The mechanisms of chronic nadolol treatment on the regulation of bronchial tone in a murine model of asthma

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A Dissertation Presented To

The Department of Pharmacological and Pharmaceutical Sciences

College Of Pharmacy, University Of Houston

In Partial Fulfillment Of The

Requirement for the Degree Of

Doctoral of Philosophy in Pharmacology

By

Rui Lin

August 2008

The mechanisms of chronic nadolol treatment on the regulation of bronchial tone in a murine model of asthma

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Rui Lin

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Richard A. Bond, Ph.D.
Advisor
Associate Professor, Department of Pharmacological and Pharmaceutical Sciences, University of Houston

_____
Brian J. Knoll, Ph.D.
Associate Professor of Pharmacology

_____
Carlos H. Pedemonte, Ph.D.
Professor of Pharmacology

_____
Gregory M. Cahill, Ph.D.
Associate Professor, Department of Biology and Biochemistry

_____
Robert H. Moore, M.D.
Associate Professor of Pediatrics, Baylor College of Medicine

_____

_____
Sunny E. Ohia, Ph.D.
Dean, College of Pharmacy

ACKNOWLEDGEMENTS

I am deeply indebted to the people who have assisted me, directly and indirectly, in completing my dissertation successfully.

Firstly, I would like to express my deep appreciation to my major professor, Dr. Richard Bond. This dissertation would not have been written without him, and it is he who provided me this great chance to get involved in this challenging project. As my advisor, Dr. Bond set up a great example for me both academically and personally. I am very grateful to him for all the encouragement and challenge throughout my entire academic program. I also thank Dr. Brian Knoll, Dr. Carlos Pedemonte, Dr. Gregory Cahill, and Dr. Robert Moore, who have each given me valuable guidance and suggestions for the project.

Secondly, I want to thank the entire faculty who have shared their invaluable knowledge with me. It would also have been impossible for me to accomplish this work without having taken all the courses and having set up a good knowledge background. Particularly, I want to thank Dr. Knoll, who has attended all our group meetings and shared a lot of laboratory resources with us. I also thank Dr. Tahir Hussain (Associate Professor, Department of Pharmacological and Pharmaceutical Sciences) who kindly let me use his equipment. I especially want to thank all my present and past lab mates for their support and friendship. Although their names are too many to be listed here, I will always cherish every single contribution. I cannot leave out my gratitude to my fellow graduate students who help and support me all the time, as well as to all the staff members who provide academic and administrative assistance. I really appreciate the chance given by the department for me to study and finish my research here.

Finally, I would like to express my warmest gratitude to my parents, Liyuan Lin and Lide Dai, and my husband, Qinchun Rao, for their continuous priceless support and their belief in me throughout my life. Without their support and understanding, I would not have been able to get through all the difficult times and keep going up to the last moment. I love you all!

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Abstract

Statement of the problem: Using a murine model of asthma, chronic beta blocker treatment was found to improve airway hyperresponsiveness (AHR). This finding completed a paradigm shift in the treatment of asthma using β adrenoceptor (AR) ligands, which was an analogy of the paradigm shift that occurred in congestive heart failure. Therefore, we hypothesized that chronic beta blocker treatment altered the cellular signaling modulating airway smooth muscle tone.

Procedure or methods: Radioligand binding assays were used to measure β AR number and further identify the subtype of β AR regulated by chronic beta blocker treatment. To evaluate the effect of chronic nadolol treatment on Gs signaling pathways, cAMP accumulation in the lung homogenates was measured by direct enzyme immunoassay; the cAMP-PKA dependent signaling was evaluated by isoproterenol induced tracheal relaxation; Gs, GRK2, GRK3, PDE4D and β arrestin 2, important proteins regulating the Gs signaling and playing important roles in lung physiology were measured by immunoblotting. To evaluate the effect of chronic nadolol treatment on Gi and Gq signaling pathways, $G\alpha_{i3}$, $G\alpha_{i2}$, Gq, GRK5, PLCβ1, PKCα were also measured by immunoblotting. To evaluate the effect of chronic nadolol treatment on arachidonic acid metabolism, cysteinyl leukotrines were measured in bronchoalveolar lavage fluid (BALF), while cPLA2, COX2 and 5-LO were also measured by immunoblotting. In addition, β_2 AR knockout mice were treated with the same protocol as the wildtype mice to confirm that the effects of chronic nadolol treatment on PDE4D, $G\alpha_{i3}$ and PLCβ1 were β_2 AR dependent.

Results: Chronic beta blocker treatment increased β_2 AR density and decreased PDE4D expression. Although chronic beta blocker treatment showed no effect on cAMP-PKA dependent airway smooth muscle relaxation, the regulation of Gi expression and Gi signaling in airway smooth muscle were found to be altered with chronic nadolol treatment. Also, chronic nadolol treatment decreased PLCβ1 expression and PKCα, 5-LO translocation. While a reduction in AHR, mucous metaplasia, and inflammatory cell counts in bronchoalveolar lavage fluid (BALF) was observed in wildtype mice, chronic nadolol treatment had no effect on PDE4D, Gi3 and PLCβ1 expression in β_2 AR null mice.

Conclusion: Chronic nadolol treatment decreased airway tone in a murine model of asthma by decreasing cAMP breakdown and Gi, Gq signaling. These effects are possibly dependent on its chronic blockade of β_2 AR.

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Abbreviations

4C3M	4-cyano-3-methylisoquinoline
5-LO	5-lipoxygenase
AC	Andenylyl cyclase
AHR	Airway hyperresponsiveness
BALF	Bronchoalveolar lavage fluid
CysLTs	Cysteinyl leukotrines
GPCR	G protein coupled receptor
GRK	G protein coupled receptor kinase
IBMX	3-Isobutyl-1-methylxanthine
ICI	ICI 118,551
Nad Chr, Nd Ch	s/c mice with 28 days nadolol treatment
NS/NC, ctrl	Naïve mice without drug treatments
NTX s/c, s/c	Sensitized challenged mice
OVA	ovalbumin
PDE	Phosphodiesterase
PKA	Protein kinase A
PKC	Protein kinase C
PLA	Phospholipase A
PLC	Phospholipase C
SDS-PAGE	sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis

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CHAPTER 1 Introduction and statement of problem

β_2 adrenoceptors (β_2 ARs) are typical G protein coupled receptors (GPCRs) expressed in many tissues and cells including those of the airway. β_2 AR agonists are the most effective bronchodilators for asthma therapy (Rossing, Fanta et al. 1980; Britton, Hanley et al. 1988; Barnes 1993; Waldeck 2002). However, the use of long-acting β_2 AR agonists (LABAs) is not recommended as first line therapy by the FDA (FDA 2005), because accumulating evidence has shown that chronic use of β_2 AR agonists is associated with worsening of asthmatic symptoms and asthma-related deaths (Spitzer, Suissa et al. 1992; Cockcroft, McParland et al. 1993; Suissa, Ernst et al. 1994; Drazen, Israel et al. 1996; Taylor, Town et al. 1998; Nelson, Weiss et al. 2006). Antagonists of β_2 ARs, namely beta blockers, are contraindicated in asthma therapy because they antagonize the relaxant effect of β_2 AR on airway smooth muscle and produce a detrimental effect when used acutely (Dunn, Gerber et al. 1986; Nelson, Fraunfelder et al. 1986; Spitz 2003). However, chronic use of some beta blockers showed a protective effect against airway hyperresponsiveness in a murine model of asthma (Callaerts-Vegh, Evans et al. 2004) and in a small pilot clinical study in mild asthmatics (Hanania, Singh et al. 2006; Hanania, Singh et al. 2008). These outcomes completed the parallel between congestive heart failure and asthma in terms of the use of β AR ligands (Bond 2001): β agonists increased cardiac output acutely but also increased mortality when used chronically (Weber, Andrews et al. 1982; Nicholas, Oakley et al. 1990). Beta blockers

were also contraindicated in heart failure therapy until the late 1990's. However, chronic therapy with some beta blockers was shown to increase cardiac inotropy and decrease mortality in heart failure (Bristow, Gilbert et al. 1996; Packer, Bristow et al. 1996; Lechat, Packer et al. 1998). The mechanisms of these beneficial effects are still rarely understood.

Since upregulation of β AR density has been shown in lung membranes after chronic beta blocker treatment (Callaerts-Vegh, Evans et al. 2004), we hypothesized that chronic beta blocker treatment decreased the bronchial tone by regulating β_2 AR signaling on the airway smooth muscle. To test this hypothesis, we examined both bronchodilatory signaling and bronchoconstrictive signaling pathways in lung homogenates and tracheal ring preparations, because it has been suggested that bronchial tone is balanced by both signaling pathways (Deshpande and Penn 2006). The Gs signaling pathway is the only bronchodilating pathway currently known to be present in airway smooth muscle. The activation of pulmonary β_2 ARs can activate the Gs signaling pathway but also produces desensitization of receptors (Billington and Penn 2003). In addition, β_2 ARs have been also shown to couple with Gi protein and regulate airway smooth muscle function (Hakonarson, Herrick et al. 1995). Therefore, the function and the expression of G proteins, GRKs and other proteins involved in these two signaling pathways of β_2 AR were examined. β_2 AR signaling has also been found to crosstalk with Gq signaling (McGraw and Liggett 2005; McGraw, Elwing et al. 2007), which is the predominant

bronchoconstrictive signaling pathway. Therefore, we hypothesized that 1) chronic beta blocker treatment improves bronchodilatory signaling pathways by upregulating the expressions of β_2 ARs and downregulating cAMP degradation or β_2 AR desensitization; 2) chronic beta blocker treatment attenuates bronchoconstrictive signaling pathways by downregulating the expressions of key component proteins, such as the effector enzymes PLCβ1, PKCα, and the enzymes responsible for leukotrienes production, a potent bronchoconstrictor using Gq signaling pathway; and 3) the effect of chronic beta blocker treatment on bronchoconstrictive signaling pathways is dependent on the regulation of the β_2 AR signaling pathway.

In this dissertation, we partly review the literature related to our study in chapter 2, and list all material and methods used for this study in chapter 3. In chapter 4, we describe the effect of chronic beta blocker treatment on AHR and pulmonary β_2 AR expression. Chapter 5 discusses the effect of chronic nadolol treatment on classical β_2 AR signaling pathways, and its effects on non-classical β_2 AR signaling pathways are disused in chapter 6. In the end, our main findings are summarized in chapter 7.

CHAPTER 2 Review of literature

1 Asthma

Asthma is a chronic disease characterized by airway hyperresponsiveness (AHR) and inflammation. According to World Health Organization (WHO) estimates, in 2005, 300 million people suffered from asthma and 255,000 people died of asthma. The rate of asthma has increased in recent decades and is still increasing. It is estimated that an additional 100 million persons will suffer from asthma in 2025 (Masoli, Fabian et al. 2004). In the United States, over 22.2 million people were diagnosed with asthma, and over 12.2 million people needed hospitalization in 2005. Although death from asthma is a very uncommon event considering the large number of patients being diagnosed, there were still over 4200 asthma deaths in 2002 (Akinbami 2006), indicating that over 11 people per day die of asthma in the United States. Many of these deaths would have been preventable if asthma control had been consistent and successful. Even with good asthma control, the direct costs due to medical expenses and the indirect costs due to loss of work productivity and school days were both high because, according to (Weiss 2001), up to 90% of cases the diagnosis is made by the age of 6. In addition, studies have shown that the decline of lung function with aging was more progressive in asthmatics than non-asthmatics (Masoli, Fabian et al. 2004). Obviously, there is a demand for drug therapies which are safe and efficacious.

The hallmark features of asthma include reversible airflow obstruction, bronchial hyperresponsiveness, and airway inflammation. Airway obstruction is a respiratory problem due to a decreased amount of air inhaled in each breath. According to studies, severe airway obstruction develops in patients with persistent and fatal asthma, and is considered to result from airway remodeling. Airway remodeling involves mucous gland hyperplasia, mucous plugging, collagen deposition, basement membrane thickening, bronchial smooth muscle hypertrophy, new blood vessel growth, and goblet cell hyperplasia (Chetta, Foresi et al. 1997; Holgate, Holloway et al. 2006). Some of these structural changes in airways may also induce AHR, another universal feature of asthma. AHR is a symptom of increased sensitivity to environmentally provocative stimuli. It is also thought that the narrowness of the airway tract, the increase of inflammatory mediators, altered bronchial epithelial integrity, and neurohumoral influences all have input to the severeness of airway hyperresponsivenss (Jeffery, Wardlaw et al. 1989; Lambert, Wiggs et al. 1993; Boulet 2003). Airway inflammation is thought to induce almost all other clinical manifestations. The presence of numerous eosinophils, mast cells, activated T cells, neutrophils, and macrophages in asthmatic airways predispose the release of cytokines and inflammatory mediators, which in turn induce epithelium injury, vascular leakage, mucus secretion and the other pathogenesis (Barnes, Chung et al. 1998; Hanania 2008).

There are several biological pathways, mediators and different cell types that play varying roles in the pathogenesis of asthma. Although the pathogenesis of asthma varies among patients, the therapeutic strategies can be classified into two categories: those which adjust the imbalance of the contractile and relaxant function of the airway, and those which decrease inflammation in the lung. The abnormality of airway smooth muscle has been considered to be largely responsible for airway obstruction and hyperresponsiveness. The smooth muscle cells in asthmatic subjects are more sensitive to provocative stimuli, and more susceptible to airway remodeling, smooth muscle hypertrophy and hyperplasia (Rodger 1992; Black and Johnson 1996; Holgate, Holloway et al. 2006). Current knowledge about airway smooth muscle indicates that it has immunomodulatory functions aside from contractile function (Hershenson, Brown et al. 2008; Tliba, Amrani et al. 2008). In our study, we focus on the effect of drug treatments on the contractile function of airway smooth muscle.

Although there is no cure for asthma, asthma control is achieved with medications such as β_2-adrenergic agonists, inhaled corticosteroids, leukotriene modifiers, and anti-IgE antibodies (Barnes 2006). Until now, short acting β_2-adrenergic agonists have been the best rescue bronchodilators. However, chronic use of β_2-adrenergic agonists has aroused widespread debate as increased mortality and morbidity have been shown to be associated with regular use of β_2-adrenergic agonists (Currie, Lee et al. 2006; Walters, Gibson et al. 2007). The mechanisms of the detrimental effect of β_2-adrenergic agonists

have been rather elusive. The majority of the asthma research community agrees that the downregulation of β_2-adrenergic receptors was one of the main causes for the regular use of β_2-adrenergic agonists to exacerbate the mortality and morbidity of asthma (Hancox 2006) in the long run. It was also recognized that certain single nucleotide polymorphisms of the β_2-adrenergic receptor gene correlate with a higher susceptibility to desensitization than the other alleles (Small, McGraw et al. 2003), though there has been some controversy between in vitro and in vivo studies (Leineweber and Brodde 2004). However, the effect of beta blocker treatment on asthmatics had not been extensively studied because the acute treatment by beta blocker exacerbated airway hyperresponsiveness.

2 Temporal hormesis of drug therapy

GPCRs are members of the biggest superfamily of proteins targeted by marketed drugs. From the observations on the dose response of GPCR ligands, hormesis was characterized as a phenomenon of low dose stimulation and high dose inhibition (Calabrese and Baldwin 2001). However, from a temporal standpoint, GPCR ligands show a beneficial effect acutely, but a detrimental effect chronically can be frequently found in various diseases, such as the increased morbidity and mortality of using β AR agonists in heart failure (Shipley and Hess 1995) and asthma (Nelson, Weiss et al. 2006), the establishment of tolerance of morphine, barbiturates, the loss of response of dopamine

antagonist used for Parkinson's disease therapy (Gerlach and Riederer 2003), and alcohol addiction (Hack and Christie 2003). This phenomenon can be defined as a temporal hormesis. Of note, the temporal hormesis was not only a characteristic of agonists but also a trait of antagonists: certain antagonists of β adrenergic receptors showed an acutely detrimental effect, but a chronic beneficial effect, such as the chronic use of some beta blockers in heart failure (CIBIS 1994). According to our findings, in an animal model of asthma, some beta blockers also showed a beneficial effect when given chronically (Callaerts-Vegh, Evans et al. 2004). Although this paradigm shift is currently limited to the animal model of asthma, we believe it could be employed in many other models if the mechanism can be better understood.

3 **Receptor theory and inverse agonism**

An inverse agonist belongs to a subgroup of antagonists which also decrease the constitutive physiological response in biological systems. Inverse agonists were classified as a subset of antagonists because they could block the effect of agonists. However, Costa and Hertz (1989) first demonstrated the negative intrinsic activity of some antagonists, which were later defined as inverse agonists. Enlightened by this, the constitutive activity of receptors had been discovered. It turned out that the characteristics of receptors varied in different biological systems in terms of certain pharmacological readouts: some conventional agonists became partial agonists or even

antagonists in a system where the receptors showed high constitutive activity. In contrast, partial agonists became full agonists in systems with low constitutive activity (Milligan 2003). And those antagonists showing negative intrinsic activity, which actually inhibited the constitutive activities, were defined as inverse agonists because they stimulated the inhibition of constitutive activities in a dose-dependent manner, exactly mirroring the way of agonists to stimulate the "visible" receptor responses (Figure 1). The use of constitutively active receptor systems and transgenic animals with overexpressed receptors revealed that the inverse agonism was a common phenomenon (de Ligt, Kourounakis et al. 2000; Milligan 2003). 85% of competitive antagonists are inverse agonists (Kenakin 2004; Costa and Cotecchia 2005).

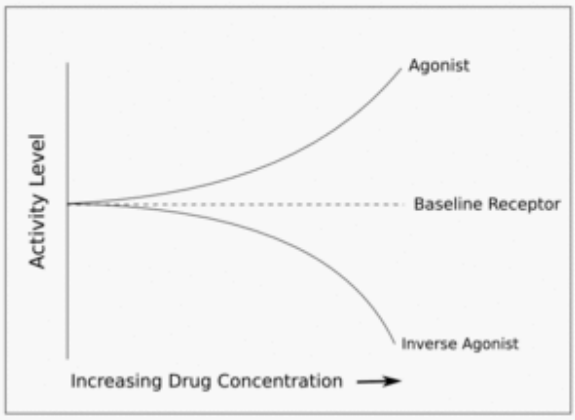


Figure 1. Inverse agonist versus agonist

The effects of increasing concentration of agonists and inverse agonists are exactly mirror symmetry.
(http://upload.wikimedia.org/wikipedia/commons/thumb/6/6d/Inverseagonist.gif/300px-Inverseagonist.gif, accessed on May 13 2008)

The concept of inverse agonism and constitutive receptor activity revolutionized receptor theory. In the early 1900's, an occupation theory was originally established to explain the relationship between receptor occupancy and responses. In a specific system, the occupancy of a receptor by agonists induces the conformational changes directing a response, whereas occupancy by antagonists produces no response. Without the occupancy of agonists, receptors are inactive and show no response (Kenakin 2004).

Borrowing from the study of ion channels, a simple two-state model was set up to better summarize the diverse responses of one single receptor in response to agonists with different strengths. The two-state model proposes an equilibrium of active and inactive states, in which agonists could drive the equilibrium to the active state and inverse agonists worked in an opposite manner. To incorporate the effect of G proteins, an extended ternary model (Figure 2 (a)) was proposed to elaborate on the simple ternary complex model (De Lean, Stadel et al. 1980; Samama, Cotecchia et al. 1993). With the understanding that the binding of receptor and G protein was the first step of signaling, the ability to activate receptors was considered to be the ability to elicit the binding of receptor and G proteins, namely, efficacy. In the extended ternary model, two states of receptors (Ra and Ri) were also proposed to coexist even without the presence of ligands. The presence of agonists shifted the balance of two states towards active state (Ra) to form more receptor-agonist complexes (ARa). Inverse agonists drove the equilibrium of receptor to inactive state (Ri) and formed a receptor-inverse agonist complex, while

neutral antagonists just kept the balance of the two states. A cubic ternary complex model suggested that the inactive receptors could also bind with the G protein but just do not signal (Figure 2 (b)). This model fit better with thermodynamic principles and was also supported by some concrete data (Kenakin 2004a; Rang 2006).

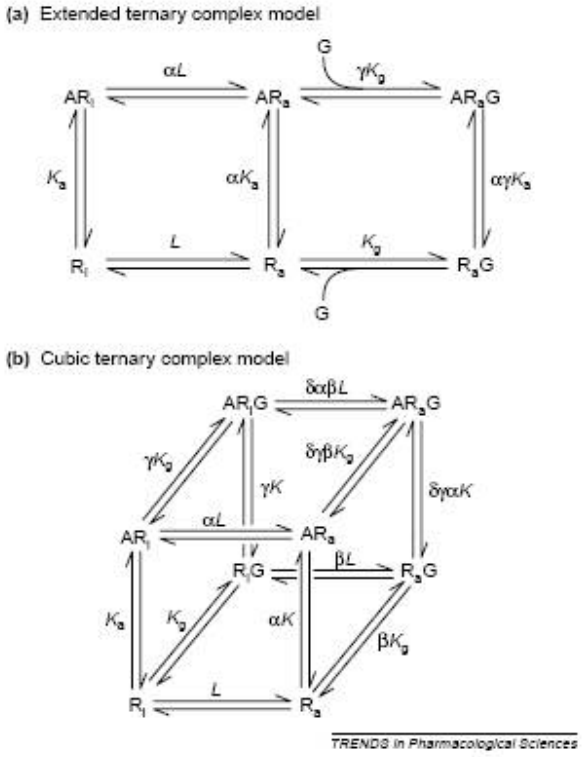


Figure 2. Extended ternary complex model and cubic ternary complex model
(a) Extended ternary complex model. Both states (Ri) and (Ra) of receptor coexist according to the allosteric constant L. The physiological response can be induced by the binding of G protein (G) with the activated receptor state Ra. Ligand A can bind with both receptor states and also with the complex of Ra and G protein. The tendency of the system to produce constitutive activity (spontaneous formation of the active state RaG species) is defined by the allosteric constant L {L= [Ra]/[Ri]}. The affinity of ligands for the receptors is given by Ka whereas the efficacy is described by two terms, α and γ. The term α is the differential affinity of the ligand for Ra and the term γ is the differential affinity of the ligand-bound ARa for G proteins. (b) Cubic ternary complex model. The inactive receptor species Ri and ARi are allowed to interact with G

proteins (but not signal) in this variant model. β refers to the differential affinity of the receptor active state (over the inactive state) for the G protein. (Kenakin 2004a)

To this date, newly discovered responses and post-receptor activities have brought a lot of challenges to the old ternary complex models. Experimental observations have revealed that one receptor can bind to more than one G protein and simulate multiple signaling pathways. Instead of the two-state model, a single receptor may reserve a collection of micro-conformations, each of which signals certain molecular reactions, such as coupling to the G protein, internalization, phosphorylation, and dimerization or interaction with other membrane proteins. In other words, receptor efficacy extends to a spectrum of rate constants denoting ligand-selective conformations. Additionally, the allosteric effect caused by the binding of auxiliary coupling proteins on a site distinct from the natural binding locus of endogenous ligands also enriches the spectrum of efficacy (Kenakin 2001; Kenakin 2003; Kenakin 2004a; Kenakin 2004b; Kenakin 2005; Kenakin 2006). Therefore, some inverse agonists could also be classified as protean agonists or allosteric agonists in certain cellular milieu. Using these theories as tools, we have tried to screen the potential mechanisms of certain beta blockers showing temporal hormesis in an animal model of asthma.

4 Potential clinical significance of inverse agonists

With the discovery of inverse agonists, their physiological and pathological relevance and implication have received more and more attention. Generally speaking, the effect of inverse agonists was undetectable in physiological states. However, the inhibitory effect of inverse agonists on constitutive receptor activity might have clinical significance especially in diseases caused by constitutively active receptors (Parra and Bond 2007). For example, heart failure patients with β_1AR Gly49 polymorphism, who had a higher constitutive activity of β_1 receptor than the Ser49 variant, showed a higher survival rate than patients with that variant. Chronic treatment with beta blockers showed a better effect on individuals with the Gly49 variant than those with wild type receptors (Borjesson, Magnusson et al. 2000; Magnusson, Levin et al. 2005). These beta blockers can be classified as inverse agonists and show clinical significance.

When the period of the drug administration is taken into account, the effect of inverse agonists can alter completely. In heart failure, acute treatment by beta blockers can worsen the weak contractility of the myocardium signaled by β receptors, but chronic treatment with beta blockers reduced mortality and improved heart contractility (Waagstein, Hjalmarson et al. 1975; Swedberg, Hjalmarson et al. 1980; Anderson, Lutz et al. 1985; Bristow 2000). Carvedilol, metoprolol, and bisprolol are beta blockers categorized as inverse agonists, and have been shown to be efficacious in reducing the mortality of heart failure. Although the mechanisms of chronic beta blocker treatment in

heart failure is still ill-defined, long term treatment of inverse agonists had been shown to be associated with receptor upregulation and crosstalk with other receptors, especially receptors sharing the same signaling G proteins (de Ligt, Kourounakis et al. 2000).

4.1 Inverse agonism in heart failure

Amplified signaling in experimental models was achieved by pharmacological or genetic interventions in order to evaluate the effect of inverse agonists. Chronic treatment with beta blockers that showed inverse agonist properties, such as metoprolol and carvediol, have demonstrated beneficial effects on failing hearts with amplified signaling. However, chronic treatment with bucindolol, which was an antagonist with no inverse agonist characteristics, showed no beneficial effect (Maack, Cremers et al. 2000). This suggested that the inverse agonism, instead of neutral blockade of β AR, contributed to the beneficial effects of the chronic treatment by beta blockers. Chronic agonist treatment decreased receptor density, which was considered to be the result of receptor desensitization. In parallel, chronic treatment with inverse agonists could increase receptor density (Elfellah and Reid 1989) which may result in enhanced cellular signaling. Therefore, the temporal hormesis of inverse agonism might be an analog of the temporal hormesis of agonism, with the direction just reversed (see Figure 3).

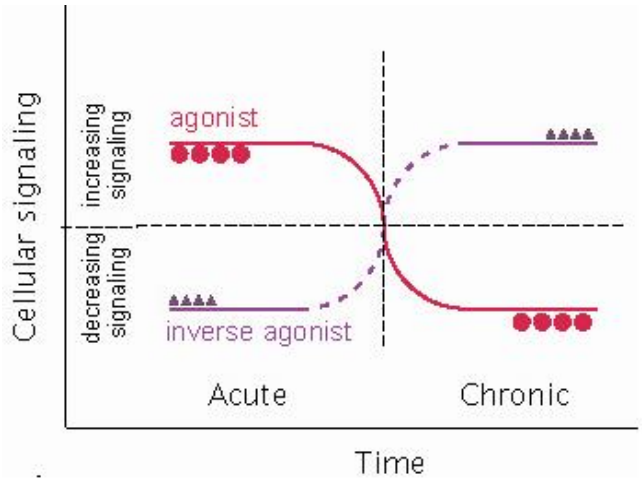


Figure 3. Hypothesis of temporal hormesis

Fundamental hypothesis of temporal hormesis, that agonists and inverse agonists have temporally reciprocal effect on cellular signaling. (Unpublished figure created by Zsuzsanna Callaerts-Vegh)

4.2 Is asthma another example?

The beneficial effect of chronic beta blocker treatment in an asthma model has been only shown in our laboratory (Callaerts-Vegh, Evans et al. 2004). However, in the temporal hormesis in β agonist therapy, a nice parallelism was shown between the use of β AR ligands in heart failure and asthma. Regardless of the subtype preferences, β AR ligands are important therapies in disease control. When used acutely, agonists showed a beneficial effect and antagonists showed a detrimental effect. When used chronically, agonists lost the beneficial effect or even resulted in a detrimental effect, and antagonists paradoxically showed a beneficial effect. Desensitization of β AR has been considered as

the mechanism of the loss of beneficial effect by chronic β AR agonist treatment in both diseases. Although it is controversial to explain the beneficial effect of chronic beta blocker treatment in heart failure by inverse agonism, it is still rational to hypothesize that the inverse agonism is one of the mechanisms of the beneficial effect in chronic beta blocker treatment (Bond, Spina et al. 2007).

5 Signal transduction of β_2 AR in airway smooth muscle cells

Airway and lung function depends on the orchestration of multiple cell types. For example, injured epithelium attracts the infiltration of inflammatory cells, and causes chronic inflammation and fibrosis of airway smooth muscles. Also, epithelial cells control mucous production and mucociliary clearance which are also related to the regulation of airway tone (Holgate, Peters-Golden et al. 2003). Therefore, the contractile state of airway smooth cells is still the main focus of a few therapeutic strategies, including β_2 AR agonists. In order to elucidate the mechanism of chronic beta blocker treatment, our studies have focused on its effect in airway smooth muscle tone. β AR related signaling transduction mechanisms involved in regulating the airway smooth muscle tone are also discussed.

5.1 Airway smooth muscle

Airway smooth muscle cells lay under the epithelium and circumferentially surround the airway tract. These cells bundle together and narrow the hollow airway tract when the airway contracts. Modest contraction occurs in a normal airway tract, but bronchoconstriction often occurs in asthmatic airways in response to certain stimuli. Although the precise pathophysiology of hyperresponsiveness of the airway smooth muscle is not fully understood, it is still considered that GPCRs play a critical role in the regulation of airway smooth muscle function. The rapid brochodilatory effect of the β_2 AR agonist is a very good example. However, over 20 receptor types expressed in airway smooth muscle cells have been identified, and this number is still increasing. They are involved in the regulation of not only contractility, but also airway inflammation, airway remodeling, and mucus hypersecretion (Billington and Penn 2003; Deshpande and Penn 2006). Contractile dysfunction has been considered to be the central role of airway smooth muscle in the pathology of asthma, though most recent research has revealed the increasing importance of the immunomodulatory function of airway smooth muscle (Hershenson, Brown et al. 2008; Tliba, Amrani et al. 2008).

5.2 The classical Gs pathway

β_2 AR occupies up to 70% of the total pulmonary β AR population in various species (Goldie, Papadimitriou et al. 1986; Mardini, Higgins et al. 1994; Abraham, Kottke et al.

2003). In airway smooth muscle cells, β_2 AR couples with a heterotrimeric G protein, $G\alpha_s$ subunit. The activation of β_2 AR induces the dissociation of the heterotrimeric G protein. In turn, the dissociated $G\alpha_s$ subunit binds to the membrane bound enzyme adenylyl cyclase (AC). Gs-AC coupling subsequently activates the enzymatic effect of AC and then induces the production of cAMP. cAMP levels are also regulated by phosphodiesterase (PDE), which degrades cAMP to a non-bioactive metabolite, 5′-AMP. The formation of cAMP leads to the characteristic cellular response via the activation of a specific protein kinase, protein kinase A (PKA), by dissociating its regulatory (inhibitory) subunits. PKA then phosphorylates serine and threonine residues on specific proteins, such as myosin light chain kinase, resulting in a direct relaxant effect on the contractile machinery. Also, cAMP and PKA dependent phosphorylation of the cAMP response element binding protein (CREB), the transcription factor, subsequently controls the expression of numerous genes; therefore, PKA-CREB regulation is also possibly involved in the function of the airway smooth muscle (Billington and Penn 2003).

However, activation of the β_2 AR-Gs coupled signaling pathway could be also cAMP independent. The study of an isoproterenol-induced calcium activated potassium channel opening has suggested that a β AR stimulated Gs protein could directly regulate the opening of a potassium channel (Kume, Hall et al. 1994). Also, Gs stimulated but cAMP and PKA independent airway smooth muscle relaxation has been observed in physiological (Spicuzza, Belvisi et al. 2001; Tanaka, Yamashita et al. 2003) and

pathological status (Song, Milanese et al. 2000), though signaling pathways in both cases were still ill-defined.

5.3 Desensitization of β_2 AR

β_2 AR is well-known to be susceptible to desensitization after exposure to agonists. Two kinds of phosphorylation have been identified to be responsible for β_2 AR desensitization. PKA mediated phosphorylation of β_2 AR doesn't need high occupancy of receptors by a strong agonist. Therefore, it is historically referred to heterologous desensitization because it could be induced by the activation of other receptors. It has shown that PKA consensus site on the third intracellular loop causes 40~60% uncoupling of receptor from Gs with a short half-life (1~2min) (Clark, Knoll et al. 1999). In contrast, GRKs mediated phosphorylation is referred to homologous desensitization because it depends on relatively high occupancy of receptor by strong agonists. Phosphorylation by GRK occurs on C-terminal of β_2 ARs, and promotes rapid binding of arrestins which finally uncouples the receptor with Gs. A high concentration of agonist is needed for switching from PKA phosphorylation to GRK phosphorylation (Clark, Knoll et al. 1999). β arrestin and GRKs mediated internalization was found to be one of the mechanisms of β_2 AR desensitization (Goodman, Krupnick et al. 1996). Internalized receptors may be sorted into endosomes and end up with resensitization or degradation (Freedman, Liggett et al. 1995; Freedman and Lefkowitz 1996). Desensitization of β_2-ARs has been considered to be one of the

mechanisms which induce the detrimental effect of chronic β AR agonist therapy for asthmatics, while it could also be the candidate intracellular event modified by chronic beta blocker treatment.

5.3.1 Homologous desensitization

GRK2, GRK3 and GRK5 have been reported to be involved in the desensitization of β_2 AR in vivo. GRK2 plays such an important role in cardiac function that no other kinases can compensate the loss of GRK2 in genetic modified animals (Metaye, Gibelin et al. 2005). The clinical significance of GRK2 has been shown by the increase of GRK2 expression and activity in heart failure, while beta blockers tend to inhibit the increase of GRK2 (Leineweber, Rohe et al. 2005). In cystic fibrosis lungs, the expression of GRK2 and GRK5 is also higher than in the healthy subjects (Mak, Chuang et al. 2002). The desensitization of β_2 AR in airway smooth muscle by chronic administration of albuterol (Finney, Belvisi et al. 2000) and IL-1 (Mak, Hisada et al. 2002) has also been associated with increased GRK2 and GRK5 activity. Although genetically altered mice lacking one copy of GRK2 (GRK2 +/ −) showed no difference in airway function compared to wildtype mice, homozygous GRK3 knockout (GRK3 −/ −) mice demonstrated a significantly higher response to methacholine (Walker, Peppel et al. 1999). GRK5 knockout mice also showed a decrease in β_2 AR induced relaxation of airway smooth muscle, suggesting the role of GRK3 and GRK5 in desensitization of muscarinic

receptors and followed by the weaker antagonism of β_2 AR induced relaxation in airway smooth muscle (Walker, Gainetdinov et al. 2004).

5.3.2 Heterologous desensitization

PKA is involved in the rapid desensitization of β_2-ARs, the stimulation of PDE activity (Houslay and Adams 2003), the regulation of numerous gene transcriptions involved in inflammation, crosstalk to tyrosine receptor signaling and $G\alpha_q$-phospholipase C (PLC) signaling (Dodge and Sanborn 1998). Cytokines and prostaglandins can cause desensitization of β_2 AR responsiveness on airway smooth muscle in a PKA-dependent manner (Guo, Pascual et al. 2005; Hu, Nino et al. 2008). PKA is involved not only in the intracellular events of airway smooth muscle relaxation, but also in heterologous desensitization of the cellular signaling induced by airway constrictors. For example, PKA mediates the downregulation of the histamine H_1 receptor (Kawakami, Miyoshi et al. 2004) and the M_2 muscarinic receptor (Rousell, Haddad et al. 1996), the inhibition of PLCβ activity (Dodge and Sanborn 1998), and the increased sensitivity of PDE4D3 (Houslay and Adams 2003). Although evidence has shown that the anti-spasmogenic effect of β_2 AR on guinea pig tracheal was not dependent on PKA (Spicuzza, Belvisi et al. 2001), as one of the most important effectors of Gs signaling pathways PKA still plays a very important role in the regulation of airway GPCR signaling.

5.3.3 β arrestin

Conventionally, β arrestins are only considered to be the adaptor proteins responsible for GRK-mediated receptor internalization. However, recent studies have revealed the diverse functions of β arrestins in cellular signaling. Besides desensitization, β arrestins have been reported to regulate the mitogen-activated protein kinases, ERK, JNK, p38, Akt, PI3 kinase, and RhoA, as well as NFkB (Lefkowitz and Whalen 2004; DeWire, Ahn et al. 2007). The β_2 AR inverse agonist was shown to traffic the signaling to ERK1/2 via a β arrestin-dependent pathway (Azzi, Charest et al. 2003). The complex of β arrestin and PDE4 regulates the phosphorylation of PKA, then results in the switch of β_2 AR signaling and T cell migration (Baillie and Houslay 2005; Houslay and Baillie 2005). More intriguing, the importance of β arrestin in the pathogenesis of asthma has also been demonstrated in vivo using β arrestin 2 knockout mice (Walker, Fong et al. 2003). Therefore, β arrestin might play a unique role in the regulation of airway smooth muscle function besides its function in potentiating β_2 AR desensitization.

5.4 The role of PDE4D in the regulation of airway tone

The intracellular content of cAMP is determined by both AC activity and phosphodiesterase (PDE) activity, which degrade cAMP into non-functional 5'-AMP. Among the 11 mammalian PDE families, the PDE4 family is most important to airway function (Houslay and Adams 2003). Selective PDE4 inhibitors showed a notably

appealing therapeutic profile for asthma (Torphy 1998; Sun, Deng et al. 2006). For example, cilomilast, which is ten-fold more selective for PDE4D than for PDE4A, B and C, showed better efficacy than rolipram, the non-selective PDE4 inhibitor, applied in the treatment of asthma and chronic obstructive pulmonary disease (COPD) (Singh, Barrett et al. 2003). In support of clinical studies, PDE4D has been identified as the major PDE species in airway epithelia (Barnes, Livera et al. 2005). Also, a cAMP-driven upregulation of PDE4D5, a splice variant in PDE4D family, has been identified in human airway smooth muscles (Le Jeune, Shepherd et al. 2002). Therefore, PDE4D, the major isoform expressed in the airway, is the most relevant to the airway function. PDE4D expression and activity has been shown to increase in BALB/cJ mice under cigarette smoke exposure, *in utero* (Singh, Barrett et al. 2003). PDE4D knockout mice that did not express PDE4D protein were resistant to cholinergic airway contraction, with or without antigen challenge (Hansen, Jin et al. 2000). A more recent study from the same group confirmed that the resistance to cholinergic airway contraction in PDE4D knockout mice was related to the enhanced level of cAMP accumulation, in response to endogenous prostanoids (Mehats, Jin et al. 2003). Therefore, PDE4D might also be an important target protein for the regulation of chronic beta blocker treatment.

5.5 Non-classical β_2 AR signaling pathways

5.5.1 β_2 AR Gi coupling in airway smooth muscle

The coupling of β_2 AR and Gi protein has been extensively studied in heart failure. In myocardium, β_2 AR was discovered to couple to both Gs and Gi proteins. In failing hearts, β_1 AR-Gs signaling was found to be decreased and therefore the branch of Gi signaling by β_2 AR stimulation was accentuated (Xiao 2001). Selective blockade of Gi signaling can only increase β_2 AR mediated inotropic function but not β_1 AR mediated inotropic activity (Xiao, Zhang et al. 2003). The stimulation of the L-type Ca^{2+} channel may be one of the mechanisms (He, Balijepalli et al. 2005). Increased expression of Gi protein was found in heart failure (El-Armouche, Zolk et al. 2003), and a beta blocker was shown to work as an agonist of Gi signaling so that it could antagonize the Gs signaling of β_2 ARs in a failing human heart (Harding and Gong 2004).

However, studies on β_2 AR and Gi coupling in airway smooth muscle have been relatively few. It has been demonstrated on the relaxation of rabbit tracheae sensitized by human serum from allergic human beings (Hakonarson, Herrick et al. 1995), where the Gi3 protein showed higher expression in sensitized tracheal rings than in the non-sensitized ones. The higher expression of the Gi3 subunit has also been reported on the bronchial smooth muscle of rats, which showed hyperresponsiveness to acetylcholine (Chiba, Sakai et al. 2001). In mice, a strain which is hyperresponsive to intravenous

acetylcholine challenge showed higher expression of Gi and higher affinity with muscarinic receptors than a strain which is hyporesponsive (Gavett and Wills-Karp 1993). The study on human CD4 cells also showed that Gi3 expression was increased in asthmatic subjects (Kim and Agrawal 2002). The Gi2 subunit has also been shown to regulate airway function. Transgenic mice overexpressing Gi2 showed lower airway resistance than wildtype mice. Conversely, mice expressing Gi2 inhibitory peptide in airway smooth muscle showed higher airway resistance (McGraw, Elwing et al. 2007). However, in vitro study using airway smooth muscle cells has shown that the blockade of Gi2 actually abolished carbachol-induced actin reorganization, which is one of the hallmarks denoting the activation of airway smooth muscle cells (Hirshman and Emala 1999).

It is still unclear whether β AR signaling in airway smooth muscle is regulated by the Gi protein. There is evidence showing the involvement of Gi signaling in M2 receptor (Hirshman, Lande et al. 1999) mediated airway smooth muscle contraction. It has also been shown that Gi mediated the decrease of tracheal relaxation and cAMP production in response to β_2 AR stimulation (Hakonarson, Herrick et al. 1995; McGraw, Elwing et al. 2007). In addition, Gi seemed to play a role in regulating the PDE4D gene and its expression, which would also affect the cAMP production and heterologous desensitization (Hu, Nino et al. 2008).

5.5.2 Crosstalk of Gs and Gq signaling transduction in airway smooth muscle

Gs signaling and Gq signaling in airway smooth muscle cells mutually affect and antagonize each other in order to keep the balance of airway tone. The variety of Gq-coupled receptors on airway smooth muscles is more than that of Gs-coupled receptors. They are principle mediators of contraction. Followed by receptor activation, Gq activation induces phospholipase C activation, which subsequently hydrolyzes the phosphoinositol 4,5-bisphosphate (PIP2) into 1,2-diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3), followed by the release of intracellular Ca^{2+} and the activation of PKC. Both consequences contribute to airway smooth muscle contraction. Competitive Gs and Gq signalings regulate the airway smooth muscle tone, so that the downstream signaling molecules in each pathway are mutually regulated (Billington and Penn 2003; Deshpande and Penn 2006; Penn and Benovic 2008). For example, a decrease of adenylyl cyclase activity (Schears, Clancy et al. 1997) induced by carbachol pretreatment can be blocked by PKC inhibitor. PKC activation can potentiate both heterologous and homologous β_2 AR desensitization (Boterman, Smits et al. 2006) in airway smooth muscle. Paradoxically, β_2 AR knockout mice showed less airway responsiveness to constrictors than mice with a moderate overexpression of β_2 AR, while PLC β1 expression was lower in β_2 AR knockout mice than β_2 AR overexpression mice (McGraw, Almoosa et al. 2003). Therefore, the mutual regulation of downstream signaling molecules between two pathways have been considered to be therapeutic targets in airway hyperresponsiveness. In mice the expression of $G\alpha_{i2}$ was

downregulated by an inhibiting peptide, the Gs signaling was potentiated and PKCα expression in airway smooth muscle was increased. In contrast, mice overexpressing $G\alpha_{i2}$ showed dampening Gs signaling but lower expression of PLCβ3 (McGraw, Elwing et al. 2007). A pharmacological strategy to decrease $G\alpha_{i2}$ activity while decreasing PKC related signaling, or a strategy to increase $G\alpha_{i2}$ activity while increasing PLCβ3 related signaling both have therapeutic implications.

6 Crosstalk of Gs and the arachidonic acid pathways on airway smooth muscle tone

Leukotrienes (LTs) are known to be one of the most highly potent contractile agonists of airway smooth muscle. Higher concentrations of LTs have been found in the blood, urine and bronchoalveolar lavage fluid (BALF) of asthmatic patients compared with that of non-asthmatic individuals (Lam, Chan et al. 1988; Wenzel, Larsen et al. 1990; Drazen, O'Brien et al. 1992). Moreover, inhalation of LTs produced airflow obstruction (Drazen 1986). Therefore, agents that block the synthesis or activity of LTs have been shown to be effective in the treatment of asthma. LT receptor antagonists could prevent the dysfunction of β_2 AR (Song, Crimi et al. 1998) and therefore have already been used clinically for asthma therapy. LT inhibition reduced bronchial hyperresponsiveness, cell recruitment, fibroblast growth, and mucus production in the airways (Vargaftig and Singer 2003).

As we know, the initial step in LT pathway is the activation of cPLA2. When cPLA2 is activated and translocated into the cell membrane, cPLA2 selectively cleaves arachidonic acid on the pernuclear cell membranes and converts it into 5-HPETE and then to leukotriene A4. 5-lipoxygenase (5-LO) and the 5-LO activating protein (FLAP) are critical to the synthesis of LTA4. LTA4 is unstable and may be transformed into LTB4 or cysteinyl-LTs (cysLTs) by LTA4 epoxid hydrolase or LTC4 synthase. In cardiac myocytes, the β_2 AR/Gi/cPLA2 signaling pathways have been demonstrated to be relevant to contractility (Pavoine, Behforouz et al. 2003; Pavoine and Defer 2005).

Furthermore, CysLTs bind to two distinct receptors that have been identified as CysLT1 and CysLT2 receptors. They are G protein coupled receptors (GPCR) which use both PTX sensitive or non-sensitive G proteins for signaling. The CysLT1 receptor has been reported to desensitize β_2 AR through activation of PKC in human airway smooth muscle cell culture (Rovati, Baroffio et al. 2006). Although PKC inhibition has been reported to attenuate desensitization of CysLT receptor in transgenic mice expressing human CysLT receptor (Deshpande, Pascual et al. 2007), PKC-aided calcium release and signaling cannot be underestimated in an asthmatic phenotype in which desensitization of contractile signaling is not a major issue (Deshpande and Penn 2006). CysLT receptors were also reported to bind to a PTX-sensitive G protein and to be involved in the regulation of airway smooth muscle cell proliferation, EGF receptor transactivation and

ERK1/2 phosphorylation (Ravasi, Citro et al. 2006). An EGF receptor tyrosine kinase inhibitor decreases LT-induced bronchial hyperresponsiveness, eosinophil counts and MUC5AC release in BALF (Vargaftig and Singer 2003). Therefore, these effects of LT stimulation were believed to be signaled by CysLT-Gi pathway. Nevertheless, we have reported that chronic nadolol treatment decreased PLCβ1 and Gi expression (Lin, Peng et al. 2008), which might subsequently suppress PKC activation and calcium release, as well as EGFR trans-activation and ERK1/2 phosphorylation, and then result in the bronchoprotective effect *in vivo*.

CHAPTER 3 Material and methods

1 Animals

BALB/cJ male mice aged 5 weeks and FVB/NJ female mice aged 6~8 weeks (Jackson Animal Laboratory, Bar Harbor, Maine) were housed under specific pathogen-free conditions and fed with a chicken ovalbumin free diet. β_2 AR-deficient mice were a gift from Dr. Brian Kobilka (Stanford University) and bred in a local facility. Female β_2 AR deficient mice age 6~8 weeks were used for the experiments. Approval of the University of Houston Institutional Animal Care and Use Committee for the experiments was obtained.

2 Antigen sensitization and challenge protocol

Mice were sensitized with ovalbumin adsorbed to aluminum hydroxide and were challenged with the same antigen. Sensitization involved injection of 25μg of ovalbumin on protocol days 2, 9 and 16. Challenging involved intranasal administration of ovalbumin (25μg) on a daily basis for protocol days 23 to 27. Mice received ovalbumin sensitization and challenge were denoted as s/c mice in the context. Normal mice were sensitized and challenged with normal saline, used as control mice.

3 Drug administration

For chronic treatment, s/c mice received 250 ppm of nadolol in chow for 28 days and on the 28th day they were subjected to the measurements . For the subtype study, ICI 118,551 (10 mg/Kg/day), or metoprolol (10 and 20 mg/Kg/day) were given from protocol days 21 to 28 using an osmotic mini-pump (Alzet, #2004, Durtech Corporation, Cuperinto, CA) by subcutaneous implantation at protocol day 21. For the dexamethasone combined study, dexamethasone (5mg/kg) was given half an hour before the challenge via i.p. for 5 days. Salbutamol was delivered for 28 days at a dose of 0.5 mg/kg/day using a minipump because of its short half-life. The drug treatment time line is shown in Figure 4. These doses were chosen based on the previous reports in mice (Costall, Naylor et al. 1985; Kubota, Yamazaki et al. 1990). Control groups received normal feed for 28 days and the same amount of saline injection. This study used mainly nadolol instead of ICI 118,551 because of the tremendous cost differential (ICI118,551 is over 500 times more expensive than nadolol) and ease of administration.

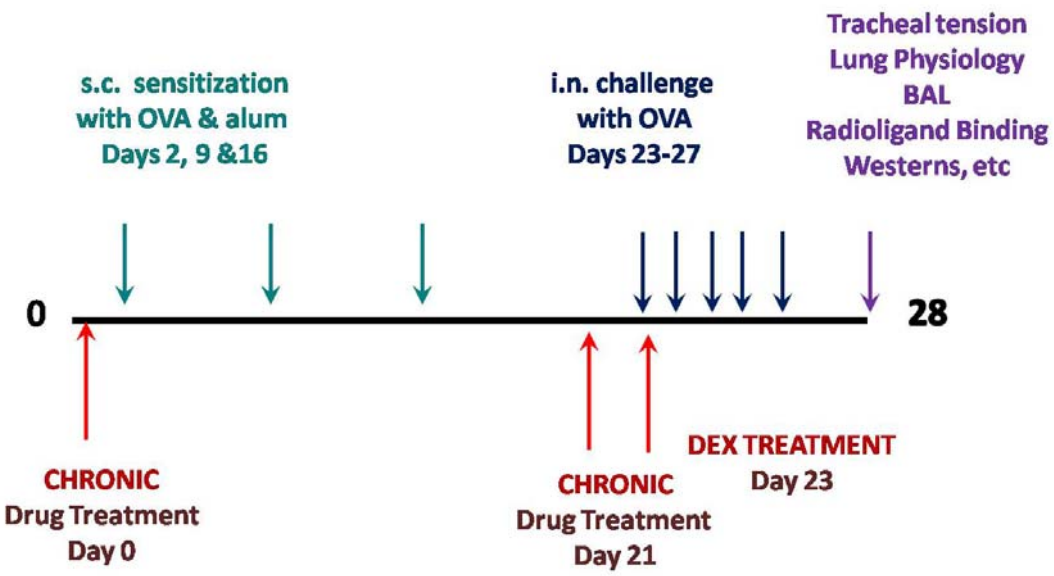


Figure 4. Experiment design of drug treatment

4 Lung membrane preparation

On the last day of treatment protocol, mice were anesthetized by 0.1 ml of 65 mg/ml pentobarbital. After perfusion by saline, the lungs were dissected out and flash frozen in liquid nitrogen. The frozen lung tissue was homogenized in ice-cold homogenization buffer (25 mM Tris-HCl, 0.32M sucrose, pH7.4), containing one complete protease inhibitor tablet (Roche Applied Science, Indianapolis, IL) per 25ml, by a polytron (Pro-200, Pro-scientific, and Inc. Monroe, CT) with three 30 second bursts at a setting of 3. Homogenates were centrifuged at 1000g for 10 minutes at 4°C (Sorvall SS-34, Newton, CT). The supernatant was collected and filtered using through cotton gauze. This filtered

supernatant was centrifuged at 40,000g for 20 minutes at 4°C. The supernatant was collected as the cytosolic fraction. The pellets were suspended in an ice-cold 25mM Tris-HCl buffer (pH7.4) and centrifuged at 40,000g for 20 minutes at 4°C again. The final pellets were suspended in 200µl of the same buffer and the protein concentration was determined using a BCA protein assay kit (Pierce, Rockford, IL).

5 Radioligand binding

β-AR density was determined by incubation of membranes with increasing concentrations (5–1,000 pmol) of (-)3-[^{125}I]- iodocyanopindolol (ICYP) (Amersham Pharmacia). Propranolol (1µM) (Sigma) was used to determine total β-AR densities. ICI 118,155 (0.1µM) and CGP20712A (0.3µM) were used to determine β_1 AR and β_2 AR respectively. Specific binding was calculated by subtracting non-specific binding from the total binding data and fitted to a hyperbolic equation to estimate Kd and Bmax. Specific binding to each subtype was calculated by subtracting the total binding from ICI118,551 or CGP20712A binding respectively. All experiments were performed in triplicate.

6 Lung function and airway hyperresponsiveness

On protocol day 28, the mice were anesthetized using a 1:2 dilution of the mixture of Ketamine 42.86 mg/ml, Xylazine 8.57 mg/ml, and Acepromazine 1.43 mg/ml per 0.1 ml,

tracheotomized, and connected to a computer-controlled small animal ventilator (Flexivent, Scientific Respiratory Equipment, Montreal). Airway resistance (Raw) was measured by using the forced oscillation technique (Fust, Bates et al. 2004; Wagers, Lundblad et al. 2004). The complex input impedance of the respiratory system was computed as previously described (Evans, Bond et al. 2003), and the value of the real part of respiratory system impedance at 19.75 Hz was taken to reflect the magnitude of Raw. To induce airway constriction, a solution containing 150 $\mu g \cdot ml^{-1}$ of acetyl-ethylcholine chloride (methacholine) (Sigma, St. Louis, MO) was infused intravenously, using a syringe infusion pump (Raze Scientific Instruments, Stanford, CT, USA). The methacholine infusion was started at 0.008 $ml \cdot min^{-1}$, and its rate was doubled stepwise up to a maximum of 0.136 $ml \cdot min^{-1}$. Each methacholine dose was administered for 5 min, during which data were sampled at 1 min intervals and then averaged. To examine the degree of airway responsiveness of each animal, the values for Raw as a function of methacholine doses were plotted.

7 Bronchoalveolar lavage fluid

Cold phosphate buffer saline (1 ml) was infused and drawn back through the tracheal cannula from euthanized (pentobarbital 0.1 ml of 65 mg/ml) mice, and repeated once. The total leukocyte count in 20 µl BALF was determined using a Coulter counter. Cytospin preparations were prepared from 200 µl of BALF at 500 rpm for 5 min and

stained with Hema-3 for enumeration of cell types in order to count the percentage of eosinophils. Eosinophils were identified by standard morphologic criteria. At least 200 cells were counted from cytospin preparations. The number of eosinophils is the product of the total cell count and the percentage of eosinophils.

8 cAMP accumulation

On the last day of protocol, the lungs were harvested and cut into small pieces (1×1mm) in the cold and aerated Krebs–Henseleit solution containing following components (mM): NaCl 120, KCl 4.7, $MgCl_2 \cdot 6H_2O$ 1.2, $NaHCO_3$ 25, $NaH_2PO4 \cdot H_2O$ 1.2, D-Glucose 11, $CaCl_2$ 2.5, IBMX (3-Isobutyl-1-methylxanthine) 0.1. The tissue was equally divided into 6 Eppendorf tubes and incubated with vehicle, 0.1, 1, 10, and 100μM isoproterenol respectively. The incubation was conducted in 37ºC water bath for 30min with continuous shaking. After incubation, 500μl 0.1M HCl was added to terminate the reaction. Then, the tissue was homogenized with a polytron (Pro-200, Pro-scientific, and Inc. Monroe, CT) with three 30 second bursts at a setting of 3. The supernatant was separated and subjected to the assay after 10 min centrifugation at 600g (Jouan MR22i). The pallets were weighed after drying at 60ºC overnight. The content of cAMP in the supernatant was measured using Assay Designs™ Direct Cyclic AMP Enzyme Immunoassay (EIA) kit.

9 Quantitative immunoblotting

To measure the expression of Gαi, PDE4D, Gαs, Gαq, 5-LO, COX2, cPLA2, PLCβ1, PKCα, β arrestin2, GRK2, GRK3 and GRK5 in lung homogenates, the membranous or cytosolic fractions were dissolved in SDS sample buffer to 30 μg/42 μl and heated at 95°C for 5 minutes. BALF samples contained much fewer proteins, so that 15 μg/42 μl samples were prepared instead. This was followed by SDS PAGE electrophoresis through 10% Tris-HCl gels (Bio-Rad, ready gel). Proteins were electroblotted to immobilon filters (Millipore) and blocked by 5% non-fat milk for 1 hour. The blot was then incubated with anti-Gαi3 (Santa Cruz, 1:1,000), anti-Gαi2 (Santa Cruz, 1:1,000), anti-PDE4D (Febgennix, 1:500), anti-Gαs (Santa Cruz, 1:1000), anti-Gαq (Santa Cruz, 1:1000), anti-GRK2 (Santa Cruz, 1:500), anti-GRK5 (Santa Cruz, 1:200), anti-GRK3 (Santa Cruz, 1:100), anti-PKCα (Santa Cruz, 1:1000), anti-cPLA2 (Santa Cruz, 1:500), anti-PLCβ1 (Santa Cruz, 1:500), anti-COX2 (Cayman, 1:1000), anti-5-LO (Cayman, 1:500), and anti-β arrestin2 (Santa Cruz 1:1000) in 5% non-fat milk (Carnation) overnight. Again, the blots were washed and blocked by non-fat milk, and incubated with a secondary antibody (Donkey anti rabbit, goat anti mouse, Jackson immunoresearch, 1:10,000). The bands were visualized by chemiluminescence (Pierce, IL), and the digital images were collected by a CCD camera (FlurochemTM). The blot was then stripped and re-probed by anti- actin (Santa Cruz, 1:1,000), which was used as a loading control. A monoclonal antibody to smooth muscle α-actin (clone 1A4, 1:1000) was used on airway smooth muscle cells. The densities of Gαi3, Gαi2, PDE4D, Gαs, Gαq, 5-LO, COX2,

cPLA2, PLCβ1, PKCα, β arrestin2, GRK2, GRK3 and GRK5 bands were normalized to actin bands and quantified using AlphaEase software (Alpha InnoTech, San Leandro, CA).

10 Airway smooth muscle cultures

Tracheal smooth muscle cells were cultured from explants of excised tracheas. The trachea was removed at the last day of the protocol, placed in a sterile Petri dish and washed with Hanks' balanced saline solution (HBSS) containing 2X concentration of antibiotic-antimycotic solution. Additional surrounding tissue was removed with the aid of a dissecting microscope, trachea minced into 2 to 3-mm pieces and kept for 2-3 h in 60 mm dishes at 37ºC in HBSS. The explants were allowed to adhere; HBSS was aspirated and replaced with 2.5 ml of Dulbecco's modified Eagle's medium with 20% fetal bovine serum (FBS) and 2X antibiotic-antimycotic. The explants were incubated at 37 °C in a humidified environment of 95% air, 5% CO_2. After 3 days the concentration of FBS was changed to 10%, and the antibiotic-antimycotic was reduced to 1X. For cultures from chronically treated mice, nadolol (10^{-6}M) was present in the cultures till the time they were harvested. When smooth muscle cells were observed growing out of the explants, the explants were aspirated. The cultures were passaged when confluent (about 450,000 to 600,000 cells per ml), about once per week at 1:5 ratios, and harvested using the lysis buffer (PBS containing 1 % Nonidet P-40, 0.5 % sodium deoxycholate, 0.1 % SDS and

Complete Protease Inhibitor). Passage 2~7 were used for preparing the lysate.The cell extract was centrifuged at 4°C for 5 mins at full speed and the supernatant removed. 50 µl of the supernatant was used for protein assay and the remaining volume mixed with ¼ volume of 4X SDS sample buffer and stored at -80°C until used for immunoblotting. With each passage, a small number of cells were seeded onto a glass cover slip in growth medium. After one day of incubation at 37°C, the attached cells were fixed with 4% paraformaldehyde. The fixed cells were labeled with a monoclonal antibody to smooth muscle α-actin (clone 1A4) and FITC secondary antibody, and then viewed by fluorescence microscopy. 4', 6-diamidino-2-phenylindole (DAPI) was used to stain the nuclei of smooth muscle cells. The morphology of the cells is shown in Figure 5.

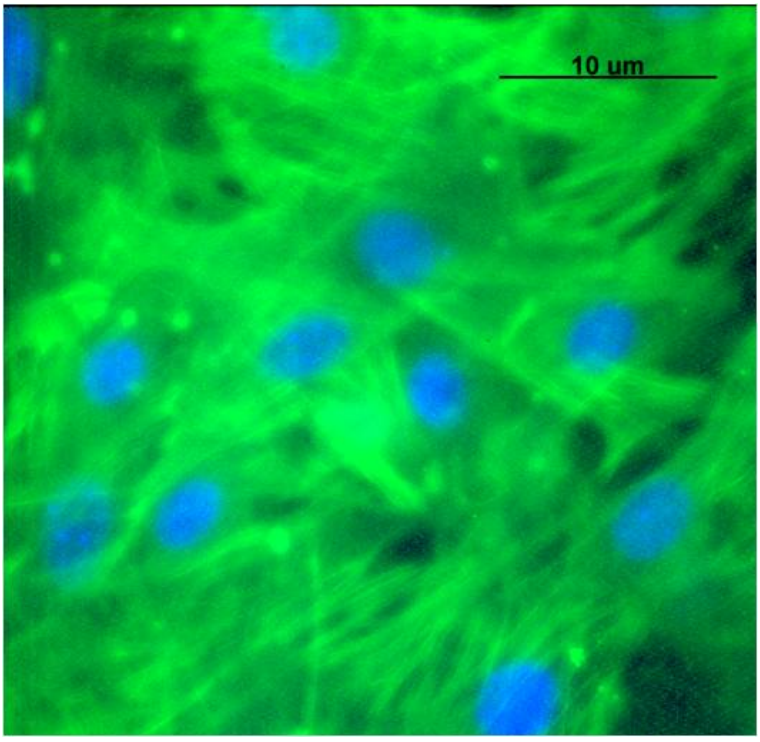


Figure 5. Immunofluorescent labeling of smooth muscle actin and nuclei

Primary cultures of tracheal smooth muscle cells were derived from tracheal explants. To confirm that the cells were of smooth muscle origin, immunohistochemistry was performed using an anti-smooth muscle α-actin antibody and an FITC-conjugated secondary antibody. As shown in this representative experiment, >90% of the cells stain positively for smooth muscle α-actin. (unpublished data from Vikas Arora)

11 Isometric tension studies on tracheal rings

On the last day of protocol, the mice were killed with a lethal dose of pentobarbital (75µl of 65 mg/ml). Tracheas were carefully isolated, then dissected free of connective tissue and placed in cold and gassed (5% CO_2 in O_2) Krebs–Henseleit solution (K–H) (mM): NaCl 120, KCl 4.7, $MgCl_2 \cdot 6H_2O$ 1.2, $NaHCO_3$ 25, $NaH_2PO_4 \cdot H_2O$ 1.2, D-Glucose 11, $CaCl_2$ 2.5. Approximately equal size tracheal rings were suspended in organ baths (13 ml) with one end attached to an isometric force transducer (Harvard Apparatus, Inc., Holliston, MA) and the other to a tissue holder. After setting the resting tension at 0.5 gram, the tissues were equilibrated in K–H at 37ºC and bubbled with 5% CO_2 in O_2 for 1h. The bath solution was changed every 15 min. After equilibration, 3µM indomethacin was added to remove the mechanical tone, then the tracheas were contracted with increasing concentrations of methacholine (10^{-8} to 10^{-4}M) to construct the dose response curve, and each dose was accumulatively added with a 2-minute interval. To construct the isoproterenol cumulative dose response curve, the trachea was pre-contracted by 10 µM methacholine (approximately the EC80) for 10 minutes after a complete wash. Then, the increasing concentrations of isoproterenol (10^{-9} to 10^{-4}M) were accumulatively added

into the bath at 2-minute intervals. The changes in tone were recorded using Chart 4.21 for windows (Powerlab data acquisition system, ADinstruments, USA). For the pertussis toxin study, the trachea was incubated in the buffer with or without 0.3μg/ml pertussis toxin (RBI, Natick, MA, USA) for 16 hrs before constructing the dose response curves. For trachea from chronically treated mice, the K-H solution was supplemented with nadolol (10^{-6}M) to maintain exposure of the receptor to the drug.

12 Measurement of cysteinyl leukotrines in BALF

The fresh BALF was centrifuged at 360g, 4ºC for 10 min. The supernatant was stored under -80ºC for less than 72 hrs before the assay. The concentration of cys-LTs was measured by a commercial kit (Amersham Pharmacia Biotech, Piscataway, New Jersey). The assay was based on the competition between unlabelled Leukotriene C4 and a fixed quantity of peroxidase labeled Leukotriene C4 for a limited number of binding sites on a peptide leukotriene specific antibody. With fixed amounts of antibody and peroxidase labeled Leukotriene C4 the amount of peroxidase labeled ligand bound by the antibody was inversely proportional to the concentration of added unlabeled ligand. The peroxidase ligand that was bound to the antibody was immobilized on to polystyrene microplate wells precoated with second antibody. Unbound ligand was thus removed from the well by simple washing procedures. The amount of peroxidase labeled Leukotriene C4 bound to the antibody was determined by addition of

tetramethylbenzidine (TMB)/hydrogen peroxide single pot substrate. The reaction was stopped by addition of an acid solution, and the resultant color read at 450 nm in a microplate spectrophotometer. The concentration of unlabelled Leukotriene C4 in a sample is determined by interpolation from a standard curve. The detection limit for LTC4/D4/E4 was 10pg/ml.

CHAPTER 4 Effect of chronic nadolol treatment on β_2 AR receptors

1 Result

In this chapter, we demonstrate the effect of chronic nadolol treatment on pulmonary β_2 AR density and discuss the relationship between the regulation of β_2 AR density with lung function.

1.1 Effect of chronic beta blocker treatment on β AR expression

Chronic beta blocker treatment was shown to increase β AR expression in the animal model of asthma. To identify which subtype of β AR is more affected by the chronic treatment with beta blocker, both β_1 and β_2 AR numbers were measured on mouse lung membranes. To further elucidate the relationship between the increased β AR number and pulmonary function, the combined effect of dexamethasone with chronic nadolol treatment on β AR density on lung membranes was also measured by radioligand binding assay.

1.1.1 β AR subtype

To identify the effect of chronic beta blocker treatment on β_2 AR number, ICI118,551 (10mg/kg), a highly selective beta blocker to β_2 AR, and metoprolol (10 and 20mg/kg), a beta blocker preferential to β_1 AR was given to s/c mice for 7 days. S/c mice showed a lower level of β_2 AR expression than did the control mice (156.8±36.78 vs. 1245.2±321.0

pmol/mg, n=4 vs. n=3 respectively, P<0.01), while all chronic beta blocker treatment could all increase the density of β_2 AR. This is shown in Figure 6 (ICI 682.3±127.6, Met 20 602.3±42.86, Met 10 707.8±38.24 vs. s/c 156.8±36.78 pmol/mg, respectively, n=4~5, P<0.05).

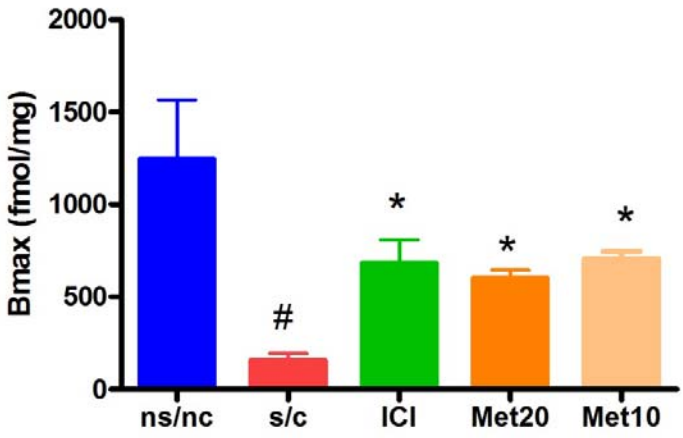


Figure 6. Effect of chronic beta blocker treatment on β_2 AR expression

The receptor density in s/c mice (n=3) is significantly less than in control mice (Ctrl, n=4). ICI treatment for 7 days treatment (ICI, n=5), and metoprolol 10mg/kg (n=4) and 20mg/kg (n=5) treatment for 28 days all increased the receptor density in lung membranes from NTX s/c mice. Bmax is presented as mean ± SEM. * P < 0.05 compared with NTX s/c, # P < 0.001 compared with ctrl (Student's t-test).

β_1 AR density was also measured in all treatment groups. S/c mice showed a smaller β_1 AR number than the control mice did, but the difference was not statistically significant (568.6±179.0 vs.1037.28±258.0). Beta blocker treatments, which increased β_2 AR densities, did not affect the expression of β_1 AR (see Figure 7; ICI 542.4±5.155, Met 20 501.0±100.9, Met 10 401.5±36.95 vs. s/c 156.8±36.78 pmol/mg, respectively, n=4~5).

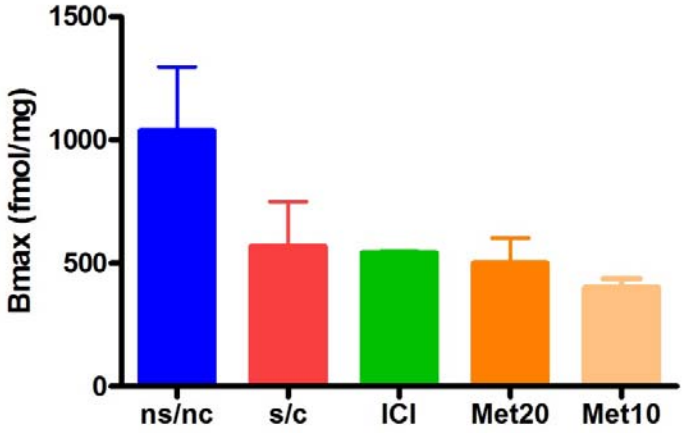


Figure 7. Effect of chronic beta blocker treatment on β_1 AR expression

The receptor density in s/c mice (n=6) is not significantly less than that in control mice (Ctrl, n=4). ICI treatment for 7 days (ICI, n=5), and metoprolol 10mg/kg (Met10, n=4) and 20mg/kg (Met 20, n=5) treatment for 28 days did not affect the receptor density in lung membranes. Bmax is presented as mean ± SEM.

1.1.2 Effect of the combination of dexamethasone and chronic nadolol treatment on total β AR expression

As shown in Figure 8, s/c mice showed significantly lower β AR density than control mice (379.15±55.71 vs. 857.75±84.73 pmol/mg respectively, $P<0.05$, n=4 vs. n=6), while chronic nadolol treatment increased β AR density (547.6±44.72 vs. 379.15±55.71pmol/mg respectively, $P<0.05$, n=6). Dexamethasone treatment (5mg/kg, i.p. 5 days) dramatically increased β AR density in s/c mice. Figure 7 also shows that the effect of dexamethasone was not only validated by the increase of β AR density (952.58±96.75 vs. 379.15±55.71 pmol/mg respectively, n=5 vs. n=6), but also validated

by a dramatic drop in eosinophil cell counts in BALF (see also Figure 9, 108.1±25.69 vs. 833.4±168.0 ×1000 cells per ml respectively, n=6 vs. n=9). To the same extent as dexamethasone treatment alone, dexamethasone increased βAR density when combined with chronic nadolol treatment (see Figure 8; 1000.68±97.1 vs. 952.58±96.75 pmol/mg respectively, n=5 vs. n=6). However, βAR density did not further increase when chronic nadolol treatment and dexamethasone treatment were combined. Dexamethasone could also increase βAR density when combined with salbutamol acute and chronic treatment (data not shown). As far as we observed, the effect of dexamethasone on increasing β AR was so intensive that the effect of all other treatments was overwhelmed. All treatments showed similar Kd value except chronic nadolol treatment (Table 1). Kd value represents the affinity of receptors to their ligands.

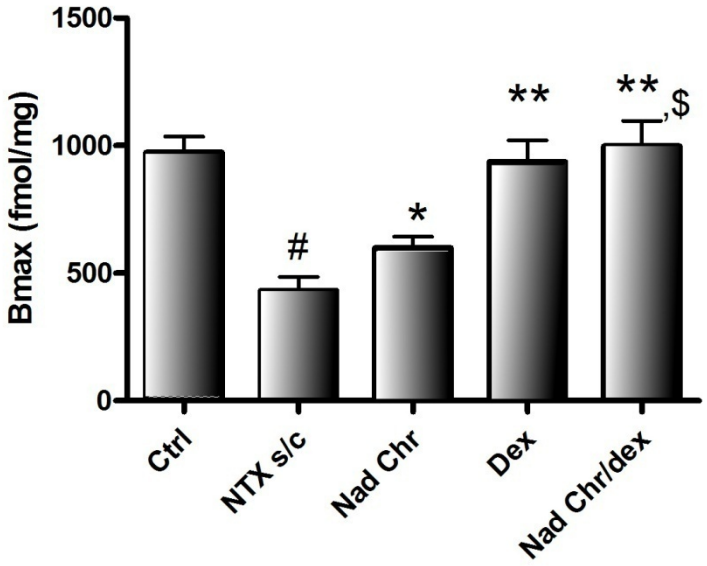


Figure 8. Effect of dexamethasone treatment combined with or without chronic nadolol treatment on β AR density

The receptor density in non-treated sensitized and challenged mice (NTX s/c, n=6) was significantly less than in the control mice (Ctrl, n=4). Both 5 days treatment with dexamethasone (dex, n=5) and 28 days treatment with nadolol (Nad Chr, n=6) increased the receptor density in lung membranes from NTX s/c mice. Co-treatment of dex with chronic nadolol treatment (Nad Chr/dex, n=5) did not further increase βAR density when compared to dex treatment. Bmax is presented as mean ± SEM. * $P < 0.05$, ** $P<0.001$ compared with NTX s/c, # $P < 0.001$ compared with ctrl, $ $P<0.01$ compared with Nad Chr (Student's t-test).

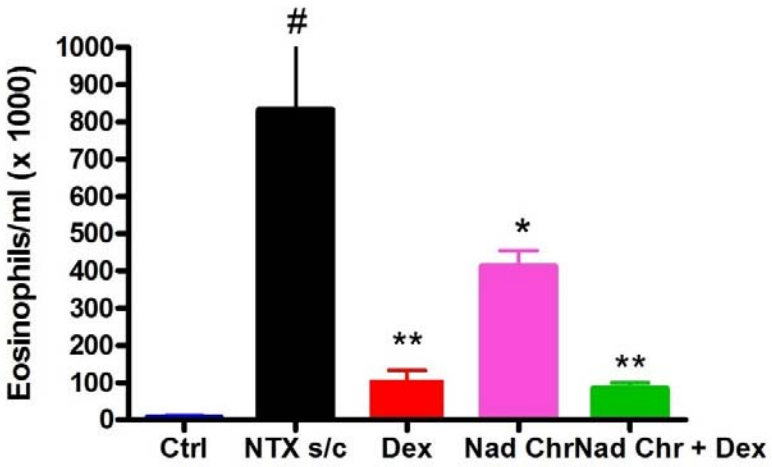


Figure 9. Effect of drug treatment on eosinophil cell counts in BALF

The eosinophil cell count in BALF was determined to be the product of the total cell count and the percentage of eosinophils. Non-treated sensitized and challenged mice (NTX s/c) showed much higher cell counts than control mice did (NS/NC). Nadolol chronic treatment (Nad Chr), dexamethasone treatment (Dex) with or without any combined treatments all decreased the leukocyte counts significantly. No additive effect was shown in the combined treatment of dexamethasone and chronic nadolol treatment. Values are mean±SEM, * $P<0.05$ compared with NS/NC, ** $P<0.01$ compared with NTX s/c, #$P<0.01$ compared with NTX s/c. (unpublished data from Sergio Parra)

Table 1. Effect of drug treatment on Kd value of β AR on lung membranes

Treatment	Kd
Ctrl	21.09±2.41
NTX s/c	18.91±2.03
Nad Chr	34.94±5.60*
Dex	22.13±6.24
Nad Chr/dex	21.77±5.53

Values are presented as mean±SEM, * $P < 0.05$ compared with NTX s/c
Ctrl: no treatment, NTX s/c: no treatment on s/c mice, Nad Chr: 28days treatment of nadolol in s/c mice, Dex: 5 days treatment of dexamethasone on s/c mice, Nad Chr/dex: combined treatment of Nad Chr and Dex.

Chronic nadolol treatment decreased receptor affinity 1.5 times, as shown in Table 1, when compared to s/c mice (34.94±5.60 vs. 18.91±2.03, P<0.05). However, 1.5-fold decrease of receptor affinity would not significantly change the saturation and EC50 of receptors, which may be due to residual nadolol in the membrane preparation.

Pulmonary β AR numbers can be regulated by different agents and different mechanisms. We have also tested the β AR number on animals given guanadrel for 28 days (5mg/kg/day), a drug that deplete the endogenous catecholamine in animals. Chronic guanadrel treatment increased the airway resistance in s/c mice. However, the β AR number increased significantly, as shown in Figure 10; (588.72±40.14 vs. 497.6±80.15pmol/mg respectively, P<0.05, n=5 vs. n=3). This increase was so moderate

that it might be due to a homeostatic effect. Therefore, the increase of β AR numbers might go through different mechanisms, some of which may not be responsible for a bronchoprotective effect.

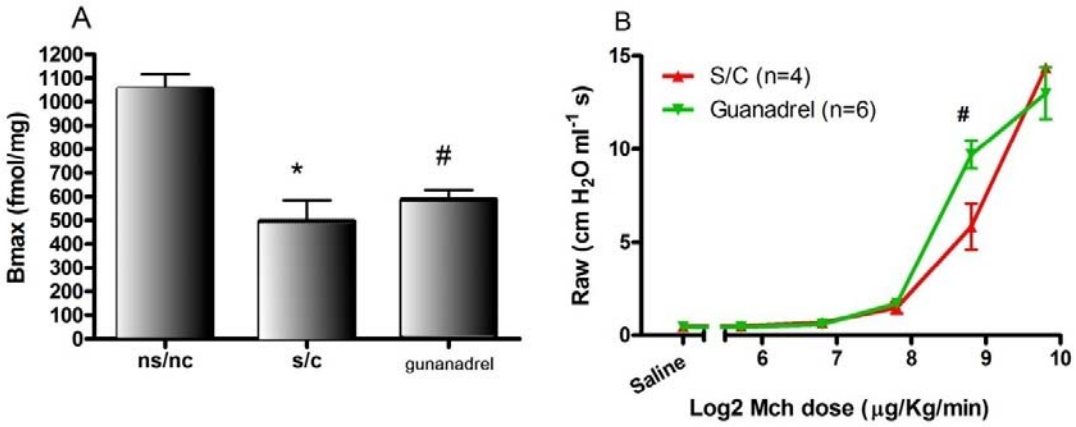


Figure 10. Effect of chronic guanadrel treatment on βAR number and airway resistance

The βAR number was measured on mice lung membranes using radioligand binding (A). The airway resistance was measured *in vivo* using forced oscillation technique (B) (unpublished data from Sergio Parra). Chronic guanadrel treatment (n=5) increased β AR number and airway resistance in s/c mice (n=4). Values are mean±SEM, * P<0.05 compared with Ctrl, # P<0.05 compared with NTX s/c

Collectively, this showed that chronic treatment with beta blocker preferentially increased the expression of the β_2 subtype of adrenoceptors in our mouse model of asthma. However, chronic beta blocker treatment might increase β AR through mechanisms different from dexamethasone or guanadrel.

2 Discussion

To study the cellular mechanisms of the bronchoprotective effect of chronic beta blocker treatment, we first shed light on β_2 AR density. Pulmonary β_2 AR has been considered to be the major target in the therapy of asthma, because no other bronchodilator has shown a better effect than the β AR agonist in ameliorating asthma (Barnes 2006). In human and mice lungs, up to 70% of β ARs are the β_2 subtype (Henry, Rigby et al. 1990; Sano, Yoshimasa et al. 1993). No changes in the β_2-AR densities have been found in lungs of severe asthmatics (Spina, Rigby et al. 1989), probably because these measurements were not made during, or just after, an asthma attack. Reductions in β-ARs have been shown in lymphocytes after bronchial provocation with an antigen (Gamboa, de la Cuesta et al. 1990), and in acute asthma (Hataoka, Okayama et al. 1993). Plenty of evidence has shown that desensitization and a decrease of β_2 AR density is associated with the chronic treatment of β agonists (Kelsen, Anakwe et al. 1997; Broadley 2006).

Since chronic treatment with beta blocker decreased hyperresponsiveness in a murine model of asthma, we hypothesized that the resensitization and increase of β_2 AR may be associated with chronic beta blocker treatment. As an increase in total β AR number has been already observed (Callaerts-Vegh, Evans et al. 2004), we next identified which subtype was affected by chronic beta blocker treatment. By using the selective β_2 AR inverse agonist ICI 118,551 (β_2/ β_1=550:1), the hyperresponsiveness in our mouse model of asthma was decreased by only 7 days of treatment. The preferential inverse agonists

for β_1 AR metoprolol (β_1/ β_2=2.3:1) could also decrease bronchial hyperresponsiveness in a higher dose (20mg/kg/d), but showed no effect in the lower dose (10mg/kg/d) (Lin, Peng et al. 2008). This discrepancy may due to the low selectivity of metoprolol to β_2 AR. However, as shown in Figure 6, all chronic beta blocker treatments correlated with a significant increase of β_2 AR number, while Figure 7 showed none of the treatments changed the β_1 AR numbers. This suggests that 10mg/kg/d metoprolol is enough to affect β_2 AR density. However, a higher dose and more selectivity were required to show the bronchoprotective effect. These data show that the reduction of airway hyperresponsiveness which resulted from chronic β-blocker administration is associated with marked increases of β_2-AR densities, suggesting that the beneficial effect of β-AR antagonists/inverse agonists is mediated by the β_2-AR.

The relationship of β AR density and airway function has not yet been determined. The transgenic mice with a 75-fold overexpression of β_2 AR on epithelial cells have shown hyporesponsiveness to methacholine (McGraw, Forbes et al. 2000). However, transgenic mice with a 10-fold overexpression of β_2 AR on airway smooth muscles have shown hyperresponsiveness to bronchoconstrictors (McGraw, Almoosa et al. 2003). Dexamethasone has also been shown by different labs using different measurements to increase the β_2 AR density in airway smooth muscle cells (Seco, Salgueiro et al. 1995; Kalavantavanich and Schramm 2000); this has been considered to be one of the mechanisms of its synergistic effect with the β AR agonist. Chronic nadolol treatment

increased β AR density and decreased eosinophils only modestly, while the effect of dexamethasone was dramatic. Therefore, the effect of chronic nadolol treatment on β AR density might be masked by the dramatic increase of dexamethasone when two treatments were combined. As seen in Table 1, the Kd value, which represents the affinity of the ligand for the receptor, did not change with treatments except for the chronic nadolol treatment. Nevertheless, the increase of Kd value induced by chronic nadolol treatment was only 1.5-fold, which might be due to residual nadolol in membrane preparation. Although significant, this modest change of Kd value did not indicate a significant change in β AR characteristics. However, with the dramatic increase of the β AR number, dexamethasone still did not decrease airway hyperresponsiveness (data not shown) while the chronic nadolol treatment did. Dexamethasone has been suggested to upregulate β AR density by nuclear receptor signaling and reverse the downregulation of β AR resulting from chronic β AR agonist treatment (Barnes 1995; Mak, Nishikawa et al. 1995). Another mechanism of dexamethasone might be that it potentiates the coupling efficiency of the β AR receptor to the Gs protein (Kalavantavanich and Schramm 2000; Aksoy, Mardini et al. 2002; Mak, Hisada et al. 2002). Chronic nadolol treatment increased β AR number modestly, whereby neither mechanism might be involved.

Guanadrel is a drug that depletes catecholamines, and produces a modest increase in β AR number with long term treatment. This might be only due to a homeostatic lowering of β AR signaling in the airway and the subsequent desensitization. The magnitude of

the chronic nadolol treatment in increasing β AR density was close to that of guanadrel treatment. However, chronic nadolol treatment showed an improvement in airway function while guanadrel did not, which was shown in Figure 10. This implies that the increase of β AR density by chronic nadolol treatment was not a homeostatic reflection of the loss of β AR signaling. Alternative pathways regulated by chronic nadolol treatment appear to be required.

CHAPTER 5 Effect of chronic nadolol treatment on the classical β_2 AR signaling pathway

1 Results

In this chapter, we discuss the classic signaling pathway of β_2 AR and examine how chronic nadolol treatment improves lung function by regulating the function of β_2 AR. β_2 AR is a Gs coupled GPCR. It can activate a Gs-cAMP signaling pathway and result in airway smooth muscle relaxation, which is considered as the classical signaling pathway in airway smooth muscle. To evaluate the effect of chronic nadolol treatment on the function of β ARs, we first examined the effect of chronic nadolol treatment on the cAMP signaling pathway, including cAMP production, PKA-dependent trachea relaxation, and the expression of the Gs protein. Expressions of GRK2, GRK3 and β arrestin2 were measured to examine the effect of chronic nadolol treatment on the desensitization of β_2 AR-Gs-cAMP signaling. In addition, PDE4D is most responsible for intracellular cAMP breakdown was also involved in the desensitization of cAMP signaling, therefore PDE4D expression was also examined.

1.1 Effect of chronic nadolol treatment on cAMP production

To investigate the effect of chronic nadolol treatment on cAMP production, cAMP accumulation was firstly measured using fresh lung pieces incubated with a phosphodiesterase inhibitor and an increasing concentration of isoproterenol. The cAMP

accumulation increased in the lung homogenates of the control mice in a dose-dependent manner. However, this increase did not occur in s/c mice with or without chronic nadolol treatment (Figure 11). This implies that cAMP production in s/c mice was impaired, and was not affected by chronic nadolol treatment.

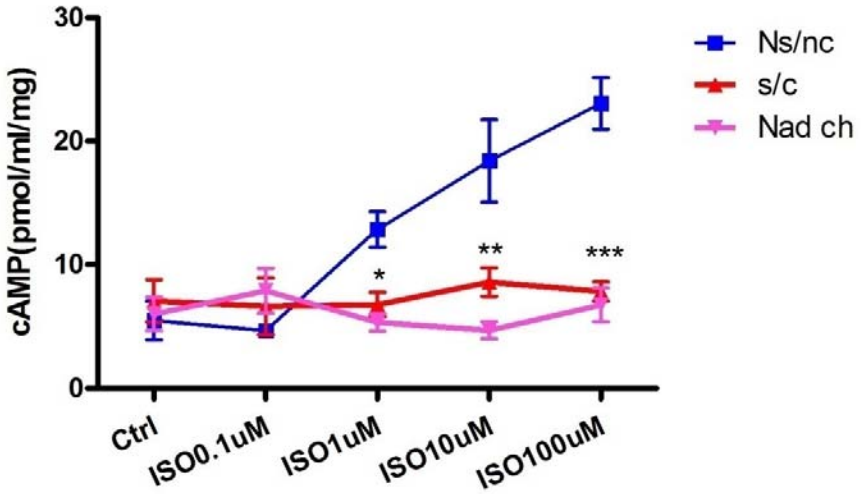


Figure 11. Effect of chronic nadolol treatment on cAMP accumulation in lung homogenate

cAMP accumulation in control mice (Ns/nc, n=8) and animal model of asthma (s/c, n=8) with or without chronic nadolol treatment (Nad ch) showed no difference with and without the presence of 0.1 μM isoproterenol (ISO). However, the cAMP production was much higher in control mice than s/c mice with or without chronic nadolol treatment at higher isoproterenol concentrations. cAMP concentration (pmol/ml/mg) is normalized by the dry weight of tissues and presented as mean ± SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ compared with ctrl (Student's t-test).

Isoproterenol-induced cAMP production was known to be signaled by the coupling of activated Gs and adenylyl cyclase. Unfortunately, the activity of adenylyl cyclase was very low and undetectable in lung homogenates. Only Gs expression on lung membranes was measured by western blotting. However, as shown in Figure 12, no difference was

detected not only between the control and s/c mice (0.0654±0.0067 vs. 0.0616±0.009, n=3), but also between the presence and absence of chronic nadolol treatment in s/c mice (0.0616±0.009 vs. 0.0617±0.013, n=3).

β AR agonists bind to β AR and activate Gs, then activate adenylyl cyclase to initiate cAMP synthesis. Since the Gs expression did not change, and the cAMP accumulation was lower in s/c mice, the adenylyl cyclase activity was probably impaired in s/c mice. Nevertheless, chronic nadolol treatment has no effect on Gs expression or cyclase activity, or cAMP production.

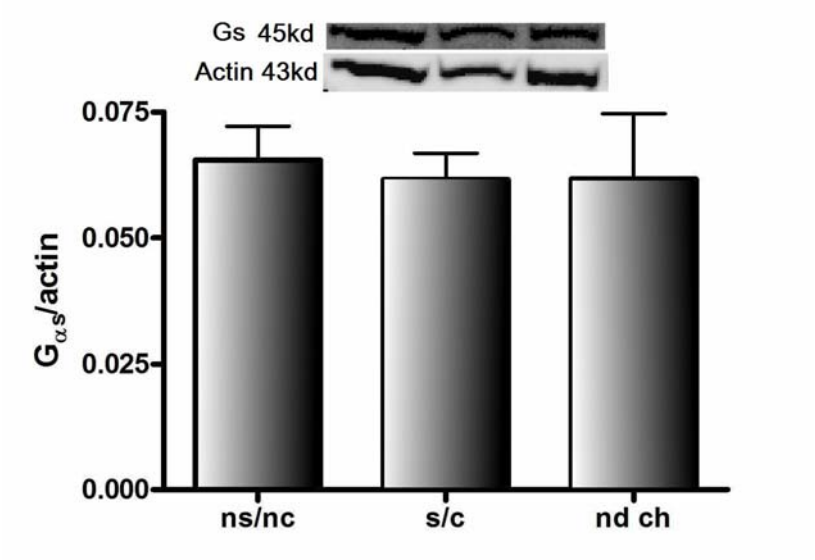


Figure 12. Immunoblotting of Gαs in lung membranes

Expression of Gαs in membranous fraction of lung homogenate was measured by western blotting. Expression of actin was also measured as a loading control. Representative band images are shown above the bar graph.Densitometry analysis of Gαs/actin ratios are shown in the lower panels as mean±SEM (n=3). ns/nc (control), s/c (OVA sensitized and challenged), Nd ch (nadolol chronic). No difference is shown between experimental groups.

1.2 Effect of chronic nadolol treatment on PDE4D expression

1.2.1 Expression of PDE4D in cytosolic fraction of lung homogenates

Although chronic nadolol treatment showed no effect on impaired cAMP accumulation in s/c mice globally, discrete cAMP concentrations may be modulated by PDE in different subcellular compartments (Jin, Bushnik et al. 1998). As we know, PDE is the only enzyme controlling cAMP breakdown. In particular, PDE4 is the major isoform of PDEs expressed in the mouse airway (Mehats, Jin et al. 2003; Richter, Jin et al. 2005). The PDE4D expression in s/c mice showed no significant difference compared with control mice, though an insignificant lower level of expression was observed as seen in Figure 13 (1.475±0.235 vs. 0.924±0.162, n=12 vs. n=13); after chronic treatment with nadolol, PDE4D was significantly decreased (0.924±0.162 vs. 0.201±0.064, $P<0.01$, n=13 vs. n=6).

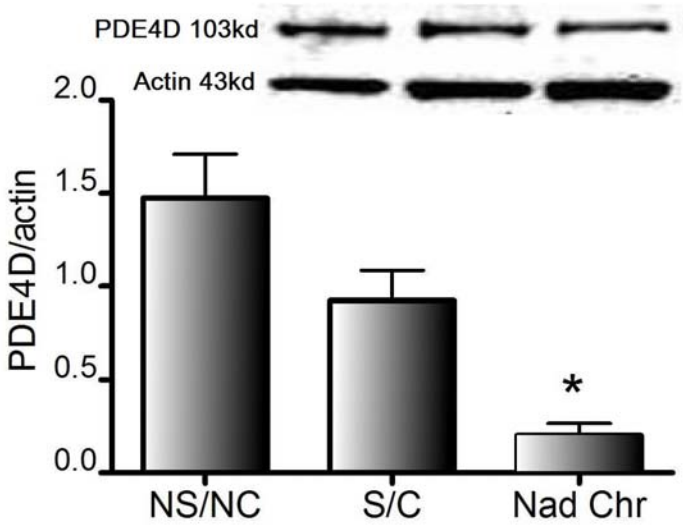


Figure 13. Immunoblotting of PDE4D in lung homogenates

The expression of PDE4D in cytosolic fraction of lung homogenate was measured by western blotting. The expression of actin was also measured as a loading control. Representative band images are shown above the bar graph. Densitometry analysis of PDE4D/actin ratios are shown in the bar graph as mean±SEM (n=6-13). NS/NC (control), S/C (OVA sensitized and challenged), Nad Chr (chronic nadolol treatment). Chronic nadolol treatment decreased PDE4D expression in lung homogenates. * $P<0.05$ compared with S/C.

To further confirm that the effect of chronic nadolol treatment on PDE4D is mediated by β_2 AR signaling, PDE4D expression was measured in lung homogenates from β_2 AR knockout mice. Similar to wildtype mice, β_2 AR knockout mice did not show significant difference in PDE4D expression over mice with or without OVA sensitization and challenge, though an insignificantly lower expression was shown in mice with OVA sensitization and challenge. However, as seen in Figure 14, chronic nadolol treatment decreased PDE4D expression in wildtype mice (0.16±0.02 vs. 0.30±0.04, $P<0.05$, n=4),

but not in knockout mice (0.24±0.07 vs. 0.21±0.07, P>0.05, n=4). This result indicated that β_2 AR gene knockout eliminated the effect of chronic nadolol treatment on decreasing PDE4D, implying the effect of chronic nadolol treatment was mediated by β_2 AR signaling.

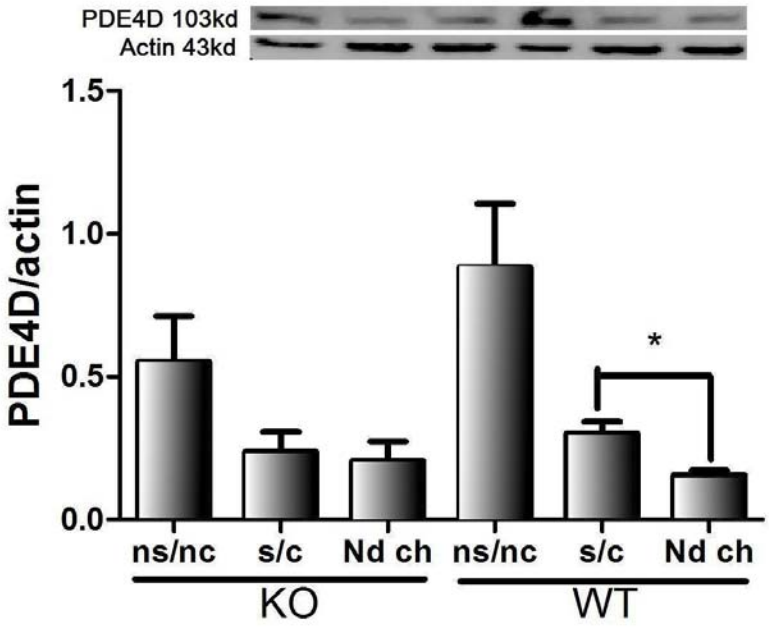


Figure 14. Immunoblotting of PDE4D on β_2 AR knockout mice

Western blotting of PDE4D in the cytosolic fraction of lung homogenate. The expression of actin was also measured as a loading control. Representative band images are shown above the bar graph. Densitometry analysis of PDE4D/actin ratios are shown in the bar graph as mean ±SEM (n=3~6). KO (β_2 knockout mice), WT (wildtype mice), Nd Ch (nadolol chronic). Chronic nadolol treatment decreased PDE4D expression in wildtype mice, but not in knockout mice. * P<0.05 compared to s/c, # P<0.05 compared to Nd Ch.

1.2.2 Splice variant of PDE4

PDE4D can hardly be detected in trachea and large bronchi homogenate (data not shown). Therefore, it might not be the predominant isoform of PDE4 expressed in airway smooth muscle. PDE4A is also an important subtype of PDE4 expressed in mouse lung (Mehats, Jin et al. 2003). Inhibitors to both PDE4A and PDE4D subtypes showed a prominent effect in lowering airway hypersensitivity in the ovalbumin-sensitized and challenged animal model (Huang, Dias et al. 2007). Meanwhile, PDE4A expression can be measured using the cell lysate of a primary culture of airway smooth muscle. However, no difference was observed between control mice and s/c mice with or without chronic nadolol treatment, as seen in Figure 15, implying that chronic nadolol treatment specifically affected PDE4D subtype in cell types other than airway smooth muscle cells.

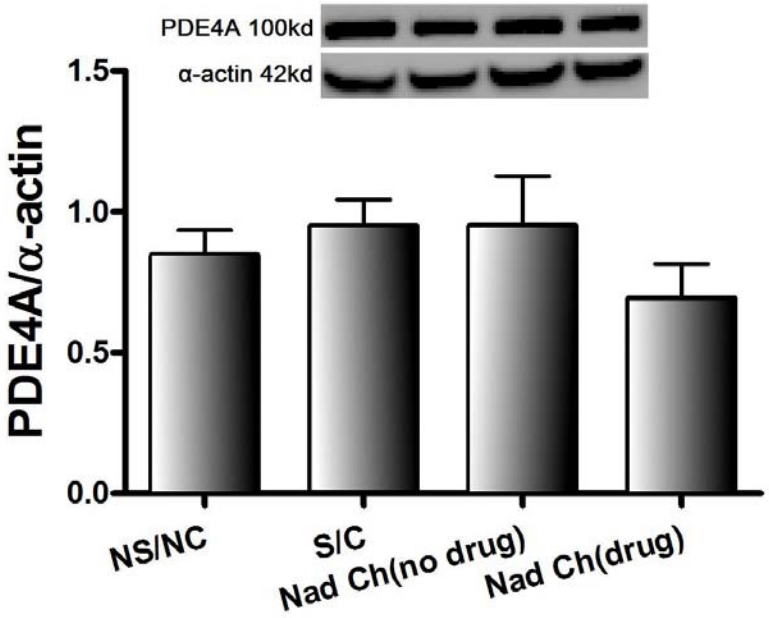


Figure 15. Immunoblotting of PDE4A in airway smooth muscle cells

The expression of PDE4A in airway smooth muscle cell culture was measured by western blotting. The expression of α-actin was also measured as a loading control. Representative band images are shown above the bar graph. Densitometry analysis of PDE4A/α-actin ratios is shown as mean±SEM (n=4-8). NS/NC (control), S/C (OVA sensitized and challenged), Nad Ch (no drug): cells growing without nadolol in culture medium, Nad Ch (drug): cells growing with presence of nadolol 10^{-6}M. No difference was shown between experimental groups.

To further identify the splice variant responsible for the effect of chronic nadolol treatment on PDE4D, we obtained an antibody specifically for PDE4D3 from the same company providing the pan-PDE4D antibody, and ran the same assay. However, no signal was detected at the corresponding molecular weight (99kD) of PDE4D3. We also

obtained tissue samples of wildtype and PDE4D knockout mice from Dr. Marco Conti's lab (Stanford University), and ran parallel experiments to identify the PDE4D splice variant expressing in our samples. Both PDE4D3 and PDE4D4 were detected in the wildtype mouse sample from Dr. Conti, while our sample showed a single band corresponding to PDE4D4 (119kD) (see Figure 16). This result suggested that PDE4D4 was the major splice variant in BALB/cJ mice used for constructing the asthma model.

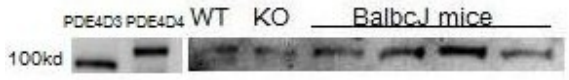


Figure 16. Splice variant of PDE4D in BALB/cJ and C57BL/6 mice

Expression of PDE4D splice variant were detected using pan-PDE4D antibody on samples from Dr. Wito Ritcher (Stanford University, Dr. Marco Cant's laboratory) (WT and KO, representing wildtype and PDE4D knockout C57BL/6 mice respectively) and samples using in our study. WT mice showed two bands around 100kd, but the one around 99kd was knockout in the KO mice. However, BALB/cJ mice do not show expression of the 99kd band.

Since PDE4D was not predominantly expressed in airway smooth muscle, we wondered if PDE4D mainly expressed in other cell types. Because PDE inhibitors showed an anti-inflammatory effect in the airway, the inflammatory cells might express more PDE4D than airway smooth muscle cells. Therefore, the expression of PDE4D in BALF was also examined in order to investigate the effect of chronic nadolol treatment on PDE4D that expressed in BALF cells. However, not enough protein concentration was obtained in

BALF from control mice, and actin could not be detected in all BALF samples. Therefore, the density of PDE4D bands was normalized by the band density of the sample from the control mouse (the only sample which produced enough protein amounts for the assay) instead of actin band density. Nevertheless, the same amount of protein was loaded to each lane, so that the band density was still comparable. Pan-PDE4D antibody detected a single band around 80kD in BALF samples, which was different from what we observed in lung extracts. As seen in Figure 17, the density of this band showed a difference between s/c mice with and without chronic nadolol treatment (4.387±0.50 vs. 2.604±0.18, $P<0.01$, n=3 vs. n=5), though the exact splice variant of this band remained to be verified.

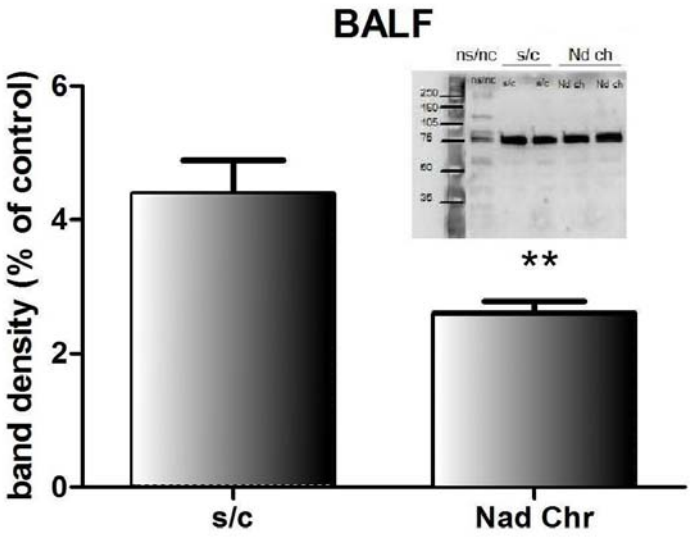


Figure 17. Immunoblotting of PDE4D in BALF

Expression of PDE4D in BALF was measured by western blotting. Representative band images are shown in the inset diagram. Percentages of PDE4D band density for S/C (OVA sensitized and challenged) and Nad Chr (chronic nadolol treatment) over the density of control are shown in the bar graph as mean±SEM (n=3~5). Chronic nadolol treatment decreased the unknown splice variant of PDE4D in BALF. ** P<0.01 compared with S/C.

In summary, chronic nadolol treatment specifically decreased PDE4D expression in mouse lung and BALF, though the specific splice variant of PDE4D in BALF was not fully identified. The effect on downregulation of PDE4D by chronic nadolol treatment can be eliminated by β_2 AR gene knockout. This implied that the presence of β_2 AR was critical for the effect of chronic nadolol treatment on the downregulation of PDE4D. In contrast, PDE4A expression was not affected by chronic nadolol treatment on airway smooth muscle cells.

1.3 Effect of chronic nadolol treatment on isoproterenol induced trachea relaxation

Airway smooth muscle relaxation is conventionally considered to be regulated by β AR-cAMP-PKA signaling pathway. Using an *ex vivo* model, β AR function was evaluated by measuring the tone of mouse trachea in organ chamber. Mice with or without chronic nadolol treatment were used to compare the effect of treatment. To study PKA activation in mouse trachea, a specific inhibitor for phosphorylation by PKA was used to identify the PKA activity.

Methacholine pre-contracted tracheae were used to evaluate the relaxant effect of isoproterenol with or without prior chronic nadolol treatment. In our study, we constructed methacholine dose response curves on each tissue preparation before starting the measurement of isoproterenol induced relaxation. To our surprise, the maximum contraction induced by methacholine was higher in the control mice than in the s/c mice as shown in Figure 18A (425.5±77.36 vs. 294.2±63.7 % resting tension, $P<0.05$, n=4), and nadolol chronic treatment increased the maximum response in the s/c mice (see Figure 18A; 358.3±29.74 vs. 294.2±63.7 % resting tension, $P<0.05$, n=6 vs. n=4). However, the methacholine dose response curves showed similar EC80 and no significant differences among the three groups when analyzed by two way ANOVA (Figure 18B).

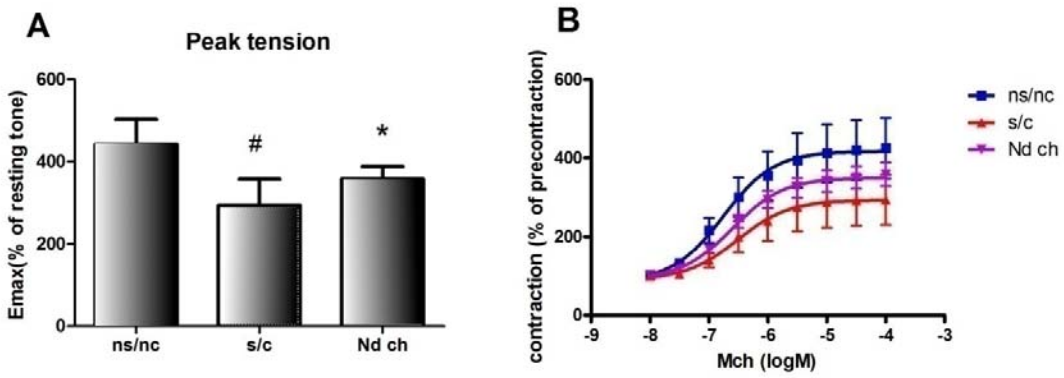


Figure 18. Cumulative concentration response to methacholine in the mouse isolated trachea

The maximum contraction induced (Emax) by 10^{-4}M methacholine in s/c mice (n=4) was lower than the control mice (ns/nc, n=4). Chronic nadolol treatment (Nd ch, n=7) increased Emax of methacholine induced tracheal contraction (A). Cumulative concentration response curve to methacholine in mouse isolated trachea (B) shows that EC80 in all experimental groups are 10^{-}

^{6}M. Each point represents mean and vertical lines at each point show SEM. No difference was shown between experimental groups (two-way ANOVA). *P<0.05 compared with s/c, #P<0.05 compared with ns/nc.

To normalize the initial contraction, we used 10^{-6}M methacholine (EC80) in all three treatment groups to precontract the trachea for the relaxation study. Isoproterenol (ISO) induced a larger relaxant effect on the control mice than on the s/c mice. The presence of nadolol (10^{-6}M) in the bath solution shifted the curve to the right. However, the maximal relaxation showed no significant difference compared with the s/c mice (see Figure 19A). After the equilibrium, nadolol was removed from the bath solution and the ISO-induced tracheal relaxation was measured again. As shown in Figure 19B, no difference was shown between the s/c mice with and without chronic nadolol treatment.

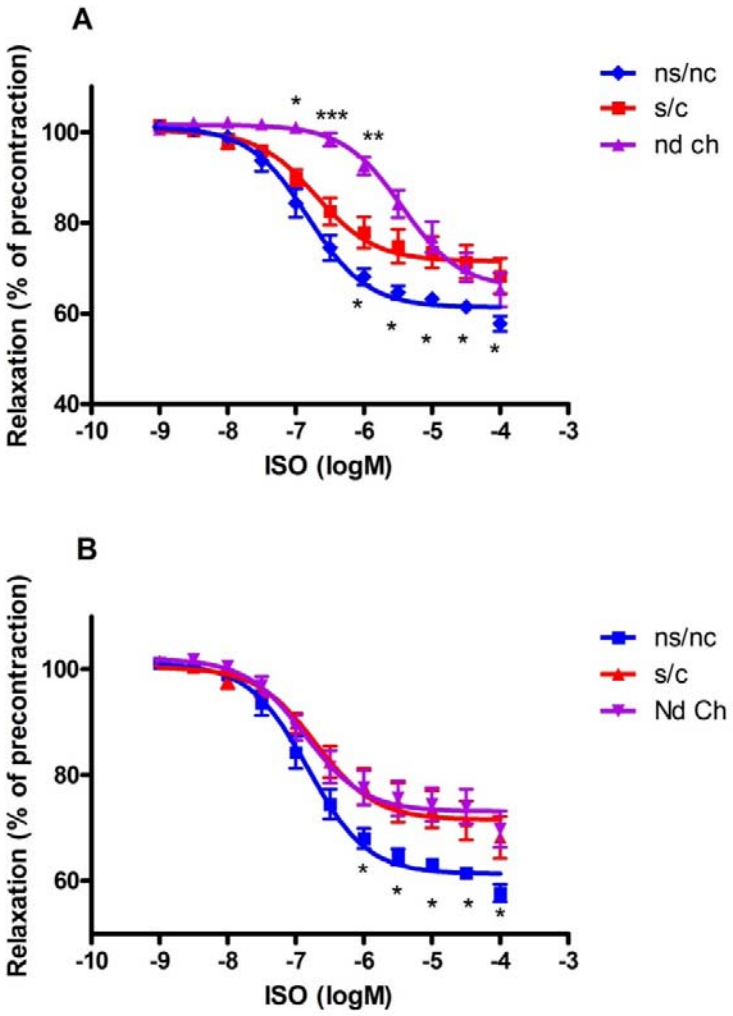


Figure 19. Isoproterenol induced relaxation of methacholine pre-contracted mouse trachea

Cumulative concentration-response curves to isoproterenol in the mouse isolated trachea after methacholine pre-contraction with (A) or without (B) the presence of nadolol (1 μM) in the bath solution. Each point represents mean and vertical lines at each point show SEM (n=4-7). The initial contraction was obtained by the addition of 1μM methacholine to attain the approximate EC80. Ns/nc (control), s/c (OVA sensitized and challenged), Nd Ch (nadolol chronic). S/c mice showed decreased sensitivity of tracheal relaxation in response to isoproterenol, while chronic nadolol treatment showed no effect. *P<0.05, **P<0.01, ***P<0.001 compared with S/C, two-way ANOVA.

High concentration of KCl can depolarize tracheal smooth muscle and also cause contraction. KCl contracted tissue preparation circumvents the variable sensitivity to methacholine in different treatment groups. The trachea was contracted with 60mM KCl for 10 minutes until the tone reached the maximum plateau. The plateau tensions of the control (n=4), the s/c mice (n=4) and the Nd ch (n=2) group were 0.85±0.11g, 0.72±0.04g and 0.88±0.22g, respectively, which showed no significant difference. Increasing concentrations of isoproterenol were added into the organ chamber to construct dose response curves (Figure 20). No difference was observed either between the control mice and the s/c mice, or between the s/c mice with or without chronic nadolol treatment.

Although the resting tension of the trachea showed no difference in the KCl pre-contracted model, s/c mice showed no impairment in isoproterenol-induced relaxation. Therefore, the impairment of isoproterenol-induced relaxation in s/c mice was specific in response to the methacholine-induced tracheal contraction. Studies of tracheal relaxation were conducted by pre-contracting the tissue using both methacholine and KCl. Both studies showed that chronic nadolol treatment did not affect the isoproterenol-induced relaxation in trachea strips.

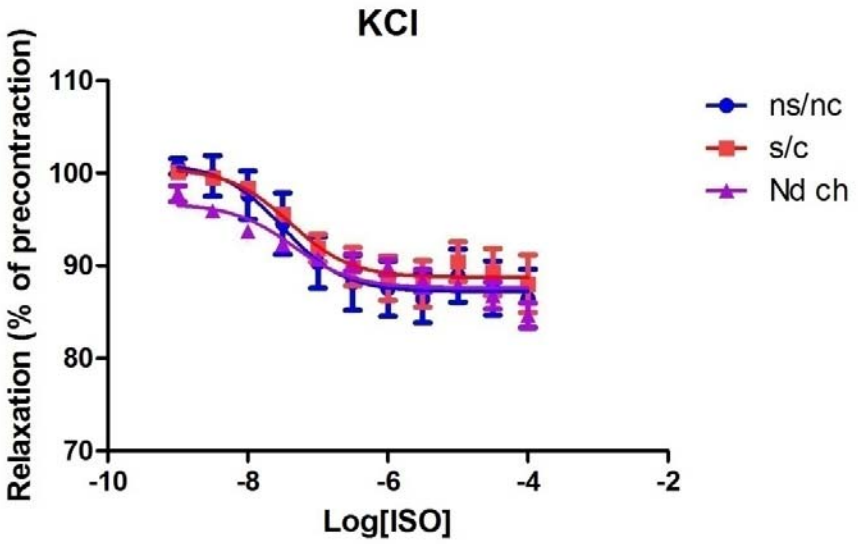


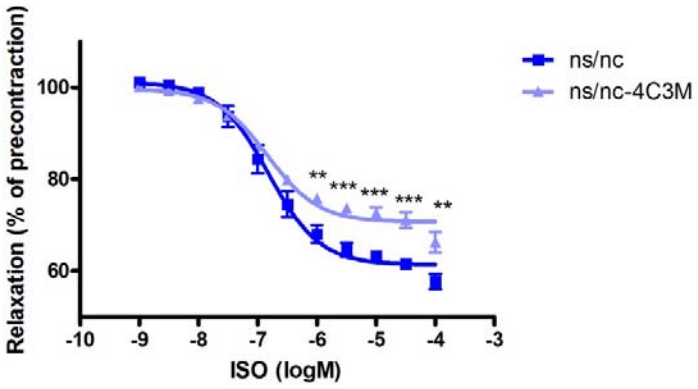
Figure 20. Isoproterenol induced relaxation of KCl pre-contracted mouse trachea

Cumulative concentration-response curves to isoproterenol in the mouse isolated trachea after potassium chloride (60mM) precontraction. Each point represents mean and vertical lines at each point show SEM (n=2~4). No difference was detected among three groups.

1.4 Isoproterenol-induced trachea relaxation in the presence of a PKA inhibitor

Isoproterenol-induced tracheal relaxation may be PKA-dependent and PKA-independent (Spicuzza, Belvisi et al. 2001). To investigate cAMP-PKA-dependent tracheal relaxation and the effect of chronic nadolol treatment, isoproterenol-induced tracheal relaxation was measured after a 30-minute pretreatment with 30μM 4C3M, a specific PKA inhibitor. 4C3M specifically blocks the phosphorylation induced by activated PKA and therefore has been shown to decrease the relaxation in treacheal smooth muscle (Algara-Suarez and

Espinosa-Tanguma 2004). In our study, isoproterenol induced tracheal relaxation was significantly decreased in the trachea from control mice at concentrations higher than 0.3μM with the presence of 4C3M (see Figure 21). However, the pretreatment with 4C3M did not change the isoproterenol-induced relaxant effect in s/c mice with or without chronic nadolol treatment. The data suggest that the cAMP-PKA signaling responsible for the tracheal relaxation is impaired in s/c mice, and chronic nadolol treatment had no effect.


Relaxation (% of precontraction)
100
80
60
-10
-9
-8
-7
-6
-5
-4
-3
ISO (logM)
**
***
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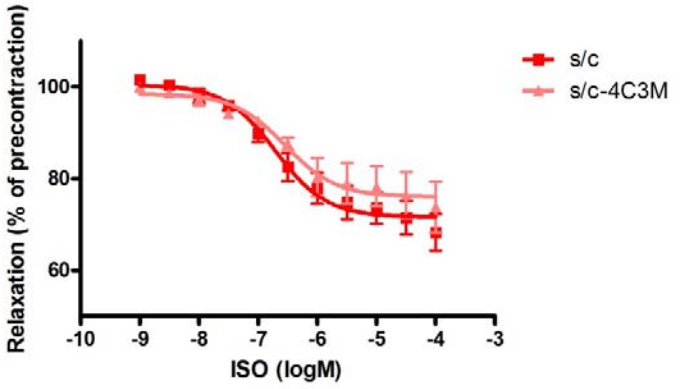
**
ns/nc
ns/nc-4C3M


Relaxation (% of precontraction)
100
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-6
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ISO (logM)
s/c
s/c-4C3M

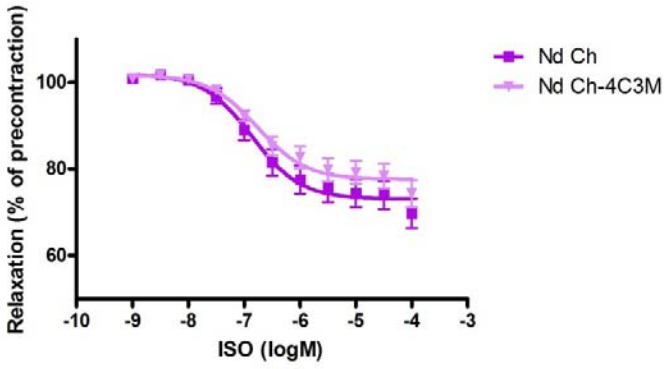
Relaxation (% of precontraction)
100
80
60
-10
-9
-8
-7
-6
-5
-4
-3
ISO (logM)
Nd Ch
Nd Ch-4C3M

Figure 21. Isoproterenol induced PKA dependent relaxation of methacholine pre-contracted mouse trachea

Cumulative concentration-response curves to isoproterenol in the mouse isolated trachea after methacholine pre-contraction with or without the pretreatment of 4C3M in the bath solution (30 μM). Each point represents mean and vertical lines at each point show SEM (n=4-7). The initial contraction was obtained by the addition of 1μM methacholine to attain the approximate EC80. Ns/nc (control), ns/nc-4C3M (control trachea pretreated with 4C3M, the same way for the following groups), s/c (OVA sensitized and challenged), Nd Ch (nadolol chronic). 4C3M reduced isoproterenol-induced tracheal relaxation in Ns/nc mice, but showed no effect in s/c and Nd Ch mice. *P<0.05, **P<0.01, ***P<0.001 compared with ns/nc, two way ANOVA.

1.5 Effect of chronic nadolol treatment on desensitization of Gs signaling

As above mentioned, β_2 AR undergoes both heterologous and homologous desensitization. Since we did not observe any effect of chronic nadolol treatment on PKA specific activity by using trachea rings, we focused on homologous desensitization. Increased GRK expression is known to be associated with heart failure and COPD (Mak, Chuang et al. 2002; Hata and Koch 2003). GRK2 and GRK3, as well as β arrestins, are known to play important roles in β_2 AR desensitization. To examine the effect of chronic beta blocker treatment in GRKs mediated desensitization, GRK2, GRK3 and β arrestin 2 expression in lung homogenates were measured by immunoblotting.

1.5.1 GRK2 expression

GRK2 expression was measured by western blotting. A band around 80kd was identified as GRK2, using the positive control purchased from Santa Cruz Inc.. In a membranous fraction, the GRK2 band was not reproducible because another band around 70kd was

more pronounced than that of GRK2. This 70kd band could also be detected in the cytosolic fraction, though it did not show as strong and reproducible signals as the GRK2 band did (80kd). Therefore, only the expression of GRK2 in cytosolic fraction was taken into account. As shown in Figure 21, GRK2 expression in s/c mice was much less than in the control (0.12±0.014 vs. 0.037±0.011, $P<0.05$, n=3), and chronic nadolol treatment did not affect GRK expression (Figure 22, 0.035±0.009 vs. 0.037±0.011, n=3).

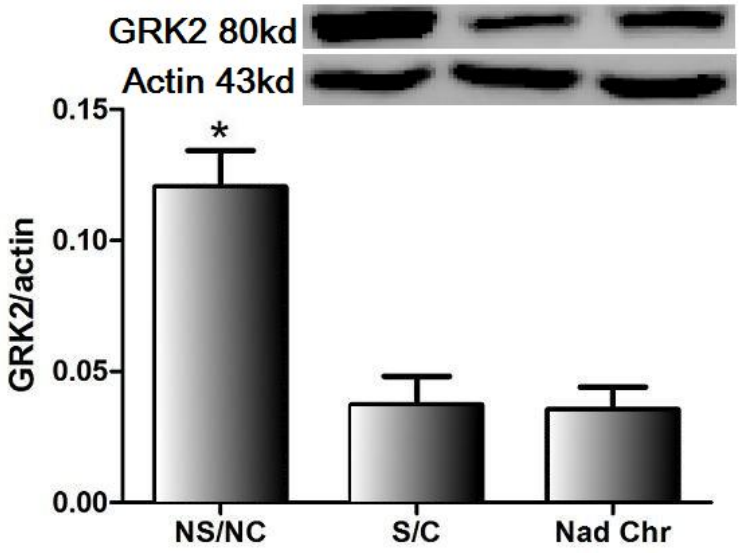

Figure 22. Immunoblotting of GRK2 in lung homogenates

The expression of GRK2 in cytosolic fraction of lung homogenate was measured by western blotting. The expression of actin was also measured as a loading control. Representative band images are shown above the bar graph. Densitometry analysis of GRK2/actin ratios are shown in the bar graph as mean±SEM (n=3). NS/NC (control), S/C (OVA sensitized and challenged), Nad Chr (nadolol chronic). GRK2 expression was lower in s/c mice than in control mice, while chronic nadolol treatment did not affect GRK2 expression. * $P<0.05$ compared with S/C, Student t-test.

1.5.2 GRK3 expressions

A very high concentration of the GRK3 antibody was necessary to obtain reproducible signals from membranous fraction. The bands on the cytosolic fraction were very faint and not reproducible. The expression of GRK3 in the membranous fraction was reproducible but showed large variation. As a result, no statistical significance was noted between groups; as shown in Figure 23, s/c mice showed a higher level of expression than the control (0.25±0.09 vs. 0.089±0.04, n=5 vs. n=6), and chronic nadolol treatment showed a tendency to decrease the higher expression of GRK3 in s/c mice (0.10±0.05 vs. 0.25±0.09, n=6).

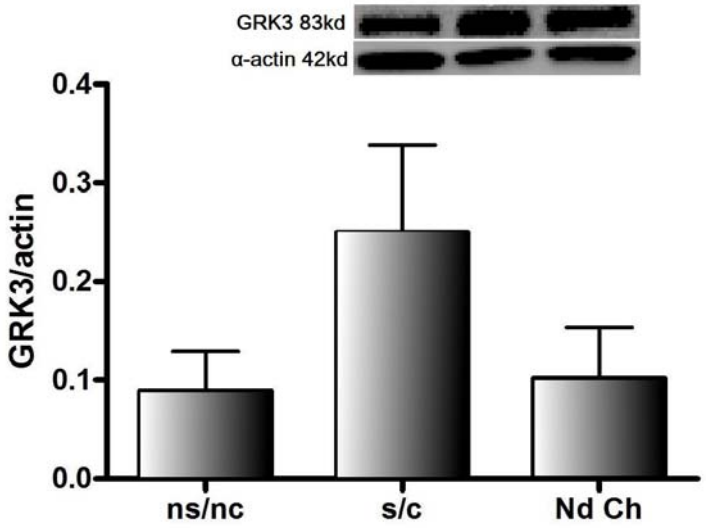

Figure 23. Immunoblotting of GRK3 in lung membranes

The expression of GRK3 in a membranous fraction of lung homogenate was measured by western blotting. Expression of actin was also measured as a loading control. Representative band images are shown in the inset diagram. Densitometry analysis of GRK3/actin ratios are shown in bar

graph as mean±SEM (n=5~6). NS/NC (control), S/C (OVA sensitized and challenged), Nad Chr (nadolol chronic). No significant difference was found.

GRK3 expression in the lysate of airway smooth muscle cell culture was also measured. Much clearer and more stable bands were shown in the assays using cell lysate samples. However, lysates of cell culture from s/c mice showed no difference from control mice. As seen in Figure 24, GRK3 expression was significantly increased in the cells cultured with nadolol in the medium (0.19±0.02 vs. 0.12±0.02, P<0.05, n=8).

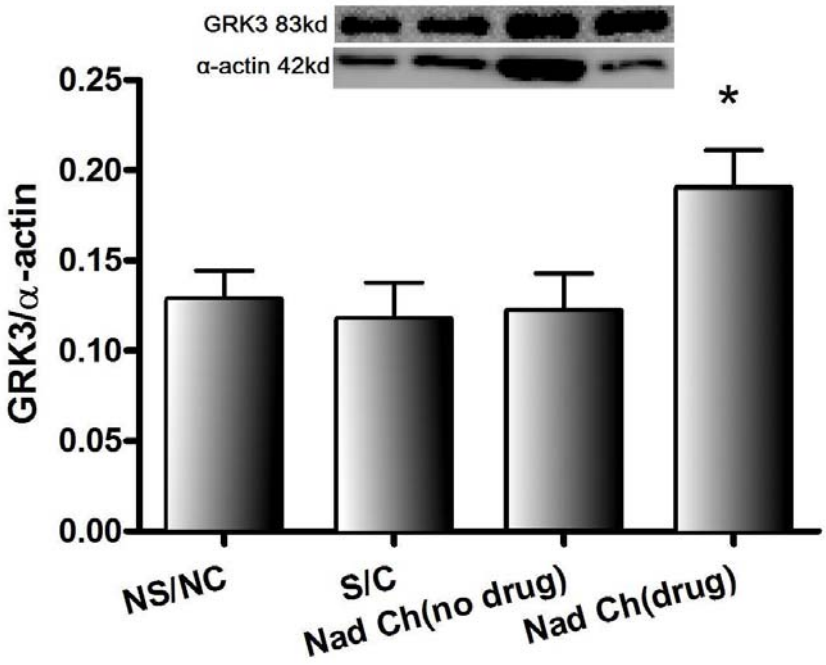

Figure 24. Immunoblotting of GRK3 in airway smooth muscle cells

Expression of GRK3 in airway smooth muscle cell culture was measured by western blotting. Expression of α-actin was also measured as a loading control. Representative band images are shown above the bar graph. Densitometry analysis of GRK3/α-actin ratios are shown in the bar

graph as mean±SEM (n=4~8). NS/NC (control), S/C (OVA sensitized and challenged), Nad Ch (no drug): cells growing without nadolol in culture medium, Nad Ch (drug): cells growing with presence of nadolol 10^{-6}M. Chronic nadolol treatment increased GRK3 expression in primary culture of airway smooth muscle cells. * P<0.05 compared with S/C.

GRK3 expression in lung homogenates was very low. The effect of chronic nadolol treatment was a slight decrease of GRK3 expression, which was not significant. However, chronic nadolol treatment increased GRK3 expression in airway smooth muscle cells, where the detection of GRK3 expression was more robust and the effect was significant.

1.5.3 β arrestin 2 expressions in lung membranes

β arrestins have been known to function as adaptor proteins in GRK mediated β_2 AR internalization (Goodman, Krupnick et al. 1996). β arrestin 2 knockout mice showed resistance to ovalbumin induced airway hyperresponsiveness (Walker, Fong et al. 2003). Therefore, we measured the expression of β arrestin 2 in lung homogenates from our mouse model of asthma. As seen in Figure 25, in a membranous fraction of lung homogenates, the expression of β arrestin 2 expression in s/c mice was dramatically higher than in the control mice (2.193±0.42 vs. 0.10±0.02, P<0.001, n=6 vs. n=5) though chronic nadolol treatment showed no significant effect (1.898±0.13 vs. 2.193±0.42, n=5~6).

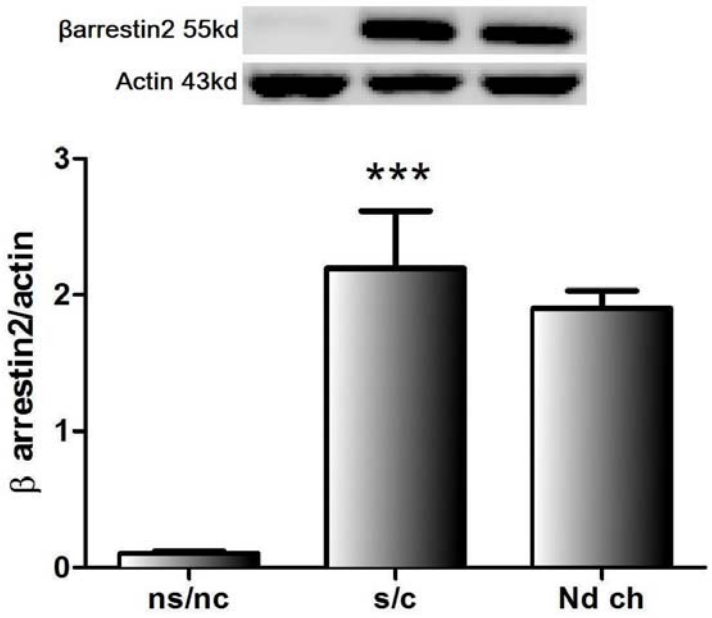

Figure 25. Effect of chronic nadolol treatment on β arrestin 2 expression

The expression of β arrestin 2 in a membranous fraction of lung homogenate measured by western blotting. The Expression of actin was also measured as a loading control. Representative band images are shown in the inset diagram. Densitometry analysis of β arrestin 2/actin ratios are shown in bar graph as mean±SEM (n=5~6). S/c mice showed a pronounced increase of β arrestin 2 comparable to the control mice, while mice with chronic nadolol treatment did not show any significant change. NS/NC (control), S/C (OVA sensitized and challenged), Nad Chr (nadolol chronic). ***P<0.001, student t-test.

In summary, although s/c mice showed pronounced difference in the expression of GRK2 and β arrestin 2 compared with control mice, chronic nadolol treatment showed no effect on GRK2, GRK3 and β arrestin 2 expressions on lung extracts.

2 Discussion

2.1 Importance of airway smooth muscle contractility

Activation of β_2 AR in airway smooth muscle cells results in bronchodilation which has been taken advantage of in developing asthma therapies. The anti-inflammatory effect of β_2 AR activation in inflammatory cells and epithelial cells has also been considered to be part of the mechanisms of the bronchodilatory effect of β AR agonists (Hanania and Moore 2004). When compared to airway mast cells with regards to the β_2 AR-induced inhibition of histamine release, airway smooth muscle cells have had a greater receptor reserve of β_2 AR for smooth muscle relaxation (Chong and Peachell 1999). Also, β_2 AR in airway smooth muscle cells was much less susceptible to GRK-induced desensitization than airway epithelial cells and mast cells (McGraw and Liggett 1997). Based on these studies, β_2 AR signaling in airway smooth muscle cells seems to be more important. It is certain that the contraction of airway smooth muscle plays an important role in inducing airway hyperresponsiveness, and thereby increasing the burden of breathing, deranging the normal distribution of ventilation, and in its most severe manifestation leads to ventilatory failure and death. This study is trying to delineate the mechanism of the bronchoprotective effect of chronic beta blocker treatment on methacholine-induced hyperresponsiveness. Therefore, the first priority in our focus of study is the central role that airway smooth muscle contractile function plays in the regulation of bronchial tone.

In congestive heart failure, chronic use of a β AR agonist has increased mortality and morbidity of the disease, and the β AR agonist acutely increases heart contractility; however, chronic use of certain beta blockers decreases the mortality and increases heart contractility, even though acute use of beta blockers was found to be detrimental to the failing heart. In parallel, the chronic use of the β AR agonist has also been reported to increase the mortality and morbidity of asthma, while the β AR agonist is still the best bronchodilator when used acutely; acute use of beta blockers was found to be detrimental to asthmatics but chronic use of certain beta blockers did show a bronchoprotective effect to the methacholine-induced hyperresponsiveness in the animal model (Callaerts-Vegh, Evans et al. 2004). In congestive heart failure, chronic treatment with some β-AR inverse agonists has increased β-AR density and adenylyl cyclase coupling (Bristow, Larrabee et al. 1992), and decreased G-protein-coupled receptor kinase (GRK) activity in the human myocardium (Leineweber, Rohe et al. 2005), denoting a reversal of desensitized β-AR signaling in the failing heart. Perhaps the usefulness of certain beta blockers in asthma models is also due to the reversal of desensitized β-AR signaling in hyperresponsive airways.

2.2 The effect of chronic beta blocker treatment on the Gs-cAMP signaling pathway

The Gs-cAMP signaling pathway became the first candidate to identify the signaling pathways regulated by chronic nadolol treatment. As shown in chapter 4, we have already found that chronic beta blocker treatment increased β AR density. It has been

shown that basal adenylyl cyclase activity increases when β AR density increases (McGraw and Liggett 2005). In heart failure, chronic carvedilol (non-selective beta blocker) treatment has been shown to increase adenylyl cyclase coupling in the myocardium (Bristow, Larrabee et al. 1992). Therefore, we expected the analogous effect of chronic beta blocker treatment on airway smooth muscles. In contrast, long term treatment with the β_2 agonist albuterol has been shown to decrease the brochoprotective effect on airway hyperresponsiveness *in vivo*, decrease the cAMP accumulation and PKA activity *ex vivo*, decrease β_2 AR density, Gs expression and function, and also increase PDE and GRK2 activity (Finney, Bevis et al. 2000). However, in our mouse model of asthma, we did not find any change in cAMP accumulation, Gs protein expression in lung homogenates, or isoproterenol-induced trachea relaxation in s/c mice after chronic nadolol treatment.

To some extent, cAMP accumulation reflects the integrity of β AR-Gs-AC signaling. As shown in Figure 11, in control mice, the cAMP accumulation increased in response to isoproterenol in a dose dependent manner, implying the functional β AR-Gs-AC signaling, while this response was impaired in s/c mice. Gs dysfunction has been suggested to induce impaired relaxation of sensitized and challenged human bronchi rings in response to salbutamol (Song, Milanese et al. 2000), without any change in its expression. Since no difference in Gs expression was observed in our animal model (Figure 12), we also measured β AR agonist induced trachea relaxation in order to

evaluate Gs function. Consistent with cAMP accumulation, Figure 18 shows that isoproterenol induced tracheal relaxation was impaired in s/c mice as compared to control mice, while no change was found in s/c mice with chronic nadolol treatment.

β AR agonists induced airway smooth muscle relaxation is also known to be mediated by PKA. Downstream Gs-AC-cAMP signaling, activated PKA can phosphorylate myosin light chain kinase (MLCK), resulting in the decrease of its capability to phosphorylate myosin light chain, thus reducing contractile activity (Billington and Penn 2003). After pretreatment with a specific inhibitor for PKA phosphorylation, isoproterenol induced tracheal relaxation significantly decreased in control mice, demonstrating the involvement of a PKA-dependent mechanism in tracheal relaxation. However, the significant decrease in tracheal relaxation was not observed in s/c mice with or without chronic nadolol treatment (Figure 21). These experiments further confirmed that chronic nadolol treatment does not affect Gs-cAMP-mediated airway smooth muscle relaxation.

On the other hand, as shown in Figure 13, we have found that chronic nadolol treatment decreased the expression of PDE4D. This finding was repeated using two mouse strains and abolished by β_2 AR gene deletion, implying that chronic nadolol treatment decreased PDE4D expression in a β_2 AR dependent manner (Figure 15). PDE is the only enzyme that degrades cAMP inside the airway smooth muscle cells. PDE inhibitors can potentiate isoproterenol induced relaxation, and also have shown a synergistic effect with

PGE2 on reducing TNFα release in macrophages and monocytes (Torphy, Undem et al. 1993; Seldon, Barnes et al. 1995; Sinha, Semmler et al. 1995). Therefore, the regulation of PDE4 expression has significance in the pathogenesis and treatment of asthma (Torphy 1998). Theoretically, a downregulation of PDE4D should contribute to the augmentation of cAMP signaling. However, chronic nadolol treatment did not increase the PKA-dependent tracheal relaxation in s/c mice (Figure 21), though it decreased PDE4D expression (cAMP accumulation was measured in the presence of PDE inhibitor, therefore the PDE activity was not reflected by cAMP assay). This conflict suggested two possibilities: 1) the decrease of PDE4D in lung homogenates might reflect the expression of PDE4D from cell types other than airway smooth muscle cells; 2) the decrease of PDE4D did affect intracellular cAMP gradient, but did not affect PKA-mediated tracheal relaxation.

To elucidate the first possibility, PDE4D expression was measured in BALF, which contains mainly leukocytes. Figure 17 shows a decrease of BALF PDE4D expression in mice after chronic nadolol treatment, though the immunoblotting result shows a banding pattern very different from the lung homogenate samples, suggesting that a different splice variant of PDE4D4 is highly expressed in leukocytes compared with the whole lung homogenates. PDE4D expression can hardly be detected in trachea and bronchial extract from our animal model (data not shown), which might be a species difference from the human airway smooth muscle cells (Le Jeune, Shepherd et al. 2002). Of note,

different strains of mice also showed different patterns of PDE4D splice variants (Figure 16). Another isoform, PDE4A, can be detected in airway smooth muscle cells as seen in Figure 15, but chronic nadolol treatment did not decrease PDE4A expression. Collectively, these data suggest that chronic nadolol treatment decreases a certain PDE4D splice variant that might play an important role in cAMP mediated anti-inflammatory effect. To further identify the splice variant of PDE4D in lung homogenates, PDE4D3 and PDE4D4 positive controls were purchased from the same company producing the pan-antibody of PDE4D. The results suggested that the highest expressed splice variant in lung homogenates was PDE4D4 (Figure 16). In addition, samples of mouse brain homogenate of PDE4D3 knockout mice were kindly provided as a gift by Dr. Wito Richter from Dr. Marco Conti's laboratory (Stanford University) and were run in parallel to compare the splice variants. The team from Dr. Conti's laboratory has published the result that the PDE4D3 gene knockout mice show less airway hyperresponsiveness to methacholine (Hansen, Jin et al. 2000; Mehats, Jin et al. 2003). Our results confirmed that the knockout mice used for their study showed little PDE4D3 expression, though PDE4D4 expression was intact in the knockout mice (Figure 16). Therefore, the actual role of PDE4D4 in regulating airway smooth muscle contraction remains undefined, while PDE4D3 was unfortunately not detectable in lung homogenates of our animal model.

The results of PDE4D splice variant expression in BALF and the low PDE4D expression in trachea and large bronchi suggest the bronchoprotective effect of chronic nadolol treatment might be partly due to its anti-inflammatory function instead of the improved brochodilatory function. PDE4D4, the major PDE4D splice variant decreased by chronic nadolol treatment, has also been shown to be expressed on the pulmonary microvascular endothelial cells and regulate PKA-mediated microtubule reorganization (Creighton, Zhu et al. 2008). However, we still could not rule out the possibility that the decreased breakdown of cAMP due to the downregulation of PDE4D might induce the increase of cAMP mediated airway smooth muscle relaxation. Chronic nadolol treatment might regulate airway smooth muscle relaxation in a PKA-independent pathway, and exchange protein directly activated by cAMP (Epac) might be involved (Spicuzza, Belvisi et al. 2001). In addition, the subcellular localization of PDE4D has been discovered in thyroid cells (Jin, Bushnik et al. 1998), and AKAP (A-kinase anchoring protein), RACK (receptor for activated C kinase), β arrestins have been found to be scaffold proteins that control the proximity of PDE4D with its interacting proteins and influence the compartmentalization of cAMP (Houslay and Adams 2003; Barnes, Livera et al. 2005). Therefore, the reduction of PDE4D induced by chronic nadolol treatment might contribute to the decreased airway hyperresponsiveness via some indirect mechanisms rather than via PKA-dependent smooth muscle relaxation.

To normalize the isoproterenol induced relaxation, two airway smooth muscle constrictors were compared. However, all of the data about tracheal relaxation were collected from tracheae precontracted with a certain concentration of methacholine. Cumulative dose response curves were constructed to determine the appropriate concentrations of methacholine that induce 80% of maximal response. The maximal contraction of the trachea has limited value for studying β AR agonist induced relaxation because the potency of β AR agonist have been shown to be reduced in maximally contracted trachea (Lemoine and Overlack 1992). The cumulative dose response curve showed no significant difference, not only between s/c mice and control mice, but also between s/c mice with and without chronic nadolol treatment (Figure 18B). This finding was not surprising because airway smooth muscle excised from asthmatics have been reported to show similar responses as normal subjects, in response to constrictors such as histamine and methacholine (Hershenson, Brown et al. 2008). In the mouse model, hyperresponsiveness could not be detected in tracheal smooth muscle, while bronchial smooth muscle showed hyperresponsiveness (Chiba, Ueno et al. 2004). As shown in Figure 18B, the dose response curve of tracheal contraction obtained from s/c mice was slightly lower than control mice, while chronic nadolol treatment slightly increased the contraction. The ratio of peak contraction to resting tension surprisingly showed that the maximal contraction of s/c mice was less than control mice and chronic nadolol treatment increased the maximal contraction in response to methacholine (Figure 18A). This observation may be due to the lower expression of Gq protein in s/c mice (Figure 30),

and the effect of chronic nadolol treatment on increasing Gq expression in airway smooth muscle cells (Figure 31). Nevertheless, we found that the EC80 of methacholine-induced tracheal contraction showed no significant difference among all three experimental groups (ns/nc: 355.6±61.82, n=4; s/c: 239.7±49.58, n=4; chronic nadolol treatment: 294.9±21.84, n=6). Therefore, we used the EC80 to stimulate the contraction of trachea for all samples in order to investigate the effect of isoproterenol induced relaxation.

We also used KCl to precontract the tracheal rings. KCl can depolarize the cell membrane on airway smooth muscle so it is another constrictor commonly used for studying the contraction and relaxation of smooth muscle. The tracheal contraction induced by KCl mainly depends on external calcium (Sanderson, Delmotte et al. 2008). No difference was observed in KCl induced peak contraction of tracheal rings from s/c or control mice (Figure 19). Isoproterenol induced tracheal relaxation was not impaired in KCl precontracted tracheal ring from s/c mice, implying that isoproterenol induced tracheal relaxation was not impaired in antagonizing extracellular calcium sensitive contraction at least in tracheal smooth muscle.

2.3 The effect of chronic beta blocker treatment on GRKs mediated β_2AR desensitization

Increasing evidence shows that the level of GRK expression can correlate with the pathological conditions. Different levels of GRK2 expression in lung cell types have been correlated to distinct β_2 AR desensitization and internalization patterns (McGraw and Liggett 1997; Menard, Ferguson et al. 1997). With the help of transgenic animal models, the roles of GRK3, GRK5 and β arrestin 2 in the regulation of airway hyperresponsiveness have been studied (Walker, Peppel et al. 1999; Walker, Fong et al. 2003; Walker, Gainetdinov et al. 2004). Therefore, we also examined the expression of GRKs and β arrestin 2 in our animal model of asthma. To our surprise, we found that GRK2 expression was much higher in control mice than in s/c mice. We discovered that GRK2 expression in control mice was nearly 2 fold greater than s/c mice (Figure 21). This magnitude of difference was almost the same as the difference of β_2 AR density between control and s/c mice measured by radioligand binding (Figure 5). These observations imply that the β_2 AR density is directly proportional to GRK2 in s/c and control mice, while it did not occur in Nd ch mice. Yet, chronic nadolol treatment increased β AR density (Figure 7), but did not affect GRK2 expression (Figure 21). This implies that GRK2-induced β AR desensitization might be relatively less in s/c mice after chronic nadolol treatment.

GRK3 was very hard to detect in membranous fraction of lung homogenates. With a 5 times increase in antibody titer and longer exposure time, we still could not observe significant difference in GRK3 expressions between the experimental groups (Figure 22). On the other hand, Figure 23 showed that in primary culture of airway smooth muscle cells chronic nadolol treatment increased GRK3 expression. GRK3 gene knockout mice have shown higher airway sensitivity to methacholine, and altered heart rate recovery from methacholine induced bradycardia, suggesting a GRK3 mediated muscarinic receptor desensitization (Walker, Peppel et al. 1999) in airway smooth muscle and myocardium. Therefore, the increase in levels of GRK3 by chronic nadolol treatment in primary airway smooth muscle cell culture suggested a higher potential of muscarinic receptor desensitization in airway smooth muscle. The increase of GRK3 expression was not seen in whole lung homogenates, implying that the increase of GRK3 may be specifically localized in airway smooth muscle.

The importance of β arrestins in desensitization mechanisms and in novel cellular signalings has been broadly reviewed (Reiter and Lefkowitz 2006; Vroon, Heijnen et al. 2006; DeWire, Ahn et al. 2007; Premont and Gainetdinov 2007). In particular, the pathological role of β arrestin in asthma has also been studied using β arrestin 2 knockout mice (Walker, Fong et al. 2003). Figure 24 showed that β arrestin 2 expression in s/c mice was over 25 times higher than control mice. This huge difference was not comparable with what we observed in GRK2 and GRK3 expressions. On the whole,

chronic nadolol treatment did not seem to affect the expressions of GRK2 and GRK3, as well as β arrestin 2. This implies that GRKs/ β arrestin mediated β_2 AR desensitization might not be influenced by chronic nadolol treatment. The huge difference of β arrestin 2 expression between the s/c mice and control mice might reflect the involvement of β arrestin 2 in higher chemotaxis activity in s/c mice, since the downregulation of chemotaxis in β arrestin 2 knockout mice has been shown to be associated with the loss of airway hyperresponsiveness after ovalbumin sensitization and challenge (Walker, Fong et al. 2003).

CHAPTER 6 Effect of chronic nadolol treatment on non-classical signaling pathways of β_2 AR

1 Results

β_2 AR has been known to couple with Gi signaling in the myocardium (Xiao, Zhang et al. 2003). Impaired β_2 AR-induced tracheal relaxation in sensitized subjects has also been shown to be rescued by Gi inhibition (Hakonarson, Herrick et al. 1995). Therefore, we investigated the effect of chronic nadolol treatment on Gi protein and Gi coupled β_2 AR signaling. β_2 AR has also been known to couple with Gi and cPLA2 in myocardium (Pavoine and Defer 2005). cPLA2 is important enzyme to metabolize arachidonic acid. The bioactive metabolites of arachidonic acid also play important roles in regulating lung function. In addition, decreased PLCβ1 expression has been found in β AR null mice (McGraw, Almoosa et al. 2003), indicating the crosstalk of β_2 AR signaling with Gq signaling. In this chapter, the effect of chronic nadolol treatment in Gi, Gq and arachidonic acid signaling were examined.

1.1 Effect of chronic nadolol treatment on Gi signaling pathway

1.1.1 Gi expression on lung membranes

$G\alpha_{i3}$ and $G\alpha_{i2}$ expressions were measured in lung membranes from different groups of mice. As seen in Figure 26A, an increased expression of $G\alpha_{i3}$ was shown in s/c mice (3.914±0.86 vs. 1.325±0.30, $P<0.05$, n=6 vs. n=5), and chronic nadolol treatment

decreased $G\alpha_{i3}$ expression in s/c mice (1.656±0.54 vs. 3.914±0.86, P<0.05, n=6 vs. n=5); however, $G\alpha_{i2}$ expression showed no significant difference in all three experimental groups (see Figure 26B).

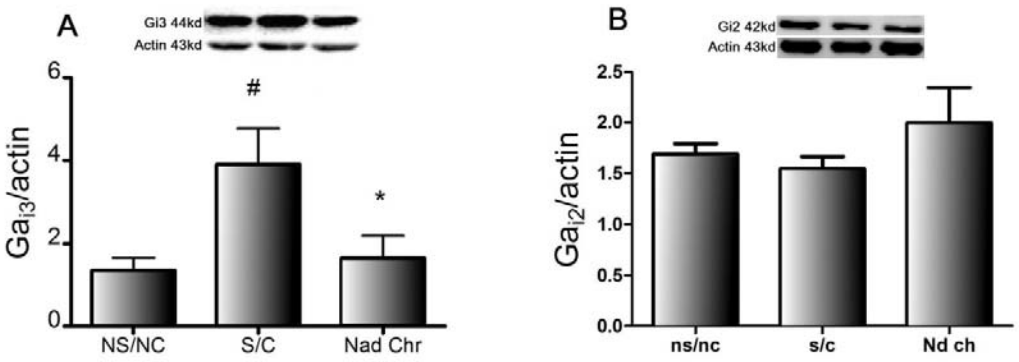

Figure 26. Immunoblotting of $G\alpha_{i3}$ and $G\alpha_{i2}$ in lung membranes

The expression of $G\alpha_{i3}$ and $G\alpha_{i2}$ in membranous fraction of lung homogenate was measured by western blotting. The expression of actin was also measured as a loading control. Representative band images are shown in the inset diagrams. Densitometry analysis of $G\alpha_{i3}$/actin and $G\alpha_{i2}$/actin ratios are shown in the bar graphs as mean±SEM (n=6). S/c mice showed higher expression of $G\alpha_{i3}$ than control mice, and chronic nadolol treatment decreased $G\alpha_{i3}$ expression in s/c mice. No difference of $G\alpha_{i2}$ was detected among all experimental groups. NS/NC (control), S/C (OVA sensitized and challenged), Nad Chr (nadolol chronic). * P<0.05 compared with S/C, # P<0.05 compared with NS/NC.

To further evaluate our hypothesis that chronic nadolol treatment decreased $G\alpha_{i3}$ expression by blocking β_2 AR signaling, $G\alpha_{i3}$ expression was measured in lung membranes and trachea and large bronchi homogenates from β_2 AR knockout and wildtype mice, a different mouse strain from the strain used above. The results are presented in Figure 27. No difference of $G\alpha_{i3}$ expression was observed in lung membranes (Figure 27A). However, $G\alpha_{i3}$ expression in trachea and large bronchi

homogenates was higher in wildtype s/c mice compared with β_2 AR knockout s/c mice (Figure 27B, 0.2653±0.029 vs. 0.1587±0.032, P<0.05, n=5). Wildtype s/c mice also showed a significant higher expression of $G\alpha_{i3}$ than non-sensitized non-challenged mice did (Figure 27B, 0.2653±0.029 vs. 0.1409±0.023, P<0.05, n=5 vs. n=4). This difference was absent in knockout mice. This implies that the genetic elimination of β_2 AR signaling in airway smooth muscle mimics the above-mentioned effect of chronic nadolol treatment on $G\alpha_{i3}$ expression reduction. However, chronic nadolol treatment did not decrease $G\alpha_{i3}$ expression in wildtype FVB/NJ mice the same way as in wildtype BALB/cJ mice, which may be due to the strain variation.

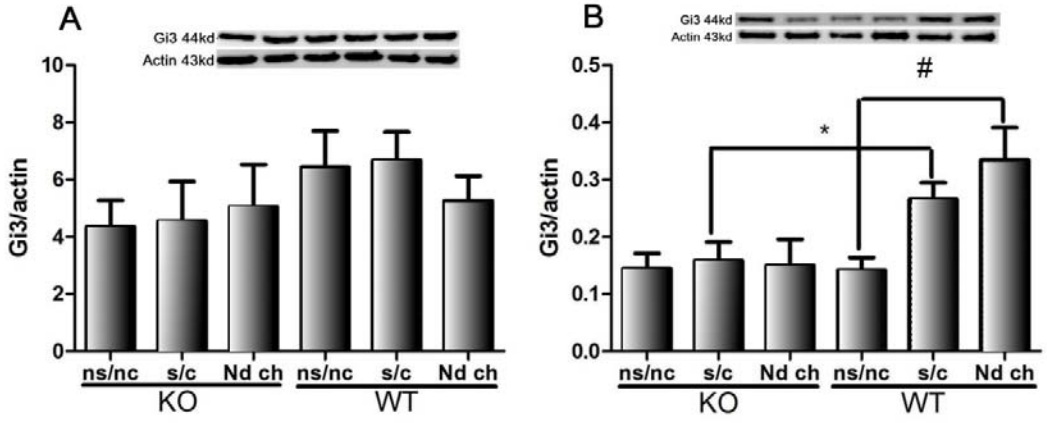

Figure 27. Immunoblotting of $G\alpha_{i3}$ on β_2 knockout FVB/NJ mice

Western blotting of Gαi3 in lung membranes (A) and trachea and large bronchi homogenate (B). Expression of actin was also measured as a loading control. Representative band images are shown above the bar graphs. Densitometry analysis of $G\alpha_{i3}$/actin ratios are shown in the bar graphs as mean ± SEM (n=3~6). KO (β_2 knockout mice), WT (wildtype mice), Nd Ch (nadolol chronic). No difference of $G\alpha_{i3}$ expression can be detected in lung membranes (A). In wildtype mice, $G\alpha_{i3}$ expression in trachea and large bronchi homogenates was higher in s/c mice than in non-sensitized and challenged mice (ns/nc), while no difference was detected in knockout mice.

Chronic nadolol treatment showed no effect on $G\alpha_{i3}$ expression in either wildtype or knockout mice (B). * $P<0.05$ compared to s/c, # $P<0.05$ compared to Nd Ch.

1.1.2 Effect of chronic nadolol treatment on pertussis toxin pretreated trachea

Isolated mouse trachea was used to further evaluate the β_2 AR and Gi coupled signaling in airway smooth muscle. To investigate β_2 AR-Gi signaling in trachea, 0.3 μg/ml pertussis toxins, a specific Gi inhibitor was added into the bath solution and incubated with trachea for 16 hours before measurement. The trachea incubated with or without pertussis toxin in bath solution for 16 hours was then pre-contracted by 10^{-6}M methacholine. When the tracheal tone reached the plateau, a cumulative concentration response curve to isoproterenol was constructed. Pretreatment of the pertussis toxin increased isoproterenol-induced tracheal relaxation in s/c mice as shown in Figure 28B, but showed no significant effect in control mice and s/c mice with chronic nadolol treatment (see Figure 28A and C). This result is consistent with the result of $G\alpha_{i3}$ expression on lung membranes from BALB/cJ, indicating that chronic nadolol treatment functionally decreased β_2 AR-Gi signaling in airway smooth muscle.

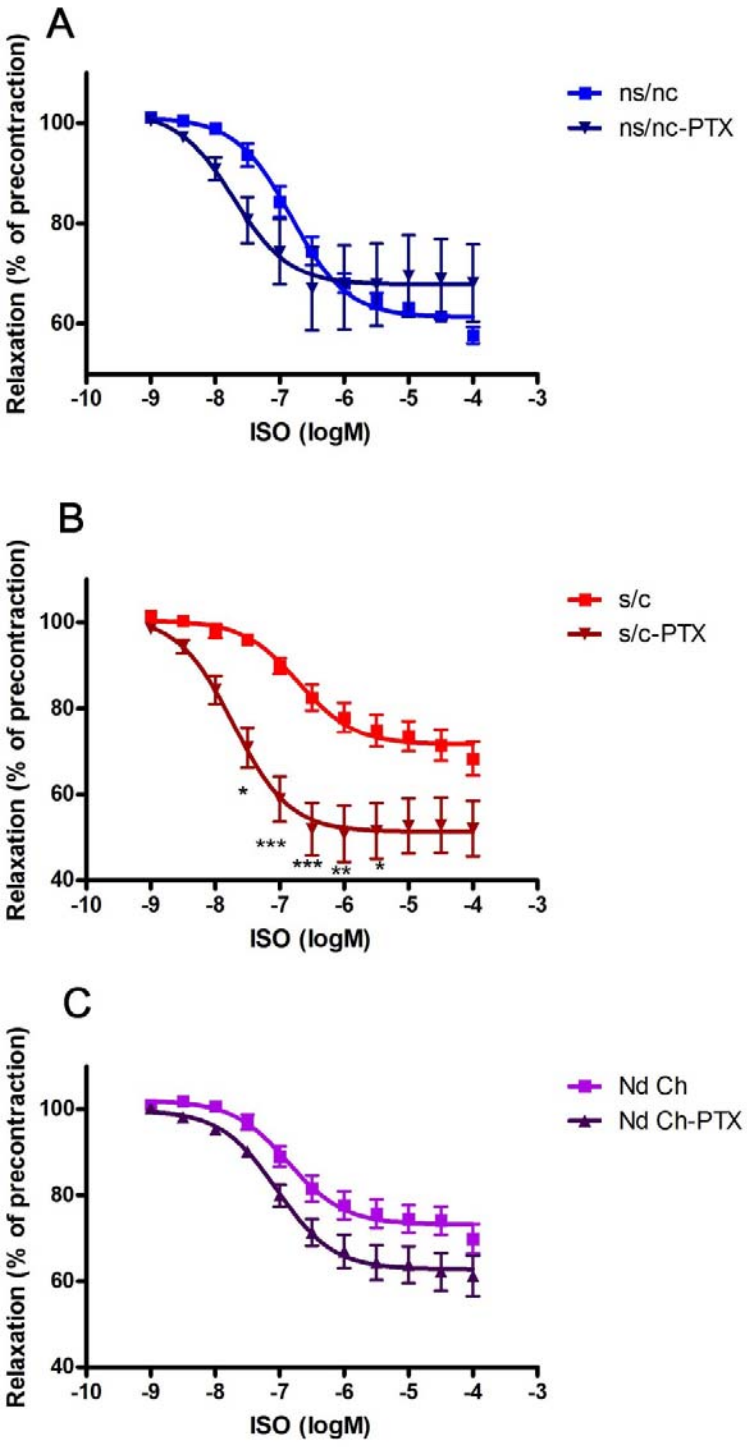
A
Relaxation (% of precontraction)
100
80
60
-10
-9
-8
-7
-6
-5
-4
-3
ISO (logM)
ns/nc
ns/nc-PTX
B
Relaxation (% of precontraction)
100
80
60
40
*
***
***
**
*
-10
-9
-8
-7
-6
-5
-4
-3
ISO (logM)
s/c
s/c-PTX
C
Relaxation (% of precontraction)
100
80
60
40
-10
-9
-8
-7
-6
-5
-4
-3
ISO (logM)
Nd Ch
Nd Ch-PTX

Figure 28. Effect of pertussis toxin on isoproterenol induced relaxation of methacholine pre-contracted mouse trachea

Cumulative concentration-response curves to isoproterenol in methacholine pre-contracted mouse isolated trachea with or without the pretreatment of pertussis toxin in the bath solution (0.3μg/ml). Each point represents mean and vertical lines show SEM (n=4-7). The initial contraction was obtained by the addition of 1μM methacholine to attain the approximate EC80. Ns/nc (control), ns/nc-PTX (control trachea pretreated with pertussis toxin, the same for the following groups), s/c (OVA sensitized and challenged), Nd Ch (s/c mice with nadolol chronic treatment). Pertussis toxin increased isoproterenol sensitive tracheal relaxation in s/c mice, while it showed no effect on control mice and s/c mice with chronic nadolol treatment. *P<0.05, **P<0.01, ***P<0.001 compared with s/c. Two way ANOVA

Studies on mice overexpressing $G\alpha_{i2}$ have shown a compensating decrease in bronchial tone (McGraw, Elwing et al. 2007). Our finding is comparable: a higher $G\alpha_{i3}$ expression (instead of $G\alpha_{i2}$ expression) was detected in s/c mice (Figure 26), accompanied by a lower maximum tracheal contraction induced by methacholine (Figure 18A). However, after pretreatment with pertussis toxin, methacholine-induced tracheal contraction in s/c mice was higher than in control mice at methacholine concentrations higher than 10^{-5}M (Figure 29). This is probably because pertussis toxin removed Gi induced compensatory reduction in maximum tracheal contraction to methacholine. In the presence of pertussis toxin pretreatment, chronic nadolol treatment reduced the contraction in s/c mice to the same level of control mice in trachea (Figure 29), demonstrating the reduction of Gi dependent tracheal contraction without any compensatory effect. In contrast, in the absence of pertussis toxin pretreatment, chronic nadolol treatment increased maximum tracheal contraction in s/c mice (Figure 18A), probably because of a lower compensatory contractile effect associated with the reduction of Gi signaling.

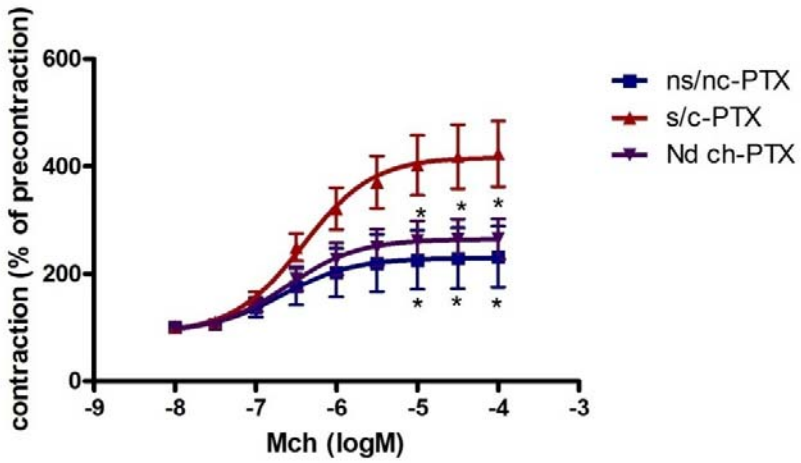

Figure 29. Cumulative concentration response to methacholine in the mouse isolated trachea pretreated with pertussis toxin

Cumulative concentration response to methacholine in the mouse isolated trachea pretreated with pertussis toxin. Each point represents mean and vertical lines at each point show SEM. S/c mice (s/c-PTX, n=6) showed a higher contraction at concentration 10^{-5} to 10^{-4}M than in the control mice (ns/nc-PTX, n=6). Chronic nadolol treatment (Nd ch-PTX, n=8) decreased higher contraction in response to high concentration of methacholine in s/c mice. *P<0.05 compared with s/c.

1.1.3 Effect of chronic nadolol treatment on GRK5 expression

GRK5 gene knockout mice have shown a decrease in β_2 AR stimulated airway smooth muscle relaxation (Walker, Gainetdinov et al. 2004). GRK5 is involved in the desensitization of Gi coupled receptors. To investigate the effect of chronic nadolol treatment on GRK5 expression, immunoblotting of GRK5 was performed on both membranous and cytosolic fractions of lung homogenates. As shown in Figure 31A, chronic nadolol treatment increased GRK5 expression on membranous fraction (0.1096±0.019 vs. 0.022±0.01, P<0.05, n=3) of lung homogenates from s/c mice, though

it had no effect on the GRK5 expression on cytosolic fraction as shown in Figure 30B (0.071±0.01 vs. 0.06±0.02, P>0.05, n=3). The expression of GRK5 in s/c mice was significantly lower than in control mice in cytosolic fraction, seen in Figure 30B (0.06±0.02 vs. 0.31±0.01, P<0.01, n=3). According to Figure 30A, it was also lower than control mice in membranous fraction though the difference was not statistically significant (0.022±0.01v.s. 0.1103±0.05, P>0.05, n=3 vs. n=4). This implies that in our asthma model, GRK5 expression was lower than in normal subjects, and chronic nadolol treatment increased the membrane-bound GRK5, which may contribute to a higher degree of Gi coupled M2 receptors desensitization (Walker, Gainetdinov et al. 2004).

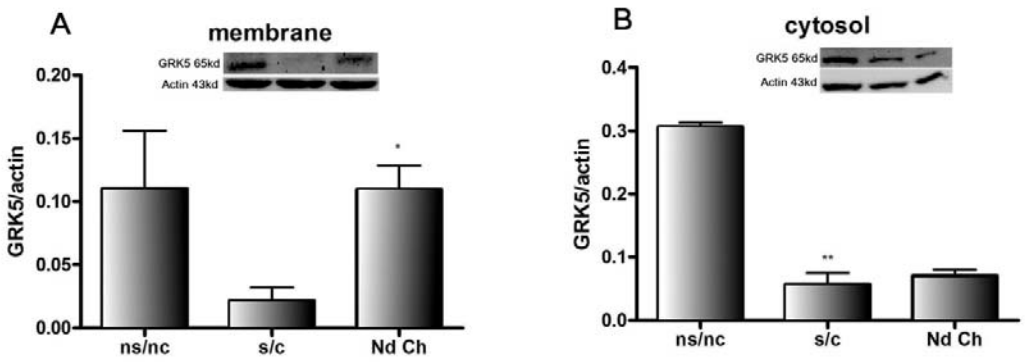

Figure 30. Immunoblotting of GRK5 in lung homogenates

Expressions of GRK5 in membranous and cytosolic fractions of lung homogenate were measured by western blotting. Expression of actin was also measured as a loading control. Representative band images are shown above the bar graphs. Densitometry analysis of GRK5/actin ratios are shown in the bar graphs as mean±SEM (n=3). Ns/nc (control), s/c (OVA sensitized and challenged), Nd Ch (nadolol chronic treatment). Chronic nadolol treatment increased GRK5 expression in membranous fraction, but showed no effect in the cytosolic fraction. S/c mice showed lower GRK5 expression than the control mice did in the cytosolic fraction. * P<0.05 compared with S/C, ** P<0.01 compared with ns/nc, Student t-test.

Collectively, this suggests that chronic nadolol treatment not only decreases Gi expression, but also decreases Gi-dependent signaling. Gi-dependent tracheal contraction can be also decreased by chronic nadolol treatment. The effect of chronic nadolol treatment on increasing the desensitization of the M2 receptor by GRK5 may be one of the mechanisms. As a result, the Gi signaling decreased with the higher desensitization of Gi coupled receptors. The compensatory effect of lowering contractile signaling has been observed using mice with an overexpressed Gi protein (McGraw, Elwing et al. 2007). This is consistent with our finding in the lower methacholine-induced tracheal contraction of s/c mice which showed higher Gi3 expression. McGraw et al have shown that PLCβ or PKCα expressions are also affected by genetic modification of Gi in mouse airway smooth muscle. Therefore, we also investigated the effect of chronic nadolol treatment on Gq signaling pathways.

1.2 Effect of chronic nadolol treatment on Gq signaling pathways

Airway smooth muscle tone has been known to be regulated by the balance of Gq signaling contraction and Gs signaling relaxation (Deshpande and Penn 2006). Chronic nadolol treatment has been found to decrease PLCβ1 expression (Lin, Peng et al. 2007). Gq, PLCβ1 and PKCα are three most important proteins involved in Gq signaling. Therefore, the expressions were examined to further investigate how chronic nadolol treatment affects the Gq signaling pathway in airway smooth muscle.

1.2.1 Effect of chronic nadolol treatment on Gq expression

Gq expression in lung membranes and the primary culture of airway smooth muscle cells was measured by immunoblotting. As shown in Figure 31, s/c mice showed a lower expression of Gq in a membranous fraction of lung homogenates than control mice (0.61±0.02 vs. 0.87±0.06, $P<0.01$, n=3); chronic nadolol treatment did not show any effect on the Gq expression in lung membranes (0.59±0.02 vs. 0.61±0.02, n=3). Figure 32 shows that in the primary culture of airway smooth muscle cells, s/c mice also showed less expression of Gq than did the control mice did (0.60±0.01 vs. 0.76±0.05, n=7), but the difference was not statistically significant. However, it can also be seen in Figure 32 that chronic nadolol treatment increased Gq expression significantly in the primary culture of airway smooth muscle cells (0.94±0.06 vs. 0.60±0.01, $P<0.05$, n=8 vs. n=7), unlike what we observed in lung membranes. Chronic nadolol treatment increased Gq expression only 1.6 times on airway smooth muscle cells. It can be noted that airway smooth muscle cells only occupy a small fraction of whole lung homogenate, so that we cannot see the increase of Gq expression in the whole lung extract.

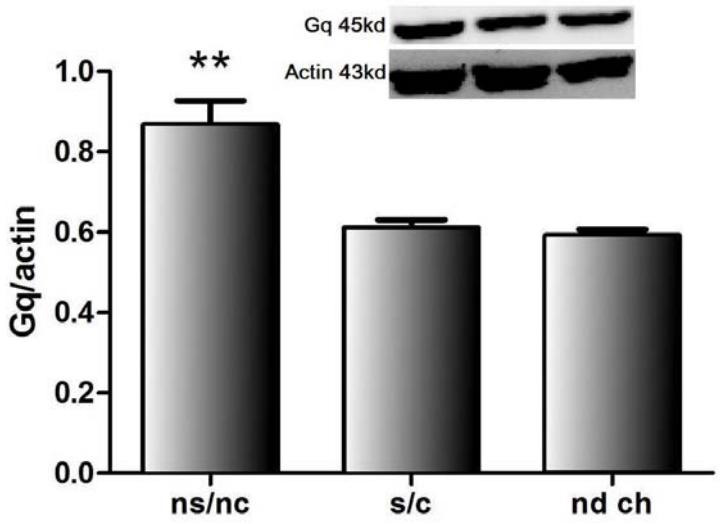

Figure 31. Immunoblotting of Gq in lung membranes

The expression of Gq in membranous fraction of lung homogenate was measured by western blotting. The expression of actin was also measured as a loading control. Gq expression in control mice (ns/nc) was higher than s/c mice with or without chronic nadolol treatment (nd ch). Chronic nadolol treatment showed no effect on Gq expression in s/c mice. Representative band images are shown above the bar graph. Densitometry analysis of Gq/actin ratios are shown in the bar graph as mean±SEM (n=3). **P<0.01 compared with S/C.

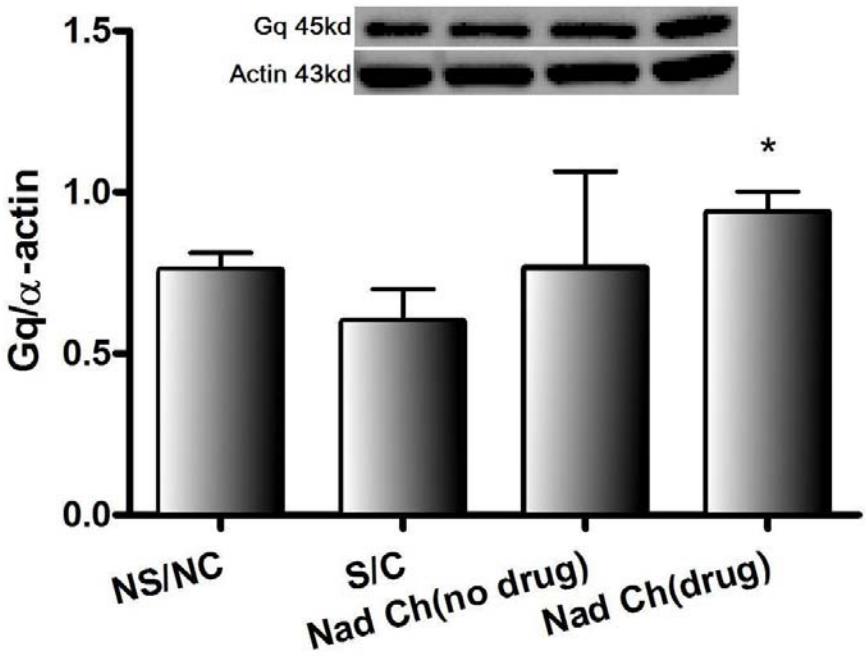

Figure 32. Immunoblotting of Gq in airway smooth muscle cells

The expression of Gq in airway smooth muscle cell culture was measured by western blotting. The expression of α-actin was also measured as a loading control. Chronic nadolol treatment increased Gq expression in the airway smooth muscle cells cultured from s/c mice. Representative band images are shown above the bar graph. Densitometry analysis of Gq/α-actin ratios are shown in the bar graph as mean±SEM (n=4-8). NS/NC (control), S/C (OVA sensitized and challenged), Nad Ch (no drug): cells growing without nadolol in culture medium, Nad Ch (drug): cells growing with presence of nadolol 10^{-6}M.

1.2.2 PLCβ1 expression on β_2 AR knockout mice

To further evaluate our hypothesis that chronic nadolol treatment decreased PLCβ1 expression by blocking β_2 AR signaling, PLCβ1 expression was measured in trachea and large bronchi homogenates from β_2 AR knockout mice and wildtype littermates. The

PLCβ1 expression was higher in wildtype s/c mice compared with β_2 AR knockout s/c mice, as seen in Figure 33 (1.098±0.18 vs. 0.2899±0.026, $P<0.01$, n=6). Figure 33 also shows that in wildtype mice, s/c mice showed a significantly higher expression of PLCβ1 than non-sensitized non-challenged mice did (1.098±0.18 vs. 0.6601±0.073, $P<0.05$, n=6). This difference was absent in knockout mice, implying that the genetic elimination of β_2 AR and its signaling mimics the effect of chronic nadolol treatment on PLCβ1 expression, as previously reported (Lin, Peng et al. 2008). However, chronic nadolol treatment did not decrease PLCβ1 expression in wildtype FVB/NJ mice, while it decreased PLCβ1in wildtype BALB/cJ mice. This conflict may be due to the strain variation.

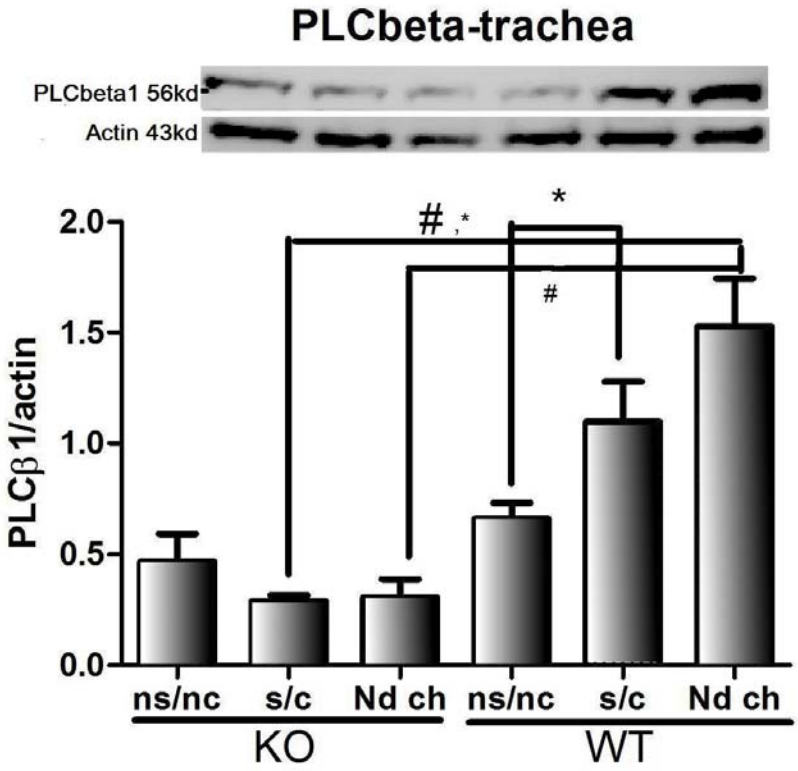

Figure 33. Immunoblotting of PLCβ1 on β_2 knockout FVB/NJ mice

Western blotting of PLCβ1 in trachea and large bronchi homogenates. Expression of actin was also measured as a loading control. Representative band images are shown above the bar graph. Densitometry analysis of PLCβ1/actin ratios are shown in the bar graph as mean ± SEM (n=3~6). KO (β_2 knockout mice), WT (wildtype mice), Nd Ch (nadolol chronic). In wildtype mice, PLCβ1 expression was higher in s/c mice than non-sensitized and challenged mice (ns/nc); in knockout mice, this difference did not appear. Chronic nadolol treatment showed no effect on either wildtype or knockout mice. * $P<0.05$ compared to s/c, # $P<0.05$ compared to Nd Ch.

1.2.3 Effect of chronic nadolol treatment on PKCα expression

Conventionally, PKCα is considered to be the most important effector enzyme involved in the Gq signaling pathway. PKCα is a Ca^{2+} sensitive kinase which regulates not only

the contractile machinery in the smooth muscle cell, but also the phosphorylation of some GPCRs using other signaling pathways (Boterman, Smits et al. 2006; Deshpande, Pascual et al. 2007). Particularly, a study on mice with genetically modified Gi expression showed that the increase of PKCα expression was associated with lower Gi expression. Chronic nadolol treatment was shown to affect the expression of Gi and PLCβ1, the component in Gq signaling pathway upstream PKC. Therefore, the PKCα expression in lung homogenates was further examined here.

In a single experiment, the expression of PKCα in s/c mice with and without chronic nadolol treatment, and also control mice were measured. No significant difference was observed in PKCα expressions in either the membranous or the cytosolic fraction of lung homogenates among three experimental groups (see Figure 34).

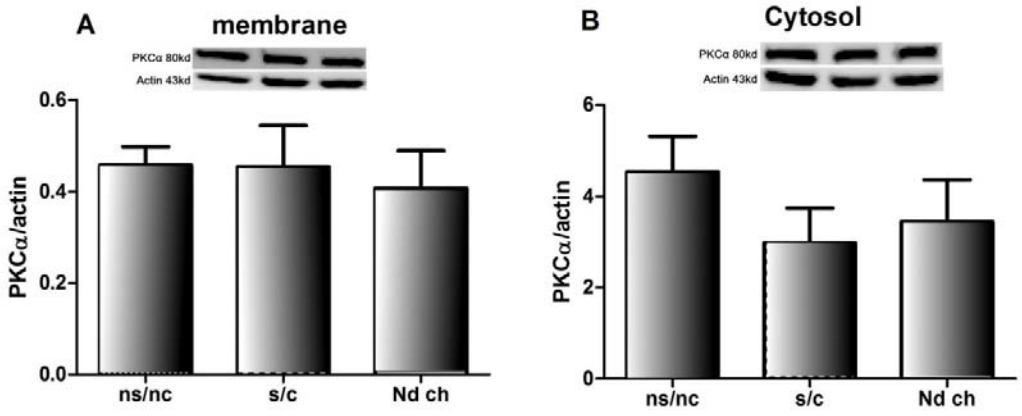

Figure 34. Immunoblotting of PKCα in lung homogenates

Expressions of PKCα in membranous (A) and cytosolic (B) fraction of lung homogenate were measured by western blotting. Expression of actin was also measured as a loading control. No significant difference was detected either between control mice (ns/nc) and s/c mice, or s/c mice with and without chronic nadolol treatment (Nd ch). Representative band images are shown above the bar graphs. Densitometry analysis of PKCα/actin ratios are shown in the bar graphs as mean±SEM (n=9). *P<0.01 compared with S/C.

The activation of PKCα was known to be associated with the translocation from cytosol to membrane (Dempsey, Cool et al. 2007). Therefore, the expression of PKCα on membrane was divided by the sum of PKCα expression on membrane and cytosol, which gives PKCα translocation. Although s/c mice did not showed significantly more translocation than control mice as shown in Figure 35 (0.12±0.003 vs. 0.09±0.011, n=5 vs. n=6), chronic nadolol treatment decreased the translocation of PKCα in s/c mice (0.07±0.008 vs. 0.12±0.003, P<0.05, n=5). Although insignificant, s/c mice showed slightly higher translocation of PKCα to cell membrane than that in control mice, implying that the activation of this enzyme in s/c mice was higher than control mice. Chronic nadolol treatment decreased PKCα translocation, implying that it could downregulate the constrictive signaling by decreasing PKC activation.

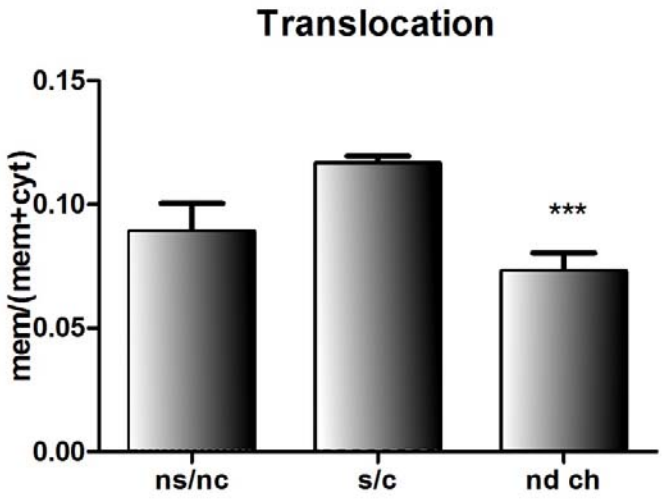

Figure 35. Translocation of PKCα in lung homogenates

The PKCα/actin ratio from lung membranes of each sample was divided by the sum of PKCα/actin from the membranous and cytosolic fraction of the same sample. The quotients were considered to be an index of PKCα translocation and shown as mean±SEM (n=5~6). Chronic nadolol treatment decreased PKCα translocation though s/c mice showed no significant difference from control mice (ns/nc). ***P<0.001 compared with S/C.

1.3 Effect of chronic nadolol treatment on arachidonic acid pathways

β_2 AR has been known to couple with Gi and cPLA2 in the myocardium (Pavoine and Defer 2005), providing evidence for a switch from the β_2 AR-Gs-cAMP pathway to the β_2 AR stimulated arachidonic acid pathway. We have observed that chronic nadolol treatment decreased Gi signaling and increased tracheal relaxation in response to the EP2 receptor agonist in our mouse model of asthma (Lin, Peng et al. 2007). The investigation cPLA2 expression was then conducted. In addition, leukotrienes are important arachidonic acid metabolites present in the airway, which have also been shown to be

regulated by prostanoid synthesis (Holgate, Peters-Golden et al. 2003). To investigate the effect of chronic nadolol treatment on the production of leukotrienes, the levels of cysLTs production and the expressions of enzymes controlling their production were also examined.

1.3.1 cPLA2 expression in lung homogenates

Phospholipase A2 (PLA2) regulates the production of both prostanoids and leukotrienes. Particularly, cPLA2 was shown to decrease in rats after OVA sensitization and challenge, following by decreased production of PGE2 in the BALF. This induced a negative feedback on leukotriene synthesis (Offer, Yedgar et al. 2005). Therefore, cPLA2 expression in lung homogenates was measured to evaluate the effect of chronic nadolol treatment on cPLA2-mediated activity. However, no difference in cPLA2 expression was observed in our s/c mice. Chronic nadolol treatment also showed no effect on the expression of cPLA2 in either membranous or cytosolic fractions of lung homogenates (shown in Figure 36).

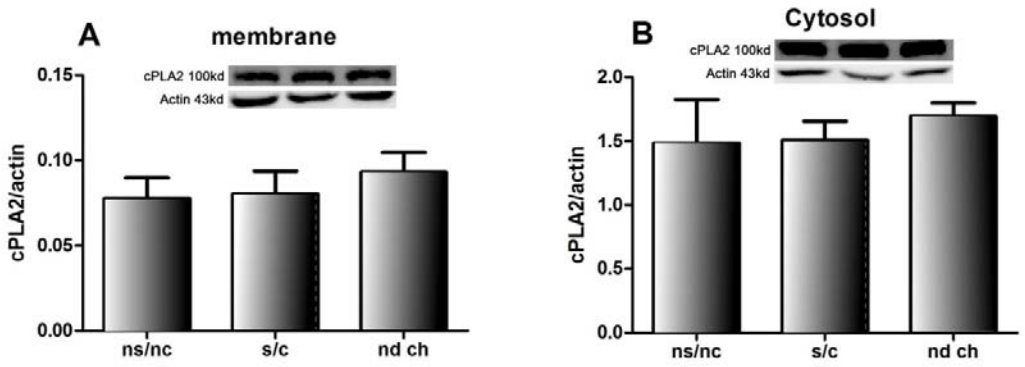

Figure 36. Immunoblotting of cPLA2 in lung homogenates

Expressions of cPLA2 in membranous (A) and cytosolic (B) fraction of lung homogenate was measured by western blotting. Expression of actin was also measured as a loading control. No difference of cPLA2 expression was shown between s/c mice and control mice (ns/nc) in both membranous and cytosolic fractions. Chronic nadolol treatment (nd ch) showed no effect on cPLA2 expression in s/c mice. Representative band images are shown above the bar graphs. Densitometry analysis of cPLA2/actin ratios are shown in the bar graphs as mean±SEM (n=6).

1.3.2 5-lipoxygenase (5-LO) expression and cys-LT production in BALF

5-LO is the enzyme which metabolizes arachidonic acids and then synthesizes leukotrienes (LTs), some of which are potent constrictors in airways. Apart from mast cells, human bronchial epithelial cells also express 5-LO and generate bioactive LTs (Jame, Lackie et al. 2007). To measure 5-LO expression in s/c mice and the effect of chronic nadolol treatment, mouse lung extracts were used to measure 5-LO by western blotting. No significant difference was observed in 5-LO expressions in either membranous or cytosolic fractions of lung homogenates among three experimental groups (see Figure 37).

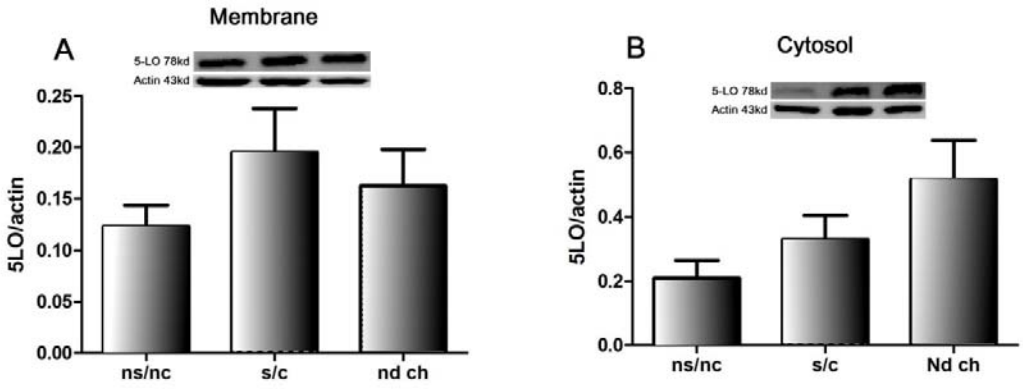

Figure 37. Immunoblotting of 5-LO in lung homogenates

The expression of 5-LO in membranous (A) and cytosolic (B) fractions of lung homogenate was measured by western blotting. The expression of actin was also measured as a loading control. No significant difference was detected among control mice (ns/nc) and s/c mice, or s/c mice with and without chronic nadolol treatment (Nd ch). Representative band images are shown above the bar graphs. Densitometry analysis of 5-LO/actin ratios are shown in the bar graphs as mean±SEM (n=9).

However, when comparing the ratio of the membranous expression of 5-LO over the sum of membranous and cytosolic expression, chronic nadolol treatment decreased the ratio of membrane bound 5-LO (0.26±0.03 vs. 0.39±0.05, $P<0.05$, n=9), denoting a lower translocation of 5-LO from cytosol to membrane; this is shown in Figure 38.

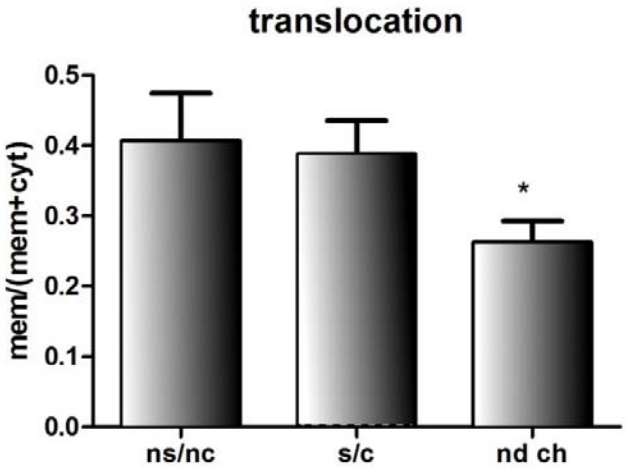

Figure 38. Translocation of 5-LO in lung homogenates

5-LO/actin ratio from lung membranes of each sample was divided by the sum of 5-LO/actin from membranous and cytosolic fractions of the same sample. The quotients were considered as an index of 5-LO translocation and shown as mean±SEM (n=9). Chronic nadolol treatment decreased 5-LO translocation though s/c mice showed no significant difference from control mice (ns/nc). *P<0.05 compared with S/C.

Therefore, we also measured the effect of chronic nadolol treatment in leukotrienes (LTs) production in BALF from FVB/NJ female mice. As seen in Figure 39, s/c mice produced more leukotrienes in BALF than control mice (12.64±0.6556 vs. 10.25±0.6656 pg/ml, P<0.05, n=5 vs. n=4). Figure 39 also shows that after chronic nadolol treatment, leukotriene production in s/c mice slightly increased (14.88±0.8385 vs. 12.64±0.6556 pg/ml, n=5). This implies that chronic nadolol treatment had no effect on leukotriene production in BALF from FVB/NJ female mice.

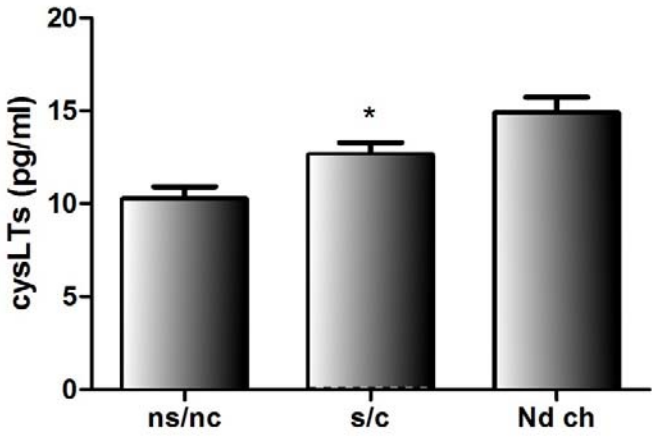

Figure 39. Cysteine-leukotrienes in BALF from FVB/NJ mice

Concentration of CysLTs in BALF is shown in mean±SEM (n=4~5). S/c mice produced more CysLT than control mice (ns/nc). Chronic nadolol treatment (Nd ch) showed no significant difference from s/c mice. *P<0.05 compared with ns/nc.

1.3.3 COX2 expression on lung membranes

Both leukotrienes and prostanoids are metabolites of arachidonic acids. We have shown that chronic nadolol treatment increases prostanoid-induced tracheal relaxation, (Lin, Peng et al, 2008), implying that chronic nadolol treatment increases prostanoid production and signaling. Increased prostanoid production is controlled by cyclooxygenases (COX). Lower COX activity has been associated with higher 5-LO activity (Peters-Golden and Feyssa, 1993). Cyclooxygenase 2 (COX2) is an inducible enzyme becoming abundant at a site of inflammation. Our previous study showed that

chronic nadolol treatment decreased eosinophil cell count, IL-5, IL-13, IL-10 and TGF-β in BALF, showing an anti-inflammatory effect in s/c mice (Nguyen, Omoluabi et al. 2008). Therefore, COX2 expression has the potential to be regulated by chronic nadolol treatment. To investigate the effect of chronic nadolol treatment on COX2 expression, lung extract was used to measure COX2 expression. COX2 expressed mainly in a membranous fraction of lung homogenates. As shown in Figure 40, s/c mice showed higher expression of COX2 than control mice did (0.22±0.02 vs. 0.10±0.03, $P<0.05$, n=6 vs. n=5); however, it also can be seen that chronic nadolol treatment showed no effect on COX2 expression in s/c mice (0.22±0.02 vs. 0.19±0.03, n=6).

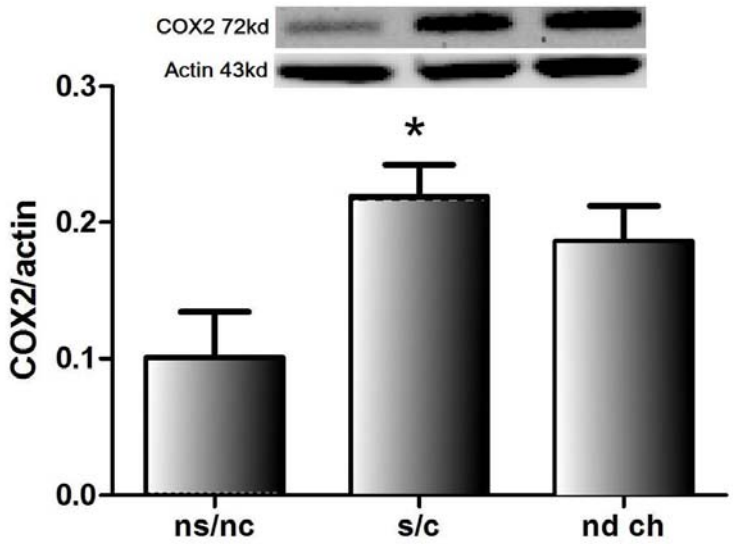

Figure 40. Immunoblotting of COX2 in lung membranes

The expression of COX2 in membranous fraction of lung homogenate was measured by western blotting. The expression of actin was also measured as a loading control. COX2 expression in s/c mice was higher than control mice, but chronic nadolol treatment (nd ch) showed no effect on

COX2 expression in s/c mice. Representative band images are shown above the bar graph. Densitometry analysis of Gq/actin ratios are shown in the bar graph as mean±SEM (n=5~6). *P<0.05 compared with S/C.

In summary, chronic nadolol treatment showed no effect on cPLA2 and COX2, but it did show decreased 5-LO translocation, implying that another mechanism of chronic nadolol treatment on asthmatic airways may involve the modulation of the arachidonic acid pathway. However, cysteinyl-leukotrines production in BALF did not seem to be affected by chronic nadolol treatment.

2 Discussion

2.1 The effect of chronic nadolol treatment on Gi signaling

The role of Gi signaling in airway smooth muscle cells is not well defined. The expression of Gi protein reported in different studies has been conflicting. In the lung membranes of our animal model of asthma, $G\alpha_{i3}$ expression was higher in s/c mice than control mice; $G\alpha_{i2}$ was not (as shown in Figure 27). As shown in Figure 28A, this difference cannot be detected in lung membranes from FVB/NJ mice treated with the same protocol of OVA sensitization and challenge. However, Figure 28B shows that Gi3 expression in trachea and large bronchi homogenates were higher in s/c FVB/NJ mice than in control mice (FVB/NJ mice underwent the same protocol as s/c BALB/cJ mice used for Figure 27). In β_2 AR knockout mice, this difference was not seen. Chronic nadolol treatment decreased $G\alpha_{i3}$ expression in lung membranes from BALB/cJ mice. Unexpectedly, chronic nadolol treatment did not decrease $G\alpha_{i3}$ expression in FVB/NJ

female mice treated with the same sensitization and challenge protocol (Figure 28B). FVB/NJ mice have been reported to be the most sensitive strain to ovalbumin sensitization and challenge in developing allergic asthma responses (Whitehead, Walker et al. 2003). Therefore, compared to BALB/cJ mice, in which chronic nadolol treatment decreased $G\alpha_{i3}$ expression, the same dose of nadolol might not be enough to induce the same effect. In addition, nadolol was administered by mixing it with the chow. We observed that female FVB/NJ mice ingested less food than the male BALB/cJ mice, which may be another reason that chronic nadolol treatment did not manifest the same effect on $G\alpha_{i3}$ expression in this mouse strain. In contrast, chronic nadolol treatment decreased AHR, mucous production and inflammatory cell counts in BALF from FVB/NJ male mice, showing the same effect as in BALB/cJ mice (unpublished data from the laboratory). Therefore, the regulation of Gi protein by chronic nadolol treatment showed mouse strain variation. In the mouse strain with higher sensitivity to OVA, Gi protein expression was higher mainly in airway smooth muscle, thus a higher dose of chronic nadolol treatment might be necessary to decrease Gi expression.

β_2 AR is known to couple with both Gs and Gi protein, though the two signaling pathways have opposite effect on cAMP generation. The regulation of the duplex G protein binding with β_2 AR has been considered to be a therapeutic target in heart failure (Xiao, Ji et al. 1995; Xiao, Avdonin et al. 1999; Kilts, Gerhardt et al. 2000; Xiao 2001; Harding and Gong 2004). In airway smooth muscle cells, β_2 AR/Gi coupling has also

been shown to affect airway smooth muscle relaxation (Hakonarson, Herrick et al. 1995). Therefore, we examined Gi signaling in isoproterenol induced airway smooth muscle relaxation by blocking Gi activity with pertussis toxin. As shown in Figure 28, pertussis toxin. a Gi specific inhibitor, produced up to 20% increase in isoproterenol induced relaxation in tracheal rings from s/c mice, which was significantly higher than the pertussis toxin specific increase of relaxation in control mice and s/c mice with chronic nadolol treatment. This functional assay supported our finding on the reduction of $G\alpha_{i3}$ expression by chronic nadolol treatment (Figure 26A). The pertussis toxin sensitive increase of airway smooth muscle relaxation was less in the chronic nadolol treatment group than in the control group (compared Figure 28C with A). Because that chronic nadolol treatment reduced $G\alpha_{i3}$ to the level as low as control mice, β_2 AR/Gi signaling mediated airway smooth muscle relaxation might be only a part of the $G\alpha_{i3}$ mediated functions. Aside from β_2 AR, there are several other Gi coupled receptors, such as M2 receptor and adenosine receptors which play roles in airway function. The M2 receptor has been found to be the major subtype of muscarinic receptors expressed on airway smooth muscle. In addition, it has also been discovered that M2 receptor contributes to the airway smooth muscle contraction at constant calcium transients (Hirshman, Lande et al. 1999). β_2 AR stimulation has been reported to downregulate M2 receptors in human embryonic lung 299 cells (Rousell, Haddad et al. 1996). However, M2 receptor mediated airway smooth muscle contraction has also been shown to be desensitized in a GRK5-dependent manner (Walker, Gainetdinov et al. 2004). Figure 30A shows that GRK5

expression increased in s/c mice after chronic nadolol treatment. Therefore, it is possible that the lower AHR after chronic nadolol treatment can be associated with the decreased β_2 AR/Gi signaling and increased GRK5 dependent M2 receptor desensitization.

In the membrane fraction, GRK5 expression was lower in s/c mice than control mice, and chronic nadolol treatment increased GRK5 expression significantly (Figure 30A). GRK5 contains lipid binding domain in both carboxyl and amino-terminals. Phosphatidylinositol 4,5-biphosphate (PIP2) binding with GRK5 was suggested to enhance GRK5 mediated receptor phosphorylation (Penela, Ribas et al. 2003). Therefore, the increase of membranous GRK5 expression may imply a higher potential of GRK5 mediated desensitization. A study on GRK5 knockout mice suggested that M2 receptor mediated antagonism of airway smooth muscle relaxation was desensitized by GRK5 (Walker, Gainetdinov et al. 2004). Our results suggest that s/c mice may be mimicking GRK5 knockout mice and therefore demonstrated less desensitization in constrictive signaling (Figure 30B), while chronic nadolol treatment increased GRK5 in s/c mice and may increase the potential of M2 receptor desensitization.

Isoproterenol induced relaxation pertussis toxin pretreated trachea was measured in trachea precontracted by methacholine at EC80. To identify the concentration of EC80, cumulative response curves were also constructed on each tracheal ring. Interestingly, unlike the trachea without pertussis toxin pretreatment, the contraction of trachea from

s/c mice was significantly greater than control mice, while chronic nadolol treatment decreased the contraction significantly (Figure 29). This may not be due to the blockade of M2 receptor-Gi signaling, because s/c mice showed highest $G\alpha_{i3}$ expression and pertussis toxin would produce a decrease in M2/Gi dependent tracheal contraction in s/c mice. The blockade of Gi signaling would normally be associated with increased airway smooth muscle relaxation instead of constriction (Figure 29). However, a recent study using genetically modified mice of Gi2 expression suggested that Gi inhibition is associated with a compensatory increase of bronchial hyperactivity (McGraw, Elwing et al. 2007), which involves increased Gq signaling. Therefore, the increase of methacholine-stimulated tracheal contraction induced by pertussis toxin may support this hypothesis. In our animal model, s/c mice expressed higher Gi protein than control mice. Therefore, pertussis toxin showed the greatest inhibitory effect on Gi signaling and compensatorily potentiating airway smooth muscle contraction to a higher degree than control mice, while chronic nadolol treatment decreased Gi expression, as well as the capacity of pertussis toxin to quench Gi signaling and to potentiate airway smooth muscle contraction.

2.2 The effect of chronic nadolol treatment on Gq signaling

While Gs signaling is the primary pathway responsible for airway smooth muscle relaxation, Gq signaling is the chief signaling pathway producing airway smooth muscle

contraction. The balance between these two signaling pathways controls airway tone in pathological and physiological conditions. Although chronic nadolol treatment was hypothesized to regulate airway tone through β_2 AR signaling, its effect on Gq signaling was also examined because crosstalk between the two signaling pathways has been observed in a variety of experimental models. Consequently, we investigated the effect of chronic nadolol treatment on key components in Gq signaling pathways.

First, we measured Gq expressions in lung membranes. Unexpectedly, Gq expression in control mice was significantly higher than in s/c mice (Figure 31). This was consistent with our results of higher methacholine-induced maximal contraction of tracheal rings in control mice (Figure 18A). Chronic nadolol treatment increased Gq expressions in airway smooth muscle cell culture (Figure 32), a finding consistent with its effect on maximal contraction of tracheal rings. This effect was not observed in lung membranes, probably because the reduced proportion of airway smooth muscle cells in whole lung homogenates. However, the tracheal rings, which have a larger proportion of airway smooth muscle cells than the whole lung, reflected the effect of chronic nadolol treatment on Gq expression through the increase in maximal contraction (Figure 18A). This has not been demonstrated in rat bronchial rings, in which both the acetylcholine induced contraction and the Gq expression are increased after antigen sensitization and challenge (Chiba, Sakai et al. 2000). Besides the difference in species, the bronchus preparation used in Chiba, et al study might be more suitable than tracheal preparation to mimic

airway hyperresponsiveness (Chiba, Ueno et al. 2004). On the other hand, the study of β_2 AR signaling components has provided the evidence that the G proteins are present in large stoichiometric excess relative to both receptor and effectors (Post, Hilal-Dandan et al. 1995). Therefore, the airway smooth muscle with higher Gq expressions show higher contractions only at the highest concentration of methacholine, while the cumulative contraction curves to methacholine showed no significant difference among experimental groups. In terms of the magnitude of signaling amplification, Gq protein may not be as important as its downstream effectors, such as phospholipase C β (PLCβ) and PKC.

Lower expressions of PLCβ1, the effector downstream of Gq proteins and related to calcium release in airway smooth muscle cells, has been shown in control mice compared with s/c mice, while chronic nadolol treatment reduced PLCβ1 expressions (Lin, Peng et al. 2008). We also measured PLCβ1 expression in β_2 AR knockout mice and wildtype littermates. FVB/NJ β_2 AR gene knockout eliminated the difference in PLCβ1 expression between s/c mice and control mice, showing a similar effect as chronic nadolol treatment of s/c BALB/cJ mice, while nadolol chronic treatment had no effect on β_2 AR knockout s/c mice. However, chronic nadolol treatment did not decrease PLCβ1 expressions in wildtype FVB/NJ mice (Figure 33) which was not consistent with the result in BALB/cJ mice. We speculate that it shared the same strain dependence as the effect of chronic nadolol treatment on $G\alpha_{i3}$ expression. These results suggest that

chronic nadolol treatment decreases proteins that are important in regulating bronchial tone.

PKC is another effector enzyme involved in Gq signaling and even downstream PLCs. The activation of PKC could increase the tone of human airway smooth muscle cells (Cheng, Xu et al. 2007). PKC has also been shown to potentiate the β AR/Gi coupling in cardiac myocytes (Belevych, Juranek et al. 2004) and the desensitization of β_2 AR in tracheal smooth muscle (Boterman, Smits et al. 2006). β AR agonist induced downregulation of M2 receptor and chronic carbochol treatment induced decreased adenylyl cyclase activity were both PKC dependent (Rousell, Haddad et al. 1996; Schears, Clancy et al. 1997). McGraw et al. have observed an increase of PKCα in mice expressing Gi inhibiting peptide, suggesting the crosstalk of Gi signaling with PKCα (McGraw, Elwing et al. 2007). Therefore, we also measured PKCα in lung homogenates from our animal model. Although we did not detect significant difference of PKCα in lung homogenates as shown in Figure 35, we found that the ratio of membrane-bound PKCα could be decreased by chronic nadolol treatment as seen in Figure 36. PKCα is a calcium sensitive isoform of PKCs. It would be translocated to the membrane from the cytosol when activated (Dempsey, Cool et al. 2007). Therefore, the translocation of PKCα reflects the activation of this enzyme, while chronic nadolol treatment decreased PKCα activation. Collectively, chronic nadolol treatment could regulate Gq signaling pathways by decreasing PLCβ1 expression and PKCα activation.

2.3 The effect of chronic nadolol treatment on arachidonic acid signaling

β_2 ARs are abundant in epithelial cells and are upregulated by chronic beta blocker treatments (Lin, Peng et al. 2008). These results suggest that epithelial cells may be an important target of chronic nadolol treatment. In asthma, chronic inflammation is associated with damaged epithelium and the activation of the proliferative process of airway smooth muscle cells, resulting in airway hyperresponsiveness (Holgate, Peters-Golden et al. 2003). The release of cysteinyl leukotrienes by epithelium or mast cells during the chronic inflammation plays an important role in regulating airway smooth muscle function. Leukotrienes are not only produced by mast cells, but also in epithelial cells (Jame, Lackie et al. 2007). Leukotriene C4 and D4 are 1000-fold more potent than histamine in contracting human airway smooth muscle cells (Barnes and Costello 1984) in addition to their important roles in airway remodeling (featured by excess smooth muscle accumulation). Therefore, agents that block the formation or activity of leukotrienes are effective in the treatment of asthma (Buccellati, Fumagalli et al. 2002). Hence, we considered that leukotrienes were good candidate mediators to produce epithelial dysfunction and airway hyperresponsiveness.

We first measured cytosolic phospholipase A2 (cPLA2) expression in lung homogenates. An inhibitor of cPLA2 has been reported to prevent airway hyperresponsiveness and

chronic inflammation in ovalbumin sensitized and challenged BALB/cJ mice (Malaviya, Ansell et al. 2006), indicating a pivotal role of cPLA2 in allergic asthma. Moreover, cPLA2 has been suggested to use a specific arachidonic acid pool for leukotriene productions, while sPLA2 (secretory PLA2), another isoform of PLA2, is specifically responsible for prostaglandin E2 (PGE2) production (Offer, Yedgar et al. 2005). However, as shown in Figure 37, we did not observe difference of cPLA2 expression among three experimental groups, neither in membranous nor in cytosolic fractions. Next, we measured 5-lipoxygenase (5-LO) expressions in lung homogenates, because 5-LO is the enzyme downstream PLA2 to synthesize leukotrienes (Figure 41). The expressions of 5-LO showed no difference in homogenates (Figure 36). However, when calculating the ratio of membrane-bound 5-LO to the total 5-LO expression, chronic nadolol treatment significantly decreased the ratio (Figure 38). 5-LO is also a calcium sensitive enzyme that can be translocated to membranous cellular structures where it can bind with the 5-lipoxygenase-activating protein (FLAP) and begin leukotriene synthesis (Brock, McNish et al. 1998). Therefore, scaffold proteins for 5-LO, which promote 5-LO membrane binding, can regulate the capacity of 5-LO in leukotriene biosynthesis (Rakonjac, Fischer et al. 2006). Our result suggests that chronic nadolol treatment decreases the translocation of 5-LO, which might result in less leukotriene production.

We next measured the cysteinyl leukotrienes (cysLTs) in BALF because they are potent constrictors of airway smooth muscle cells. However, cysLTs in BALF from BALB/cJ

mice was undetectable. We then measured cysLTs in BALF from FVB/NJ mice. CysLT concentration was higher in s/c mice than control mice, while chronic nadolol treatment showed no significant effect (Figure 39), consistent with our findings on Gi3 and PLCβ1 expressions.

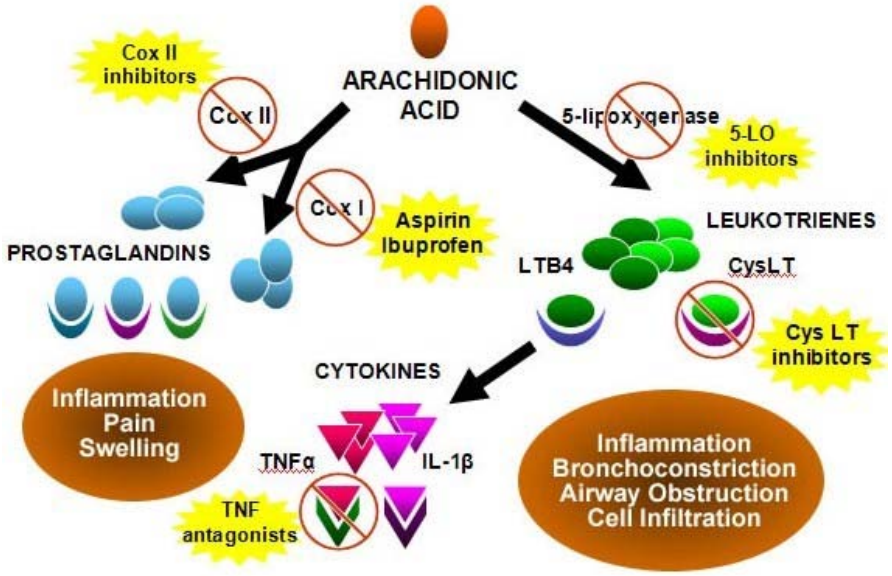

Figure 41. Arachidonic acid pathway

Arachidonic acid can be transformed into inflammatory messengers known as prostaglandins and leukotrienes, which can be key drivers of inflammation. Free arachidonic acid may be metabolized by 5-lipoxygenase (5-LO) to leukotrienes, or by one of the two types of enzymes known as cyclooxygenase, (COX I or COX II) to form prostaglandins and thromboxanes. (http://www.mydietaryfats.org/index.php/info/content/113/, accessed on June 5, 2008)

Leukotriene production from epithelial cells may also be affected by PGE2. PGE2 is the predominant eicosanoid product of pulmonary epithelial cells, showing anti-inflammatory, anti-fibrotic and bronchodilatory effects. The study of coculture of

alveolar epithelial cells and alveolar macrophage cells has shown that PGE2 has a transcellular interaction on leukotriene synthesis (Peters-Golden and Feyssa 1993). This competitive activation of either PGE2 or leukotriene synthesis has also been shown in a rat model of asthma (Offer, Yedgar et al. 2005). Yet, it is still not conclusive if PGE2 production is correlated with leukotriene production. For example, in a very recent study, cysLTs and PGE2 concentrations have been both found to increase in sputum of asthma patients (Sastre, Fernandez-Nieto et al. 2008). Using our animal model of asthma, we measured cyclooxygenase 2 (COX2) expressions in lung homogenates. COX2 expression could be only detected on lung membranes, while s/c mice showed higher COX2 expression than control mice, and chronic nadolol treatment showed no effect (Figure 40). The higher COX2 expression in s/c mice is consistent with the study of COX2 expression in the airway of asthmatic patients (Barnes, Chung et al. 1998). However, chronic nadolol treatment showed no effect on COX2 expression, indicating that chronic nadolol treatment might not affect PGE2 production. Chronic nadolol treatment may increase prostanoid induced tracheal relaxation by upregulating EP2 or IP receptors or decreasing the desensitization of prostanoid receptors (Lin, Peng et al. 2008).

In summary, chronic nadolol treatment showed no effect on expressions of cPLA2 and COX2. However, the decreased translocation of 5-LO induced by chronic nadolol treatment suggested that the long term treatment of nadolol may decrease 5-LO activation.

CHAPTER 7 Summary and conclusions

In this study, we investigated the mechanisms of the bronchoprotective effect of chronic nadolol treatment on a murine model of asthma. From our results, the main findings are:

1. Chronic beta blocker treatment affected airway resistance, BALF cellularity, and important signaling molecules in a β_2 AR specific pathway.
2. Chronic nadolol treatment had no effect on global Gs-cAMP signaling in whole lung, while chronic nadolol treatment decreased PDE4D expression.
3. Chronic nadolol treatment decreased Gi expression and Gi specific signaling in airway smooth muscle.
4. Chronic nadolol treatment decreased PLCβ1 expression, PKCα and 5-LO translocation, implying that the beneficial effect of chronic nadolol treatment results from regulation of multiple signaling pathways.

Chronic beta blocker treatment showed multivariate effects on the signaling pathways regulating airway smooth muscle contractile function (Table 2). This is not surprising given the prevalence of β_2 AR on a vast number of cell types.

Table 2. Summary of the effect of chronic nadolol treatment on some components in different signaling cascades

	Candidate proteins	Effect	Implication
Gs signaling pathway	β_2 AR	↑	With mechanism different from dexamethasone induced β_2AR upregulation.
	Gs	–	
	cAMP	–	
	PKA	–	
	PDE4D	↓	This effect is β_2AR dependent; non-airway smooth muscle PDE4D4.
	GRK2	–	
	GRK3	–	
	β arrestin 2	–	
Gi signaling pathway	Gi3	↓	This effect is β_2AR dependent, and mainly localizes in airway smooth muscle.
	Gi2	–	
	GRK5	↑	More potential to increase β_2AR and Gi signaling desensitization
Gq signaling pathway	Gq	–	
	PLCβ1	↓	This effect is β_2AR dependent; doesn't show in FVB/NJ female mice
	PKC	↓	The translocation can be downregulated
Arachidonic acid pathway	cPLA2	–	
	COX2	–	
	5-LO	↓	The translocation can be downregulated; but cannot decrease cysLTs concentration in BALF from FVB/NJ mice were

↓: decrease
↑: increase
–: do not change

References

Abraham, G., C. Kottke, et al. (2003). "Pharmacological and biochemical characterization of the beta-adrenergic signal transduction pathway in different segments of the respiratory tract." Biochem Pharmacol **66**(6): 1067-81.

Akinbami, L. "Asthma Prevalence, Health Care Use and Mortality: United States, 2003-05." Retrieved May 4 2008, from http://www.cdc.gov/nchs/products/pubs/pubd/hestats/ashtma03-05/asthma03-05.htm.

Aksoy, M. O., I. A. Mardini, et al. (2002). "Glucocorticoid effects on the beta-adrenergic receptor-adenylyl cyclase system of human airway epithelium." J Allergy Clin Immunol **109**(3): 491-7.

Algara-Suarez, P. and R. Espinosa-Tanguma (2004). "8Br-cGMP mediates relaxation of tracheal smooth muscle through PKA." Biochem Biophys Res Commun 314(2): 597-601.

Anderson, J. L., J. R. Lutz, et al. (1985). "A randomized trial of low-dose beta-blockade therapy for idiopathic dilated cardiomyopathy." Am J Cardiol **55**(4): 471-5.

Association, A. L. (2006) "Trends in asthma morbidity and mortality." **Volume**, 1-40 DOI:

Azzi, M., P. G. Charest, et al. (2003). "Beta-arrestin-mediated activation of MAPK by inverse agonists reveals distinct active conformations for G protein-coupled receptors." Proc Natl Acad Sci U S A **100**(20): 11406-11.

Baillie, G. S. and M. D. Houslay (2005). "Arrestin times for compartmentalised cAMP signalling and phosphodiesterase-4 enzymes." Curr Opin Cell Biol **17**(2): 129-34.

Barnes, A. P., G. Livera, et al. (2005). "Phosphodiesterase 4D forms a cAMP diffusion barrier at the apical membrane of the airway epithelium." J Biol Chem **280**(9): 7997-8003.

Barnes, N. C. and J. F. Costello (1984). "Mast-cell-derived mediators in asthma. Arachidonic acid metabolites." Postgrad Med **76**(5): 140-5, 148-51.

Barnes, P. J. (1993). "Beta-adrenoceptors on smooth muscle, nerves and inflammatory cells." Life Sci **52**(26): 2101-9.

Barnes, P. J. (1995). "Beta-adrenergic receptors and their regulation." Am J Respir Crit Care Med **152**(3): 838-60.

Barnes, P. J. (2002). "Scientific rationale for inhaled combination therapy with long-acting beta2-agonists and corticosteroids." Eur Respir J **19**(1): 182-91.

Barnes, P. J. (2006). "Drugs for asthma." Br J Pharmacol **147 Suppl 1**: S297-303.

Barnes, P. J., K. F. Chung, et al. (1998). "Inflammatory mediators of asthma: an update." Pharmacol Rev **50**(4): 515-96.

Belevych, A. E., I. Juranek, et al. (2004). "Protein kinase C regulates functional coupling of beta1-adrenergic receptors to Gi/o-mediated responses in cardiac myocytes." FASEB J **18**(2): 367-9.

Billington, C. K. and R. B. Penn (2003). "Signaling and regulation of G protein-coupled receptors in airway smooth muscle." Respir Res **4**(1): 2.

Black, J. L. and P. R. Johnson (1996). "Airway smooth muscle in asthma." Respirology **1**(3): 153-8.

Bond, R. A. (2001). "Is paradoxical pharmacology a strategy worth pursuing?" Trends Pharmacol Sci **22**(6): 273-6.

Bond, R. A., D. Spina, et al. (2007). "Getting to the heart of asthma: can "beta blockers" be useful to treat asthma?" Pharmacol Ther **115**(3): 360-74.

Borjesson, M., Y. Magnusson, et al. (2000). "A novel polymorphism in the gene coding for the beta(1)-adrenergic receptor associated with survival in patients with heart failure." Eur Heart J **21**(22): 1853-8.

Boterman, M., S. R. Smits, et al. (2006). "Protein kinase C potentiates homologous desensitization of the beta2-adrenoceptor in bovine tracheal smooth muscle." Eur J Pharmacol **529**(1-3): 151-6.

Boulet, L. P. (2003). "Physiopathology of airway hyperresponsiveness." Curr Allergy Asthma Rep **3**(2): 166-71.

Bristow, M. R. (2000). "beta-adrenergic receptor blockade in chronic heart failure." Circulation **101**(5): 558-69.

Bristow, M. R., E. M. Gilbert, et al. (1996). "Carvedilol produces dose-related improvements in left ventricular function and survival in subjects with chronic heart failure. MOCHA Investigators." Circulation **94**(11): 2807-16.

Bristow, M. R., P. Larrabee, et al. (1992). "Effects of carvedilol on adrenergic receptor pharmacology in human ventricular myocardium and lymphocytes." Clin Investig **70 Suppl 1**: S105-13.

Britton, J., S. P. Hanley, et al. (1988). "Dose related effects of salbutamol and ipratropium bromide on airway calibre and reactivity in subjects with asthma." Thorax **43**(4): 300-5.

Broadley, K. J. (2006). "Beta-adrenoceptor responses of the airways: for better or worse?" Eur J Pharmacol **533**(1-3): 15-27.

Brock, T. G., R. W. McNish, et al. (1998). "Capacity for repeatable leukotriene generation after transient stimulation of mast cells and macrophages." Biochem J **329 (Pt 3)**: 519-25.

Buccellati, C., F. Fumagalli, et al. (2002). "Leukotriene modifiers: novel therapeutic opportunities in asthma." Farmaco 57(3): 235-42.

Calabrese, E. J. and L. A. Baldwin (2001). "Hormesis: a generalizable and unifying hypothesis." Crit Rev Toxicol **31**(4-5): 353-424.

Callaerts-Vegh, Z., K. L. Evans, et al. (2004). "Effects of acute and chronic administration of beta-adrenoceptor ligands on airway function in a murine model of asthma." Proc Natl Acad Sci U S A **101**(14): 4948-53.

Cheng, D., Y. Xu, et al. (2007). "The effects of protein kinase C (PKC) on the tension of normal and passively sensitized human airway smooth muscle and the activity of voltage-dependent delayed rectifier potassium channel (Kv)." J Huazhong Univ Sci Technolog Med Sci **27**(2): 153-6.

Chetta, A., A. Foresi, et al. (1997). "Airways remodeling is a distinctive feature of asthma and is related to severity of disease." Chest **111**(4): 852-7.

Chiba, Y., H. Sakai, et al. (2000). "Gq protein level increases concurrently with antigen-induced airway hyperresponsiveness in rats." Respir Physiol **121**(1): 75-83.

Chiba, Y., H. Sakai, et al. (2001). "Possible involvement of G(i3) protein in augmented contraction of bronchial smooth muscle from antigen-induced airway hyperresponsive rats." Biochem Pharmacol **61**(7): 921-4.

Chiba, Y., A. Ueno, et al. (2004). "Hyperresponsiveness of bronchial but not tracheal smooth muscle in a murine model of allergic bronchial asthma." Inflamm Res **53**(11): 636-42.

Chong, L. K. and P. T. Peachell (1999). "Beta-adrenoceptor reserve in human lung: a comparison between airway smooth muscle and mast cells." Eur J Pharmacol **378**(1): 115-22.

CIBIS (1994). "A randomized trial of beta-blockade in heart failure. The Cardiac Insufficiency Bisoprolol Study (CIBIS). CIBIS Investigators and Committees." Circulation **90**(4): 1765-73.

Clark, R. B., B. J. Knoll, et al. (1999). "Partial agonists and G protein-coupled receptor desensitization." Trends Pharmacol Sci **20**(7): 279-86.

Cockcroft, D. W., C. P. McParland, et al. (1993). "Regular inhaled salbutamol and airway responsiveness to allergen." Lancet **342**(8875): 833-7.

Costa, T. and S. Cotecchia (2005). "Historical review: Negative efficacy and the constitutive activity of G-protein-coupled receptors." Trends Pharmacol Sci **26**(12): 618-24.

Costa, T. and A. Herz (1989). "Antagonists with negative intrinsic activity at opioid receptors coupled to GTP-binding proteins." Proc. Natl. Acad. Sci. USA **86**(19): 7321-5.

Costall, B., R. J. Naylor, et al. (1985). "Mechanism of action of apomorphine on rat gastric secretion." Eur J Pharmacol **116**(3): 279-85.

Creighton, J., B. Zhu, et al. (2008). "Spectrin-anchored phosphodiesterase 4D4 restricts cAMP from disrupting microtubules and inducing endothelial cell gap formation." J Cell Sci **121**(Pt 1): 110-9.

Currie, G. P., D. K. Lee, et al. (2006). "Long-acting beta2-agonists in asthma: not so SMART?" Drug Saf **29**(8): 647-56.

De Lean, A., J. M. Stadel, et al. (1980). "A ternary complex model explains the agonist-specific binding properties of the adenylate cyclase-coupled beta-adrenergic receptor." J Biol Chem **255**(15): 7108-17.

de Ligt, R. A., A. P. Kourounakis, et al. (2000). "Inverse agonism at G protein-coupled receptors: (patho)physiological relevance and implications for drug discovery." Br J Pharmacol **130**(1): 1-12.

Dempsey, E. C., C. D. Cool, et al. (2007). "Lung disease and PKCs." Pharmacol Res **55**(6): 545-59.

Deshpande, D. A., R. M. Pascual, et al. (2007). "PKC-dependent regulation of the receptor locus dominates functional consequences of cysteinyl leukotriene type 1 receptor activation." FASEB J **21**(10): 2335-42.

Deshpande, D. A., R. M. Pascual, et al. (2007). "PKC-dependent regulation of the receptor locus dominates functional consequences of cysteinyl leukotriene type 1 receptor activation." Faseb J.

Deshpande, D. A. and R. B. Penn (2006). "Targeting G protein-coupled receptor signaling in asthma." Cell Signal **18**(12): 2105-20.

DeWire, S. M., S. Ahn, et al. (2007). "Beta-arrestins and cell signaling." Annu Rev Physiol **69**: 483-510.

Dodge, K. L. and B. M. Sanborn (1998). "Evidence for inhibition by protein kinase A of receptor/G alpha(q)/phospholipase C (PLC) coupling by a mechanism not involving PLCbeta2." Endocrinology **139**(5): 2265-71.

Drazen, J. M. (1986). "Inhalation challenge with sulfidopeptide leukotrienes in human subjects." Chest **89**(3): 414-9.

Drazen, J. M., E. Israel, et al. (1996). "Comparison of regularly scheduled with as-needed use of albuterol in mild asthma. Asthma Clinical Research Network." N Engl J Med **335**(12): 841-7.

Drazen, J. M., J. O'Brien, et al. (1992). "Recovery of leukotriene E4 from the urine of patients with airway obstruction." Am Rev Respir Dis **146**(1): 104-8.

Dunn, T. L., M. J. Gerber, et al. (1986). "The effect of topical ophthalmic instillation of timolol and betaxolol on lung function in asthmatic subjects." Am Rev Respir Dis **133**(2): 264-8.

El-Armouche, A., O. Zolk, et al. (2003). "Inhibitory G-proteins and their role in desensitization of the adenylyl cyclase pathway in heart failure." Cardiovasc Res **60**(3): 478-87.

Elfellah, M. S. and J. L. Reid (1989). "Regulation of beta 1- and beta 2-adrenoceptors following chronic treatment with beta-adrenoceptor antagonists." Eur J Pharmacol **173**(1): 85-92.

Evans, K. L., R. A. Bond, et al. (2003). "Frequency dependence of respiratory system mechanics during induced constriction in a murine model of asthma." J Appl Physiol **94**(1): 245-52.

FDA. (2005). "Serevent, Advair, Foradil Withdrawals To Be Considered By Advisory Committee." Retrieved June 6, 2008, from www.thepinksheet.com/FDC/AdvisoryCommittee/Committees/Pulmonary-Allergy+Drugs/071305_betasafety/071305_BroncoP.htm.

Finney, P. A., M. G. Belvisi, et al. (2000). "Albuterol-induced downregulation of Gsalpha accounts for pulmonary beta(2)-adrenoceptor desensitization in vivo." J Clin Invest **106**(1): 125-35.

Freedman, N. J. and R. J. Lefkowitz (1996). "Desensitization of G protein-coupled receptors." Recent Prog Horm Res **51**: 319-51; discussion 352-3.

Freedman, N. J., S. B. Liggett, et al. (1995). "Phosphorylation and desensitization of the human beta 1-adrenergic receptor. Involvement of G protein-coupled receptor kinases and cAMP-dependent protein kinase." J Biol Chem **270**(30): 17953-61.

Fust, A., J. H. Bates, et al. (2004). "Mechanical properties of mouse distal lung: in vivo versus in vitro comparison." Respir Physiol Neurobiol **143**(1): 77-86.

Gamboa, P. M., C. G. de la Cuesta, et al. (1990). "Decrease of beta-receptors after the antigen-specific bronchial provocation test in bronchial asthma." Allergol Immunopathol (Madr) **18**(3): 115-9.

Gavett, S. H. and M. Wills-Karp (1993). "Elevated lung G protein levels and muscarinic receptor affinity in a mouse model of airway hyperreactivity." Am J Physiol **265**(5 Pt 1): L493-500.

Gerlach, M. and P. Riederer (2003). "[Current preclinical findings on substances against Parkinson's disease]." Nervenarzt **74 Suppl 1**: S2-6.

Goldie, R. G., J. M. Papadimitriou, et al. (1986). "Autoradiographic localization of beta-adrenoceptors in pig lung using [125I]-iodocyanopindolol." Br J Pharmacol **88**(3): 621-8.

Goodman, O. B., Jr., J. G. Krupnick, et al. (1996). "Beta-arrestin acts as a clathrin adaptor in endocytosis of the beta2-adrenergic receptor." Nature **383**(6599): 447-50.

Guo, M., R. M. Pascual, et al. (2005). "Cytokines Regulate beta-2-Adrenergic Receptor Responsiveness in Airway Smooth Muscle via Multiple PKA- and EP2 Receptor-Dependent Mechanisms." Biochemistry **44**(42): 13771-13782.

Hack, S. P. and M. J. Christie (2003). "Adaptations in adenosine signaling in drug dependence: therapeutic implications." Crit Rev Neurobiol **15**(3-4): 235-74.

Hakonarson, H., D. J. Herrick, et al. (1995). "Mechanism of impaired beta-adrenoceptor responsiveness in atopic sensitized airway smooth muscle." Am J Physiol **269**(5 Pt 1): L645-52.

Hanania, N. A. (2008). "Targeting airway inflammation in asthma: current and future therapies." Chest **133**(4): 989-98.

Hanania, N. A. and R. H. Moore (2004). "Anti-inflammatory activities of beta2-agonists." Curr Drug Targets Inflamm Allergy **3**(3): 271-7.

Hanania, N. A., S. Singh, et al. (2008). "The safety and effects of the beta-blocker, nadolol, in mild asthma: an open-label pilot study." Pulm Pharmacol Ther **21**(1): 134-41.

Hanania, N. A., S. Singh, et al. (2006). "Inverse beta-agonists in Mild Asthma." Proc Am Thorac Soc **3**(Abstracts Issue).

Hancox, R. J. (2006). "Concluding remarks: can we explain the association of beta-agonists with asthma mortality? A hypothesis." Clin Rev Allergy Immunol **31**(2-3): 279-88.

Hansen, G., S. Jin, et al. (2000). "Absence of muscarinic cholinergic airway responses in mice deficient in the cyclic nucleotide phosphodiesterase PDE4D." Proc Natl Acad Sci U S A **97**(12): 6751-6.

Harding, S. E. and H. Gong (2004). "beta-adrenoceptor blockers as agonists: coupling of beta2-adrenoceptors to multiple G-proteins in the failing human heart." Congest Heart Fail **10**(4): 181-5; quiz 186-7.

Hata, J. A. and W. J. Koch (2003). "Phosphorylation of G protein-coupled receptors: GPCR kinases in heart disease." Mol Interv **3**(5): 264-72.

Hataoka, I., M. Okayama, et al. (1993). "Decrease in beta-adrenergic receptors of lymphocytes in spontaneously occurring acute asthma." Chest **104**(2): 508-14.

He, J. Q., R. C. Balijepalli, et al. (2005). "Crosstalk of beta-adrenergic receptor subtypes through Gi blunts beta-adrenergic stimulation of L-type Ca2+ channels in canine heart failure." Circ Res **97**(6): 566-73.

Henry, P. J., P. J. Rigby, et al. (1990). "Distribution of beta 1- and beta 2-adrenoceptors in mouse trachea and lung: a quantitative autoradiographic study." Br J Pharmacol **99**(1): 136-44.

Hershenson, M. B., M. Brown, et al. (2008). "Airway smooth muscle in asthma." Annu Rev Pathol **3**: 523-55.

Hirshman, C. A. and C. W. Emala (1999). "Actin reorganization in airway smooth muscle cells involves Gq and Gi-2 activation of Rho." Am J Physiol **277**(3 Pt 1): L653-61.

Hirshman, C. A., B. Lande, et al. (1999). "Role of M2 muscarinic receptors in airway smooth muscle contraction." Life Sci **64**(6-7): 443-8.

Holgate, S. T., J. Holloway, et al. (2006). "Understanding the pathophysiology of severe asthma to generate new therapeutic opportunities." J Allergy Clin Immunol **117**(3): 496-506; quiz 507.

Holgate, S. T., M. Peters-Golden, et al. (2003). "Roles of cysteinyl leukotrienes in airway inflammation, smooth muscle function, and remodeling." J Allergy Clin Immunol **111**(1 Suppl): S18-34; discussion S34-6.

Houslay, M. D. and D. R. Adams (2003). "PDE4 cAMP phosphodiesterases: modular enzymes that orchestrate signalling cross-talk, desensitization and compartmentalization." Biochem J **370**(Pt 1): 1-18.

Houslay, M. D. and G. S. Baillie (2005). "Beta-arrestin-recruited phosphodiesterase-4 desensitizes the AKAP79/PKA-mediated switching of beta2-adrenoceptor signalling to activation of ERK." Biochem Soc Trans **33**(Pt 6): 1333-6.

Hu, A., G. Nino, et al. (2008). "Prolonged heterologous (Barnes)2-adrenoceptor desensitization promotes proasthmatic airway smooth muscle function via PKA/ERK1/2-mediated phosphodiesterase-4 induction." Am J Physiol Lung Cell Mol Physiol.

Huang, Z., R. Dias, et al. (2007). "L-454,560, a potent and selective PDE4 inhibitor with in vivo efficacy in animal models of asthma and cognition." Biochem Pharmacol **73**(12): 1971-81.

Jame, A. J., P. M. Lackie, et al. (2007). "Human bronchial epithelial cells express an active and inducible biosynthetic pathway for leukotrienes B4 and C4." Clin Exp Allergy **37**(6): 880-92.

Jeffery, P. K., A. J. Wardlaw, et al. (1989). "Bronchial biopsies in asthma. An ultrastructural, quantitative study and correlation with hyperreactivity." Am Rev Respir Dis **140**(6): 1745-53.

Jin, S. L., T. Bushnik, et al. (1998). "Subcellular localization of rolipram-sensitive, cAMP-specific phosphodiesterases. Differential targeting and activation of the splicing variants derived from the PDE4D gene." J Biol Chem **273**(31): 19672-8.

Kalavantavanich, K. and C. M. Schramm (2000). "Dexamethasone potentiates high-affinity beta-agonist binding and g(s)alpha protein expression in airway smooth muscle." Am J Physiol Lung Cell Mol Physiol **278**(5): L1101-6.

Kawakami, N., K. Miyoshi, et al. (2004). "Beta(2)-adrenergic receptor-mediated histamine H(1) receptor down-regulation: another possible advantage of beta(2) agonists in asthmatic therapy." J Pharmacol Sci **94**(4): 449-58.

Kelsen, S. G., O. O. Anakwe, et al. (1997). "Chronic effects of catecholamines on the beta 2-adrenoreceptor system in cultured human airway epithelial cells." Am J Physiol **272**(5 Pt 1): L916-24.

Kenakin, T. (2001). "Inverse, protean, and ligand-selective agonism: matters of receptor conformation." Faseb J **15**(3): 598-611.

Kenakin, T. (2003). "Ligand-selective receptor conformations revisited: the promise and the problem." Trends Pharmacol Sci **24**(7): 346-54.

Kenakin, T. (2004). "Allosteric modulators: the new generation of receptor antagonist." Mol Interv **4**(4): 222-9.

Kenakin, T. (2004)b. "Efficacy as a vector: the relative prevalence and paucity of inverse agonism." Mol Pharmacol **65**(1): 2-11.

Kenakin, T. (2004). "G-protein coupled receptors as allosteric machines." Receptors Channels **10**(2): 51-60.

Kenakin, T. (2004)a. "Principles: receptor theory in pharmacology." Trends Pharmacol Sci **25**(4): 186-92.

Kenakin, T. (2005). "New concepts in drug discovery: collateral efficacy and permissive antagonism." Nat Rev Drug Discov **4**(11): 919-27.

Kenakin, T. (2006). "Receptors as microprocessors: pharmacological nuance on metabotropic glutamate receptors 1alpha." Sci STKE **2006**(342): pe29.

Kilts, J. D., M. A. Gerhardt, et al. (2000). "Beta(2)-adrenergic and several other G protein-coupled receptors in human atrial membranes activate both G(s) and G(i)." Circ Res **87**(8): 705-9.

Kim, M. H. and D. K. Agrawal (2002). "Effect of interleukin-1beta and tumor necrosis factor-alpha on the expression of G-proteins in CD4+ T-cells of atopic asthmatic subjects." J Asthma **39**(5): 441-8.

Kubota, T., N. Yamazaki, et al. (1990). "Protective effects of adrenoceptor-blocking agents on myocardial injury induced by epinephrine in mice." J Toxicol Sci **15**(1): 1-13.

Kume, H., I. P. Hall, et al. (1994). "Beta-adrenergic agonists regulate KCa channels in airway smooth muscle by cAMP-dependent and -independent mechanisms." J Clin Invest **93**(1): 371-9.

Lam, S., H. Chan, et al. (1988). "Release of leukotrienes in patients with bronchial asthma." J Allergy Clin Immunol **81**(4): 711-7.

Lambert, R. K., B. R. Wiggs, et al. (1993). "Functional significance of increased airway smooth muscle in asthma and COPD." J Appl Physiol **74**(6): 2771-81.

Le Jeune, I. R., M. Shepherd, et al. (2002). "Cyclic AMP-dependent transcriptional up-regulation of phosphodiesterase 4D5 in human airway smooth muscle cells. Identification and characterization of a novel PDE4D5 promoter." J Biol Chem **277**(39): 35980-9.

Lechat, P., M. Packer, et al. (1998). "Clinical effects of beta-adrenergic blockade in chronic heart failure: a meta-analysis of double-blind, placebo-controlled, randomized trials." Circulation **98**(12): 1184-91.

Lefkowitz, R. J. and E. J. Whalen (2004). "beta-arrestins: traffic cops of cell signaling." Curr Opin Cell Biol **16**(2): 162-8.

Leineweber, K. and O. E. Brodde (2004). "Beta2-adrenoceptor polymorphisms: relation between in vitro and in vivo phenotypes." Life Sci **74**(23): 2803-14.

Leineweber, K., P. Rohe, et al. (2005). "G-protein-coupled receptor kinase activity in human heart failure: effects of beta-adrenoceptor blockade." Cardiovasc Res **66**(3): 512-9.

Lemoine, H. and C. Overlack (1992). "Highly potent beta-2 sympathomimetics convert to less potent partial agonists as relaxants of guinea pig tracheae maximally contracted by carbachol. Comparison of relaxation with receptor binding and adenylate cyclase stimulation." J Pharmacol Exp Ther **261**(1): 258-70.

Lin, R., H. Peng, et al. (2008). "Changes in beta(2)-adrenoceptor and other signaling proteins produced by chronic administration of 'beta-blockers' in a murine asthma model." Pulm Pharmacol Ther **21**(1): 115-24.

Maack, C., B. Cremers, et al. (2000). "Different intrinsic activities of bucindolol, carvedilol and metoprolol in human failing myocardium." Br J Pharmacol **130**(5): 1131-9.

Magnusson, Y., M. C. Levin, et al. (2005). "Ser49Gly of beta1-adrenergic receptor is associated with effective beta-blocker dose in dilated cardiomyopathy." Clin Pharmacol Ther **78**(3): 221-31.

Mak, J. C., T. T. Chuang, et al. (2002). "Increased expression of G protein-coupled receptor kinases in cystic fibrosis lung." Eur J Pharmacol **436**(3): 165-72.

Mak, J. C., T. Hisada, et al. (2002). "Glucocorticoids reverse IL-1beta-induced impairment of beta-adrenoceptor-mediated relaxation and up-regulation of G-protein-coupled receptor kinases." Br J Pharmacol **135**(4): 987-96.

Mak, J. C., M. Nishikawa, et al. (1995). "Glucocorticosteroids increase beta 2-adrenergic receptor transcription in human lung." Am J Physiol **268**(1 Pt 1): L41-6.

Malaviya, R., J. Ansell, et al. (2006). "Targeting cytosolic phospholipase A2 by arachidonyl trifluoromethyl ketone prevents chronic inflammation in mice." Eur J Pharmacol **539**(3): 195-204.

Mardini, I. A., N. C. Higgins, et al. (1994). "Functional behavior of the beta-adrenergic receptor-adenylyl cyclase system in rabbit airway epithelium." Am J Respir Cell Mol Biol **11**(3): 287-95.

Masoli, M., D. Fabian, et al. (2004). "The global burden of asthma: executive summary of the GINA Dissemination Committee report." Allergy **59**(5): 469-78.

McGraw, D. W., K. F. Almoosa, et al. (2003). "Antithetic regulation by beta-adrenergic receptors of Gq receptor signaling via phospholipase C underlies the airway beta-agonist paradox." J Clin Invest **112**(4): 619-26.

McGraw, D. W., J. M. Elwing, et al. (2007). "Crosstalk between Gi and Gq/Gs pathways in airway smooth muscle regulates bronchial contractility and relaxation." J Clin Invest **117**(5): 1391-8.

McGraw, D. W., S. L. Forbes, et al. (2000). "Transgenic overexpression of beta(2)-adrenergic receptors in airway epithelial cells decreases bronchoconstriction." Am J Physiol Lung Cell Mol Physiol **279**(2): L379-89.

McGraw, D. W. and S. B. Liggett (1997). "Heterogeneity in beta-adrenergic receptor kinase expression in the lung accounts for cell-specific desensitization of the beta2-adrenergic receptor." J Biol Chem **272**(11): 7338-44.

McGraw, D. W. and S. B. Liggett (2005). "Molecular mechanisms of beta2-adrenergic receptor function and regulation." Proc Am Thorac Soc **2**(4): 292-6; discussion 311-2.

Mehats, C., S. L. Jin, et al. (2003). "PDE4D plays a critical role in the control of airway smooth muscle contraction." Faseb J **17**(13): 1831-41.

Mehats, C., S. L. Jin, et al. (2003) "PDE4D plays a critical role in the control of airway smooth muscle contraction." Faseb J **Volume**, 1831-41 DOI:

Menard, L., S. S. Ferguson, et al. (1997). "Synergistic regulation of beta2-adrenergic receptor sequestration: intracellular complement of beta-adrenergic receptor kinase and beta-arrestin determine kinetics of internalization." Mol Pharmacol **51**(5): 800-8.

Metaye, T., H. Gibelin, et al. (2005). "Pathophysiological roles of G-protein-coupled receptor kinases." Cell Signal **17**(8): 917-28.

Milligan, G. (2003). "Constitutive activity and inverse agonists of G protein-coupled receptors: a current perspective." Mol Pharmacol **64**(6): 1271-6.

Nelson, H. S., S. T. Weiss, et al. (2006). "The Salmeterol Multicenter Asthma Research Trial: a comparison of usual pharmacotherapy for asthma or usual pharmacotherapy plus salmeterol." Chest **129**(1): 15-26.

Nelson, W. L., F. T. Fraunfelder, et al. (1986). "Adverse respiratory and cardiovascular events attributed to timolol ophthalmic solution, 1978-1985." Am J Ophthalmol **102**(5): 606-11.

Nguyen, L. P., O. Omoluabi, et al. (2008). "Chronic Exposure to Beta-Blockers Attenuates Inflammation and Mucin Content in a Murine Asthma Model." Am. J. Respir. Cell Mol. Biol. **38**(3): 256-262.

Nicholas, G., C. Oakley, et al. (1990). "Xamoterol in severe heart failure. The Xamoterol in Severe Heart Failure Study Group." Lancet **336**(8706): 1-6.

Offer, S., S. Yedgar, et al. (2005). "Negative feedback between secretory and cytosolic phospholipase A2 and their opposing roles in ovalbumin-induced bronchoconstriction in rats." Am J Physiol Lung Cell Mol Physiol **288**(3): L523-9.

Packer, M., M. R. Bristow, et al. (1996). "The effect of carvedilol on morbidity and mortality in patients with chronic heart failure. U.S. Carvedilol Heart Failure Study Group." N Engl J Med **334**(21): 1349-55.

Parra, S. and R. A. Bond (2007). "Inverse agonism: from curiosity to accepted dogma, but is it clinically relevant?" Curr Opin Pharmacol **7**(2): 146-50.

Pavoine, C., N. Behforouz, et al. (2003). "beta2-Adrenergic signaling in human heart: shift from the cyclic AMP to the arachidonic acid pathway." Mol Pharmacol **64**(5): 1117-25.

Pavoine, C. and N. Defer (2005). "The cardiac beta2-adrenergic signalling a new role for the cPLA2." Cell Signal **17**(2): 141-52.

Penela, P., C. Ribas, et al. (2003). "Mechanisms of regulation of the expression and function of G protein-coupled receptor kinases." Cell Signal **15**(11): 973-81.

Penn, R. B. and J. L. Benovic (2008). "Regulation of heterotrimeric G protein signaling in airway smooth muscle." Proc Am Thorac Soc **5**(1): 47-57.

Peters-Golden, M. and A. Feyssa (1993). "Transcellular eicosanoid synthesis in cocultures of alveolar epithelial cells and macrophages." Am J Physiol **264**(5 Pt 1): L438-47.

Post, S. R., R. Hilal-Dandan, et al. (1995). "Quantification of signalling components and amplification in the beta-adrenergic-receptor-adenylate cyclase pathway in isolated adult rat ventricular myocytes." Biochem J **311 (Pt 1)**: 75-80.

Premont, R. T. and R. R. Gainetdinov (2007). "Physiological roles of G protein-coupled receptor kinases and arrestins." Annu Rev Physiol **69**: 511-34.

Rakonjac, M., L. Fischer, et al. (2006). "Coactosin-like protein supports 5-lipoxygenase enzyme activity and up-regulates leukotriene A4 production." Proc Natl Acad Sci U S A **103**(35): 13150-5.

Rang, H. P. (2006). "The receptor concept: pharmacology's big idea." Br J Pharmacol **147 Suppl 1**: S9-16.

Ravasi, S., S. Citro, et al. (2006). "CysLT1 receptor-induced human airway smooth muscle cells proliferation requires ROS generation, EGF receptor transactivation and ERK1/2 phosphorylation." Respir Res **7**: 42.

Reiter, E. and R. J. Lefkowitz (2006). "GRKs and beta-arrestins: roles in receptor silencing, trafficking and signaling." Trends Endocrinol Metab **17**(4): 159-65.

Richter, W., S. L. Jin, et al. (2005). "Splice variants of the cyclic nucleotide phosphodiesterase PDE4D are differentially expressed and regulated in rat tissue." Biochem J **388**(Pt 3): 803-11.

Rodger, I. W. (1992). "Asthma. Airway smooth muscle." Br Med Bull **48**(1): 97-107.

Rossing, T. H., C. H. Fanta, et al. (1980). "Emergency therapy of asthma: comparison of the acute effects of parenteral and inhaled sympathomimetics and infused aminophylline." Am Rev Respir Dis **122**(3): 365-71.

Rousell, J., E. B. Haddad, et al. (1996). "Beta-Adrenoceptor-medicated down-regulation of M2 muscarinic receptors: role of cyclic adenosine 5'-monophosphate-dependent protein kinase and protein kinase C." Mol Pharmacol **49**(4): 629-35.

Rovati, G. E., M. Baroffio, et al. (2006). "Cysteinyl-leukotrienes in the regulation of beta2-adrenoceptor function: an in vitro model of asthma." Respir Res **7**: 103.

Samama, P., S. Cotecchia, et al. (1993). "A mutation-induced activated state of the beta 2-adrenergic receptor. Extending the ternary complex model." J Biol Chem **268**(7): 4625-36.

Sanderson, M. J., P. Delmotte, et al. (2008). "Regulation of airway smooth muscle cell contractility by Ca2+ signaling and sensitivity." Proc Am Thorac Soc **5**(1): 23-31.

Sano, M., T. Yoshimasa, et al. (1993). "Non-homogeneous distribution of beta 1- and beta 2-adrenoceptors in various human tissues." Life Sci **52**(12): 1063-70.

Sastre, B., M. Fernandez-Nieto, et al. (2008). "Increased prostaglandin E2 levels in the airway of patients with eosinophilic bronchitis." Allergy **63**(1): 58-66.

Schears, G., J. Clancy, et al. (1997). "Chronic carbachol pretreatment decreases adenylyl cyclase activity in airway smooth muscle." Am J Physiol **273**(3 Pt 1): L640-7.

Seco, A. J., M. E. Salgueiro, et al. (1995). "Acute and chronic treatment with glucocorticosteroids, modifying the beta 2-adrenergic response of the guinea pig trachea." Lung **173**(5): 321-8.

Seldon, P. M., P. J. Barnes, et al. (1995). "Suppression of lipopolysaccharide-induced tumor necrosis factor-alpha generation from human peripheral blood monocytes by inhibitors of phosphodiesterase 4: interaction with stimulants of adenylyl cyclase." Mol Pharmacol **48**(4): 747-57.

Shipley, J. B. and M. L. Hess (1995). "Inotropic therapy for the failing myocardium." Clin Cardiol **18**(11): 615-9.

Singh, S. P., E. G. Barrett, et al. (2003). "Prenatal cigarette smoke decreases lung cAMP and increases airway hyperresponsiveness." Am J Respir Crit Care Med **168**(3): 342-7.

Sinha, B., J. Semmler, et al. (1995). "Enhanced tumor necrosis factor suppression and cyclic adenosine monophosphate accumulation by combination of phosphodiesterase inhibitors and prostanoids." Eur J Immunol **25**(1): 147-53.

Small, K. M., D. W. McGraw, et al. (2003). "Pharmacology and physiology of human adrenergic receptor polymorphisms." Annu Rev Pharmacol Toxicol **43**: 381-411.

Song, P., E. Crimi, et al. (1998). "Anti-inflammatory agents and allergen-induced beta2-receptor dysfunction in isolated human bronchi." Am J Respir Crit Care Med **158**(6): 1809-14.

Song, P., M. Milanese, et al. (2000). "G(s) protein dysfunction in allergen-challenged human isolated passively sensitized bronchi." Am J Physiol Lung Cell Mol Physiol **279**(2): L209-15.

Spicuzza, L., M. G. Belvisi, et al. (2001). "Evidence that the anti-spasmogenic effect of the beta-adrenoceptor agonist, isoprenaline, on guinea-pig trachealis is not mediated by cyclic AMP-dependent protein kinase." Br J Pharmacol **133**(8): 1201-12.

Spina, D., P. J. Rigby, et al. (1989). "Autoradiographic localization of beta-adrenoceptors in asthmatic human lung." Am Rev Respir Dis **140**(5): 1410-5.

Spitz, D. J. (2003). "An unusual death in an asthmatic patient." Am J Forensic Med Pathol **24**(3): 271-2.

Spitzer, W. O., S. Suissa, et al. (1992). "The use of beta-agonists and the risk of death and near death from asthma." N Engl J Med **326**(8): 501-6.

Suissa, S., P. Ernst, et al. (1994). "A cohort analysis of excess mortality in asthma and the use of inhaled beta-agonists." Am J Respir Crit Care Med **149**(3 Pt 1): 604-10.

Sun, J. G., Y. M. Deng, et al. (2006). "Inhibition of phosphodiesterase activity, airway inflammation and hyperresponsiveness by PDE4 inhibitor and glucocorticoid in a murine model of allergic asthma." Life Sci **79**(22): 2077-85.

Swedberg, K., A. Hjalmarson, et al. (1980). "Beneficial effects of long-term beta-blockade in congestive cardiomyopathy." Br Heart J **44**(2): 117-33.

Tanaka, Y., Y. Yamashita, et al. (2003). "Evidence for a significant role of a Gs-triggered mechanism unrelated to the activation of adenylyl cyclase in the cyclic AMP-independent relaxant response of guinea-pig tracheal smooth muscle." Naunyn Schmiedebergs Arch Pharmacol **368**(5): 437-41.

Taylor, D. R. and R. J. Hancox (2000). "Interactions between corticosteroids and beta agonists." Thorax **55**(7): 595-602.

Taylor, D. R., G. I. Town, et al. (1998). "Asthma control during long-term treatment with regular inhaled salbutamol and salmeterol." Thorax **53**(9): 744-52.

Tliba, O., Y. Amrani, et al. (2008). "Is airway smooth muscle the "missing link" modulating airway inflammation in asthma?" Chest **133**(1): 236-42.

Torphy, T. J. (1998). "Phosphodiesterase isozymes: molecular targets for novel antiasthma agents." Am J Respir Crit Care Med **157**(2): 351-70.

Torphy, T. J., B. J. Undem, et al. (1993). "Identification, characterization and functional role of phosphodiesterase isozymes in human airway smooth muscle." J Pharmacol Exp Ther **265**(3): 1213-23.

Vargaftig, B. B. and M. Singer (2003). "Leukotrienes mediate part of Ova-induced lung effects in mice via EGFR." Am J Physiol Lung Cell Mol Physiol **285**(4): L808-18.

Vroon, A., C. J. Heijnen, et al. (2006). "GRKs and arrestins: regulators of migration and inflammation." J Leukoc Biol **80**(6): 1214-21.

Waagstein, F., A. Hjalmarson, et al. (1975). "Effect of chronic beta-adrenergic receptor blockade in congestive cardiomyopathy." Br Heart J **37**(10): 1022-36.

Wagers, S., L. K. Lundblad, et al. (2004). "The allergic mouse model of asthma: normal smooth muscle in an abnormal lung?" J Appl Physiol **96**(6): 2019-27.

Waldeck, B. (2002). "Beta-adrenoceptor agonists and asthma--100 years of development." Eur J Pharmacol **445**(1-2): 1-12.

Walker, J. K., A. M. Fong, et al. (2003). "Beta-arrestin-2 regulates the development of allergic asthma." J Clin Invest **112**(4): 566-74.

Walker, J. K., R. R. Gainetdinov, et al. (2004). "G protein-coupled receptor kinase 5 regulates airway responses induced by muscarinic receptor activation." Am J Physiol Lung Cell Mol Physiol **286**(2): L312-9.

Walker, J. K., K. Peppel, et al. (1999). "Altered airway and cardiac responses in mice lacking G protein-coupled receptor kinase 3." Am J Physiol **276**(4 Pt 2): R1214-21.

Walters, E. H., P. G. Gibson, et al. (2007). "Long-acting beta2-agonists for chronic asthma in adults and children where background therapy contains varied or no inhaled corticosteroid." Cochrane Database Syst Rev(1): CD001385.

Weber, K. T., V. Andrews, et al. (1982). "Cardiotonic agents in the management of chronic cardiac failure." Am Heart J **103**(4 Pt 2): 639-49.

Weiss, S. T. (2001). "Epidemiology and heterogeneity of asthma." Ann Allergy Asthma Immunol **87**(1 Suppl 1): 5-8.

Wenzel, S. E., G. L. Larsen, et al. (1990). "Elevated levels of leukotriene C4 in bronchoalveolar lavage fluid from atopic asthmatics after endobronchial allergen challenge." Am Rev Respir Dis **142**(1): 112-9.

Whitehead, G. S., J. K. Walker, et al. (2003). "Allergen-induced airway disease is mouse strain dependent." Am J Physiol Lung Cell Mol Physiol **285**(1): L32-42.

Xiao, R. P. (2001). "Beta-adrenergic signaling in the heart: dual coupling of the beta2-adrenergic receptor to G(s) and G(i) proteins." Sci STKE **2001**(104): RE15.

Xiao, R. P., P. Avdonin, et al. (1999). "Coupling of beta2-adrenoceptor to Gi proteins and its physiological relevance in murine cardiac myocytes." Circ Res **84**(1): 43-52.

Xiao, R. P., X. Ji, et al. (1995). "Functional coupling of the beta 2-adrenoceptor to a pertussis toxin-sensitive G protein in cardiac myocytes." Mol Pharmacol **47**(2): 322-9.

Xiao, R. P., S. J. Zhang, et al. (2003). "Enhanced G(i) signaling selectively negates beta2-adrenergic receptor (AR)--but not beta1-AR-mediated positive inotropic effect in myocytes from failing rat hearts." Circulation **108**(13): 1633-9.