenylethanolamine N-methyltransferase. The alternate pathway involves the conversion of dopamine to epinine by dopamine N-methyltransferase followed by conversion to epinephrine by N-methyl dopamine β-hydroxylase.

Reserpine-treated mice lacked circulating epinephrine and norepinephrine compared to WT mice. The lack of circulating norepinephrine and epinephrine was expected because reserpine leads to depletion of all catecholamine and is not specific for epinephrine depletion. Reserpine acts on VMAT and prevents the incorporation of several catecholamines into the vesicles for storage and protection from metabolic enzymes and results in depletion of all catecholamines.

The effect of genetic and pharmacological depletion of epinephrine on the asthma phenotype was studied by measuring the three cardinal features of asthma. Depletion of epinephrine prevented the increase in inflammatory cell infiltration into the airways compared to WT mice with Ova S/C (Figure 23). Eosinophils form the majority of the cells that infiltrated the airways. Epinephrine depletion also led to a loss in the ability of the airway epithelium to undergo mucous metaplasia and produce mucus (Figure 24). These data suggest the importance of epinephrine in development of the inflammatory responses of the asthma phenotype. Mucus is made up a number of mucins; in mice the primary mucins are Muc5ac and Muc5b. In addition, depletion of epinephrine by either genetic or pharmacological means resulted in the loss of AHR compared to WT mice with Ova S/C. This data showed the importance of epinephrine in development of the hyper-reactivity to broncho-constrictors.

In the absence of epinephrine, by genetic or pharmacological methods, the mice were resistant to development of any of the cardinal features of the asthma phenotype. In addition, we have shown that restoration of β_2AR signaling by administration of formoterol in the Epi-KO mice results in restoration of the asthma phenotype (Thanawala *et al.*, 2013). It is ironic how the first widely accepted drug used in the therapy of asthma (epinephrine) is required for development of asthma, and a drug currently used in the therapy of asthma (formoterol) can restore the asthma phenotype in mice resistant to developing the asthma phenotype. These data further highlight the problems associated with the chronic use of β_2AR agonists in the therapy of asthma. Formoterol and salmeterol, the two long-acting β_2AR agonists in the market today carry 'black-box' warnings from the FDA. Studies comparing the chronic use of formoterol or salmeterol have shown an increased risk of severe adverse effects in clinical studies (Cates *et al.*, 2008; Cates *et al.*, 2012). The FDA has eliminated monotherapy with either formoterol or salmeterol. These drugs can only be used in combination with a corticosteroid, in patients where a corticosteroid alone is not sufficient for adequate asthma control. While, β_2AR agonists still remain the best drugs for rescue medication in the event of an asthma attack, their chronic use has been associated with loss of asthma control and mortality.

This current study shows that epinephrine or ligand-activation *was* required for development of the asthma phenotype. The data from the current experiments appears to be consistent with the findings that chronic use of long-acting β_2AR agonist is detrimental in asthma, and can explain the requirement of epinephrine in development of the asthma phenotype. However, the requirement for ligand-activation does not support our hypothesis that constitutive activity of the β_2AR was required for development of the asthma phenotype. Moreover, our results fail to explain why inverse agonists like nadolol but not an antagonist like alprenolol were effective in attenuating the asthma phenotype. To resolve the issue further understanding the complexities of β_2AR signaling was required.

Conclusion

Constitutive activity of the β_2AR is not sufficient, and ligand activation of β_2AR is required for development of the asthma phenotype in murine models of asthma.

5. Chapter 2

Biased signaling at the β_2AR in a murine model of asthma

5.1 Rationale

Our previous studies have shown the requirement of β_2AR in development of the asthma phenotype (Nguyen *et al.*, 2009). β_2AR activation can be constitutive or ligand-induced. In the previous chapter we used Epi-KO mice to show that ligand-activation of the β_2AR was required for development of the asthma phenotype in a murine model of asthma (Thanawala *et al.*, 2013). In addition, certain beta-blockers with inverse agonist properties such as nadolol are beneficial in murine asthma models compared to β_2AR antagonist such as alprenolol (Callaerts-Vegh *et al.*, 2004; Nguyen *et al.*, 2008; Nguyen *et al.*, 2009). Inverse agonists and not antagonists can inactivate constitutively active receptors. These data highlight a discrepancy; while constitutive activity of β_2AR was not required in development of the asthma phenotype, only certain inverse agonists were beneficial in murine models of asthma.

Beta-blockers are conventionally classified into inverse agonists and antagonists based on their ability to inhibit the constitutive activity of β_2AR via the canonical $G\alpha_s$ -adenylyl cyclase pathway. However, in addition to the activation of adenylyl cyclase via $G\alpha_s$ G protein leading to accumulation of cAMP, the β_2AR

can also activate alternate signaling pathways. One of the alternate signaling pathways of the β_2AR leads to activation of various MAPKs like ERK 1/2 via arrestin (Figure 27A). The addition of an alternate signaling pathway allows for the possibility of a variety of different signaling profiles by ligands that act on β_2AR. A ligand can now exhibit a 'bias' towards one of the two signaling pathways. The preferential activation of one pathway over another by a ligand is referred to as 'biased signaling'.

5.2 Hypothesis and objective

From the previous studies, it was evident that inverse agonism at the $G\alpha_s$ signaling pathway of the β_2AR was not enough for the beneficial effect of beta-blockers in murine asthma models. This required further understanding of β_2AR-signaling pathways. The objective of this study was to understand the role of the alternate signaling pathway: activation of MAPK by arrestin via β_2AR in development of the asthma phenotype.

Using arrestin-3 (βarrestin-2) knockout mice, Walker and colleagues have shown the requirement of arrestin-3 in development of the asthma phenotype. Arrestin-3 KO mice did not develop an increase in the asthma phenotype parameters compared to their respective WT mice in response to Ova S/C model of asthma (Walker *et al.*, 2003). In this study, we tested the hypothesis 'Activation

of ERK 1/2 (arrestin-mediated) via β_2AR by beta-blockers was required for development of the asthma phenotype.'

5.3 Approach

Mice depleted of the endogenous ligand for the β_2AR, epinephrine (Epi-KO mice or pharmacologic sympathectomy with reserpine), do not develop inflammatory infiltration, mucous metaplasia or AHR in response to Ova S/C compared to wild-type mice (Thanawala *et al*., 2013). Epi-KO mice provide a valuable tool to study β_2AR signaling in the absence of its endogenous ligand epinephrine, because Epi-KO mice have an empty β_2AR that can now be occupied by our choice of ligands. The signaling cascade and downstream signaling events observed by administration of ligand to an empty β_2AR, allows us to isolate the signaling pathways stimulated or inhibited by the ligand alone. The aim of this study was to test the role of ERK1/2 activation via β_2AR.

We used six different compounds with differing signaling profiles at the β_2AR to test the role of β_2AR activation of ERK1/2 in the Ova S/C model of asthma (Figure 27A-E):

- Epinephrine (100 μg/kg/day, s.c. via osmotic pump for 14 days)
- Salmeterol (3 μg/kg/day, i.p. for 14 days)
- Alprenolol (7200 ppm, orally in chow for 28 days)

- Propranolol (80-140 mg/ml, orally in water for 28 days)
- Carvedilol (2400 ppm, orally in chow for 28 days)
- Nadolol (250 ppm, orally in chow for 28 days)

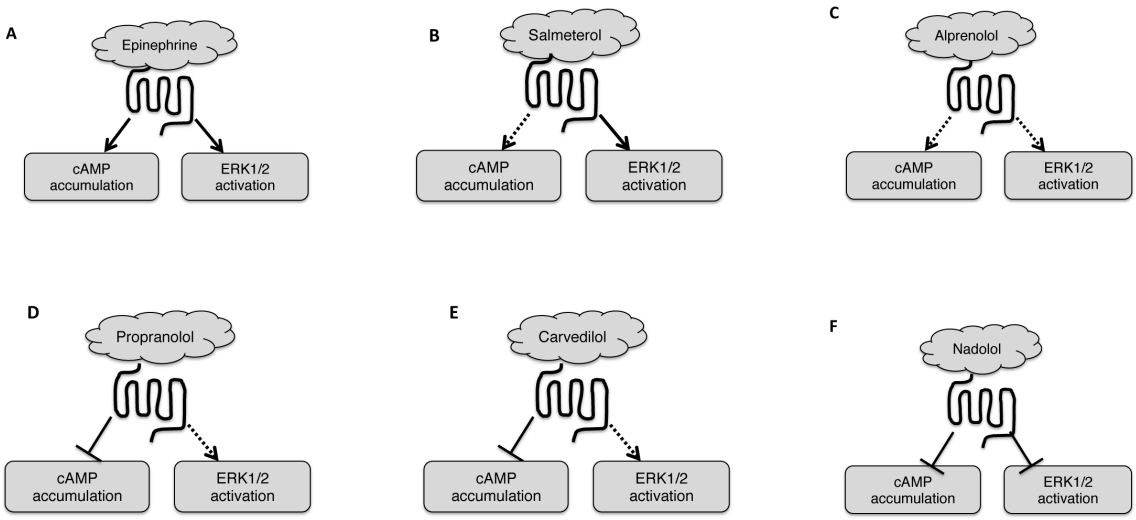

Figure 27. Signaling profiles of different β_2 adrenergic receptor ligands: The figures illustrate the signaling profiles of different β_2 adrenergic receptor (β_2AR) ligands. A) Epinephrine activates both the G protein-dependent cyclic adenosine monophosphate (cAMP) accumulation and the ERK1/2 activation pathways. Epinephrine is assigned as a full agonist for both the β_2AR signaling pathways. B) Salmeterol is a partial agonist for the cAMP activation pathway but a full agonist for the ERK1/2 activation pathway. C) Alprenolol is a partial agonist for both the β_2AR signaling pathways. D) Propranolol is an inverse agonist at cAMP accumulation but a partial agonist at the ERK1/2 activation pathway E) Carvedilol is an inverse agonist at cAMP activation but a partial agonist at the ERK1/2 activation pathway. F) Nadolol is an inverse agonist at both the β_2AR signaling pathways.

For canonical β_2AR activation, epinephrine and salmeterol are classified as agonists and alprenolol, propranolol, carvedilol and nadolol are classified as beta-blockers.

Epinephrine is the endogenous β_2AR agonist and by restoring β_2AR signaling in Epi-KO mice using epinephrine, the role of epinephrine in the asthma phenotype could be characterized completely. It was also important to test replacing epinephrine compared to the results of a WT mouse with endogenous epinephrine to see if deletion of the PNMT gene had other effects that could confound our results. Epinephrine also becomes a reference ligand to which all the other ligands are compared for their signaling profiles. Epinephrine is a full agonist at both pathways and by convention, is considered the standard ligand with no 'bias' towards either pathway. The existence of biased signaling by other ligands is measured or determined in comparison to epinephrine being considered as the reference ligand. As shown in figure 27A, epinephrine is assigned as a full agonist at the both the signaling pathways of β_2AR.

Salmeterol was the other β_2AR agonist chosen for the study. Salmeterol like formoterol has been shown to possess a bias for the ERK 1/2 signaling pathway of β_2AR compared to its activation of the cAMP pathway (Rajagopal *et al.*, 2011). As shown in figure 27B, salmeterol is also a partial agonist at the cAMP pathway and has an efficacy only about 10% of epinephrine. However, at ERK1/2

pathway, salmeterol has an efficacy about 80% of epinephrine (January *et al.*, 1998; Rajagopal *et al.*, 2011). This preferential activation defines salmeterol as 'biased' towards the activation of the ERK1/2 pathway compared to the cAMP pathway. The bias of salmeterol has been shown and discussed in detail by Rajagopal and colleagues (Rajagopal *et al.*, 2011). These properties made salmeterol an excellent choice to study the effect of the signaling bias towards ERK1/2 pathway in our study

Four beta-blockers with distinct signaling properties at the $G\alpha_s$-cAMP pathway and the arrestin-ERK1/2 were chosen for this study also using epinephrine as a reference ligand. Alprenolol, propranolol, carvedilol and nadolol were tested in the absence (Epi-KO mice) and presence (WT mice) of epinephrine in the Ova S/C model of asthma. Canonically, alprenolol is classified as an antagonist at the $G\alpha_s$-cAMP of β_2AR. However, in the absence of epinephrine, alprenolol acts a partial agonist at both the β_2AR signaling pathways as shown in figure 27C. In the presence of epinephrine, alprenolol becomes an antagonist because it 'appears' to antagonize the efficacy of epinephrine. Epinephrine has higher efficacy than alprenolol at the β_2AR cAMP accumulation. Therefore, co-administration (alprenolol to WT mice with endogenous epinephrine) of epinephrine and alprenolol results in a net efficacy at accumulation of cAMP via β_2AR, which is lower than the efficacy of epinephrine.

Propranolol is an inverse agonist at the canonical cAMP pathway but a partial agonist at ERK1/2 activation pathway (Figure 27D). Similarly, carvedilol is also an inverse agonist at the Gαs-cAMP pathway but a partial agonist at the arrestin-ERK1/2 pathway via β_2AR (Figure 27E). Propranolol and carvedilol are ERK1/2 biased ligands at the β_2AR. Propranolol and carvedilol become unique compounds to be chosen for this study because while they inhibit one pathway (cAMP), they activate the other pathway (ERK1/2). Also, propranolol has been recently studied in a pilot clinical study for the therapy of mild-asthmatics. This study showed that propranolol was not effective in lowering the FEV1 of the patients and compared the results to the study with nadolol in mild-asthmatics ((Hanania *et al.*, 2008; Short *et al.*, 2013b)

Nadolol is an inverse agonist at the $G\alpha_s$-cAMP pathway and does not activate the arrestin-ERK1/2 pathway (Figure 27F). Nadolol has been the most effective beta-blocker studied in murine and human asthma studies (Callaerts-Vegh *et al.*, 2004; Hanania *et al.*, 2008; Nguyen *et al.*, 2008; Hanania *et al.*, 2010).

All the 6 compounds were tested in the presence (WT mice) and absence (Epi-KO mice) of epinephrine; and the 3 cardinal features of asthma: inflammatory cell infiltration, mucous metaplasia and AHR were used to quantify

the asthma phenotype in the allergen-driven Ova S/C murine model of asthma (Figure 21B and 21C).

5.4 Results:

5.4.1 Effect of β_2AR ligands on inflammatory cell infiltration in absence and presence of epinephrine

The total number of cells infiltrating the airways was measured by using broncho-alveolar lavage fluid (BALF) from the mice. As seen in our previous study, Epi-KO mice did not show an increase in the number of total cells or eosinophils in the BALF with Ova S/C (Figure 28A and 28B) (Thanawala *et al.*, 2013). In Ova S/C Epi-KO mice, administration of epinephrine to restore the normal circulating level of plasma epinephrine caused an increase in the total cell and eosinophil count (Figure 28A and 28B). Restoring agonist signaling in Ova S/C Epi-KO mice with salmeterol did not cause a significant increase in total cells or eosinophils but it was also not significantly different from the restoration effect of epinephrine on total cells and eosinophils. However, administration of alprenolol, propranolol and carvedilol to Ova S/C Epi-KO mice resulted in significant increases in the total cell and eosinophil counts. Whereas, nadolol administration to Ova S/C Epi-KO mice did not result in any change from control Epi-KO mice.

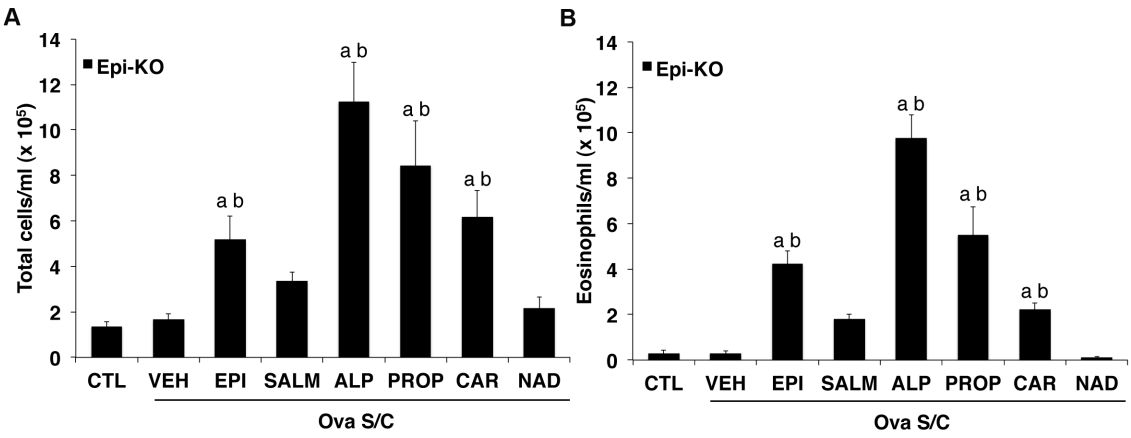

Figure 28. Effect of different β_2 adrenergic receptor ligands on inflammatory cell infiltration in epinephrine knock out mice: The graphs show the A) total and B) eosinophil cell counts in the broncho-alveolar lavage fluid (BALF) of epinephrine knock out (Epi-KO) mice. Epi-KO mice were administered epinephrine (EPI) (100 μg/kg/day s.c. for 14 days), salmeterol (SALM) (3 μg/kg/day i.p. for 14 days), alprenolol (ALP) (7200 ppm orally for 28 days), propranolol (PROP) (80-140 mg/ml, orally for 28 days), carvedilol (CAR) (2400 ppm orally for 28 days) and nadolol (NAD) (250 ppm orally for 28 days) on with ovalbumin sensitization and challenge (Ova S/C) compared to vehicle-treated Ova S/C (VEH). Control (CTL) mice were sensitized with ovalbumin and challenged with saline. Data represent mean $\pm$ SEM from 4-10 mice in each group. $^{a}P<0.05$ compared to CTL mice and $^{b}P<0.05$ compared to VEH mice.

In WT mice, Ova S/C produced a significant increase in the eosinophil infiltration and total cell count compared to control WT mice (Figure 29A and 29B). Administration of salmeterol and carvedilol to Ova S/C WT mice caused a significant increase in the total and eosinophil cell counts compared to control (sensitized to ovalbumin and challenged with saline) WT mice but was not different from Ova S/C vehicle-treated WT mice. Administration of alprenolol, propranolol or nadolol did not cause any increase in the total cell or eosinophil

counts compared to control WT mice and the alprenolol-, propranolol- or carvedilol-mediated effect on total cell and eosinophil counts was not significantly different from the vehicle-treated Ova S/C WT mice (Figures 29A and 29B). However, nadolol significantly reduced the total cell and eosinophil counts in vehicle-treated Ova S/C WT mice (Figures 29A and 29B).

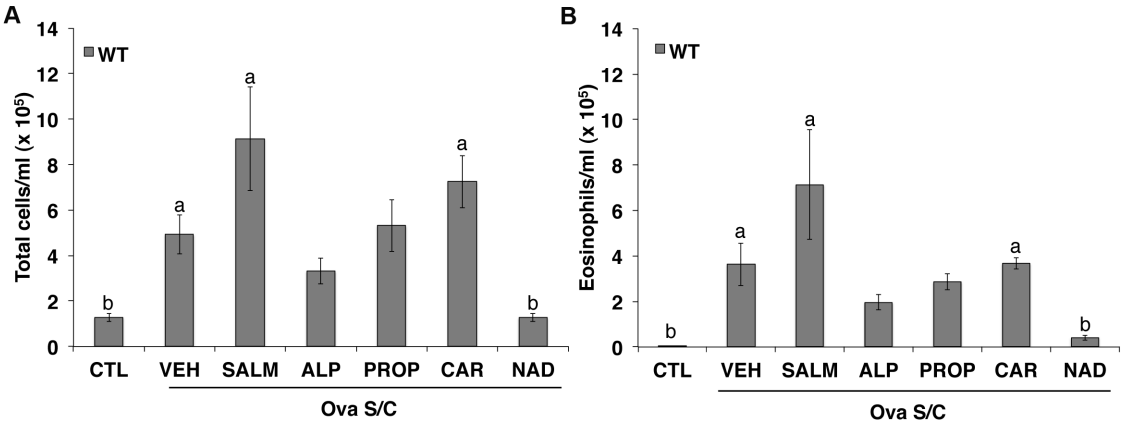

Figure 29. Effect of different β_2 adrenergic receptor ligands on inflammatory cell infiltration in wild type mice: The graphs show the A) total and B) eosinophil cell counts in the broncho-alveolar lavage fluid (BALF) of wild-type (Sv/129J) (WT) mice. WT mice were administered salmeterol (SALM) (3 μg/kg/day i.p. for 14 days), alprenolol (ALP) (7200 ppm orally for 28 days), propranolol (PROP) (80-140 mg/ml, orally for 28 days), carvedilol (CAR) (2400 ppm orally for 28 days) and nadolol (NAD) (250 ppm orally for 28 days) on with ovalbumin sensitization and challenge (Ova S/C) compared to vehicle-treated Ova S/C (VEH). Control (CTL) mice were sensitized with ovalbumin and challenged with saline. Data represent mean $\pm$ SEM from 4-10 mice in each group. $^{a}P<0.05$ compared to CTL mice and $^{b}P<0.05$ compared to VEH mice.

5.4.2 Effect of β_2AR ligands on mucin production in absence and presence of epinephrine

Epi-KO mice did not show an increase in mucin production with Ova S/C compared to control Epi-KO mice (Figures 30A and 30B). Restoration of agonist signaling in Ova S/C Epi-KO mice with either epinephrine or salmeterol did not result in significant restoration of mucin production (Figures 30A and 30B). Administration of alprenolol, propranolol or carvedilol to Ova S/C Epi-KO mice, resulted in about significant increases in the mucin volume density compared to control Epi-KO mice (Figures 30A and 30B). However, administration of nadolol to Ova S/C Epi-KO mice showed no significant changes in mucin volume density from control or vehicle-treated Epi-KO mice (Figures 30A and 30B).

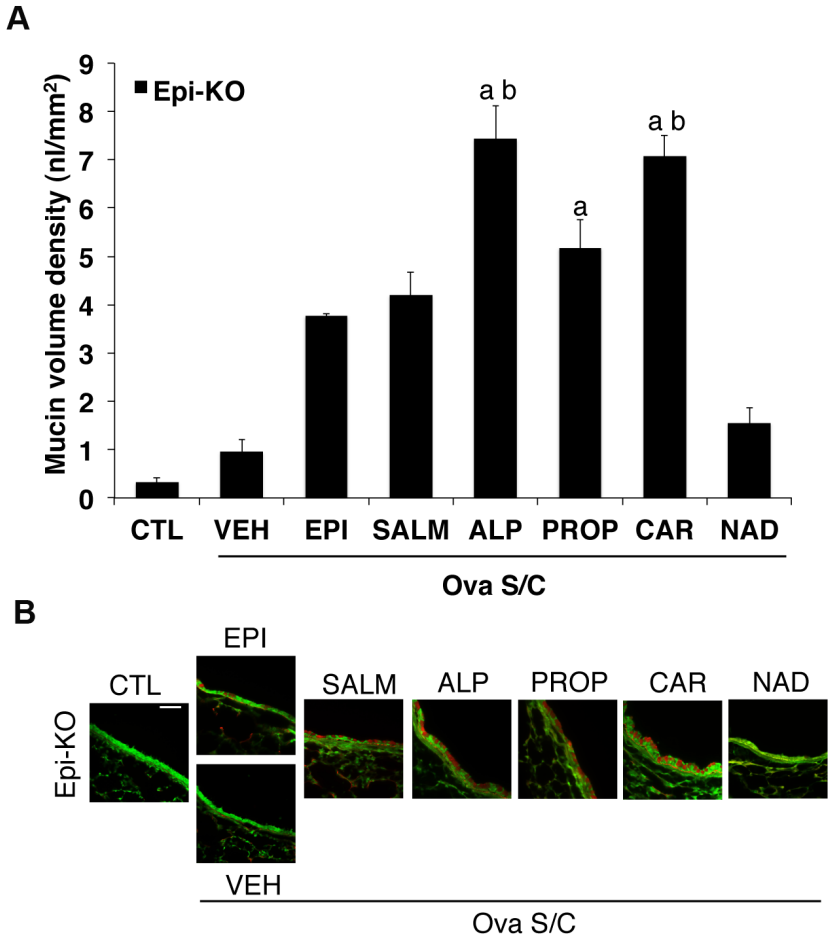

Figure 30. Effect of different β_2 adrenergic receptor ligands on mucin production in epinephrine knock out mice: The figure shows the A) morphometric quantification of mucin volume density and B) mucin (red) in the airway epithelium (green) with periodic acid fluorescent Schiff's (PAFS) stain of epinephrine knock out (Epi-KO) mice. Epi-KO mice were administered epinephrine (EPI) (100 μg/kg/day s.c. for 14 days), salmeterol (SALM) (3 μg/kg/day i.p. for 14 days), alprenolol (ALP) (7200 ppm orally for 28 days), propranolol (PROP) (80-140 mg/ml, orally for 28 days), carvedilol (CAR) (2400 ppm orally for 28 days) and nadolol (NAD) (250 ppm orally for 28 days) on with ovalbumin sensitization and challenge (Ova S/C) compared to vehicle-treated Ova S/C (VEH). Control (CTL) mice were sensitized with ovalbumin and challenged with saline. Scale bar represents 100 μm. Data represent mean $\pm$ SEM from 4-10 mice in each group. $^{a}P<0.05$ compared to CTL mice and $^{b}P<0.05$ compared to VEH mice.

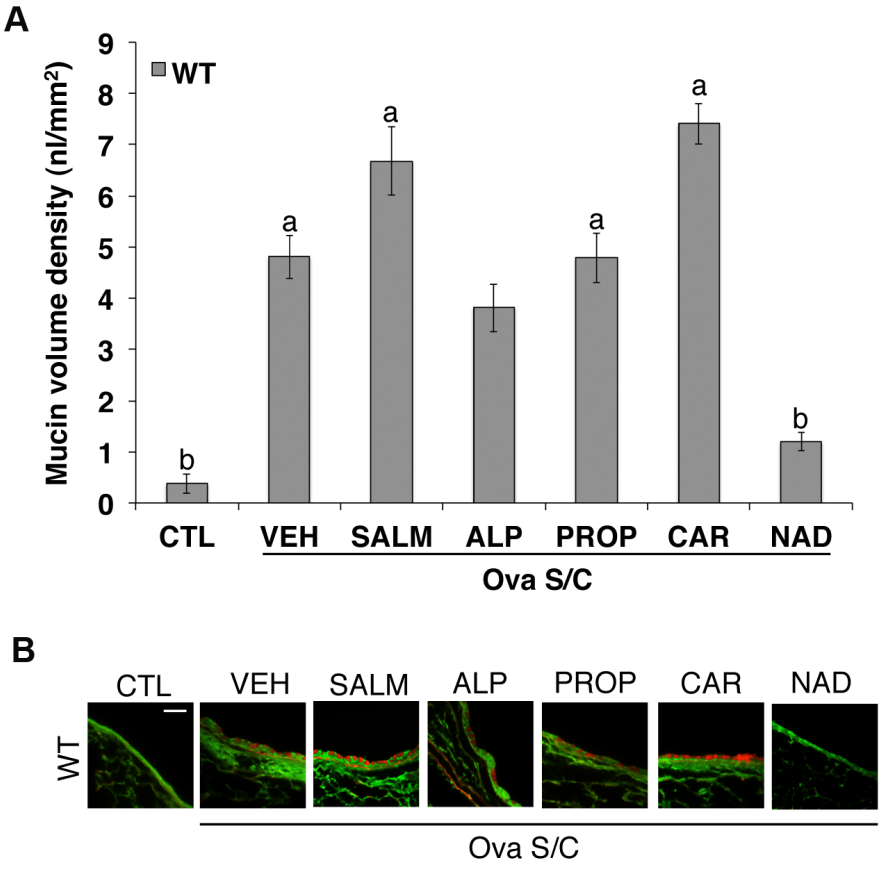

Figure 31. Effect of different β_2 adrenergic receptor ligands on mucin production in wile-type mice: The figure shows the A) morphometric quantification of mucin volume density and B) mucin (red) in the airway epithelium (green) with periodic acid fluorescent Schiff's (PAFS) stain of wild-type (Sv/129/J) (WT) mice. WT mice were administered salmeterol (SALM) (3 μg/kg/day i.p. for 14 days), alprenolol (ALP) (7200 ppm orally for 28 days), propranolol (PROP) (80-140 mg/ml, orally for 28 days), carvedilol (CAR) (2400 ppm orally for 28 days) and nadolol (NAD) (250 ppm orally for 28 days) on with ovalbumin sensitization and challenge (Ova S/C) compared to vehicle-treated Ova S/C (VEH). Control (CTL) mice were sensitized with ovalbumin and challenged with saline. Scale bar represents 100 μm. Data represent mean $\pm$ SEM from 4-10 mice in each group. $^{a}P<0.05$ compared to CTL mice and $^{b}P<0.05$ compared to VEH mice.

WT mice showed approximately 4-fold increase in mucin volume density with Ova S/C (Figures 31A and 31B). Administration of salmeterol, propranolol and carvedilol to Ova S/C WT mice resulted in a 7-fold, 5-fold and 8-fold increase in mucin production respectively compared to control WT mice (Figures 31A and 31B). Alprenolol administration did not significantly increase mucin production as compared to control WT mice (Figures 31A and 31B). However, mucin production with alprenolol treatment was also not different from vehicle-treated Ova S/C WT mice (Figures 31A and 31B). Nadolol administration to Ova S/C WT mice resulted in significantly lower mucin production compared to vehicle-treated Ova S/C WT mice, and was not different than control WT mice (Figures 31A and 31B). Figures 30B and 31B show the representative images for the mucin volume density analyses for Epi-KO and WT mice respectively.

5.4.3 Effect of β_2AR ligands on airway hyperresponsiveness in absence of epinephrine

AHR is the hyper-reactivity of the airway to a bronchoconstrictor in an asthmatic compared to a normal subject. AHR was measured by an invasive forced oscillation technique using FlexiVent® (Scireq, Montreal, Canada). Using this technique, we measured three different parameters in response to increasing doses of the bronchoconstrictor methacholine: total respiratory resistance, airway

sensitivity (PC100) and airway reactivity (K) from the different treatment groups. An increase in AHR is indicated by an increase in airway resistance, an increase in airway reactivity (K), and a decrease in PC100 value.

Epi-KO mice with or without Ova S/C showed no increase in AHR (Figure 32A-C). Replacement of epinephrine or administration of salmeterol, alprenolol or nadolol did not change AHR in Ova S/C Epi-KO mice compared to control Epi-KO mice (Figure 32A-C). However, propranolol and carvedilol restored airway resistance in Ova S/C Epi-KO mice comparable to Ova S/C WT mice. Carvedilol increased peak airway resistance and reactivity, but did not produce significant reduction in PC_{100} in Ova S/C Epi-KO mice compared to control Epi-KO mice (Figure 32A-C). Whereas, propranolol only increased peak airway resistance compared to control Epi-KO mice (Figures 32A-C).

In WT mice, Ova S/C resulted in an increase in all three AHR characteristics compared to control WT mice (Figure 33A-C). Administration of salmeterol to Ova S/C WT mice resulted in a reduction in peak airway resistance and reactivity and an increase in PC_{100} with Ova S/C in comparison to Ova S/C WT mice (Figure 33A-C). Alprenolol resulted in an increase in airway resistance, and reduction in PC_{100} with Ova S/C in WT mice compared to control WT mice (Figure 33A-C). Propranolol did not significantly increase airway resistance compared to control WT mice but was also not significantly lower than Ova S/C

WT mice (Figure 32A). Propranolol did significantly lower the PC_{100} compared to control WT mice but had no significant effect on airway reactivity compared to WT control or Ova S/C mice. (Figures 32B and 32C). Carvedilol and nadolol administration lowered peak airway resistance but had no effect on reactivity and sensitivity with Ova S/C in WT mice compared to vehicle-treated Ova S/C WT mice (Figure 33A-C).

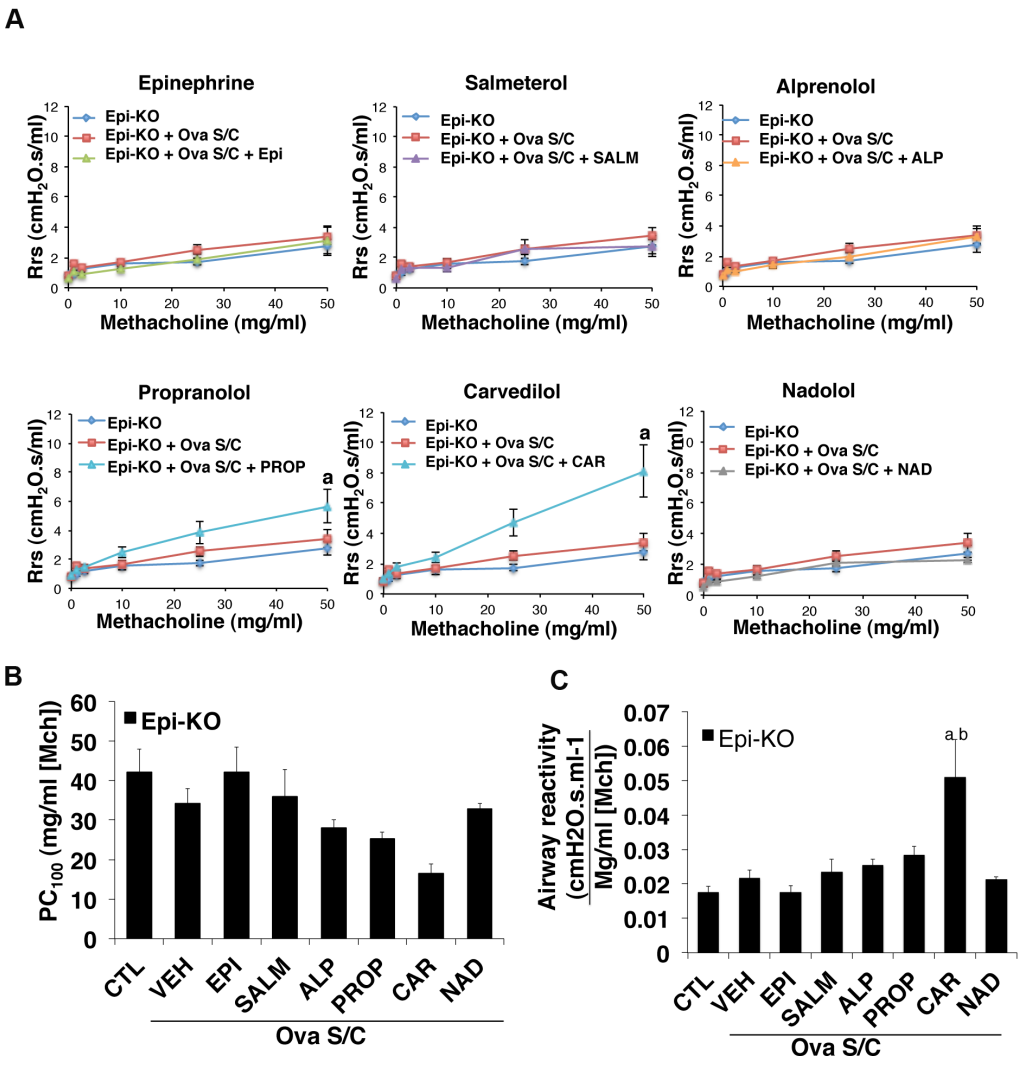

Figure 32. Effect of different β_2 adrenergic receptor ligands on airway hyperresponsiveness in epinephrine knock out mice: The figure shows the A) total respiratory resistance (Rrs) B) airway sensitivity (PC100) and C) airway reactivity (K) of epinephrine knock out (Epi-KO) mice to increasing doses of methacholine (Mch) measured by an invasive forced oscillation technique. Epi-KO mice were administered epinephrine (EPI) (100 μg/kg/day s.c. for 14 days), salmeterol (SALM) (3 μg/kg/day i.p. for 14 days), alprenolol (ALP) (7200 ppm orally for 28 days), propranolol (PROP) (80-140 mg/ml, orally for 28 days), carvedilol (CAR) (2400 ppm orally for 28 days) and nadolol (NAD) (250 ppm orally for 28 days) on with ovalbumin sensitization and challenge (Ova S/C) compared to vehicle-treated Ova S/C (VEH). Control (CTL) mice were sensitized with ovalbumin and challenged with saline. Data represent mean $\pm$ SEM from 4-10 mice in each group. [a]P<0.05 compared to CTL mice and [b]P<0.05 compared to VEH mice.

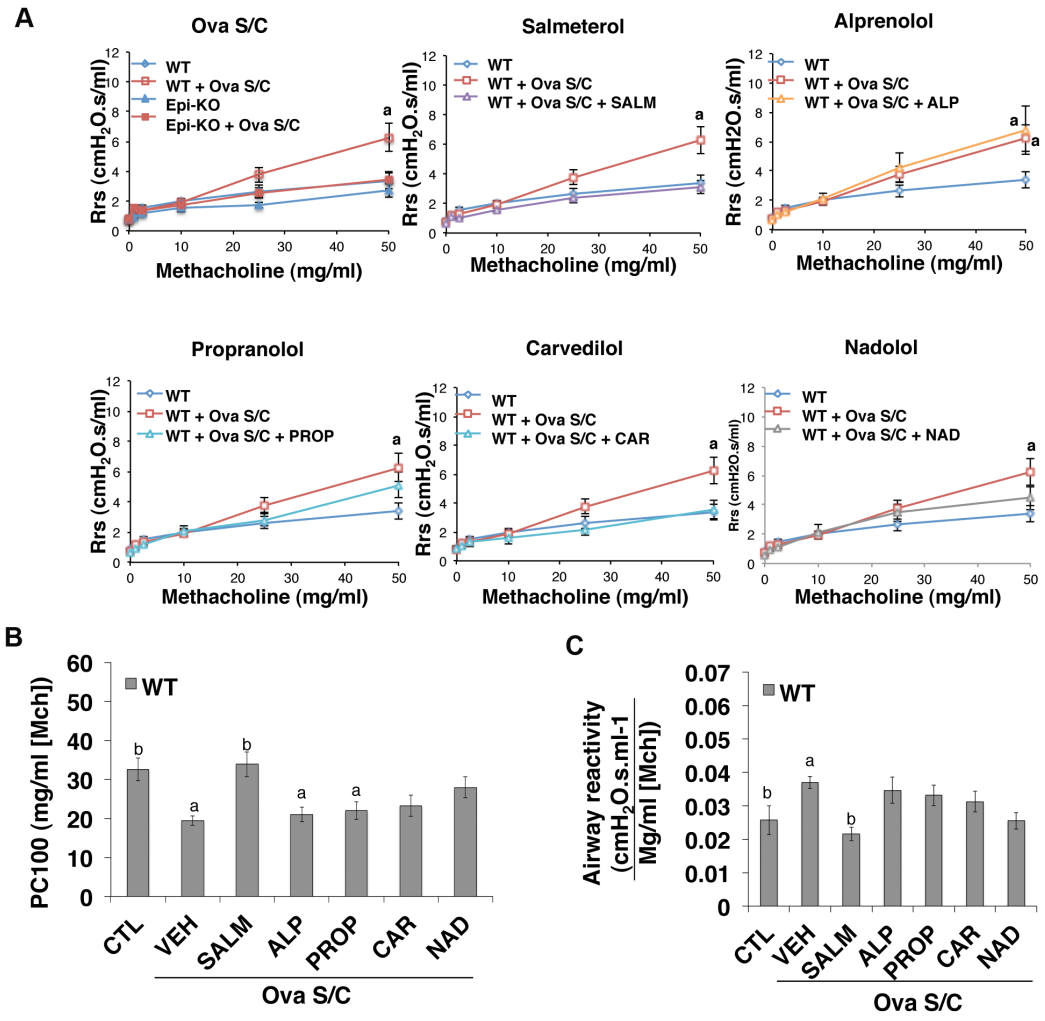

Figure 33. Effect of different β_2 adrenergic receptor ligands on airway hyperresponsiveness in wild-type mice: The figure shows the A) total respiratory resistance (Rrs) B) airway sensitivity (PC100) and C) airway reactivity (K) of wild-type (Sv/129J) (WT) mice to increasing doses of methacholine (Mch) measured by an invasive forced oscillation technique. WT mice were administered salmeterol (SALM) (3 *μ*g/kg/day i.p. for 14 days), alprenolol (ALP) (7200 ppm orally for 28 days), propranolol (PROP) (80-140 mg/ml, orally for 28 days), carvedilol (CAR) (2400 ppm orally for 28 days) and nadolol (NAD) (250 ppm orally for 28 days) on with ovalbumin sensitization and challenge (Ova S/C) compared to vehicle-treated Ova S/C (VEH). Control (CTL) mice were sensitized with ovalbumin and challenged with saline. Data represent mean ± SEM from 4-10 mice in each group. * ^{a}P<0.05 compared to CTL mice and ^{b}P<0.05 compared to VEH mice.

5.5 Discussion

5.5.1 Variability in the therapeutic efficacy of beta-blockers in asthma

Beta-blockers have been contraindicated in the therapy of asthma, however recent murine and clinical evidence indicate that a sub-group of beta-blockers have a beneficial effect by reducing inflammation, mucous metaplasia, and airway hyper-reactivity associated with asthma upon chronic administration (Callaerts-Vegh *et al.*, 2004; Hanania *et al.*, 2008; Lin *et al.*, 2008; Nguyen *et al.*, 2008; Hanania *et al.*, 2010). We have previously shown the beneficial effects of chronic administration of βAR inverse agonists like nadolol, ICI-118-551 and metoprolol in murine models of asthma compared to a beta-blocker without inverse agonist properties like alprenolol (Callaerts-Vegh *et al.*, 2004; Lin *et al.*, 2008; Nguyen *et al.*, 2008). In addition, another inverse agonist carvedilol reduced peak airway resistance but caused a leftward shift in the airway resistance curve to the bronchoconstrictor- methacholine in mice (Callaerts-Vegh *et al.*, 2004). A pilot clinical study done using nadolol in mild-asthmatics done by Hanania and colleagues show the beneficial effect of nadolol to reduce AHR (Hanania *et al.*, 2008). Recently, another small clinical study done by Short et al, showed the ineffectiveness of propranolol (a βAR inverse agonist) to reduce AHR in a subset of asthmatic patients (Short *et al.*, 2013b).

These studies exhibit a discrepancy in the effectiveness of beta-blockers in the therapy of asthma where certain members of the class beta-blockers are beneficial whereas others are not. While it seemed that inverse agonism was the desired property for therapeutic efficacy in asthma, the inverse agonists carvedilol (murine data) and propranolol (human data) were not effective in a murine asthma model or in asthmatics on inhaled corticosteroids (Callaerts-Vegh *et al.*, 2004; Short *et al.*, 2013b). Carvedilol resulted in a leftward-shift in the AHR dose response curve in the murine asthma model, and propranolol had no beneficial effect on FEV1 in asthmatic patients. These results were in sharp contrast to studies using nadolol, which resulted in a reduction in the asthma phenotype in murine models and a reduction in AHR in mild asthmatics (1999b; Hanania *et al.*, 2008; Hanania *et al.*, 2010). These data suggested inverse agonism does not distinguish drugs from being beneficial versus ineffective in asthma, and the key properties producing this difference needed to be identified in order to ensure that only promising drugs are chosen for clinical testing.

5.5.2 Importance of β_2AR, epinephrine and Epi-KO mice

In our previous studies, we showed the requirement of β_2AR in development of the asthma phenotype by using β_2AR null mice that showed an attenuated asthma phenotype. We have also used mice that lack the enzyme

phenylethanolamine N-methyltransferase to synthesize epinephrine from norepinephrine (Ebert *et al.*, 2004). We showed that Epi-KO mice did not develop an asthma phenotype in the Ova S/C model of murine asthma (Thanawala *et al.*, 2013). Those results did not support our hypothesis that constitutive activity was essential for development of the asthma phenotype, but at the same time provided us with an extremely useful tool. The only endogenous ligand for β_2AR is epinephrine and Epi-KO mice have no circulating epinephrine in their plasma, which meant these mice have empty (β_2AR) receptors. The empty receptor makes Epi-KO mice an important pharmacological tool because we can administer any ligand to these mice to act through β_2AR without confounding interference from epinephrine.

5.5.3 β_2AR signaling pathways and biased signaling

The β_2AR can signal mainly via at least two pathways, 1) the canonical pathway that leads to synthesis of cAMP by $G\alpha_s$-activated adenylyl cylcase and 2) the arrestin-mediated pathway leading to activation of MAP kinases like ERK1/2. The possibility of activation of several pathways by ligands via the β_2AR, allows for the possibility of biased signaling (also called ligand-directed trafficking). Biased signaling refers to the ability of a ligand to direct a receptor to signal preferentially through one pathway over another pathway. The extent of

the 'bias' is usually quantified by assigning the endogenous ligand as the reference for activation at all pathways i.e. without bias. Several studies have shown the bias that different β_2AR ligands can exhibit for activating the cAMP-dependent and arrestin-dependent pathways (Galandrin *et al.*, 2006; Wisler *et al.*, 2007). Biased signaling also shows that ligands have more complexity in signaling than their simple classification into agonists, antagonists and inverse agonists based on their efficacy at the canonical pathway (Thanawala *et al.*, 2014).

5.5.4 Rationale for the choice of test ligands

The aim of this study was to identify the role of the arrestin-dependent pathway that leads to ERK1/2 activation in development of the asthma phenotype. Previous studies showed the role of arrestin in development of the asthma phenotype by using arrestin-3 (or β arrestin-2) KO mice in a murine model of asthma (Walker *et al.*, 2003). Arrestin3-KO mice showed no increase in cellular infiltration or AHR compared to control mice with Ova S/C, indicating the requirement of arrestin in development of the asthma phenotype (Walker *et al.*, 2003). These data indicate the possible role of arrestin-mediated pathway in development of the asthma phenotype. The signaling profiles of different β_2AR ligands help identify their role at the arrestin pathway.

As shown in figure 18, Nadolol, ICI-118,551 and metoprolol are inverse agonists at the $G\alpha_s$-cAMP pathway and do not activate the arrestin-ERK1/2 pathway and based on our previous studies they attenuated development of asthma (Callaerts-Vegh *et al.*, 2004; Lin *et al.*, 2008; Nguyen *et al.*, 2008). Alprenolol is a weak agonist at the Gαs-cAMP pathway and the arrestin-ERK1/2 pathway, did not attenuate the asthma phenotype (Callaerts-Vegh *et al.*, 2004; Nguyen *et al.*, 2009).

Carvedilol and propranolol are both inverse agonists at the Gαs-cAMP pathway but weak agonists at the ERK1/2 pathway. Carvedilol was not beneficial in attenuation of the asthma phenotype with the exception of lowering peak airway resistance in mice and propranolol was not effective in mild-asthmatics based on a recent clinical study (Callaerts-Vegh *et al.*, 2004; Short *et al.*, 2013b). It should be noted that Wisler and colleagues suggests that while propranolol does activate ERK1/2, this activation is independent of arrestin (Wisler *et al.*, 2007). In addition, formoterol, a full agonist at both pathways restored asthma phenotype in Epi-KO mice, and albuterol, a full agonist at both pathways increased the asthma phenotype in WT mice (Callaerts-Vegh *et al.*, 2004; Lin *et al.*, 2008; Thanawala *et al.*, 2013). All these factors indicate that only ligands that did not activate the arrestin-ERK1/2 pathway were beneficial in murine asthma models. Moreover, coupled with data from arrestin-3 KO mice, the possible role

of arrestin-ERK1/2 pathway activation in development of asthma phenotype can be hypothesized (Walker *et al.*, 2003; Thanawala *et al.*, 2014).

In order to be able to distinguish the role of the arrestin-dependent pathway and test the hypothesis, ligands with varying signaling profiles were used. Five different β_2AR ligands were used: epinephrine (full agonist at both pathways), salmeterol (weak cAMP agonist and strong ERK1/2 agonist), alprenolol (weak agonist at both pathways), propranolol and carvedilol (inverse agonists at cAMP and weak agonists at ERK1/2) and nadolol (inverse agonist at cAMP and ERK1/2).

5.5.5 Epinephrine

To study the involvement of the two signaling pathways, Epi-KO mice became an important tool. At endogenous concentrations, epinephrine can activate all nine adrenergic receptors, whereas norepinephrine activates 8 of the 9 adrenergic receptors. β_2AR is the only adrenergic receptor norepinephrine cannot activate at endogenous concentrations. Using the Epi-KO mice, the effect of each of the ligands could be studied in the absence of epinephrine. Epinephrine is the endogenous ligand for β_2AR and we wanted to restore β_2AR signaling by epinephrine in Epi-KO mice. In order to ensure replacement of the endogenous level of epinephrine, it was necessary to only 'restore' the plasma

concentration of epinephrine in Epi-KO mice comparable to the endogenous concentration in WT mice. The plasma concentration of WT mice was determined using HPLC and two different doses of epinephrine, 50 μg/kg/day and 100 μg/kg/day were administered to the Epi-KO mice using osmotic pumps. The plasma levels of epinephrine (Table 5) resulting from administration of two different doses of epinephrine were measured. As shown in Table 5, the dose (100 μg/kg/day) achieved plasma levels closest to WT plasma levels was chosen for epinephrine replacement. Osmotic pumps were used to administer epinephrine because of its short half-life. After 14 days, ~20 % epinephrine can be degraded even in with 0.2% ascorbic acid and administration for duration longer than 14 days does not allow accurate dosing so 14 days of dosing was done (Khasar *et al.*, 2003; Khasar *et al.*, 2005).

Epinephrine replacement in Epi-KO mice resulted in restoration of the total cell count and eosinophil count but not a significant increase in mucin production compared to control or vehicle-treated Ova S/C Epi-KO mice (Figures 28A and 28B, 30A and 30B). Total cell count, eosinophil count and mucin production in the epinephrine-replaced Ova S/C Epi-KO mice is not different from WT mice with Ova S/C (Figures 28A-B, 29A-B, 30A-30B, 31A-31B). The restoration of eosinophilia and total cell counts was similar to the data from formoterol administration in Epi-KO as shown previously (Thanawala *et al.*, 2013). β_2AR

signaling was restored using formoterol at a dose (10 μg/kg/day) much lower than the therapeutic dose intended only to replace epinephrine (Thanawala *et al.*, 2013). However, there was no increase in AHR with epinephrine replacement in Epi-KO mice in comparison to WT mice that have circulating endogenous epinephrine (Figure 32A-C). In contrast, formoterol administration to Epi-KO mice as shown previously, restored AHR in Epi-KO mice (Thanawala *et al.*, 2013). Formoterol did restore AHR whereas epinephrine failed to do so; this could be explained by the longer duration of action of formoterol at β_2AR. The difference between WT mice and epinephrine-replaced Epi-KO could also be explained by the differences in the endogenous epinephrine physiology compared to administered epinephrine.

Endogenous epinephrine level rapidly varies based on the environmental, physiological and psychological state of the subject and is not constant. Administration of epinephrine using an osmotic pump results in infusion of a constant dose of epinephrine and will not account for the variations in plasma levels in a subject that would occur with changes in the environmental, physiological or psychological state. However, it is the closest method to mimic circulating plasma epinephrine for 14 days. Moreover, with the limit in the total number of days that epinephrine can be administered, the possibility arises that a longer duration of treatment with epinephrine could possibly restore AHR as well.

Table 5. Plasma and adrenal content of epinephrine and norepinephrine in epinephrine-replaced epinephrine knock out mice

	Plasma (pg/μl)		Adrenals (ng/mg of tissue)	
	Norepinephrine	**Epinephrine**	**Norepinephrine**	**Epinephrine**
WT	117.45 ± 12.15	80.06 ± 7.96	929.42 ± 114.93	102.04 ± 12.163
Epi-KO	76.69 ± 9.27	BLQ	795.45 + 127.49	8.19 ± 1.0284
Epi-KO + Epi 50 μg/kg/day	91.32 ± 11.32	BLQ	667.22 ± 134.98	5.48 ± 1.61
Epi-KO + Epi 100 μg/kg/day	88.84 ± 11.96	66.87 ± 11.61	1195.57 ± 77.93	8.42 ± 1.30

The table shows the norepinephrine and epinephrine levels in the mice measured using high performance liquid chromatography (HPLC) coupled with coulometric detection. WT- wild type mice (Sv/129J), Epi-KO- Epinephrine null mice, Epi 50 μg/kg/day or Epi 100 μg/kg/day – mice that received epinephrine 50 μg/kg/day or 100 μg/kg/day via osmotic pumps respectively and BLQ- below limits of quantification (50 pg/μl)

5.5.6 Salmeterol

The second ligand studied in our model was salmeterol. Salmeterol is a long acting beta-agonist with a half-life of about 12 hours and like formoterol is currently used in the therapy of asthma. However, salmeterol as described previously also carries a FDA black-box warning like formoterol, indicating small but significant mortality and cannot be administered without a corticosteroid (Aaronson, 2006).

Salmeterol was administered at 3 μg/kg/day based on the doses used in mice (Maris *et al.*, 2004; Singam *et al.*, 2006; Riesenfeld *et al.*, 2010; Qian *et al.*, 2011). This dose was chosen to only restore β_2AR signaling and not to mimic the therapeutically viable dose. The 14 days duration of the treatment was based on the duration of treatment used for epinephrine. Salmeterol did not cause a significant increase in total cell count, eosinophil count or mucin production or AHR in Epi-KO mice compared to control or vehicle-treated Ova S/C Epi-KO mice (Figure 28A and 28B, 30A-30B, 32A-C). In WT mice, salmeterol increased total cell, eosinophil count and mucin production compared to WT control mice but not different from vehicle-treated Ova S/C WT mice (Figure 29A and 29B, 31A and 31B). Salmeterol also had no effect on AHR in WT mice compared to control WT mice (Figure 33A-C).

Salmeterol is an arrestin-biased ligand for the β_2AR; that is, salmeterol activates the arrestin-dependent pathway more preferentially than the cAMP pathway compared to epinephrine. Formoterol is also an arrestin-biased ligand. However, salmeterol is different from formoterol in that, formoterol is a full agonist at the cAMP pathway whereas salmeterol is a weak agonist at the cAMP pathway (Rajagopal *et al.*, 2011). Previously we had shown that formoterol is able to restore inflammatory cell infiltration, mucin production and AHR in Epi-KO mice (Thanawala *et al.*, 2013). However, here it is shown that salmeterol cannot cause a significant increase in cellular infiltration, mucin production or AHR in Epi-KO mice (Figure 29A and 29B, 31A and 31B, 33A-C).

These data indicate that the ERK1/2 activation of salmeterol was not enough for restoration of mucin production, cellular infiltration or AHR in the Epi-KO mice. The data could also indicate the possible role of the cAMP-dependent pathway in development of the asthma phenotype since the activation profiles of formoterol and salmeterol mainly differ at the cAMP-pathway. In addition, the data indicate that different pathways may be involved in the development of inflammatory cell infiltration and mucus production.

5.5.7 Alprenolol

Previously, we had studied the effect of alprenolol on AHR, cellular infiltration and mucin production in BALB/c mice (Callaerts-Vegh *et al.*, 2004; Nguyen *et al.*, 2009). We showed that alprenolol did not have any beneficial effect on the Ova S/C mice with chronic administration (Callaerts-Vegh *et al.*, 2004). Here, the role of alprenolol in absence (Epi-KO mice) and presence (WT mice) of epinephrine was studied. The same dose as our previous studies (7200 ppm) of alprenolol was used (Callaerts-Vegh *et al.*, 2004). This dose was chosen based on the affinity of alprenolol for the β_2AR.

Alprenolol has been historically classified as an antagonist with intrinsic sympathomimetic activity (Jasper *et al.*, 1990; Lima, 1996). The intrinsic sympathomimetic activity refers to its property to activate the β_2AR canonical signaling in the absence of a stronger agonist. However, upon studying its activity at the two signaling pathways in the absence of epinephrine, it was revealed that alprenolol was in fact a partial agonist for both pathways (Figure 27C). This made adding alprenolol to the current study even more important because it is a ligand whose relative efficacies at both pathways in the presence and absence of epinephrine varies. While in the absence of epinephrine, alprenolol would act as a partial agonist at both pathways, in the presence of epinephrine it would act as an antagonist at both pathways.

In Epi-KO mice, alprenolol was able to restore the inflammatory cellular infiltration and mucin production (Figure 28A and 28B, 30A and 30B). However, it did not increase AHR in Epi-KO mice (Figure 32A-C). This again brings to fore the discrepancy observed in the inflammatory responses such as cellular infiltration and mucin production against AHR. Alprenolol acting as a partial agonist for both the signaling pathways in the absence of epinephrine was able to restore the cellular infiltration and mucin production in the Epi-KO mice compared to control mice. In addition, alprenolol worsened the mucin production and eosinophilia compared to epinephrine-replaced Epi-KO mice. This could be possibly due to the duration of treatment of alprenolol compared to epinephrine. Alprenolol was administered for 28 days to ensure chronic treatment, whereas epinephrine was administered only for 14 days due to the limitations discussed previously. Chronic activation of the β_2AR by alprenolol may have led to sustained activation of the two signaling pathways resulting in the worsening of the asthma phenotype. However, it indicates that activation of the ERK1/2 activation pathway was required for the restoration of the mucin and cellular infiltration of the asthma phenotype

The data in WT mice was similar to that seen previously, no significant effect on cellular infiltration and mucin production or AHR compared to vehicle-treated Ova S/C WT mice (Figure 29A and 29B, 31A and 31B, 33A-C) (Callaerts-

Vegh *et al.*, 2004; Nguyen *et al.*, 2009). This data indicates that while alprenolol was intended to act as an antagonist to epinephrine in the WT mice, it may not have been able to exert its effects in the presence of the strong agonist epinephrine hence we do not see any difference compared to vehicle-treated ET mice in any of the parameters of the asthma phenotype with alprenolol administration.

5.5.8a Propranolol

Propranolol is an inverse agonist at the cAMP pathway but a partial agonist at activating ERK1/2. Propranolol was used in the pilot clinical study by Short et al., and was not efficacious in reducing the FEV1 in mild-asthmatics (Short *et al.*, 2013b). Propranolol like nadolol is an inverse agonist and a prototype beta-blocker and its ineffectiveness in the clinical study caused confusion in understanding why nadolol was beneficial in reducing FEV1 but propranolol was not (Hanania *et al.*, 2008; Short *et al.*, 2013b). We added propranolol to this study along with carvedilol to understand the role of the ERK1/2 activation pathway of the β_2AR in development of the asthma phenotype. It should be noted that while carvedilol and propranolol have similar signaling profiles in terms of the pathways they activate, there exist differences between these beta-blockers. Wisler and colleagues showed that while carvedilol activates

the ERK1/2 pathway dependent on arrestin, whereas propranolol activates ERK1/2 independent of arrestin (Wisler *et al.*, 2007). Moreover, Van der Westhuizen and colleagues showed that the ERK1/2 activation by propranolol is through the β_2AR and α adrenergic receptors, whereas, carvedilol activates ERK1/2 only via the β_2AR (van der Westhuizen *et al.*, 2014).

Propranolol administration to Epi-KO mice caused a significant increase in the total cells, eosinophil counts, mucin production and airway resistance compared to control Epi-KO mice (Figure 28A and 28B, Figure 30A and 30B, Figure 32A). However it caused no significant differences in PC_{100} or airway reactivity compared to control Epi-KO mice (Figure 32B and 32C). These data indicate that replacing β_2AR signaling (ERK1/2 pathway) with propranolol resulted in a significant increase in the cellular infiltration, mucin production and airway resistance.

In WT mice, the effect of propranolol administration on total cell or eosinophil cell counts compared to control WT mice was not significantly different but was also not different from vehicle-treated WT mice (Figure 29A and 29B). While, mucin production with propranolol administration was significantly increased compared to WT control mice but not different from vehicle-treated WT mice (Figure 31A and 31B). Further, propranolol only significantly reduced PC_{100} (no effect on airway resistance or reactivity) compared to control WT mice but not

different from vehicle-treated WT mice (Figure 33A-C). Indicating that propranolol was not beneficial in attenuating total cell counts, eosinophilic infiltration, mucin production or PC_{100} in Ova S/C WT mice.

5.5.8b Carvedilol

The next ligand tested, carvedilol was important to study because like propranolol, it is an inverse agonist at the $G\alpha_s$-cAMP pathway it activates the ERK1/2 activation pathway via the β_2AR (van der Westhuizen *et al.*, 2014) (Figure 27D). All other compounds tested in the current study (except nadolol), activated both pathways to some extent but carvedilol and propranolol became 'cleaner' compounds to study because they lack activation of $G\alpha_s$-cAMP pathway. Carvedilol restored the cellular infiltration, mucin production, airway resistance and airway reactivity to methacholine in the Epi-KO mice compared to control Epi-KO mice. These data suggest that by activation of the arrestin-ERK pathway alone, even with the $G\alpha_s$-cAMP pathway shut down we were able to restore the asthma phenotype in Epi-KO mice. The mucin production with carvedilol in Epi-KO mice was comparable to the mucin production in the Epi-KO by alprenolol. However, alprenolol showed worsening of the cellular infiltration that was absent with carvedilol compared to epinephrine-replaced Epi-KO mice. This further indicates a role of the cAMP pathway in the cellular infiltration

because the difference between carvedilol and alprenolol is in their activation of the cAMP pathway. While alprenolol is a partial agonist at the cAMP pathway, carvedilol is an inverse agonist.

The cellular infiltration data in WT mice was similar to what we had previously seen, no beneficial or detrimental effect on cellular infiltration (Callaerts-Vegh *et al.*, 2004). However, the leftward shift in AHR dose response curve to methacholine as was seen previously was not observed in these mice (Callaerts-Vegh *et al.*, 2004). This can be attributed to strain differences in mice. The WT mice used in this study Sv/129J mice have a relatively lower AHR response compared to BALB/cj mice used in our previous studies (Zosky *et al.*, 2009). In our previous studies, we studied the effect of carvedilol on inflammatory infiltration and AHR in WT mice; here we show that carvedilol significantly worsens the mucin production compared to control Ova S/C WT mice (Callaerts-Vegh *et al.*, 2004).

Carvedilol can activate other adrenergic receptors apart from the β_2AR. However, Van der Westhuizen and colleagues have shown that carvedilol activates ERK1/2 via the β_2AR and does not activate ERK1/2 via the β_1 or α adrenergic receptors (van der Westhuizen *et al.*, 2014). Moreover, carvedilol also has antioxidant properties but the role of the antioxidant properties in

development of the asthma phenotype cannot be determined based on the current data.

5.5.9 Nadolol

Previous murine and clinical studies in mild asthmatics have shown nadolol to be a promising drug for the therapy of asthma (Callaerts-Vegh *et al.*, 2004; Lin *et al.*, 2008; Nguyen *et al.*, 2008; Nguyen *et al.*, 2009). ICI-118,551 and high-dose metoprolol were also therapeutically beneficial in our murine models but owing to the difficulty in clinical translation with ICI-118,551 and the need for a higher dose of metoprolol, nadolol became the better choice to proceed with for the clinical studies (Callaerts-Vegh *et al.*, 2004; Lin *et al.*, 2008; Nguyen *et al.*, 2008; Nguyen *et al.*, 2009). Nadolol has the ability to shut down both signaling pathways in the presence and absence of epinephrine (Figure 27E). This allows the current model to be studied with a negative control. Our data with chronic nadolol (250 ppm) administration in WT mice was consistent with our previous studies, showing an attenuation of inflammatory cellular infiltration, mucin production and AHR with Ova S/C (Callaerts-Vegh *et al.*, 2004; Lin *et al.*, 2008; Nguyen *et al.*, 2008; Nguyen *et al.*, 2009). In the Epi-KO mice, nadolol did not restore the cellular infiltration, mucin production or AHR with chronic administration (Figures 28A and 28B, 30A and 30B, 32A-C). Moreover, the

absence of a phenotype in the Epi-KO mice with Ova S/C prevented any observations or conclusions to be drawn about the effect of nadolol in this model at baseline.

To test the effect of nadolol in the Epi-KO mice it was necessary to observe an asthma phenotype. As is discussed in chapter 4, we used another model of murine asthma to study the effects of nadolol in the Epi-KO mice.

5.6 Cellular impedance assay

The data in chapter 2 show that while beta-blockers are grouped together, they can have varying effects on downstream signaling pathways of the β_2AR and by extension on disease therapeutics. Hence the classification of these compounds as a single group 'beta-blockers' is archaic and needs to be revisited (Thanawala *et al.*, 2014). Studies done by Bouvier lab have shown that the different β_2AR ligands can be classified into 5 different groups using a cellular impedance assay (Stallaert *et al.*, 2012; van der Westhuizen *et al.*, 2014). The following β_2AR ligands were used in this study: isoproterenol, epinephrine, salbutamol (albuterol), salmeterol, labetalol, bucindolol, alprenolol, pindolol, propranolol, carvedilol, xamoterol, bisoprolol, nadolol, ICI-118,551, metoprolol, timolol and atenolol. The studies used a cellular impedance assay that was able

to classify these compounds into 5 different groups based on cluster analysis. Each group possessed a specific impedance signature.

The cellular impedance assay was done using xCELLigence system (ACEA Biosciences) that measures cellular impedance after stimulation with different ligands. The cells used in the study (HEK293T) overexpressing the β_2AR were cultured such that they adhered to microelectrodes on the bottom of the wells. The system is then able to measure small changes in the ionic microenvironment of the cells that it records as a signature (Figure 34). This assay is a holistic readout of the changes in the microenvironment of the cells that may result from or indicate physiological changes occurring within the cell. However, since it is a holistic readout it is difficult to isolate the intracellular changes that result in particular signatures. In spite of this limitation, the authors have used different signaling pathway blockers to isolate the signaling events that contribute to the impedance signature. Further details about the cellular impedance assay can be found in the paper by Stallaerts et al (Stallaert *et al.*, 2012).

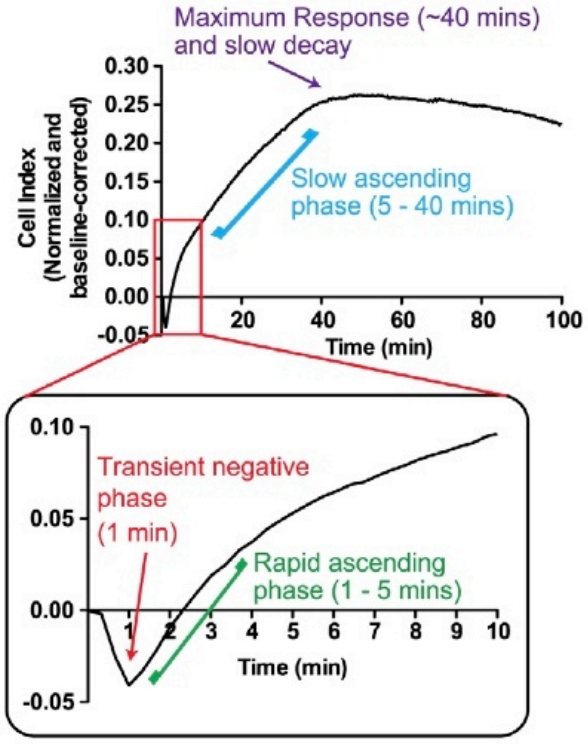

Figure 34. Cellular impedance signature of isoproterenol: The figure depicts the cell index as a measure of the cellular impedance of isoproterenol (50 μM) obtained from xCelligence system (ACEA biosciences). The signature can be divided into four distinct parts: Transient negative phase (inset red), rapid ascending phase (inset green), slow ascending phase (blue) and maximum response with slow decay (purple). Each of these parts represent different signaling pathways activated in the cell in response to isoprotereol treatment. For different ligands the signature differs based on the pathways being activated and the intensity of the activations. Adapted from (Stallaert *et al.*, 2012)

While, there appears to be no logical connection between how the impedance signature is formed and how that relates to ligand signaling, there is a way this assay is able to classify the different β_2AR ligands into groups that signal with similar profiles. As shown in Table 6, the assay was able to classify the ligands into 5 distinct groups. Each of these groups had a distinct signature for impedance and with our knowledge of the signaling profiles of these ligands we can appreciate how it is able to predict the similarities between these

compounds. Classical pharmacology, including the two-state model of receptor activation would allow us to distinguish the compounds into 4 of the 5 groups- the agonists, partial agonists, antagonists, inverse agonists. However, this assay is able to further divide the inverse agonists into groups 4 and 5, based on their activation of the arrestin-mediated pathway.

The application of this method to be able to distinguish compounds can be tremendous, especially in drug discovery where newer methods for screening multiple compounds are always needed. Here we have described an assay that may help screen compounds and group ones with similar signaling profiles from which desired signaling compounds could be used for testing in therapeutic conditions.

Table 6. cAMP accumulation and ERK1/2 activation by β_2AR ligands

Group	Ligand	cAMP	ERK 1/2
1	Isoproterenol	Agonist	Agonist
	Epinephrine	Agonist	Agonist
1/2*	Salbutamol (albuterol)	Agonist/partial agonist	Agonist/partial agonist
2	Salmeterol	Partial agonist	Partial agonist
3	Labetalol	Partial agonist/Antagonist	Partial agonist/Antagonist
	Bucindolol	Partial agonist/Antagonist	Partial agonist/Antagonist
	Pindolol	Partial agonist/Antagonist	Partial agonist/Antagonist
	Alprenolol	Partial agonist/Antagonist	Partial agonist/Antagonist
4	Carvedilol	Inverse agonist	Partial agonist
	Propranolol	Inverse agonist	Partial agonist
	Xamoterol	Inverse agonist	Partial agonist
5	Nadolol	Inverse agonist	Inverse agonist
	ICI-118,551	Inverse agonist	Inverse agonist
	Metoprolol	Inverse agonist	Inverse agonist
	Timolol	Inverse agonist	Inverse agonist
	Atenolol	Inverse agonist	Inverse agonist
	Bisoprolol	Inverse agonist	Inverse agonist

* salbutamol was shown as group 1 in Stallaert et al and group 2 in van der Westhuizen et al.

5.7 Summary:

The results have been summarized in table 7

Table 7. Summary of results from chapter 2

		Epi-KO					Wild-type			
Drug	**Pathway**	**Total cells**	**Eos**	**Mucin**	**AHR**		**Total cells**	**Eos**	**Mucin**	**AHR**
VEH	-	-	-	-	-		↑	↑	↑	↑
EPI	Both	↑	↑	-	-		N/D	N/D	N/D	N/D
SALM	ERK>caMP	-	-	-	-		↑	↑	↑	-
ALP	Both	↑	↑	↑	-		-	-	-	↑
PROP	ERK	↑	↑	↑	↑		-	-	↑	-
CAR	ERK	↑	↑	↑	↑		↑	↑	↑	-
NAD	None	-	-	-	-		-	-	-	-

*all responses indicate statistically significant increase compared to respective control mice
Eos- eosinophil count in airways
ERK- ERK1/2 activation pathway
cAMP- cAMP accumulation pathway
AHR- airway resistance
N/D- not determined
VEH- vehicle-treated (Ova S/C)
EPI- Epinephrine
SALM- salmeterol
ALP- Alprenolol
PROP- Propranolol
CAR- Carvedilol
NAD- Nadolol

Inflammatory cell infiltration:

Epinephrine, alprenolol, propranolol and carvedilol restore the cellular infiltration in airways in the Epi-KO mice compared to control Epi-KO mice. Salmeterol does not significantly restore the cellular infiltration and nadolol does not restore cellular infiltration in the Epi-KO mice.

In WT mice, salmeterol, alprenolol, propranolol and carvedilol do not significantly reduce the inflammatory cell infiltration seen with Ova S/C whereas nadolol results in the significant reduction of the cellular infiltration.

Mucin production:

Alprenolol, propranolol and carvedilol restore the mucin production in Epi-KO mice with Ova S/C, whereas epinephrine, salmeterol or nadolol do not restore mucin production.

In WT mice, salmeterol, alprenolol propranolol and carvedilol have no beneficial effects on mucin production on Ova S/C but nadolol attenuates the mucin production in Ova S/C WT mice.

AHR:

Epinephrine, salmeterol, alprenolol and nadolol do not restore airway resistance in Epi-KO mice with Ova S/C whereas; propranolol and carvedilol restore the airway resistance in Epi-KO mice.

In WT mice, salmeterol, carvedilol and nadolol can attenuate the airway resistance whereas alprenolol cannot attenuate the AHR with Ova S/C.

5.8 Conclusion:

Activation of the ERK1/2 pathway can restore the asthma phenotype and ligands that do not activate the ERK1/2 pathway prevent the restoration of the asthma phenotype in Epi-KO mice with Ova S/C.

6. Chapter 3

Three-state receptor theory modeling

6.1 Rationale

With the discovery of constitutively active receptors and inverse agonism, it was necessary to expand classical receptor theory to include the two-state model of receptor activation. The two-state model can explain the existence of constitutively active receptors in addition to the classical ligand-activated receptors (Bond *et al.*, 1995; Leff, 1995). Both the constitutively (or spontaneously) active receptors and the ligand-activated receptors can activate the downstream signaling pathway.

In our murine model of asthma, we have shown that only certain inverse agonists like nadolol, ICI-118,551 and high-dose metoprolol were beneficial in attenuating the asthma phenotype. Inverse agonists and not antagonists can inactivate the activity of constitutively active receptors. The efficacy of only inverse agonists in the murine asthma phenotype led to the hypothesis that 'constitutively active β_2ARs were required for the development of the asthma phenotype'. However, the Epi-KO mice were resistant to the asthma phenotype in the Ova S/C model of asthma, indicating the need for a ligand-activated receptor rather than a constitutively active receptor for asthma phenotype.

Moreover, inverse agonists such as carvedilol could restore the asthma phenotype in the mice. The discrepancy in the effects of inverse agonists at the β_2AR required further understanding of the β_2AR signaling.

In the previous study, we have seen that β_2AR can activate more than one signaling pathway. Moreover, β_2AR ligands differentially regulate the two signaling pathways. The activation profiles of the β_2AR ligands can determine their beneficial or detrimental effect on the murine asthma phenotype. The two-state model of receptor activation cannot explain the differential effects of the β_2AR ligands on the murine asthma phenotype.

To account for the two distinct signaling pathways of the β_2AR and their regulation by the β_2AR ligands, the two-state theory needed further extension to a three-state model. With the addition of a second signaling pathway, it became necessary to add another receptor state to be able to explain the effects of these ligands at the two signaling pathways of the β_2AR. Thus, arose the need for implementing the three-state model of receptor activation as is discussed in the literature review section.

6.2 Hypothesis and objective

The β_2AR can activate the $G\alpha_s$-cAMP-dependent signaling pathway and an arrestin-dependent ERK1/2 activation pathway. Since at least two

independent pathways are known for the β_2AR and there exists reversal of potency/efficacy reversal, it is necessary to add another active state (conformation) of the receptor and test the three-state model of receptor activation. The three-state model of receptor activation posits that receptors exist in three different conformations: an inactive state (R) and two active states (R* and R**). Each of the two active states can stimulate one of the two signaling pathways of the receptor. However, addition of another active state of the receptor allows for the possibility of variations in equilibria between the three states. To account for this complexity, the three-state model of receptor activation describes two types of interactions between the two signaling pathways mediated by their respective active conformations of the receptor. The two systems are called the 'intact system' and the 'isolated system'.

In an intact system the different receptor states (pathways) are interdependent, wherein, the activation of one pathway can affect the activation of another pathway by affecting the receptor equilibria. In the isolated system the receptor states (pathways) are independent and the activation of one pathway is independent of the activation of the other pathway and vice versa. Consequently, in the isolated system, the two active states of the receptor are not affected by each other. To simplify, the isolated system can be considered as 2 different two-state models for the same receptor. Details of these models have been

discussed in the literature review section. Briefly, the inactive conformation of the receptor in the intact system is in a direct equilibrium with both the active conformations, while in the isolated system, the inactive receptor conformation is in separate equilibria with each of the active conformations (Leff *et al.*, 1997).

While the two signaling pathways for β_2AR can be independent of each other, there is evidence to show overlap between the $G\alpha_s$-cAMP-dependent signaling pathway and an arrestin-dependent ERK1/2 activation pathway. Apart from the arrestin-dependent ERK1/2 activation, $G\alpha_s$-cAMP can also lead to ERK1/2 activation via the activation of the enzyme PKA (Taylor *et al.*, 2008; Pidoux *et al.*, 2010). However, there is a spatial and temporal difference in ERK1/2 activation by cAMP and arrestin. The cAMP-dependent ERK1/2 activation occurs near the plasma membrane and is a transient event with a short half-life, whereas, the ERK1/2 activation by arrestin is a cytosolic event that has a slow onset of action but is sustained for a longer duration (DeWire *et al.*, 2007). In the previous study, it was hypothesized that the ERK1/2 activation via the arrestin pathway was responsible for development of the asthma phenotype. This hypothesis was based on the known signaling profiles of different β_2AR ligands tested in the murine model for asthma. Figure 18 shows different ERK1/2 activation profiles of β_2AR ligands as shown by Wisler and colleagues (Wisler *et al.*, 2007).

Apart from ERK1/2 activation, there is more cross talk between these two pathways. Arrestin is required to shutdown the activation of cAMP by desensitization of the β_2AR; it participates in the internalization of β_2AR, in addition to acting as a scaffold for other cytosolic proteins (Figure 9, Figure 35) (DeWire *et al.*, 2007). Even though the two pathways are 'independent' there may still exist a correlation between the downstream signaling of the cAMP pathway and the arrestin pathway.

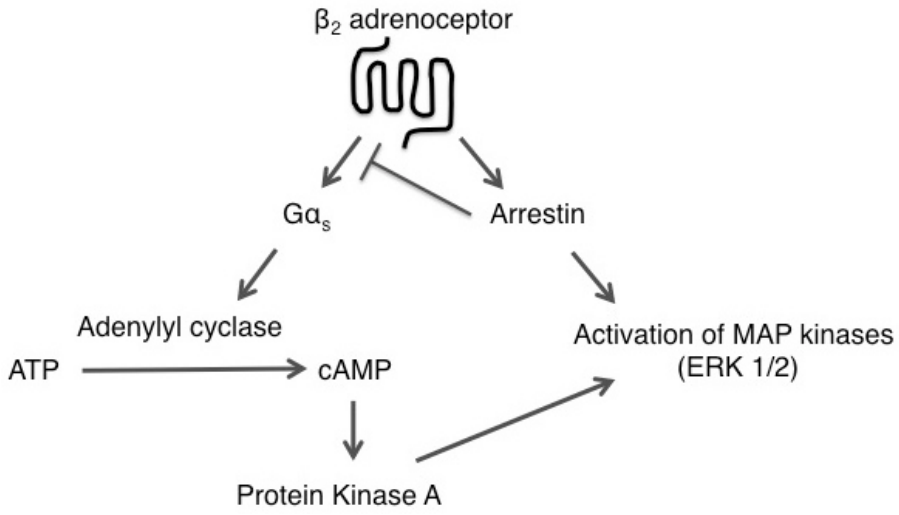

Figure 35. Cross talk between the signaling pathways of the β_2 adrenergic receptor: β_2 adrenergic receptor (β_2AR) can activate two signaling pathways: the canonical G protein signaling pathway and the arrestin-dependent pathway. Activation of $G\alpha_s$ leads to activation of adenylyl cyclase which converts adenosine mono phosphate (ATP) to cyclic adenosine monophosphate (cAMP). cAMP activates protein kinase A (PKA). PKA can further result in activation of mitogen-activated protein kinases (MAPKs) like extracellular signal-regulated kinases (ERK1/2). PKA can also phosphorylate β_2AR which can lead to signaling via $G\alpha_i$ to activate ERK1/2. Arrestin results in desensitization of β_2AR and can lead to internalization of the receptor. Further, arrestin can scaffold and allow activation of MAPKs such as ERK1/2.

In the current study, we use mathematical modeling based on the three-state model of receptor activation to understand the signaling of the β_2AR. Based on the existence of two independent signaling pathways of the β_2AR and the known cross talk between the two pathways, we tested the hypotheses, 'Regulation of the β_2AR signaling pathways by the β_2AR ligands follows the intact system of the three-state model of receptor activation'.

6.3 Approach

The two types of the three-state model of receptor activation are:

- Intact system
- Isolated system

The aim of the current study was to determine the type of three-state model of receptor activation is followed by the β_2AR ligands at the β_2AR. In order to achieve this, experimental data that quantified the activation of the two pathways by different β_2AR ligands at the β_2AR was required.

For this purpose, we chose the data from Galandrin and colleagues (Galandrin *et al.*, 2006). In this paper, the authors have shown the dose response curves for different β_2AR ligands via the β_2AR for both the signaling pathways, cAMP-dependent and arrestin-dependent pathways. The β_2AR ligands studied include:

- Isoproterenol
- Bucindolol
- Labetalol
- Propranolol
- Carvedilol
- Metoprolol
- Atenolol
- Bisoprolol

The experimental data was compared to the data obtained by mathematical modeling of the β_2AR ligands to activate the two different pathways using both the intact and isolated system formulae of the three-state model. The modeling data that matched the experimental data indicated the type of three-state receptor activation followed by β_2AR ligands at the β_2AR.

In order to do the mathematical modeling, we estimated the affinities of these β_2AR ligands for the different receptor states based on experimental evidence. It is important to note that the affinities are only theoretical estimates. However, once they are chosen, the affinities for each ligand and receptor conformation are fixed and remain constant for use in the different formulae for the mathematical modeling as discussed by Leff and colleagues (Leff *et al.*, 1997):

$$L = \frac{[\mathrm{R}]}{[\mathrm{R}^*]}$$

$$M = \frac{[\mathrm{R}]}{[\mathrm{R}^{**}]}$$

Where,

[R]- concentration of the inactive state of the receptor

[R*]- concentration of the receptor active state 1 (activates pathway 1 or cAMP)

[R**]- concentration of the receptor active state 2 (activates pathway 2 or arrestin)

$$K_A = \frac{[\mathrm{A}].[\mathrm{R}]}{[\mathrm{AR}]}$$

$$K_{A^*} = \frac{[\mathrm{A}].[\mathrm{R}^*]}{[\mathrm{AR}^*]}$$

$$K_{A^{**}} = \frac{[\mathrm{A}].[\mathrm{R}^{**}]}{[\mathrm{AR}^{**}]}$$

Where,

[A]- concentration of the ligand A

[R]- concentration of the inactive state of the receptor

[R*]- concentration of the receptor active state 1 (activates pathway 1 or cAMP)

[R**]- concentration of the receptor active state 2 (activates pathway 2 or arrestin)

K_A- equilibrium dissociation constant that determines affinity of the ligand for R

K_A*- equilibrium dissociation constant that determines affinity of the ligand for R*

K_A**- equilibrium dissociation constant that determines affinity of the ligand for R**

Intact three-state system:

$$f_{R^*} = \frac{\frac{1}{L} + \left[\frac{1}{L.K_{A^*}}\right].[\mathrm{A}]}{\left[1 + \frac{1}{L} + \frac{1}{M}\right] + \left[\frac{1}{K_{A^*}} + \frac{1}{L.K_{A^*}} + \frac{1}{M.K_{A^{**}}}\right].[\mathrm{A}]}$$

$$f_{R^{**}} = \frac{\frac{1}{M} + \left[\frac{1}{M.K_{A^{**}}}\right].[\mathrm{A}]}{\left[1 + \frac{1}{L} + \frac{1}{M}\right] + \left[\frac{1}{K_{A^*}} + \frac{1}{L.K_{A^*}} + \frac{1}{M.K_{A^{**}}}\right].[\mathrm{A}]}$$

Where,

f_{R^*} - fraction of receptors in the active state R* (it is also indicative of the activity of the ligand at R*)

$f_{R^{**}}$ - fraction of receptors in the active state R**(it is also indicative of the activity of the ligand at R**)

L- Ratio of R to R*

M- Ratio of R to R**

Isolated three-state system:

$$f_{R^*} = \frac{\frac{1}{L} + \left[\frac{1}{L.K_{A^*}}\right].[\mathrm{A}]}{\left[1 + \frac{1}{L}\right] + \left[\frac{1}{K_{A^*}} + \frac{1}{L.K_{A^*}}\right].[\mathrm{A}]}$$

$$f_{R^{**}} = \frac{\frac{1}{M} + \left[\frac{1}{M.K_{A^{**}}}\right].[\mathrm{A}]}{\left[1 + \frac{1}{M}\right] + \left[\frac{1}{K_{A^*}} + \frac{1}{M.K_{A^{**}}}\right].[\mathrm{A}]}$$

The above formulae for determining the fR* and fR** for the intact and isolated systems were used for the mathematical modeling. A more detailed description of the models can be found in the literature review section on the three-state model of receptor activation. L and M (receptor ratios) were set at a standard value of 1 (Leff *et al.*, 1997).

The values used from the experimental data for the modeling is shown in Table 8 and 9. These values were obtained from the study done by Galandrin and colleagues (Galandrin *et al.*, 2006).

Table 8: Experimental data for accumulation of cAMP by β_2AR from Galandrin et al. for dose-response curves

No.	Ligand	E_{max}	EC_{50}
1	Isoproterenol	100	4.99×10^{-9}
2	Bucindolol	-2.8	1×10^{-5}
3	Labetalol	31.5	6.76×10^{-9}
4	Propranolol	-43.9	2.32×10^{-9}
5	Carvedilol	-6.7	1×10^{-5}
6	Metoprolol	-59.2	2.942×10^{-7}
7	Atenolol	-59.5	2.4008×10^{-6}
8	Bisoprolol	-59.4	3.055×10^{-7}

Table 9: Experimental data for activation of ERK1/2 by β_2AR from Galandrin et al. for dose-response curves

No.	Ligand	E_{max}	EC_{50}
1	Isoproterenol	100	2.14×10^{-7}
2	Bucindolol	64.8	9.5×10^{-10}
3	Labetalol	64.1	1.165×10^{-8}
4	Propranolol	40.6	8.64×10^{-9}
5	Carvedilol	38.12	6.88×10^{-9}
6	Metoprolol	-41.2	ND
7	Atenolol	-55.8	ND
8	Bisoprolol	-51.6	ND

The values used for the mathematical modeling are shown in Table 10. These values were estimated based on the literature known about the activation patterns of these ligands. For example if a ligand ‘A’ is a full agonist at both signaling pathways, the affinity of A for active states R* and R** is greater than its affinity for the inactive state R. ‘A’ would bind preferably to R* and R** in comparison to R and lead to a shift in equilibrium towards formation of more R*

and R**. An increase in R* and R** would lead to an increase in the signaling via both the active states.

Table 10: Receptor affinities for mathematical simulation of the three-state model

No.	Ligand	K_A	K_{A^*}	$K_{A^{**}}$
1	Isoproterenol	1×10^{-4}	2.5×10^{-9}	$1 \times 10^{-7.3}$
2	Bucindolol	1×10^{-9}	1.1×10^{-9}	2.5×10^{-10}
3	Labetalol	1.1×10^{-8}	$1.6 \times 10^{-8.5}$	$0.8 \times 10^{-8.5}$
4	Propranolol	$1 \times 10^{-8.5}$	$1.25 \times 10^{-8.2}$	$1 \times 10^{-8.85}$
5	Carvedilol	$1 \times 10^{-8.5}$	$1.1 \times 10^{-8.5}$	$1 \times 10^{-8.825}$
6	Metoprolol	$1 \times 10^{-6.75}$	$1 \times 10^{-6.15}$	$1 \times 10^{-6.25}$
7	Atenolol	$1 \times 10^{-5.75}$	$1 \times 10^{-5.15}$	$1 \times 10^{-5.25}$
8	Bisoprolol	$1 \times 10^{-6.75}$	$1 \times 10^{-6.15}$	$1 \times 10^{-6.25}$

K_A: Affinity of the ligand for the inactive state of the receptor (R)
K_{A^*}: Affinity of the ligand for the active state of the receptor (R*)
$K_{A^{**}}$: Affinity of the ligand for the active state of the receptor (R**)

6.4 Results

6.4.1 Experimental data

The dose response curves obtained by using the values in Table 8 are shown in figures 36A and 36B (Galandrin *et al.*, 2006). Figure 36A depicts the dose response curves of eight β_2AR ligands for accumulation of cAMP and figure 36B shows the activation of ERK1/2 by the eight β_2AR ligands. Figure 36A shows the efficacy of the different ligands at the cAMP pathway. While, isoproterenol acts as a full agonist labetalol is a partial agonist at the cAMP pathway. Bucindolol and carvedilol act as very weak inverse agonists or antagonists. Whereas, propranolol is a partial inverse agonist and metoprolol, bisoprolol and

atenolol act as strong inverse agonists. Figure 36B shows the efficacy of the ligands at the ERK1/2 activation. While, isoproterenol is a strong agonist at activating ERK1/2; bucindolol, labetalol, carvedilol and propranolol act as partial agonists. Galandrin and colleagues did not observe constitutive activity at the ERK1/2 pathway and hence could not show inverse agonism at that pathway (Galandrin *et al.*, 2006). It should be noted due to this limitation figure 36B does not have metoprolol, atenolol and bisprolol.

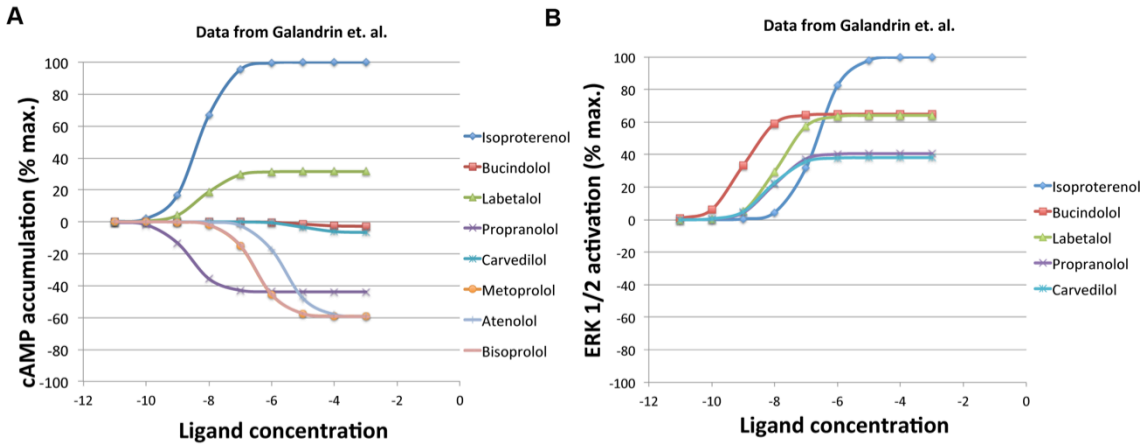

Figure 36. Dose response curves of different β_2 adrenergic receptor ligands at two of its signaling pathways: The graphs represent the dose response curves of different β_2 adrenergic receptor (β_2AR) ligands at A) cyclic adenosine monophosphate (cAMP) accumulation and B) extracellular signal-regulated kinases (ERK1/2) activation. These curves were derived based on the data from Galandrin et al. (2006) as shown in table 8 and 9 respectively.

6.4.2 Mathematical modeling

The simulations of the intact system and isolated system using the mathematical modeling as per Table 9 have been shown in figures 37 and 38 respectively. The figures 37A, 38A, 37B and 38B show the fR* and fR** against increasing concentration of the ligands respectively. An increase in R*, corresponding to a higher fR* represents a higher number of receptors in the R* conformation. A higher number of receptors in the R* conformation represents an increase in the downstream event of the R* conformation. Similarly, an increase in the R**, represented by an increase in fR** translates to a higher number of receptors in R** conformation and an increase in the downstream event of R**. Therefore, fR* is representative of the efficacy at cAMP pathway whereas, fR** indicates the efficacy at the ERK 1/2 pathway.

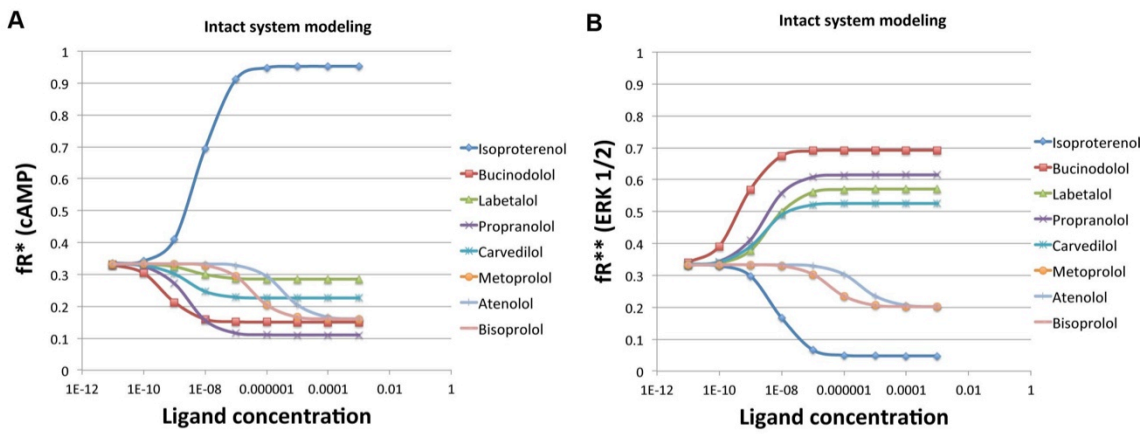

Figure 37. Three-state receptor activation based mathematical modeling of different β_2 adrenergic receptor ligands at two of its signaling pathways using the intact system: The graphs represent the dose response curves of different β_2 adrenergic receptor (β_2AR) ligands at A) cyclic adenosine monophosphate (cAMP) accumulation and B) extracellular signal-regulated kinases (ERK1/2) activation. cAMP accumulation is represented by the fraction of active receptors, R* (fR*) and ERK1/2 activation is represented by fraction of active receptors, R** (fR**). These curves were derived based on the formulae (from Leff et al (1997)) for the intact system of the three-state model of receptor activation as shown in table 10.

Figure 37A and 37B depict the dose response curves of the intact system modeling done as per the Table 8 using the formulae for the intact system. Here, figure 37A shows that only isoproterenol is a full agonist while all the other ligands are inverse agonists at fR*. However, figure 37B shows that isoproterenol is an inverse agonist at fR**, while bucindolol, labetalol, propranolol and carvedilol are agonists at fR**. In addition, metoprolol, bisoprolol and atenolol are inverse agonists at fR**.

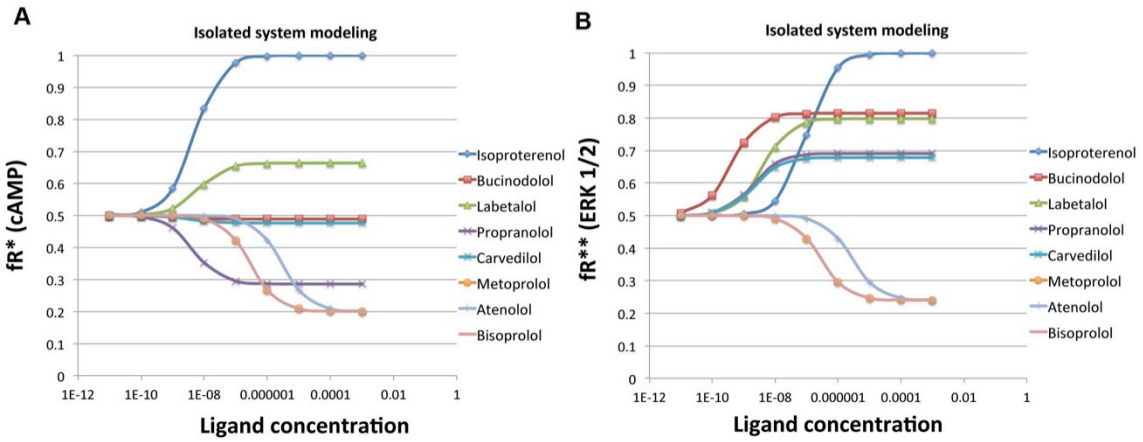

Figure 38. Three-state receptor activation based mathematical modeling of different β_2 adrenergic receptor ligands at two of its signaling pathways using the isolated system: The graphs represent the dose response curves of different β_2 adrenergic receptor (β_2AR) ligands at A) cyclic adenosine monophosphate (cAMP) accumulation and B) extracellular signal-regulated kinases (ERK1/2) activation. cAMP accumulation is represented by the fraction of active receptors, R* (fR*) and ERK1/2 activation is represented by fraction of active receptors, R** (fR**). These curves were derived based on the formulae (from Leff et al (1997)) for the isolated system of the three-state model of receptor activation as shown in table 10.

Figures 38A and 38B show the data from the isolated system modeling of the different ligands. Figure 38A shows that the fR** increases representing the increase in activity at cAMP pathway with isoproterenol acting as a full agonist and labetalol as a partial agonist. Bucindolol and carvedilol act as weak inverse agonists, very similar to being antagonists whereas propranolol, metoprolol, bisoprolol and atenolol act as inverse agonists at the cAMP pathway. At fR** or ERK1/2 activation in figure 38B, Isoproterenol acts as a strong agonist, bucindolol and labetalol are partial agonists followed by propranolol and

carvedilol. Theoretically, constitutive activity at the ERK1/2 pathway can be assumed and therefore we were able to determine the possible activity of atenolol, metoprolol, and bisoprolol as inverse agonists at the ERK1/2 pathway. However, we do not have their efficacies under experimental conditions and this remains purely a mathematical determination.

6.4.3 Comparison

Comparison of the modeling data of the intact and isolated systems with the experimental data shows that the experimental data is comparable to the isolated system of three-state receptor activation rather than the intact system. Figures 36A and 36B are identical to figures 38A and 38B. The experimental signaling profiles of each of the ligands can be plotted accurately using the isolated system modeling. This indicates that the β_2AR ligands via the β_2AR follow the isolated system of three-state receptor activation.

6.5 Discussion

The three-state model of receptor activation was warranted by the discovery of more than one signaling pathway activated by one receptor. To add the second active receptor conformation, it was necessary to fulfill another requirement. The two pathways must be independent and not sequentially

activated, meaning that the activation of one pathway must not lead to the activation of the second pathway. This can be confirmed by potency reversal. Potency reversal refers to the chance in rank order of potencies of a set of ligands between different study systems. Details of potency reversal are discussed in the literature review section.

The three-state model of receptor activation states that the three-receptor states can exist as an intact system (both active states are regulated by a common pool of inactive receptors) or isolated system (the two active states are regulated by separate respective pools of inactive receptors). The isolated system can be considered as 2 two-state models such that each active receptor conformation with its inactive receptor pool comprises of one two-state model.

Our hypothesis was that β_2AR ligands via the β_2AR would follow the intact system of three-state activation based on the extensive cross talk between the two pathways (cAMP and ERK1/2). However, the modeling data indicates that the β_2AR ligands follow the isolated system of three-state receptor activation. This finding poses an important question- are the two pathways indeed completely independent of each other despite the known overlap in downstream signaling? We have previously discussed the overlap of these two pathways as shown in figure 35. However, the modeling data suggests the two pathways

appear to be independent of each other. While, this finding disproves the hypothesis, it allows for a unique avenue in drug development.

To appreciate the practical importance of the modeling method, it is pertinent to understand the generic application of the mathematical modeling. Mathematical modeling is a great tool to test the robustness and predictive value of a study. The mathematical modeling data in this study allows us to confirm that the β_2AR signaling follows a three-state model of receptor activation. Hence, proving the first part of the hypothesis. However, it suggests that the β_2AR ligand signaling through β_2AR is not an intact system but an isolated system.

The mathematical modeling of the ligand-receptor interaction allows the possibility of not only knowing how the system behaves mathematically, but also to determine the profile of a compound with desired therapeutic efficacy. For example, from our studies in murine asthma models, we formed the hypothesis that ligands that inhibit the ERK1/2 activation of β_2AR are beneficial in murine asthma models. We can obtain experimental data on the compounds and test which of these drugs have the desired profile. In addition, knowing that the two pathways are independent in this system (β_2AR), designing drugs that selectively act on one of the two signaling pathways becomes easier since its activity at the cAMP pathway may not affect the activity at the ERK1/2 pathway. Indeed, if we can elucidate the role of the cAMP pathway of the β_2AR in asthma, we can

modify the ligands to design an ideal compound to target asthma knowing that the activity at one pathway does not necessarily interfere with the activity at the other pathway.

The application of mathematical modeling of the three-state receptor activation is not limited to β_2AR and its ligands. It can be applied to any receptor that activates more than one signaling pathway. If the receptor follows a three-state model, we can determine whether it follows an intact or an isolated system and help design better therapeutic options for that particular receptor and its related disorders. Theoretically, once a receptor can exist in more than one conformation, it can exist in an infinite number of conformations. However, Occam's razor prevails and we cannot introduce additional conformations of the receptors unless warranted by experimental data. Here, the three-state model has been discussed since there is evidence of two independent signaling pathways but the possibility of four, five or multi-state receptor models definitely exists and needs to be considered if there is evidence for more than 2 independent signaling pathways for a particular receptor. The multi-state receptor theory models can be very complex and are beyond that scope of this thesis.

6.6 Conclusion

β_2AR ligands via the β_2AR appear to follow the isolated system of the three-state model of receptor activation.

7. Chapter 4

Effect of beta-blockers in the PAR2 model of asthma

7.1 Rationale

Epi-KO mice are resistant to the Ova S/C model of murine asthma, highlighting the requirement of epinephrine in development of the asthma phenotype. We studied the effect of the different β_2AR ligands in the Epi-KO mice using the Ova S/C model. While, certain β_2AR ligands restored the asthma phenotype, we could not observe beneficial effects of the ligands due to the absence of an asthma phenotype in the Epi-KO mice. Here, we use another model of asthma to induce an asthma phenotype in the Epi-KO mice. Using the PAR2 model of asthma in the Epi-KO mice, we can study the beneficial as well as detrimental effects of the β_2AR ligands.

7.2 Hypothesis and objective

Previously, we have studied the effect of chronic administration of beta-blockers and the role of epinephrine in the Ova S/C model of murine asthma. In the current study, another model of murine asthma based on a PAR2 ligand in the Ova S/C model was used. The PAR2 model of murine asthma represents allergic-asthma and has been shown to result in a worsening of the asthma phenotype in mice compared to the Ova S/C model (Ebeling *et al.*, 2005). Certain

allergens that result in allergic-asthma have protease activity that can activate the PAR2 (Ebeling *et al.*, 2005). One of the objectives of the current study was to understand the role of epinephrine in another model of asthma using a PAR2 ligand.

We also wanted to study the beneficial or detrimental effects of beta-blockers in the PAR2 model of asthma. Alprenolol and carvedilol resulted in restoration of asthma phenotype in Epi-KO mice with Ova S/C model of asthma (Figures 28A, 28B, 30A, 30B). Nadolol did not restore the asthma phenotype in Ova S/C Epi-KO mice (Figures 28A, 28B, 30A, 30B) and attenuated the asthma phenotype in WT mice in many different strains of mice (Callaerts-Vegh *et al.*, 2004; Lin *et al.*, 2008; Nguyen *et al.*, 2008; Nguyen *et al.*, 2009). However, we do not know if nadolol can be beneficial in attenuation of the asthma phenotype in the Epi-KO mice, due to the absence of a readout. If Epi-KO mice develop an asthma phenotype with the PAR2 model, we can study the effect of different beta-blockers in the PAR2 model of asthma.

In this study we hypothesized that- 'Epi-KO mice develop asthma phenotype in the PAR2 model of asthma and nadolol attenuates the asthma phenotype'

7.3 Approach

To study the role of epinephrine and the effect of beta-blockers in the PAR2 model of asthma, we chose the Ova S/C model of murine asthma supplemented with a PAR2 ligand. PAR2 is a GPCR that is activated by serine proteases. PAR2 plays an important role in development of asthma because it is activated by serine proteases (eg: tryptase) contained in aeroallergens (Compton *et al.*, 2001; Macfarlane *et al.*, 2001; Sun *et al.*, 2001; Asokananthan *et al.*, 2002).

In this experiment, we used the Ova S/C model that was supplemented by challenge with a PAR2 ligand (SLIGRL-NH_2). The schematic of the protocol is shown in figure 20C. Epi-KO mice were intra-peritoneally sensitized to 2 mg/kg/day ovalbumin with 2 mg alum on days: 0, 7 and 14, followed by intra-nasal challenge with 1 mg/kg/day ovalbumin with or without 100 μM SLIGRL-NH_2 (Tocris Biosciences) or saline on days: 41-45. Some mice received alprenolol (7200 ppm), carvedilol (2400 ppm) or nadolol (250 ppm) *ad libitum* in chow for 28 days as shown in figures 20C and 21C. On day 46, the mice were euthanized and analyzed for inflammatory cell infiltration in the airways and mucous metaplasia.

7.4 Results

7.4.1 Inflammatory cell infiltration

Ova S/C did not result in a significant increase in total cells or eosinophils in the airways of Epi-KO mice as seen previously (Figure 39A and 39B). However, Ova and SLIGRL-NH_2 challenge (henceforth referred to as the 'PAR2' model) resulted in a significant increase in total cells as well as eosinophilic infiltration into the airways of Epi-KO mice compared to control Epi-KO mice (Figures 39A and 39B). Administration of alprenolol or carvedilol to the vehicle-treated PAR2 mice resulted in a significant increase in the eosinophilic infiltration of the airways but had no significant effect on the total cell count compared to vehicle-treated PAR2 mice (Figure 39A and 39B). In contrast, administration of nadolol did not significantly increase or decrease the total cell or eosinophil infiltration compared to vehicle-treated PAR2 mice (Figure 39A and 39B).

7.4.2 Mucous metaplasia

Epi-KO mice did not show a significant increase in mucin volume density with Ova S/C. However, challenge with PAR2 ligand resulted in an increase in the mucin volume density compared to control Epi-KO mice figures 40A and 40B. Administration of alprenolol and carvedilol resulted in a significant increase in mucin volume density compared to vehicle-treated PAR2 mice. However,

administration of nadolol did not increase or decrease the mucin volume density compared to vehicle-treated PAR2 mice.

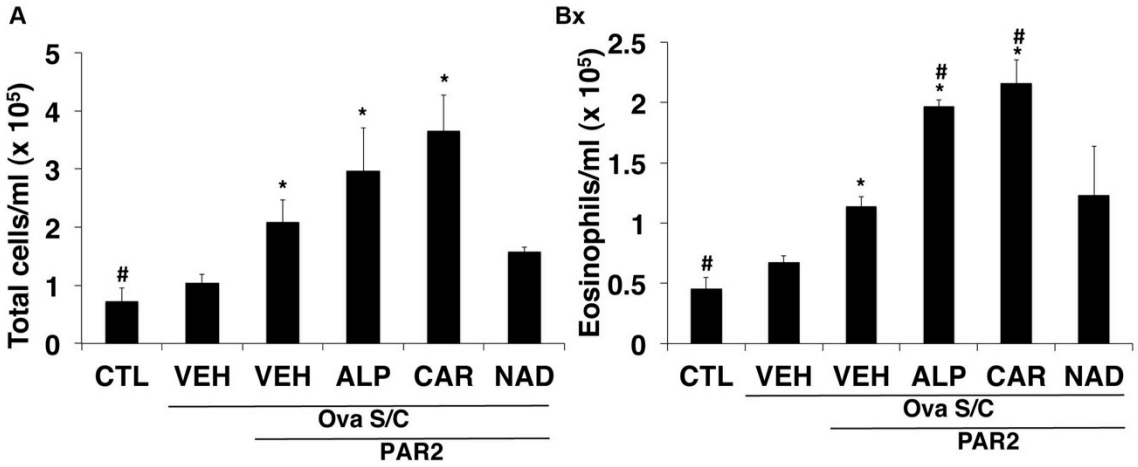

Figure 39. Effect of beta-blockers on inflammatory infiltration in epinephrine knock out (Epi-KO) mice in the protease-activated receptor-2 (PAR2) ligand model: The graphs represent total cell and eosinophil count from bronchoalveolar lavage fluid (BALF) obtained from the airways of Epi-KO mice. Ova S/C mice with PAR2 challenge were treated with either vehicle (VEH), alprenolol (ALP) (7200 ppm), carvedilol (CAR) (2400 ppm) or nadolol (NAD) (250 ppm). One group of vehicle-treated mice were challenged with ovalbumin alone. 'Control' (CTL) represents mice sensitized with ovalbumin and challenged with saline A and B) Total cell and eosinophil counts from CTL, vehicle- treated Ova S/C Epi-KO mice with or without PAR2 challenge in comparison to ALP, CAR or NAD-treated Epi-KO mice with PAR2 challenge. Data represent mean $\pm$ SEM from 4-8 mice in each group. *P<0.05 compared to CTL mice. $^{\#}$P<0.05 compared to vehicle-treated PAR2 S/C mice.

7.5 Discussion

7.5.1 PAR2 model of murine asthma

Our previous studies showed the requirement of epinephrine in the Ova S/C murine model of asthma using Epi-KO mice. However, there are other

models of murine asthma that have been shown to generate a worse asthma phenotype compared to Ova S/C (Ebeling *et al.*, 2005). In this study, a PAR2 ligand (SLIGRL-NH_2) was used to supplement the Ova S/C. Administration of the PAR2 ligand alone after ovalbumin sensitization to mice does not result in a significant increase in asthma phenotype (Ebeling *et al.*, 2005). However, co-administration of the PAR2 ligand with ovalbumin during the challenge phase resulted in the worsening of the asthma phenotype in mice (Ebeling *et al.*, 2005).

PAR2 can be activated by proteases *in vivo* and result in the activation of downstream inflammatory signaling cascades. In our model, Ova S/C was used but in addition, during the challenge phase the mice received 100 μM of a PAR2 ligand, SLIGRL-NH_2. Addition of the PAR2 ligand to the challenge phase led to development of the asthma phenotype in Epi-KO mice which were previously resistant to development of asthma phenotype in the Ova S/C model. Current data shows the susceptibility of Epi-KO mice to develop the asthma phenotype in the PAR2 model of murine asthma. Hence, the requirement of epinephrine in development of the murine asthma phenotype is model-dependent.

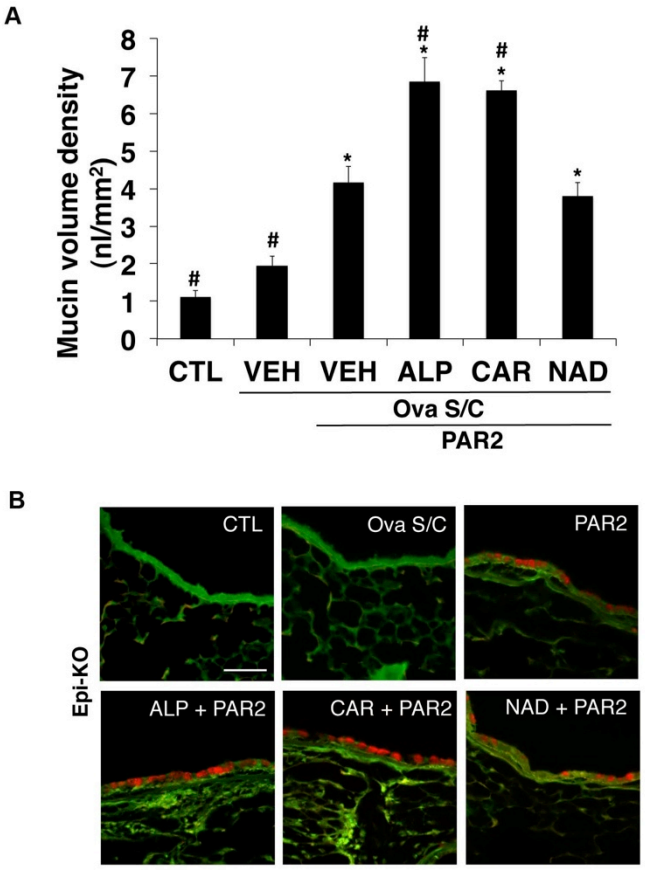

Figure 40. Effect of beta-blockers on mucin production in epinephrine knock out (Epi-KO) mice in the protease-activated receptor-2 (PAR2) ligand model: The figure shows the mucin content (red) in airway epithelia (green) after periodic acid fluorescent Schiff's (PAFS) stain of the airways of Epi-KO mice. Ova S/C mice with PAR2 challenge were treated with either vehicle (VEH), alprenolol (ALP) (7200 ppm), carvedilol (CAR) (2400 ppm) or nadolol (NAD) (250 ppm). One group of vehicle-treated mice were challenged with ovalbumin alone. 'Control' (CTL) represents mice sensitized with ovalbumin and challenged with saline A) Mucin volume density from CTL, vehicle-treated Ova S/C Epi-KO mice with or without PAR2 challenge in comparison to ALP, CAR or NAD-treated Epi-KO mice with PAR2 challenge. B) Representative images for all the groups. Scale bar (white) represents 100 μm. Data represent mean $\pm$ SEM from 4-8 mice in each group. *P<0.05 compared to CTL mice. $^{\#}$P<0.05 compared to vehicle-treated PAR2 S/C mice.

7.5.2 PAR2 and β_2AR signaling pathways and their effects on asthma phenotype

We have previously discussed at least two different signaling pathways are activated by the β_2AR: the $G\alpha_s$-dependent pathway and the arrestin-dependent ERK1/2 activation pathway. PAR2 is a GPCR that can also activate at least two downstream signaling pathways similar to the β_2AR: $G\alpha_q$-dependent pathway and the arrestin-dependent ERK1/2 activation pathway (Walker *et al.*, 2014). While, there is no evidence of a cross talk between the pathways of the β_2AR and PAR2, they both can activate ERK1/2 via arrestin-dependent pathway. Moreover, there is a paradoxical effect in the activation of both the β_2AR and PAR2 in murine asthma models. Acute activation of β_2AR and PAR2 results in relaxation of the airway smooth muscle providing relief from bronchoconstriction. However, chronic activation of β_2AR or PAR2 results in an increase inflammatory responses and loss of control of asthma. A recent review by Walker and Defea discusses the evidence that supports the role of the arrestin-dependent pathway in the inflammatory response to murine models of asthma (Walker *et al.*, 2014).

7.5.3 Administration of beta-blockers

In the current study, the effects of beta-blockers alprenolol, carvedilol and naolol were determined in the PAR2 model of murine asthma. Alprenolol and carvedilol administration to PAR2 Epi-KO mice worsened the eosinophilic

infiltration and mucous metaplasia compared to vehicle-treated PAR2 mice but had no significant effect on total cell count (Figures 39A, 39B, 40A and 40B). Nadolol had no beneficial or detrimental effect on cellular infiltration or mucous metaplasia in PAR2 Epi-KO mice compared to vehicle-treated PAR2 mice (Figures 39A, 39B, 40A and 40B). The effects of carvedilol and alprenolol on the asthma phenotype in the PAR2 model were similar to their effects in the Ova S/C model (they worsened the asthma phenotype in Epi-KO mice) but nadolol was not beneficial in the PAR2 model of murine asthma. In the Epi-KO mice with the Ova S/C model of murine asthma, nadolol did not restore the asthma phenotype and a beneficial effect could not be observed due to lack of a readout (Figures 28A, 28B, 30A, 30B). With the PAR2 model of murine asthma, Epi-KO mice showed an increase in inflammatory infiltration and mucin production, however, nadolol failed to attenuate the asthma phenotype.

The failure of nadolol can be explained by understanding the mechanism of action of nadolol and the signaling of PAR2. As discussed previously, the PAR2 is a GPCR that can activate at least two downstream pathways: G protein-dependent cAMP accumulation and arrestin dependent ERK1/2 (DeFea *et al.*, 2000; Kumar *et al.*, 2007; Ramachandran *et al.*, 2012). We have shown in our previous study, ligands that can activate ERK1/2 can lead to development of the asthma phenotype in the Epi-KO mice (Chapter 2), implicating ERK1/2 activation

in development of asthma phenotype. ERK1/2 activation by PAR2 can explain the severity of the asthma phenotype in the PAR2 model and the susceptibility of the Epi-KO in comparison to the Ova S/C model (Figure 41) (DeFea *et al.*, 2000). In addition, carvedilol and alprenolol both activate ERK1/2 downstream of the β_2AR and in the PAR2 model may lead to an additive effect on ERK1/2 activation supplementing the ERK1/2 activation by the PAR2 ligand.

Nadolol does not activate ERK1/2 via β_2AR and may not affect the ERK1/2 activation by the PAR2. Hence, we do not observe any change in the PAR2 asthma phenotype with nadolol administration to Epi-KO mice. The signaling profile of nadolol via β_2AR can explain lack of therapeutic benefit from administration of nadolol in the PAR2 model, since there is no known overlap or cross talk between the pathways of the β_2AR and the PAR2. The current study provides the possible limitations of the therapeutic benefits of nadolol in asthma therapy arising from a variety of etiological factors.

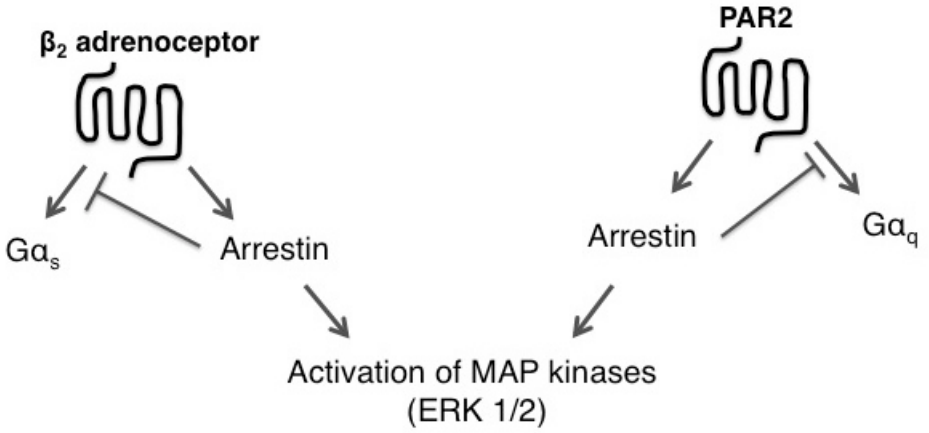

Figure 41. Signaling pathways of β_2 adrenergic receptor and protease-activated receptor-2: β_2 adrenergic receptor (β_2AR) can activate two downstream signaling pathways, the canonical G protein-dependent signaling pathway via $G\alpha_s$ and the arrestin-dependent pathway that leads to activation of mitogen-activated protein kinases (MAPKs) such as extracellular signal-regulated kinase (ERK1/2). Arrestin also inhibits the G protein-dependent signaling pathway. The protease-activated receptor 2 (PAR2) can also activate two downstream pathways via G protein $G\alpha_q$ and arrestin-dependent MAPK or ERK1/2 activation. Both β_2AR and PAR2 can activate ERK1/2.

7.6 Conclusion

Epinephrine is not required for development of the asthma phenotype in the PAR2 model of murine asthma. Alprenolol and carvedilol, ligands that activate ERK1/2 downstream of β_2AR worsen the asthma phenotype in Epi-KO mice in the PAR2 model of asthma. Nadolol does not activate ERK1/2 downstream of β_2AR and does not worsen or attenuate the asthma phenotype in Epi-KO mice in the PAR2 model of asthma.

8. Summary and conclusions

1) Genetic (using Epi-KO mice) or pharmacological (using reserpine) ablation of epinephrine prevents development of inflammatory cell infiltration, mucin overproduction and AHR in the Ova S/C model of murine asthma. The lack of an asthma phenotype suggests the requirement of ligand-activation in development of the asthma phenotype. β_2AR can be activated by a ligand or can be constitutively active in in the absence of a ligand. The requirement of epinephrine suggests that *constitutive activity at the β_2AR is not sufficient and ligand-activation is required for development of the asthma phenotype in the Ova S/C model of asthma*.

2) Moreover, administration of epinephrine or salmeterol to Epi-KO mice, to restore β_2AR signaling, results in restoration of the inflammation (mucin production for salmeterol) in the Epi-KO mice using the Ova S/C model of asthma. Epinephrine and salmeterol are β_2AR agonists that can activate both the G protein-dependent and the arrestin-dependent ERK1/2 activation signaling pathways of the β_2AR. The restoration of the asthma phenotype in Epi-KO mice by β_2AR agonists supports the previous conclusion that *ligand-activation of the β_2AR is required for development of the asthma phenotype.*

3) Chronic administration of a beta-blocker, alprenolol or carvedilol to Epi-KO mice also results in restoration of the asthma phenotype with Ova S/C model of asthma. Alprenolol is a partial agonist at the β_2AR that activates both the G protein-dependent and the arrestin-dependent signaling pathways of the β_2AR. Propranolol and carvedilol are inverse agonists (inhibit) at the G protein-dependent pathway but activate the ERK1/2 activation pathway of the β_2AR. Both alprenolol and carvedilol activate the arrestin-dependent pathway. The restoration of the asthma phenotype after alprenolol and carvedilol administration suggests that *activation of the arrestin-dependent ERK1/2 pathway is required for development of the asthma phenotype*.

4) Chronic administration of another beta-blocker nadolol does not restore the asthma phenotype in Epi-KO mice with Ova S/C model of asthma. Nadolol inhibits both the G protein-dependent and the arrestin-dependent signaling pathways of the β_2AR. This is consistent with previous murine data from other Class 5 ligands (Table 7) such as ICI-118,551 and metoprolol. Together, the data from administration of epinephrine, salmeterol, alprenolol, propranolol, carvedilol and nadolol to Epi-KO mice suggests the *role of the arrestin-dependent ERK1/2 activation pathway of the β_2AR in development of the asthma phenotype to the Ova S/C model of asthma.*

5) The classical model and the two-state model of receptor activation fail to explain the activation of the two independent signaling pathways of the β_2AR by different β_2AR ligands. The experimental data from Galandrin et al is comparable to the data generated by the mathematical modeling based on the isolated system of the three-state model of receptor activation for the activation of β_2AR by different β_2AR ligands. The similarity of the mathematical modeling to the experimental evidence suggests that *β_2AR ligands follow the isolated system of the three-state model of β_2AR activation.*

6) The Epi-KO mice develop an asthma phenotype in the PAR2 model of murine asthma. Alprenolol and carvedilol worsen the inflammation in the Epi-KO mice with PAR2 administration. Whereas nadolol is not beneficial or detrimental to the asthma phenotype in the Epi-KO mice in the PAR2 model of asthma. The susceptibility of the Epi-KO mice to the PAR2 model of asthma indicates that epinephrine is not required in the PAR2 model of asthma. PAR2 can also activate ERK1/2 via arrestin similar to β_2AR. The worsening of the inflammation with alprenolol and carvedilol in the Epi-KO mice with the PAR model can be attributed to the additive activation of ERK1/2 via both PAR2 and β_2AR.

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