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Involvement of adenosine A1 receptors in systemic inflammation and altered vascular reactivity in allergic mice

Dovenia S. Ponnoth
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**Involvement of Adenosine A₁ receptors in systemic inflammation and altered
vascular reactivity in allergic mice**

Dovenia S. Ponnoth

**Dissertation submitted to the School of Pharmacy at West Virginia University in
partial fulfillment of the requirements for the degree of**

**Doctor of Philosophy
in
Pharmaceutical and Pharmacological Sciences**

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**Keywords: Asthma, adenosine, adenosine receptor, inflammation, vascular
reactivity**

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ABSTRACT

Involvement of Adenosine A₁ receptors in systemic inflammation and altered vascular reactivity in allergic mice

Dovenia S. Ponnoth

Epidemiological studies have established that people who have lung diseases such as asthma and COPD are likely to develop adverse cardiovascular events. While the reason for this has not been elucidated, it is believed that inflammation could be the key component linking the two conditions. Adenosine, an endogenous purine nucleoside, has been strongly implicated in asthma pathophysiology and also has potent effects on the cardiovascular system. This work was undertaken to gain a better understanding of the relationship between asthma and its cardiovascular effects, and more specifically the possible role adenosine plays under these conditions.

We hypothesize that asthmatic lung inflammation translates into systemic inflammation and alters vascular responses where adenosine plays an important role. Therefore, this study investigated the effects of aerosolized adenosine, used to elevate lung adenosine levels, on vascular reactivity and inflammation in a well established mouse model of allergic asthma established in our lab. We found that allergic mice had poor vasorelaxation to adenosine and systemic inflammation, in addition to lung inflammation, and aerosolized adenosine exacerbated these effects. Data indicated a possible role for A₁ adenosine receptor (AR) in altered vascular reactivity and inflammation. Based on these findings, we next studied the effects of adenosine using genetically modified mice in which the A₁ AR gene was deleted (A₁KO) and corresponding A₁ wild-type(A₁WT) mice, in which the A₁ receptor was present.

Allergic A₁KO mice had no systemic inflammation, and the adenosine-mediated vasorelaxation responses obtained were comparable to non-allergic mice. Aerosolized adenosine also did not have any effect in A₁KO mice. On the other hand, allergic A₁WT mice had lower aortic relaxation response to non-selective adenosine analog NECA, presence of significantly higher levels of inflammatory cytokines in plasma and higher airway responsiveness to NECA, compared to non-allergic A₁WT controls and all groups of A₁KO mice, with aerosolized adenosine exacerbating all these responses. From these data, it can be concluded that asthmatic mice had poor vascular responses and inflammation, possibly mediated through the A₁ AR.

To my parents, Mildred and Kumar, and sister, Deanne

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TABLE OF CONTENTS

	Page
LIST OF TABLES.....	xi
LIST OF FIGURES.....	xii
LIST OF ABBREVIATIONS.....	xvi
CHAPTER ONE: INTRODUCTION.....	1
Generation of adenosine and its metabolism.....	1
Adenosine Receptors.....	4
Asthma.....	4
Adenosine in asthma.....	5
Reactive airway diseases and cardiovascular complications.....	7
Animal models of asthma.....	8
Murine models in asthma.....	8
Purpose and project summary.....	10
CHAPTER TWO: MATERIALS AND METHODS.....	12
Mice.....	12
Animal sensitization protocol.....	12
<i>In vitro</i> organ bath.....	14
Airway responsiveness.....	15
CHAPTER THREE: ADENOSINE-MEDIATED ALTERATION OF VASCULAR REACTIVITY AND INFLAMMATION IN A MOUSE MODEL OF ASTHMA..	18
Abstract.....	18
Introduction.....	19

Materials and Methods.....	21
Animals.....	21
Animal Sensitization.....	21
Preparation of isolated mouse aorta and isometric force measurement.....	23
Contraction/relaxation experiments.....	23
Experimental protocol....	24
Real time RT-PCR: A ₁ , A _{2A} , A _{2B} , A ₃ AR and eNOS genes expression.....	24
Assessment of systemic inflammation.....	25
Multiplex cytokine assay.....	26
C-reactive protein assay.....	26
Drugs used.....	26
Statistical analysis.....	27
Results.....	27
Systemic inflammation in control, allergic and adenosine aerosolized allergic mice.....	27
Vascular reactivity in control, allergic and adenosine aerosolized allergic mice.....	28
Endothelial function and eNOS expression in control, allergic and adenosine aerosolized allergic mice.....	29
Adenosine receptors gene expression in control, allergic and adenosine aerosolized allergic mice	30

Effect of specific A ₁ AR (DPCPX) and A _{2B} AR (alloxazine) antagonists on vascular reactivity in control, allergic and adenosine aerosolized allergic mice	30
Discussion.....	38
CHAPTER FOUR: ROLE OF A _{2A} ADENOSINE RECEPTORS IN THE REGULATION OF VASCULAR TONE.....	
Abstract.....	44
Introduction.....	45
Materials and Methods.....	47
Mice.....	47
Preparation of isolated mouse aorta and isometric force measurement.....	47
Contraction/relaxation experiments.....	48
Experimental protocol.....	49
Real time PCR: A ₁ , A _{2A} , A _{2B} and A ₃ AR genes expression.....	49
Drugs used.....	50
Statistical Analysis.....	50
Results.....	51
Response to NECA, CGS-21860 and BAY 60-6583.....	51
Endothelial function in A _{2A} WT and KO aorta.....	51
Adenosine receptors gene expression in A _{2A} WT and KO mice.....	52

Effect of specific A ₁ AR (DPCPX), A _{2A} AR (SCH 58261) and A _{2B} AR (alloxazine) antagonists on vascular reactivity in A _{2A} WT and KO mice.....	52
Discussion.....	63
CHAPTER FIVE: EVIDENCE FOR THE ROLE THE OF A ₁ ADENOSINE RECEPTOR IN ALTERED VASCULAR RESPONSES AND INFLAMMATION IN ALLERGIC MICE.....	
Abstract.....	67
Introduction.....	68
Materials and Methods.....	71
Animals.....	71
Animal Sensitization.....	71
Preparation of isolated mouse aorta and isometric force measurement.....	73
Contraction/relaxation experiments.....	74
Experimental protocol.....	74
Assessment of systemic inflammation.....	74
Multiplex Cytokine Assay.....	74
Airway responsiveness to methacholine and NECA.....	75
Drugs used.....	76
Statistical Analysis.....	76
Results.....	77

Systemic inflammation in control, allergic and adenosine aerosolized allergic mice.....	77
Vascular reactivity in control, allergic and adenosine aerosolized allergic mice	77
Endothelial function in control, allergic and adenosine aerosolized allergic mice.....	78
Airway responsiveness to methacholine and NECA.....	78
Discussion.....	86
CHAPTER SIX: CONCLUSION.....	91
PUBLICATIONS AND ABSTRACTS.....	97
REFERENCES.....	99
APPENDIX A: Selection of phenylephrine and acetylcholine concentrations for vascular reactivity experiments.....	109
APPENDIX B: Preliminary data in allergic A _{2A} WT and KO mice.....	112
APPENDIX C: Cytokine profile in BAL fluid.....	114
CURRICULUM VITAE.....	118

LIST OF TABLES

		Page
Table 3.1	Systemic inflammation as assessed by levels of inflammatory cytokines in plasma of control (CON), adenosine-aerosolized control (CON+AD), allergic (SEN) and adenosine aerosolized allergic (SEN+AD) mice.....	32
Table 5.1	Systemic inflammation as assessed by levels of inflammatory cytokines in plasma of A ₁ WT and A ₁ KO control (CON), allergic (SEN) and adenosine-aerosolized allergic (SEN+AD) mice.....	79

LIST OF FIGURES

	Page
Figure 1.1	Structure of adenosine.....2
Figure 1.2	Metabolic pathways of adenosine.....3
Figure 2.1	Ventilatory cycle from whole body plethysmography.....17
Figure 3.1	Concentration response curves for adenosine-mediated relaxation/contraction in control, adenosine aerosolized control, allergic and adenosine aerosolized allergic mice.....33
Figure 3.2	Endothelial responses in control, adenosine aerosolized control, allergic and adenosine aerosolized allergic mice.....34 (A) Effect of acetylcholine on phenylephrine-precontracted aorta.....34 (B) Expression of eNOS by real time RT-PCR..... 34
Figure 3.3	Expression of A ₁ , A _{2A} , and A _{2B} adenosine receptors in mice aorta of control, allergic and adenosine aerosolized allergic mice.....35

Figure 3.4	Effect of a specific A_1 AR antagonist, DPCPX on adenosine-mediated relaxation/contraction in aorta of allergic and adenosine aerosolized allergic mice.....	36
Figure 3.5	Effect of A_{2B} AR antagonist, alloxazine on adenosine-mediated relaxation/contraction in aorta of control mice and allergic and adenosine aerosolized allergic mice.....	37
Figure 4.1	Concentration response curves for NECA-mediated relaxation/contraction in A_{2A} WT and A_{2A} KO mice.....	54
Figure 4.2	Concentration response curves for CGS 21860-mediated relaxation/contraction in A_{2A} WT and A_{2A} KO mice.....	55
Figure 4.3	Concentration response curves for BAY 60-6583-mediated relaxation/contraction in A_{2A} WT and A_{2A} KO mice.....	56
Figure 4.4	(A) Endothelial response in A_{2A} WT and A_{2A} KO mice to acetylcholine.....	57
	(B) CGS 21860 response in A_{2A} WT endothelium-denuded (-E) aorta.....	57

Figure 4.5	Adenosine receptors gene expression in A_{2A} WT and A_{2A} KO aorta.....	58
Figure 4.6	Effect of selective A_1 antagonist DPCPX on NECA-mediated relaxation/contraction in A_{2A} WT and A_{2A} KO mice.....	59
Figure 4.7	Effect of selective A_{2A} antagonist SCH 58261 on (A) NECA and (B) CGS 21860-mediated relaxation/contraction in A_{2A} WT aorta.....	60
Figure 4.8	Effect of selective A_{2B} antagonist alloxazine on NECA- mediated relaxation/contraction in A_{2A} WT mice.....	61
Figure 4.9	Effect of selective A_{2B} antagonist alloxazine on NECA-mediated relaxation/contraction in A_{2A} KO mice.....	62
Figure 5.1	Concentration-response curves for NECA-mediated relaxation/contraction in A_1 WT and A_1 KO control, allergic and adenosine aerosolized allergic group.....	80
Figure 5.2	Comparison of NECA-mediated aortic response between A_1 KO and A_1 WT allergic mice.....	81

Figure 5.3	Comparison of NECA-mediated aortic response between A ₁ KO and A ₁ WT allergic and adenosine aerosolized allergic mice.....	82
Figure 5.4	Endothelial response to ACh in control, allergic and adenosine aerosolized allergic A ₁ WT and A ₁ KO mice.....	83
Figure 5.5	Airway responsiveness to MCh in A ₁ KO and A ₁ WT mice.....	84
Figure 5.6	Airway responsiveness to NECA in A ₁ KO and A ₁ WT mice.....	85
Figure 6.1	Proposed pathway for IL-13 signaling effects in asthma.....	96

LIST OF ABBREVIATIONS

μg	micro gram
μl	micro liter
A ₁ AR	A ₁ adenosine receptor
A _{2A} AR	A _{2A} adenosine receptor
A _{2B} AR	A _{2B} adenosine receptor
A ₃ AR	A ₃ adenosine receptor
AC	Adenylyl cyclase
ACh	Acetylcholine
ADA	Adenosine deaminase
AD	Adenosine
ADP	Adenosine diphosphate
AK	Adenosine kinase
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BAL	Bronchoalveolar lavage
cAMP	Cyclic adenosine monophosphate
CGS 21860	2- <i>p</i> -(2-carboxyethyl)phenethylamino- 5' N- ethylcarboxy amidoadenosine hydrochloride
CON	Control

COPD	Chronic obstructive pulmonary disease
CRC	Concentration response curve
CRP	C-reactive protein
CVD	Cardiovascular disease
DAG	Diacylglycerol
DMSO	Dimethyl sulfoxide
DPCPX	1,3-Dipropyl-8-cyclopentylxanthine
IgE	Immunoglobulin E
i.p.	Intraperitoneal
IL-1 β	Interleukin 1 β
IL-4	Interleukin 4
IL-5	Interleukin 5
IL-6	Interleukin 6
IL-10	Interleukin 10
IL-13	Interleukin 13
IP ₃	Inositol triphosphate
kg	Kilogram
LO	Lipoxygenase
LTB ₄	Leukotriene B ₄
MCh	Methacholine
MCP-1	Monocyte chemotactic protein-1
MIP-1 α	Macrophage inflammatory protein 1 α
mg	milligram

ml	milliliter
mm	millimeter
mM	millimole
NECA	N-ethylcarboxamide-adenosine
NP	Nucleoside phosphorylase
NO	Nitric oxide
OVA	Ovalbumin
PCR	Polymerase chain reaction
PE	Phenylephrine
Penh	Enhanced pause
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
SAH	S-Adenosyl-L-homocysteine
SAH-hydrolase	S-Adenosyl-L-homocysteine-hydrolase
SAM	S-Adenosyl-L methionine
SEM	Standard error of the mean
SEN	Sensitized
XO	Xanthine oxidase

CHAPTER ONE

INTRODUCTION

Adenosine is an endogenous purine nucleoside with multiple physiological effects and is produced by all cells. Figure 1.1 shows the chemical structure of adenosine. Some of its regulatory activities are vasodilation of the coronary vessels, including under ischemic conditions, neuromodulation especially in the sleep-wake cycle, effects on the heart including regulating inotropy, chronotropy and many others (Fredholm, 2007).

Generation of adenosine and its metabolism

Under normal physiological conditions, adenosine is formed by the intracellular conversion of S-adenosyl-L methionine (SAM) to S-adenosyl-L-homocysteine (SAH). This moiety is then converted to adenosine and homocysteine by SAH-hydrolase (Deussen et al., 1989; Pelleg and Porter, 1990). Adenosine can also be produced extracellularly through successive dephosphorylation of ATP; ATP is dephosphorylated to ADP and then AMP which is converted to adenosine via ecto-5'-nucleotidases (Bardenheuer and Schrader, 1986; Forsythe and Ennis, 1999). Adenosine thus produced can be further converted to inosine by the enzyme adenosine deaminase (ADA) and finally is broken down to uric acid which is excreted in urine. There is another pathway through which adenosine is re-converted to AMP via the enzyme adenosine kinase (AK). In addition to being metabolized, adenosine can also act on specific receptors to induce various physiological effects. Figure 1.2 shows the metabolic processes of adenosine production and breakdown. Extra-cellular concentrations of adenosine are elevated several fold during periods of increased metabolic demand, injury or stress such as ischemia (Sparks and Bardenheuer, 1986).

Figure 1.1 Structure of adenosine

Figure 1.2 Metabolic pathways of adenosine

Adenosine receptors

Adenosine produces various effects through cell surface purinergic receptors and thus far, 4 subtypes have been identified: A₁, A_{2A}, A_{2B} and A₃. All four receptors are G-protein coupled and the effects that adenosine produces depends on both the receptor subtype that is activated as well as the organ/tissue in which the receptor is present. A₁ and A₃ receptors are coupled to G_i while the A_{2A} and A_{2B} receptors are coupled to G_s. A₁ and A_{2A} have a high affinity for adenosine, while the A_{2B} and A₃ receptors are considered to be low affinity. The A₁ receptor signals through G_{i/o} G-proteins and its activation leads to an inhibition of adenylyl cyclase (AC) which causes a lowering of cyclic AMP (cAMP) levels. Signaling through the A₁ receptor can also lead to activation of IP₃/DAG through the PLC pathway (Tawfik et al., 2005). The A_{2A} receptor signals through the G_s pathway and its activation leads to stimulation of AC resulting in increased production of cAMP. Activation of the A_{2A} receptor produces mainly anti-inflammatory effects and this receptor is involved in coronary vasodilation. The A_{2B} receptor signals through G_{s/q} and its activation can either result in increased cAMP or IP₃/DAG and Ca²⁺ levels. Lastly, the A₃ receptor signals through G_i and is negatively coupled to AC.

Asthma

As previously mentioned, adenosine has varied effects. Of particular interest to this work is the role of adenosine in asthma. Asthma is a chronic lung disease characterized by inflammation and bronchoconstriction. There is a growing incidence of asthma in the U.S. such that many experts believe the country is in the midst of an asthma epidemic. Although there are many treatment options available to manage symptoms, there is no cure for this disease. Over 20 million people have been diagnosed with

asthma, with 9 million under the age of 18 years; children are particularly susceptible. Allergic asthma accounts for about half of these numbers. Several hypotheses have been put forward to explain the growing asthma epidemic including the hygiene hypothesis and genetic predisposition.

The disease consists of episodic events which manifest as hyper-responsiveness of the airways to common triggers like dust, pollen, cold air, cigarette smoke and other pollutants, resulting in bronchospasm and difficulty in breathing. It can also be induced by exercise and inhalation of cold air. Airway obstruction occurs due to inflammation, excessive mucus production, and hyperreactive bronchial smooth muscle. As a consequence, hypoxemia secondary to hypoventilation is present. Severe acute asthmatic episodes can be life-threatening and require urgent care. Failure to treat prolonged respiratory failure can result in death. Initially, the inflammation observed in asthma is acute in nature but eventually becomes chronic with the progression of the disease, and long term effects of asthma are linked its inflammatory component. Disease management consists of anti-inflammatory drugs and bronchodilators.

Adenosine and asthma

Adenosine has long been implicated in the pathogenesis of asthma. Inhalation of adenosine was shown to be a potent bronchoprovocant in asthmatic patients (Cushley et al., 1983). This was one of the first studies to demonstrate a role for adenosine in asthma and it established that guanosine, another purine nucleoside, did not have the same effect. Inosine, the deaminated adenosine metabolite, did not produce bronchoconstriction (Mann et al., 1986b), suggesting that the effect on bronchial smooth muscle was specific to adenosine and possibly involved adenosine receptors. Asthmatic patients also had

significantly higher levels of adenosine in bronchoalveolar lavage fluid (BAL) and exhaled breath condensate than normal healthy subjects (Driver et al., 1993; Huszar et al., 2002). Adenosine can also have indirect effects in the lungs; it causes degranulation of mast cells which leads to the release of histamine and subsequent cascade of events including H_1 -receptor-mediated bronchoconstriction and inflammatory response (Fozard and Hannon, 2000; Oldenburg and Mustafa, 2005). This mast-cell mediated response is believed to occur through the A_{2B} receptor although this has not been conclusively proven (Zhong et al., 2003).

Studies done using mice lacking the ADA enzyme have shown that these mice have large amounts of adenosine in their lungs and severe lung inflammation (Blackburn et al., 2000). In fact, these mice were unable to survive beyond three weeks due to respiratory distress. Further studies using ADA deficient mice demonstrated the presence of eosinophilia in the lungs and periphery, extensive mast cell degranulation and increased levels of serum IgE (Zhong et al., 2001). There is evidence that adenosine causes recruitment of inflammatory cells to the lung (Fan and Mustafa, 2002) and amplification of the inflammatory response (Spicuzza et al., 2006). Despite all of these data linking adenosine to asthma, the exact extent of adenosine implication and specific roles of the four adenosine receptors still have to be delineated.

Reactive airway diseases and cardiovascular complications

An interesting facet noted in epidemiological studies is that people suffering from asthma, chronic obstructive pulmonary disease (COPD) and reactive airway diseases are at an increased risk for developing cardiovascular disease (CVD) (Gan et al., 2004; Schanen et al., 2005; Sin and Man, 2005). There is an association between atherosclerosis and stroke with reactive airway diseases. One study found that adult-onset asthma was associated with increased carotid atherosclerosis in women (Onufrak et al., 2007). Many studies have shown that impaired lung function is a risk factor for CVD (Tockman et al., 1995). Bronchial hyperresponsiveness to methacholine is associated with increased carotid intima-media thickness (Zureik et al., 2004). One of the leading causes for hospitalizations and deaths occurring in COPD patients is cardiovascular events (Sidney et al., 2005).

Inflammation is a key event in CVD and several inflammatory mediators and markers are associated with it, including C-reactive protein (CRP), plasma fibrinogen, leucocytes, and interleukins (IL-1 β , -4, -5, -6, -8 and -13). CRP has been implicated in atherosclerosis, and levels of this protein are elevated in this condition. The level of CRP is considered one of the best predictors of the risk of CVD (Yudkin et al., 1999; Pearson et al., 2003). Interleukins, in particular IL-6 (Yudkin et al., 2000), are also important markers of CVD.

Recent evidence in animal models indicates that CV complications associated with asthma are independent of asthma therapy and could be a result of asthma itself. Myocardial ischemia-reperfusion injury was enhanced in a rabbit model of systemic allergy and asthma (Hazarika et al., 2004) and allergic inflammation in the airways

enhanced neutrophil recruitment to the myocardium and severity of ischemia-reperfusion in a murine model (Hazarika, 2005).

Asthma thus, is a risk factor for cardiovascular disease. However, its potential as a risk factor in CVD has not been widely studied with respect to mechanisms underlying the phenomenon, nor have the effects of adenosine been explored on cardiovascular parameters in asthma. Work done in this dissertation attempts to shed light on both of these aspects.

Animal models of asthma

The use of animal models has been one of the mainstays for asthma research. Species including mice, rats, rabbits, guinea pigs, sheep and dogs amongst others have been used (Abraham et al., 1983; Muccitelli et al., 1987; de Weck et al., 1997; Liu et al., 2005). There are several advantages and disadvantages in employing animal models to study asthma. The principle criticism in using animal models of asthma is that none of the species develop the disease spontaneously nor exhibit the episodic airway hyper-responsiveness of the disease that is characteristic of the human condition, i.e., it is not possible to reproduce the exact human disease in animals. The asthma phenotype generated in animal models is a Th-2 type response with acute inflammation and increased responsiveness to bronchoprovocants observed. The main advantage to using animal models is the ability to study intact systems and carrying out *in vivo* experiments. Many of these models are also cost effective and a number of species specific probes have been developed which aid greatly in research. For mechanistic purposes, animal models are extremely useful and also for drug screening purposes.

Murine models of asthma

Mice are very commonly used to study asthma, especially for modeling allergic responses in the airways and lungs. With the increasing number of genetically modified mice available, these are ideal tools for studying specific aspects of the disease. There are knock-out mice for all four adenosine receptors as well as mice in which ADA has been knocked out and double knock-outs with specific adenosine receptors. Mice in which specific cytokines have been knocked out are also available to study inflammatory signaling with respect to asthma. These include IL-13 knock out mice which exhibit a lack of airway remodeling characteristic of asthma (Kumar et al., 2002). One of the main advantages of using mice to duplicate certain asthmatic features is the ease of various tools with which allergic models can be developed and also the easy availability and cost effectiveness of such models. Mice can be sensitized to various antigens including dust-mite (O'Brien et al., 1996), (Johnson et al., 2004), ragweed extracts (Oldenburg and Mustafa, 2005; Fan and Jamal Mustafa, 2006), and one of the more commonly used allergens, ovalbumin, in a reasonable time frame (Nadeem et al., 2007).

Purpose and Project Summary

The purpose of this investigation is to understand the effects of asthma beyond the lungs, specifically to determine if there is any alteration in vascular reactivity. Since adenosine has major implications in both asthma and on the vasculature, it is important to elucidate the adenosine-mediated systemic and vascular effects in asthma. Lastly, identifying the possible adenosine receptor(s) involved in inflammatory and other changes would be able to provide further insights in understanding the profound and diverse effects of adenosine in asthma. Determining which receptor is involved in the vascular changes is necessary for developing potential therapeutic approaches to asthma, particularly with respect to targeting and reducing the commonly reported adverse vascular effects that are a consequence with asthma. With this in mind, the following aims were developed:

Aim 1: *Identify markers of systemic and local arterial inflammation in an allergic murine model of asthma.*

Hypotheses

(a): Chronic inflammatory disease of the airway and lung leads to an increase in systemic levels of several inflammatory mediators including TNF- α , IL-1 β , IL-4, and IL-

13

(b) Chronic inflammatory disease of the airway and lung can lead to local inflammation of blood vessels (aorta).

Aim 2: *Characterize the adenosine receptor sub-type(s) involved in altered vascular response and quantify their expression levels in our model of asthma.*

Hypotheses

(a) Multiple adenosine receptors (A_1 , A_{2A} , A_{2B} , and A_3) regulate vascular responses and are differentially activated in an allergic inflammatory model.

(b) Specific adenosine receptor(s) (A_1 , A_{2A} , A_{2B} , A_3) are either up-regulated or down-regulated in asthma

Aim 3: *Identify the adenosine receptor subtype(s) involved in vascular inflammation.*

Hypothesis: The interaction between inflammatory mediators and adenosine through specific adenosine receptors is central to a cascade of pro-inflammatory events.

CHAPTER TWO

MATERIALS AND METHODS

Mice

Balb/c mice, 8-10 weeks old males were obtained from Harlan Sprague Dawley (Indianapolis, IN). To study vascular effects of A_{2A} receptor-activation, A_{2A} KO and WT mice were used which were initially obtained from Dr. Catherine Ledent, University of Brussels, Belgium and maintained as a breeding colony at WVU. Male and female mice aged 12-14 weeks were used for all A_{2A} adenosine receptor experiments. A_1 KO mice were kindly provided by Dr. Stephen Tilley, University of North Carolina, Chapel Hill and mice aged 8-12 weeks were used for experiments. All mice were kept in community cages with 12-hr periods of light and dark cycles and were maintained on a ragweed-free diet with access to water *ad libitum*. All animal care and experimentation used in this study were carried out under protocols approved by the Institutional Animal Care and Use Committee of West Virginia University (Animal Use Protocol # 05-0603 and #06-1009); animal procedures were monitored by this committee and were done in accordance with the provisions of the NIH "Guide for the Care and Use of Laboratory Animals."

Animal sensitization protocol

Sensitization of mice was performed according to the previously established protocols including one described from this laboratory (Fan and Mustafa, 2002; Fan et al., 2003; Oldenburg and Mustafa, 2005; Fan and Mustafa, 2006; Mustafa et al., 2007). This model of allergic asthma has been shown to develop airway inflammation and

airway hyper-reactivity to methacholine. In brief, mice were sensitized on days 1 and 6 with i.p. injections of ragweed allergen (Greer Laboratories, Lenoir, NC), 200 µg per dose, with 200 µL Imject Alum (Pierce Laboratories, Rockford, IL). Non-sensitized control animals received only the Imject alum with the same volumes. Ten days after sensitization, the mice were placed in a Plexiglas chamber and challenged with 1% aerosolized ragweed or with 0.9% saline as a control, using an ultrasonic nebulizer (DeVilbiss, Somerset, PA) for 20 min both in the morning and afternoon for three consecutive days. The aerosolization of allergen was performed at a flow rate of 2 mL/min, and the aerosol particles had a median aerodynamic diameter of less 4 µm (DeVilbiss).

In addition, acute elevations in lung adenosine levels were produced experimentally by adenosine inhalation (6 mg/ml for 2 min on day 14) in one group of sensitized-challenged mice and one group of control mice to further enhance allergen-induced effects. This dose was chosen based on previous studies in this model (Fan and Mustafa, 2002; Fan and Mustafa, 2006). The time frame for experiments was 24-hr post-adenosine inhalation, chosen on the basis of an earlier study from this lab which showed maximum eosinophilic inflammation in airways after 24 hr of adenosine inhalation (Fan and Jamal Mustafa, 2006), which is a hallmark of asthmatic inflammation. Twenty-four hours after the last challenge, animals were sacrificed for collection of aorta and blood for further experiments.

In-vitro* organ bath: For vascular reactivity (aorta)*Preparation of mouse isolated aorta and isometric force measurement**

Mice were sacrificed by anesthesia with pentobarbitol sodium (65 mg/kg, i.p.) followed by thoracotomy and removal of the aorta. This was then carefully cleaned to remove fat and connective tissue without damaging the tissue and was then cut transversely into 3-4 mm rings. The rings were mounted vertically between two stainless steel wire hooks and then suspended in 10 mL organ baths containing Krebs-Henseleit buffer. The Krebs-Henseleit buffer (pH 7.4) containing 118 mM NaCl, 4.8 mM KCl, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 25 mM NaHCO₃, 11 mM glucose and 2.5 mM CaCl₂ was maintained at 37°C with continuous bubbling of 95% O₂ and 5% CO₂. For measurement of isometric force response, aortic rings were equilibrated for 90 min with a resting force of 1g (Tawfik et al., 2005; Ansari et al., 2007b) and change of the bathing solution at 15 min interval. At the end of equilibration period, tissues were contracted twice with 50 mM KCl to determine the contractility of individual aortic rings, which were washed out with Krebs-Henseleit buffer. Aortic rings were then constricted with phenylephrine (PE, 10⁻⁷M) to obtain a steady contraction and changes in tension were monitored continuously with fixed range precision force transducer (TSD, 125 C, BIOPAC system) connected to the differential amplifier (DA 100B, BIOPAC system). The data were recorded using a MP100 digital acquisition system and analyzed using Acknowledge 3.5.7 software (BIOPAC system). After equilibration, the responsiveness and stability of individual rings were checked by successive administration of a submaximally effective concentration of PE (10⁻⁷ M). The integrity of the vascular endothelium was assessed

pharmacologically by acetylcholine (ACh; 10^{-7} M) to produce relaxation of PE pre-contracted rings. A relaxation of $\geq 50\%$ with ACh was considered as normal. Aortic rings were then washed several times with Krebs-Henseleit solution, and allowed to equilibrate for 30 min before the experimental protocol began in which concentration-responsiveness to various drugs was measured including NECA (non-selective adenosine analog; 10^{-11} - 10^{-5} M), adenosine (10^{-11} - 10^{-4} M) and CGS 21860 (selective A_{2A} agonist; 10^{-11} - 10^{-6} M) were run parallel in aortic rings. In all cases, the drug was added to yield the next higher concentration only when the response to the earlier dose reached a steady state. In experiments where the effects of an antagonist were measured, it was added 30 min before contraction of the tissue with PE, and was present throughout the experiments.

Contraction/relaxation responses were expressed as a percentage increase/decrease in the contraction with respect to PE (alone) in response to each concentration of agonist used.

Airway responsiveness

Airway hyperresponsiveness was measured by whole body plethysmography. Unrestrained mice were placed in Plexiglass chambers (Buxco Electronics, Wilmington, NC) and pressure fluctuations during ventilatory cycles were monitored by pressure transducers. Data was calculated in the form of enhanced pause (Penh) according to a previously described protocol (Hamelmann et al., 1997).

Penh is a dimensionless index of airway obstruction, and higher the Penh value, the greater is the obstruction. The waveform (Hamelmann et al., 1997; Drazen et al., 1999) describing the process of Penh calculation is shown in figure 2.1 and

mathematically, Penh is obtained by the following equation (Hamelmann et al., 1997; Drazen et al., 1999).

$$\text{Penh} = \text{PEF}/\text{PIF} * (\text{TE}/\text{Rt} - 1)$$

PEF= Peak expiratory height

PIF= Peak inspiratory height

TE= Expiratory time

Rt= Time to expire 65% of the volume

Pause= (TE/RT)-1

Penh= PEF/PIF * Pause

Figure 2.1: Ventilatory cycle from whole body plethysmography. T_e =expiratory time;
 R_t = Time to expire 65% of the volume; PEF = Peak expiratory height; PIF = Peak
 inspiratory height

CHAPTER THREE

ADENOSINE-MEDIATED ALTERATION OF VASCULAR REACTIVITY AND INFLAMMATION IN A MURINE MODEL OF ASTHMA

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Abstract

Chronic respiratory disorders such as asthma are believed to be associated with adverse cardiovascular events. We hypothesize that asthmatic inflammation translates into systemic inflammation and alters vascular responses where adenosine (AD) plays an important role. Therefore, this study investigated the effects of aerosolized AD, used to elevate lung AD levels, on vascular reactivity and inflammation in our allergic mouse model of asthma. Balb/c mice were divided into four groups: control (CON), CON + aerosolized AD (CON + AD), allergen sensitized and challenged (SEN), and SEN + aerosolized AD (SEN + AD). The animals were sensitized with ragweed (200 µg ip) on *days 1* and *6*, followed by 1% ragweed aerosol challenges from *days 11* to *13*. On *day 14*, the CON + AD and SEN + AD groups received a single AD aerosol challenge (6 mg/ml) for 2 min, followed by the collection of the aorta and plasma on *day 15*. Organ bath experiments showed concentration-dependent aortic relaxations to AD in the CON and CON+ AD groups, which were impaired in the SEN and SEN + AD groups. Real-time PCR data showed changes in aortic AD receptors (ARs), with the expression of A₁ARs up-regulated, whereas the expression of A₂ARs and endothelial nitric oxide synthase

genes were down-regulated, resulting in an impairment of vasorelaxation in the SEN and SEN + AD groups. The A₁AR antagonist 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) reversed the impairment in vasorelaxation observed in the SEN and SEN + AD groups, whereas the A_{2B}AR antagonist alloxazine inhibited vasorelaxation in all groups. Allergen challenge caused systemic inflammation in allergic mice, with AD aerosol further enhancing it as determined by the inflammatory cytokines profile in plasma. In conclusion, asthmatic mice showed altered vascular reactivity and systemic inflammation, with AD aerosol further exacerbating these effects.

Introduction

Asthma, a chronic disease affecting 10% of the population in North America, is characterized by perpetuation and amplification of inflammation in the airways and systemic circulation (Busse and Lemanske, 2001; Braman, 2006). Over the past two decades, adenosine has been increasingly implicated in the pathophysiology of asthma. Chronic cellular stress, inflammation and tissue damage observed in asthmatic airways are associated with increases in adenosine levels (Driver et al., 1993). The inhalation of adenosine leads to bronchoconstriction in asthmatics as opposed to normal subjects who show no response to adenosine (Cushley et al., 1984). The elevation in lung adenosine levels observed in adenosine deaminase (ADA)-deficient mice lead to severe pulmonary inflammation, airway hyper-reactivity and airway remodeling (Chunn et al., 2001; Blackburn and Kellems, 2005), suggesting that a chronic elevation of adenosine levels can lead to the promotion of lung inflammation.

Several epidemiological studies have reported that people suffering from chronic respiratory diseases such as asthma are at an increased risk for developing cardiovascular complications (Drislane et al., 1987; Toren and Lindholm, 1996; Iribarren et al., 2004; Knoflach et al., 2005). Systemic inflammation in these patients is believed to be a consequence of airway inflammation (Jousilahti et al., 2002; Nadeem et al., 2003), which may be one of the reasons for altered cardiovascular parameters in these patients. Some recent studies have also reported enhanced systemic inflammation, myocardial ischemia-reperfusion injury and neutrophil recruitment to the myocardium in animal models of allergic asthma (Hazarika et al., 2004, 2007). However, no study so far has attempted to establish a link between airway inflammation, vascular reactivity and systemic inflammation, especially in relation to adenosine.

Adenosine has a well established role in the control of vascular tone, and its effects are exerted through the activation of the four different adenosine receptor (AR) subtypes: A₁, A_{2A}, A_{2B}, and A₃ (Tabrizchi and Bedi, 2001). A₁ and A₃ ARs have been shown be involved in vasoconstriction (Shepherd et al., 1996; Talukder et al., 2002b; Hansen et al., 2003; Tawfik et al., 2005) whereas A_{2A} and A_{2B} ARs cause vasorelaxation of aorta (Lewis et al., 1994; Grbovic and Radenkovic, 2003; Ansari et al., 2007b). However, the modulation of aortic ARs expression in response to allergen and adenosine aerosol challenge as an added insult in a murine model of asthma has not been previously studied.

Allergen has been shown to cause the release of adenosine among other inflammatory mediators from allergic lung and activated leukocytes (Mann et al., 1986b; Mann et al., 1986a). Adenosine generated in allergic lungs may be involved in the release

of further chemotactic and inflammatory mediators in the lung and systemic circulation by acting on its receptors present on different cells including mast cells, eosinophils, neutrophils and other inflammatory cells (Driver et al., 1991; Fan and Jamal Mustafa, 2006; Nadeem and Mustafa, 2006; Spicuzza et al., 2006). This has been confirmed by recent observations where experimentally induced temporary elevations in lung adenosine levels through the inhalation of adenosine (resulting from breakdown of adenosine 5'-monophosphate) have been shown to cause an increase in infiltration of eosinophils in patients with asthma (van den Berge et al., 2004). A recent study from our lab has also shown increased release of inflammatory cell markers in the lung and plasma after inhalation of adenosine aerosol in allergic mice (Fan and Mustafa, 2002). These studies thus suggest that adenosine inhalation amplifies allergen-induced airway inflammation in the lungs.

Therefore, this study was undertaken to investigate the effects of inhaled adenosine on vascular reactivity and systemic inflammation using our murine model of asthma. Our data suggest that asthmatic mice have altered peripheral vascular reactivity and systemic inflammation, with inhaled adenosine further exacerbating these effects.

Materials and Methods

Animals: Balb/c mice, 8-10-wk-old males, were obtained from Harlan Sprague Dawley (Indianapolis, IN). The animals were maintained on a ragweed-free diet. All experimental animals used in this study were under a protocol approved by the Institutional Animal Care and Use Committee of West Virginia University.

Animal sensitization: Sensitization was performed according to the protocol described

earlier from this laboratory (Fan and Mustafa, 2002; Fan et al., 2003; Oldenburg and Mustafa, 2005; Fan and Jamal Mustafa, 2006; Mustafa et al., 2007). This model of allergic asthma has been shown to develop airway inflammation and airway hyper-reactivity to methacholine. The study comprised of four groups of animals: 1) Control (CON), 2) Control challenged with 6 mg/ml of adenosine aerosol for 2 min on day 14 (CON + AD), 3) allergen sensitized and challenged (SEN), and 4) allergen sensitized and challenged further challenged with 6 mg/ml of adenosine aerosol for 2 min on day 14 (SEN + AD). Mice were sensitized on days 1 and 6 with i.p. injections of ragweed allergen (Greer Laboratories, Lenoir, NC), 200 µg per dose with 200 µL Imject Alum (Pierce Laboratories, Rockford, IL). Non-sensitized control animals received only the Imject alum with the same volumes. Ten days after sensitization, the mice were placed in a Plexiglas chamber and challenged with 1% aerosolized ragweed or with 0.9% saline as a control, using an ultrasonic nebulizer (DeVilbiss Somerset, PA) for 20 min both in the morning and afternoon for three consecutive days. The aerosolization of allergen was performed at a flow rate of 2 mL/min, and the aerosol particles had a median aerodynamic diameter of less 4 µm (De Vilbiss).

Acute elevations in lung adenosine levels were produced experimentally by adenosine inhalation (6 mg/ml for 2 min on day 14) to SEN+AD group to further enhance allergen-induced effects (the control for this group was CON+AD). This dose was chosen based on our previous studies in this model (Fan and Mustafa, 2002; Fan and Jamal Mustafa, 2006). Adenosine inhalation in this model has been shown to enhance allergen-induced airway inflammation and airway hyper-reactivity to adenosine (or its analog NECA) (Fan and Mustafa, 2002; Fan et al., 2003; Fan and Jamal Mustafa, 2006). ADA-

deficient mice having sustained and chronic elevations in lung adenosine levels have also been shown to have similar features (Chunn et al., 2001). We chose 24-h post adenosine inhalation for our current studies based on an earlier study from our lab which showed maximum eosinophilic inflammation in airways after 24 h of adenosine inhalation (Fan and Jamal Mustafa, 2006), which is a hallmark of asthmatic inflammation. CON and SEN groups received only saline on day 14. Twenty-four hr after the last challenge, animals were sacrificed for collection of aorta and blood for further experiments.

Preparation of isolated mouse aorta and isometric force measurement

Mice were euthanized by anesthesia with pentobarbital sodium (65 mg/kg ip) followed by thoracotomy and the removal of the aorta, which was then cut transversely into 3 to 4-mm rings. The rings were mounted vertically between two stainless steel wire hooks and then suspended in 10-ml organ baths containing Krebs-Henseleit buffer. The Krebs-Henseleit buffer (pH 7.4), containing (in mM) 118 NaCl, 4.8 KCl, 1.2 MgSO₄, 1.2 KH₂PO₄, 25 NaHCO₃, 11 glucose, and 2.5 CaCl₂, was maintained at 37°C with continuous bubbling of 95% O₂-5% CO₂. For the measurement of isometric force response, the aortic rings were equilibrated for 90 min with a resting force of 1 g and a change of the bathing solution at 15-min intervals. The resting force of 1 g has been used earlier in our laboratory (Tawfik et al., 2005; Ansari et al., 2007b). At the end of the equilibration period, the tissues were contracted with 50 mM KCl to check the contractility of individual aortic rings twice, which were washed out with Krebs-Henseleit buffer. The aortic rings were then constricted with phenylephrine (PE; 10⁻⁷ M) to obtain a steady contraction, and changes in tension were monitored continuously with a fixed-range

precision force transducer (TSD 125C; Biopac) connected to a differential amplifier (DA 100B; Biopac). The data were recorded using the MP100 Biopac digital acquisition system and analyzed using Acknowledge 3.5.7 software (Biopac).

Contraction/relaxation experiments

After equilibration, the responsiveness and stability of individual rings were checked by successive administration of a submaximally effective concentration of PE (10^{-7} M). The integrity of the vascular endothelium was assessed pharmacologically by acetylcholine (10^{-7} M) to produce the relaxation of PE-precontracted rings. The aortic rings were then washed several times with Krebs-Henseleit solution and allowed to equilibrate for 30 min before the experimental protocol began.

Experimental protocol

The concentration-response curves (CRCs) for adenosine (10^{-11} – 10^{-4} M) were run in parallel in aortic rings from all the groups. In all cases, adenosine was added to yield the next higher concentration only when the response to the earlier dose reached a steady state. In experiments where the effects of an antagonist were measured, it was added 30 min before the contraction of the tissue with PE and was present throughout the experiments.

Real time PCR: A₁, A_{2A}, A_{2B}, A₃ AR and eNOS genes expression

The aortic tissues from all experimental groups were processed for total RNA isolation using the TRIzol reagent from Life Technologies/Invitrogen, followed by DNase

treatment to eliminate potential genomic DNA contamination as described recently by our laboratory (Ansari et al., 2007b). This was followed by a conversion of 0.5 µg of total RNA into cDNA using a high-capacity cDNA archive kit (Applied Biosystems, Foster City, CA) in a total volume of 100 µl. Real-time PCR was then performed using an ABI PRISM 7300 detection system (Applied Biosystems) using Taqman Universal Mastermix (Applied Biosystems, Branchburg, NJ) according to the instructions of the manufacturer. The reaction volume (25 µl) consisted of (in µl) 12.5 2x Taqman Universal Mastermix, 1 cDNA, and 1.25 20x 6-carboxy-fluorescein-labeled Taqman gene expression assay master mix solution. For the real-time PCR of the concerned genes [A_1 -, A_{2A} -, A_{2B} -, and A_3 AR and endothelial nitric oxide synthase (eNOS)], the Taqman inventoried assays-on-demand gene expression products (assay identifications for A_3 -, A_{2B} -, A_{2A} -, and A_1 AR and eNOS, respectively, are Mm00802076_m1, Mm00839292_m1, Mm00802075_m1, Mm01308023_m1, and Mm00435204_m1) were purchased from Applied Biosystems. 18S ribosomal RNA was used as an endogenous control. The fold difference in the expression of target cDNA was determined using the comparative cycle threshold method as described earlier (Livak and Schmittgen, 2001).

Assessment of systemic inflammation

Mice were euthanized by pentobarbitone sodium (65 mg/mL, i.p.), followed by collection of blood by cardiac puncture in heparinized syringes. The blood collected from different groups was centrifuged at 800 g for 10 min at 4° C and the resulting plasma was used for cytokine and C-reactive protein (CRP) assays.

Multiplex cytokine assay: Multiplex cytokine assay of IL-1 α , IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 (p70), , IL-13, IL-15, IL-17, TNF α , IFN γ , GM-CSF, MIP-1 α , MCP-1, KC, RANTES, IP-10, G-CSF in the plasma was measured by a commercial kit from Linco Research (St. Charles, Missouri) using the Luminex 200 system (Luminex Corp., Austin, Tx), which is a multianalyte bioassay detection system capable of performing up to 100 assays simultaneously in a single microtiter plate well. This system uses polystyrene microspheres internally dyed with red and infrared fluorophores that can be individually identified. The fluorescent microspheres were coated with capturing antibodies specific for different chemokines. The chemokine-captured beads after incubation with the sample were then mixed with phycoerythrin-conjugated detection antibodies to form immune complexes. Following incubation, washing and acquisition of fluorescence data, the concentration results were generated in graphical format using the standard curve generated for each cytokine. Results were expressed in pg/mL.

C-reactive protein assay: C-reactive protein was measured in plasma with a mouse high-sensitive ELISA kit according to the instruction manual (Kamiya Biomedical Company, Seattle, WA). This kit specifically detects mouse CRP. Results were expressed in ng/mL.

Drugs used

Acetylcholine, adenosine and phenylephrine were dissolved in distilled water. 1,3-dipropyl-8-cyclopentylxanthine (DPCPX) and 2, 4-dioxobenzo[g] pteridine (alloxazine) were dissolved in 100% DMSO as a 10 mM stock solution. The final concentration of

DMSO in the organ bath which had a total volume of 10 mL, had no effect by itself on the aortic rings (Ansari et al., 2007b). Unless stated otherwise, all chemicals were of the highest grade available and were purchased from Sigma Chemicals (St. Louis, MO).

Statistical Analysis

The data were expressed as mean $\pm$ SEM. Comparisons among different groups were analyzed by ANOVA (analysis of variance) followed by Tukey's multiple comparison test/Bonferroni's selected pair test. Comparison between two groups was assessed by unpaired t-test. A *p* value of less than 0.05 was considered as the level of significance for all statistical tests. All the statistical analyses were performed using the Graph Pad Prism statistical package.

Results

Systemic inflammation in control, allergic and adenosine aerosolized allergic mice

Table 3.1 shows the multiplex cytokine profile in all the groups. Allergen challenge significantly increased plasma levels of inflammatory cytokines IL-5, IL-6, IL-13, and MCP-1 in sensitized (SEN) compared with control (CON) mice. Allergen-sensitized and challenged mice exposed to adenosine aerosol (SEN + AD) had the highest level of the pro-inflammatory cytokines including IL-1 β , IL-5, IL-13, MCP-1, MIP-1 α and TNF- α compared to other groups. Other cytokines in the panel were either undetectable or not different among the groups (data not shown). In addition, the cytokine profile was also studied in the CON + AD group. Cytokine levels were not different between this group of animals aerosolized with adenosine compared with the CON group, suggesting that

aerosolized adenosine in the absence of allergic inflammation had no effect on the inflammatory cytokine profile. CRP was also measured as an important systemic inflammatory marker. Plasma CRP levels significantly increased in the SEN compared with the CON group, with a further increase in the SEN + AD compared with the SEN group. Again, there was no difference between the CON and CON + AD groups in CRP levels. These data show that sensitized mice challenged with allergen have systemic inflammation and that aerosolized adenosine further enhances the inflammation in these mice. Earlier studies from our laboratory have also shown no difference in the lung inflammation of control mice aerosolized with adenosine (Fan and Mustafa, 2002; Fan and Jamal Mustafa, 2006).

Vascular reactivity in control, allergic and allergic + adenosine groups

The aortic relaxation/contraction responses to adenosine were studied in each of the following four groups: CON, CON + AD, SEN, and SEN + AD. Organ bath data showed a concentration-dependent relaxation response to adenosine in the CON aorta (Fig. 3.1). The maximum relaxation produced by adenosine (10^{-4} M) was 21% and 25% in the CON and CON + AD groups, respectively. There was no difference in the vasorelaxation between the CON and CON + AD groups. Adenosine-induced aortic relaxations were impaired in the SEN aorta with a maximum relaxation of 7%. The impairment of vasorelaxation response to adenosine observed in the SEN group was further aggravated in the SEN + AD aorta. Adenosine failed to elicit any relaxation response in the SEN + AD group; however, it produced a contractile response (-5%) at higher doses. These data

show that allergen sensitization and challenge altered the vascular reactivity, with adenosine aerosol further exacerbating it in allergic mice.

Endothelial function and eNOS expression in control, allergic and allergic + adenosine groups

The SEN and SEN + AD groups had significantly lower (~50% relaxation) endothelial response to acetylcholine (Fig 3.2A) compared with that of the respective controls (~75% relaxation). Real-time PCR data also showed that the expression of eNOS in the aorta was significantly decreased in the SEN and SEN + AD groups compared with the Con group (Fig 3.2B). These data show that allergen challenge probably led to dysfunctional endothelium in the SEN and SEN + AD groups.

With the aerosolization of adenosine, it is difficult to assess the exact amount of adenosine reaching the lungs. However, the dose we have chosen is based on previous work from our laboratory (Fan and Mustafa, 2002; Fan et al., 2003; Fan and Jamal Mustafa, 2006). This dose of adenosine (6 mg/ml) has no systemic effects, which has been shown by the lack of any systemic inflammation, improvement in vascular reactivity, and endothelial response in the CON + AD compared with the CON group. We also know that the half-life for adenosine is less than a minute. In fact, this was the reason why adenosine was chosen and not an adenosine analog. Most of the systemic effects of this dose would be gone within minutes. Therefore, it is unlikely that the effects of adenosine in this study are due to any hemodynamic changes in these animals.

Adenosine receptors gene expression in control, allergic and allergic + adenosine groups

To gain further insight into the pattern of the expression of ARs and their involvement in the impairment of vasorelaxation observed in the SEN and SEN + AD groups, we first measured the aortic gene expression of all ARs before conducting organ bath experiments. Real-time PCR data showed that A₁ (Fig 3.3A), A_{2A} (Fig 3.3B) and A_{2B}AR (Fig 3.3C) expression was decreased in the SEN compared with the CON group, whereas A₃AR expression remained unaffected (data not shown). The expression of A₁AR increased significantly in the SEN + AD compared with the SEN group, whereas A_{2A}- and A_{2B}ARs decreased further; however, only the decrease in A_{2B}AR expression reached statistical significance. These data show that an alteration in AR expression is responsible for the impairment of vasorelaxation observed in the SEN and SEN + AD groups.

Effect of specific A₁ AR (DPCPX) and A_{2B} AR (alloxazine) antagonists on vascular reactivity in control, allergic and allergic + adenosine groups

Based on real-time PCR data, we used the A₁AR antagonist to reverse the impairment observed in the SEN and SEN + AD groups since A₁AR activation has been shown to cause vasoconstriction in earlier studies from our laboratory (Tawfik et al., 2005). Treatment of the aorta with the A₁AR antagonist DPCPX (10^{-5} M) before concentration-response with adenosine resulted in a reversal of impaired vasorelaxation in the SEN and SEN + AD groups (Fig 3.4). DPCPX shifted the CRC to adenosine toward the left in the SEN and SEN + AD groups, with the vasorelaxation observed in these groups being similar to the CON group (Fig 3.1). A concentration-dependent relaxation was seen in

CON animals (CON and CON + AD), which was not affected by DPCPX except at the highest concentration of adenosine that was significant (data not shown).

We expected the inhibition of vasorelaxation by the blocking of A_{2B}AR since it is considered to be involved in the relaxation of the aorta (Grbovic and Radenkovic, 2003; Ansari et al., 2007b). The pretreatment of the aorta with the A_{2B}AR antagonist alloxazine (10⁻⁴ M) before CRC with adenosine resulted in the inhibition of vasorelaxation observed in the CON and CON + AD aorta. Alloxazine shifted the CRC to adenosine toward the right, indicating a contraction in the CON and CON + AD aorta (Fig 3.5A). Impaired vasorelaxation observed in the SEN and SEN + AD groups was also affected by prior treatment of the aorta with alloxazine (Fig 3.5B), with a further rightward shift of their respective CRCs. These data suggest that A₁- and A_{2B}ARs have a significant role in the impairment of vascular reactivity observed in the SEN and SEN + AD groups. DPCPX and alloxazine are selective inhibitors of A₁- and A_{2B}AR, respectively, and have been used by others and our laboratory in earlier studies (Rose Meyer et al., 2003; Tawfik et al., 2005; Ansari et al., 2007b).

Table 3.1: Systemic inflammation as assessed by levels of inflammatory cytokines in plasma of control (CON), adenosine aerosolized control (CON+AD), allergic (SEN) and adenosine aerosolized allergic (SEN+AD) mice; Values are expressed as mean $\pm$ SEM (n=5-8).

Inflammatory marker	CON	CON+AD	SEN	SEN+AD
IL-1 β (pg/ml)	3.46 $\pm$ 0.21	3.29 $\pm$ 0.09	3.2 $\pm$ 0.06	24.08 $\pm$ 10.32 ^{\$#}
IL-5 (pg/ml)	3.35 $\pm$ 0.14	4.19 $\pm$ 0.67	8.22 $\pm$ 1.65 ^{\$}	11.12 $\pm$ 1.50 ^{\$}
IL-6 (pg/ml)	10.42 $\pm$ 2.754	12.90 $\pm$ 3.79	60.47 $\pm$ 17.63 ^{\$}	43.85 $\pm$ 10.08 ^{\$}
IL-13 (pg/ml)	27.44 $\pm$ 5.57	33.19 $\pm$ 7.60	45.41 $\pm$ 9.91 ^{\$}	170.5 $\pm$ 49.97 ^{\$#}
MCP-1 (pg/ml)	37.26 $\pm$ 4.80	39.91 $\pm$ 4.59	56.48 $\pm$ 7.26 ^{\$}	119.6 $\pm$ 29.01 ^{\$#}
MIP-1 α (pg/ml)	51.06 $\pm$ 4.69	54.36 $\pm$ 8.13	53.38 $\pm$ 12.47	165.0 $\pm$ 31.57 ^{\$#}
TNF α (pg/ml)	6.66 $\pm$ 1.03	5.52 $\pm$ 0.360	6.25 $\pm$ 0.230	14.22 $\pm$ 3.60 ^{\$#}
C-reactive protein (ng/ml)	13.75 $\pm$ 0.69	14.51 $\pm$ 0.67	18.18 $\pm$ 1.83 ^{\$}	24.42 $\pm$ 2.42 ^{\$#}

^{\$} $p < 0.05$ as compared to CON; [#] $p < 0.05$ as compared to SEN

Figure 3.1 Concentration-response curves for adenosine mediated relaxation/contraction in control (CON), adenosine aerosolized control (CON+AD), allergic (SEN) and adenosine aerosolized allergic (SEN+AD) mice. Values are expressed as mean $\pm$ SEM ($n=8$). On the ordinate, positive and negative values indicate relaxation and contraction, respectively. * $P<0.05$ as compared to CON/CON+AD; # $P<0.05$ as compared to SEN.

Figure 3.2 Endothelial responses to ACh in control (CON), adenosine aerosolized control (CON+AD), allergic (SEN) and adenosine aerosolized allergic (SEN+AD) mice. (A) Effect of acetylcholine (10^{-7} M) on phenylephrine (PE) precontracted aorta ($n=8$); (B) Expression of eNOS by real time PCR ($n=4$). For gene expression by comparative C_T method using real time PCR, the CON group was used as the calibrator against which all other groups were compared. Values are expressed as mean $\pm$ SEM. * $P<0.05$ as compared to CON/CON+AD.

Figure 3.3 Expression of (A) A_1 , (B) A_{2A} , and (C) A_{2B} adenosine receptors by real time PCR in mice aorta of control (CON), allergic (SEN) and adenosine aerosolized allergic (SEN+AD) mice. For gene expression by comparative C_T method using real time PCR, the CON group was used as the calibrator against which all other groups were compared. Values are expressed as mean $\pm$ SEM ($n=4$). * $P<0.05$ as compared to CON; # $P<0.05$ as compared to SEN.

Figure 3.4 Effect of a specific A_1 AR antagonist, DPCPX (10^{-5} M) on adenosine-mediated relaxation/contraction in aorta of allergic (SEN) and adenosine aerosolized allergic (SEN+AD) mice. Values are expressed as mean $\pm$ SEM ($n=8$). On Y-axis, positive and negative values indicate relaxation and contraction respectively. * $P<0.05$ as compared to SEN/SEN+AD.

Figure 3.5 Effect of A_{2B} AR antagonist, Alloxazine (10^{-4} M) on adenosine-mediated relaxation/contraction in aorta of (A) control mice (CON); and (B) allergic (SEN) and adenosine aerosolized allergic (SEN+AD) mice. Values are expressed as mean $\pm$ SEM ($n=4-8$). On Y-axis, positive and negative values indicate relaxation and contraction respectively. * $P<0.05$ as compared to CON/CON+AD; # $P<0.05$ as compared to SEN/SEN+AD.

Discussion

The principal finding of the present study is that allergic mice had impaired vasorelaxation to adenosine and systemic inflammation, both of which were further aggravated after acute adenosine aerosol challenge in these mice. The AR expression profile was also altered in allergic mice and those aerosolized with adenosine after allergen challenge, which resulted in an impairment of vasorelaxation in these mice. Endothelium was found to be dysfunctional in both of these groups, since they had a decrease in aortic eNOS expression and endothelial relaxation to acetylcholine. The A₁AR antagonist DPCPX attenuated the impairment in vasorelaxation observed in allergic mice and those subjected to adenosine aerosol after allergen challenge, whereas the A_{2B}AR antagonist alloxazine impaired the vasorelaxation further in these groups, supporting a role for A_{2B}AR in aortic relaxation.

Airway inflammation is associated with chronic elevations in lung adenosine levels that may be due to the release of adenosine among other inflammatory mediators from sensitized lung and activated leukocytes (Mann et al., 1986b; Mann et al., 1986a; Driver et al., 1993). This is also experimentally supported by chronically increasing lung adenosine levels by knocking out ADA, an enzyme responsible for adenosine breakdown or temporarily by the inhalation of adenosine aerosol, as in this study. Mice deficient in the ADA enzyme develop severe pulmonary inflammation, airway hyperreactivity, and airway remodeling in association with the elevated levels of adenosine in the lungs (Chunn et al., 2001; Blackburn and Kellems, 2005). Adenosine inhalation to the allergic lungs has also been shown to increase further airway responsiveness and inflammatory mediators (Driver et al., 1991; Fan and Mustafa, 2002; van den Berge et al., 2004; Fan

and Jamal Mustafa, 2006), suggesting an important role for adenosine in the amplification of lung inflammation.

Systemic inflammation has also been reported earlier in humans with asthma and animal models of asthma (Jousilahti et al., 2002; Hazarika et al., 2004). Hazarika et al (Hazarika et al., 2007) recently showed an increase in neutrophils and eosinophils in the bronchoalveolar lavage and blood in a murine model of asthma. Systemic inflammatory markers have also been shown to be elevated in patients with asthma and in the allergic mouse model of asthma after the adenosine aerosol challenge performed earlier by our laboratory (Driver et al., 1991; Fan and Jamal Mustafa, 2006). We further extended these observations in the present study to investigate the cytokine profile in systemic circulation in our murine model of asthma. We found that allergic mice exposed to acute adenosine aerosol had increased systemic inflammation since several pro-inflammatory cytokines levels (IL-1 β , IL-5, IL-13, MCP-1, MIP-1 α and TNF- α) were found to be the highest in this group. A recent report from this laboratory using this model of asthma has also shown increased activities of eosinophilic peroxidase, myeloperoxidase and β -hexosaminidase not only in the lung but also in the systemic circulation of allergic mice exposed to adenosine aerosol (Fan and Jamal Mustafa, 2006). Adenosine aerosol in humans with asthma has also been shown to cause increased release of neutrophil chemotactic factor in serum (Driver et al., 1991). These studies suggest that adenosine enhances the release of systemic inflammatory mediators from sensitized inflammatory cells. Our murine model of asthma, therefore, was appropriate to study the inflammatory effects of adenosine in combination with allergen on vascular reactivity.

Asthma has been associated with an increased incidence of cardiovascular disorders (Drislane et al., 1987; Toren and Lindholm, 1996; Iribarren et al., 2004; Knoflach et al., 2005); however, there is little information regarding the role of asthmatic inflammation in the development of cardiovascular disease, especially in relation to the vascular effects of adenosine. In the present study, adenosine produced a concentration-dependent vasorelaxation in control mice, which was impaired in allergic mice. Vascular reactivity to adenosine was further altered in aorta of allergic mice that had received aerosolized adenosine. These data implicated a role for ARs in impairment of the vasorelaxation response in allergic mice and those challenged with adenosine aerosol after allergen. We have found that allergic animals subjected to theophylline inhalation before adenosine aerosol challenge had improved aortic vasorelaxation to the non-selective adenosine analog 5'-N-ethylcarboxamidoadenosine (NECA; unpublished data). This further supports a role for adenosine in impaired vasorelaxation.

As the direct effects of adenosine on hemodynamic parameters (blood pressure, oxygen saturations, and heart rate) were not assessed, it is possible that there could be adenosine-mediated effects on vasculature independent of inflammation in the allergic group (SEN + AD). The exact cause of why adenosine inhalation blunted aortic vasorelaxation to adenosine in sensitized and challenged mice, but not in control mice, is unclear. However, because the CON + AD group did not show a change in inflammatory markers and vasorelaxation to adenosine, it is likely that changes observed in the vasculature were due to allergen and adenosine in sensitized mice. Further studies are needed to completely assess the effects of adenosine on hemodynamic parameters in this model of asthma.

To gain further insight into the AR responsible for the alteration in vascular reactivity, changes in ARs expression in allergen and adenosine-aerosolized allergic mice were measured. Real time PCR data showed a down-regulation of A₁, A_{2A} and A_{2B} ARs in allergen-sensitized and challenged mice, the net result possibly being the impaired vasorelaxation observed in this group since it is known that A_{2A} AR and A_{2B} AR are involved in vasorelaxation (Grbovic and Radenkovic, 2003; Ansari et al., 2007b). A_{2A} and A_{2B} ARs expression decreased further and the A₁ AR expression increased in allergic mice exposed to adenosine aerosol compared with allergic mice, leading to aortic contraction in this group. This effect could be due to the combined net result of an increase in the A₁ AR and further decrease in the expression of A₂ ARs since the former is involved in vasoconstriction and latter in vasorelaxation, as shown in earlier studies from others and our laboratory (Lewis et al., 1994; Tawfik et al., 2005; Ansari et al., 2007b).

Real-time PCR studies of ARs prompted us to look further into the roles of A₁ AR and A_{2B} AR on vascular responses. The A_{2B} AR antagonist alloxazine blocked the vasorelaxation in controls and caused a further impairment in allergic mice and those exposed to adenosine aerosol after allergen challenge, suggesting that adenosine-mediated vasorelaxation could mainly be via the A_{2B} AR. The A₁ AR antagonist DPCPX reversed the impaired vasorelaxation in allergic mice and those exposed to adenosine aerosol after allergen challenge, suggesting that the A₁ AR has an important role in vasoconstriction, especially when the vasorelaxant A_{2B} AR is downregulated. In an unpublished observation from our lab, pretreatment of aorta with the selective A_{2A} antagonist, SCH 58261, showed a decrease in NECA-induced aortic relaxation in control

mice. However, SCH 58261 pretreatment did not have any significant effect on altered aortic relaxation to NECA observed in SEN and SEN + AD groups, suggesting a limited role in vascular reactivity for the A_{2A} AR in this particular experimental model. In conclusion, the data further support the involvement of A₁ AR in contraction and A_{2B} AR in relaxation of mice aorta in our model of allergic asthma.

The endothelium serves as an important regulator of vascular function and homeostasis by releasing mediators such as nitric oxide and prostaglandins (Mombouli and Vanhoutte, 1999); however, its role in relation to allergen exposure and adenosine challenge has not been explored. Allergen exposure probably contributed to the impairment in endothelial responses observed in allergic and adenosine aerosolized allergic mice since eNOS expression and vasorelaxation to acetylcholine was decreased equally in both of these groups. The exact cause of this dysfunction is not known but it could be possibly due to an increase in systemic inflammatory cytokines as they are reported to suppress eNOS and nitric oxide production (Cardaropoli et al., 2003; Li et al., 2004).

Endothelial dysfunction has been reported earlier after exposure to particulate matter in aorta of mice (Cozzi et al., 2006). Nurkiewicz et al (Nurkiewicz et al., 2004) have also shown impairment of endothelium-dependent arteriolar dilation in rat spinotrapezius muscle upon inhalation of particulate matter. Endothelial dysfunction caused by allergen exposure could be somewhat similar to that of particulate matter exposure in many ways. First, allergen particle size is in the same range as that of particulate matter. Second, particulate matter is known to cause lung inflammation, which spills over in systemic circulation, leading to endothelial dysfunction and other

cardiovascular complications (Nurkiewicz et al., 2006; Bai et al., 2007). It is also reported to be associated with pulmonary as well as cardiovascular disorders (Schwarze et al., 2006).

The fact that endothelial responses to acetylcholine and eNOS expression were almost equal in allergic mice and those exposed to adenosine aerosol after allergen challenge, whereas vasorelaxation responses to adenosine were different between these groups, could be due to two reasons. First, it could be due to the further increase in several pro-inflammatory cytokines found in allergic mice exposed to adenosine aerosol as some of these cytokines are reported to directly alter vascular function (Tousoulis et al., 2006). Second, it could be due to the increased A₁AR and decreased A_{2B} AR expression favoring aortic contraction in allergic mice exposed to adenosine aerosol. Further studies are required to delineate the role of each AR in alteration of vascular responses using specific AR knock-out mice.

In conclusion, our findings provide evidence for the first time that there are alterations in systemic parameters (inflammation, lower endothelial response and impaired vasorelaxation to adenosine) as a result of allergen exposure and adenosine aerosol challenge in sensitized mice. Therefore, therapeutic strategies designed to attenuate lung inflammation should also focus on the prevention of systemic inflammation and harmful systemic side effects. This strategy may be beneficial in lowering the incidence of adverse cardiovascular events in asthmatics.

CHAPTER FOUR

ROLE OF A_{2A} ADENOSINE RECEPTORS IN THE REGULATION OF VASCULAR TONE

Abstract

Adenosine mediates vascular responses through its 4 receptor subtypes: A₁, A_{2A}, A_{2B} and A₃. In the murine aorta, the A_{2B} receptor is believed to contribute to vasorelaxation while the role of the A_{2A} receptors is not well documented. The current study was undertaken to investigate the role of A_{2A} receptors on aortic tone using A_{2A} adenosine receptor knock-out (A_{2A}KO) and corresponding wild-type (A_{2A}WT) mice for experiments along with suitable pharmacological tools. Experiments included organ bath studies and real time RT-PCR for adenosine receptor (AR) gene expression studies. Isolated aortic rings prepared from A_{2A}WT and A_{2A}KO mice were precontracted with phenylephrine (10⁻⁷M) and concentration-response curves for adenosine analogs and selective agonists/antagonists were obtained. NECA (non-selective adenosine analog; EC₅₀ = 6.78μM) and CGS 21680 (A_{2A}AR selective agonist; EC₅₀ = 0.013μM) produced concentration-dependent relaxation (25% and 28% relaxation with NECA at 10⁻⁵M and CGS 21860 at 10⁻⁵M, respectively) in A_{2A}WT aorta. In A_{2A}KO aorta, NECA (EC₅₀ = 0.075μM) induced concentration-dependent contraction (47% at 10⁻⁶M; p<0.05 compared to A_{2A}WT) while CGS 21860 produced no response. SCH 58261 (10⁻⁶ M; A_{2A}AR selective antagonist) abolished vasorelaxation in A_{2A}WT (p<0.05) while no change was observed in A_{2A}KO. When DPCPX (10⁻⁵ M; A₁ selective antagonist) was used, greater

vasorelaxation was observed in A_{2A}WT (50% vs. 25% in controls; $p < 0.05$) while A_{2A}KO showed less contraction (5% vs. 47% in controls; $p < 0.05$). Aortic endothelial function, determined by the response to acetylcholine, was significantly greater in WT compared to KO (66% in WT vs. 51% in KO; $p < 0.05$). In conclusion, A_{2A}AR KO mice had significantly lower aortic relaxation and endothelial function, suggesting that the A_{2A}AR plays an important role in vasorelaxation, probably with the endothelium playing a role.

Introduction

Adenosine is a ubiquitous endogenous nucleoside that has several physiological effects which are mediated through the four adenosine receptor (AR) subtypes, i.e., A₁, A_{2A}, A_{2B} and A₃. Adenosine is known to modulate cardiac effects (Shryock and Belardinelli, 1997) and vascular responses. A₁ receptors are involved in lowering the heart rate (Evans et al., 1982; Belardinelli et al., 1989) and have a negative role in regulating blood pressure (Brown et al., 2006). All four receptors are involved in the regulation of vascular responses (Tabrizchi and Bedi, 2001). The vascular effects of adenosine differ according to the receptor subtype activated, the type of vascular bed involved and the species.

A₁ and A₃ ARs have been shown to be involved in vasoconstriction (Shepherd et al., 1996; Talukder et al., 2002b; Hansen et al., 2003; Tawfik et al., 2005) whereas A_{2A} and A_{2B} ARs cause vasorelaxation (Lewis et al., 1994; Grbovic and Radenkovic, 2003; Ansari et al., 2007b). The A_{2A}AR was found to play a major role in the mediation of coronary vasodilation caused by adenosine and adenosine agonists (Belardinelli et al., 1998). In rat kidneys, the vasoconstrictor response is mediated via the activation of A₁

receptors (Rossi et al., 1988) and both A_{2A} and A_{2B} receptors are present in renal microvasculature, contributing differentially to vasodilation (Tang et al., 1999). In the pulmonary circulation, findings suggest that adenosine induced relaxation is mediated via the A_{2B} receptor via a nitric oxide independent mechanism (Haynes et al., 1995). It has been found that in mice, adenosine-induced aortic relaxation is predominantly mediated via A_{2B} adenosine receptor activation (Talukder et al., 2002a) and the response is endothelium-mediated through nitric oxide release (Ansari et al., 2007b). In the guinea pig, the A_{2A} receptor is involved in coronary vessel relaxation whereas the A_{2B} receptor is present predominately in the aorta (Martin et al., 1993; Shryock et al., 1998). Similarly, the vascular effect of adenosine in aorta and coronary vessels of rat has been reported to be mediated by both A_{2A} and A_{2B} receptors (Lewis et al., 1994; Lewis and Hourani, 1997).

The A_{2A} receptor, though present in mouse aorta, has not been extensively studied in aortic relaxation and its role in the modulation of aortic vascular responses is unclear. This study was, therefore, undertaken to determine vascular responses mediated via the A_{2A} receptor. Both pharmacological tools and genetically modified mice in which the A_{2A} receptor was knocked out were used in the experiments. Isolated *in vitro* preparations of aortic rings from A_{2A}WT and KO mice were used to study the effects of A_{2A} adenosine receptors on vascular smooth muscle tone using adenosine analogs (NECA), selective agonists (CGS 21860-A_{2A} selective agonist; BAY 60-6583-A_{2B} selective agonist) and antagonists (SCH5830-A_{2A} selective antagonist; DPCPX-A₁ selective antagonist and alloxazine-A_{2B} selective antagonist). Gene expression of the four adenosine receptor subtypes was also determined using aorta from both A_{2A}WT and KO mice in real time

RT-PCR experiments. We hypothesized that adenosine A_{2A} receptors contributed to adenosine-mediated vascular relaxation, in part through an endothelium-dependent pathway. Our results indicate that the A_{2A} receptor does contribute to aortic relaxation, more than previously thought.

Materials and Methods

Mice

A_{2A} KO and wild-type (WT) mice were obtained from the Institute of Experimental Medicine (C. Ledent, Universite Libre de Bruxelles, Brussels, Belgium). To homogenize the genetic background of the mice, the first-generation heterozygotes were bred for 14 generations on a CD1 (Charles River) outbred background, with selection for the mutant A_{2A} gene at each generation by PCR. Fourteenth-generation heterozygotes were bred together to generate the A_{2A} KO and WT. Twelve to fourteen week old males and females were used for experiments. All experimental animals used in this study were under a protocol approved by the Institutional Animal Care and Use Committee of West Virginia University.

Preparation of isolated mouse aorta and isometric force measurement

Mice were sacrificed by anesthesia with pentobarbitol sodium (65 mg/kg, i.p.) followed by thoracotomy and removal of aorta that was then cut transversely into 3-4 mm rings. The rings were mounted vertically between two stainless steel wire hooks and then suspended in 10 mL organ baths containing Krebs-Henseleit buffer. The Krebs-Henseleit buffer (pH 7.4) containing 118 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, 1.2 mM

KH₂PO₄, 25 mM NaHCO₃, 11 mM glucose and 2.5 mM CaCl₂ was maintained at 37°C with continuous bubbling of 95% O₂ and 5% CO₂. For measurement of isometric force response, aortic rings were equilibrated for 90 min with a resting force of 1g (Tawfik et al., 2005; Ansari et al., 2007b) and change of the bathing solution at 15 min interval. At the end of equilibration period, tissues were contracted with 50 mM KCl to check the contractility of individual aortic rings twice which were washed out with Krebs-Henseleit buffer. Aortic rings were then constricted with phenylephrine (PE, 10⁻⁷M) to obtain a steady contraction and changes in tension were monitored continuously with fixed range precision force transducer (TSD, 125 C, BIOPAC system) connected to the differential amplifier (DA 100B, BIOPAC system). The data were recorded using MP100, BIOPAC digital acquisition system and analyzed using Acknowledge 3.5.7 software (BIOPAC system).

Contraction/relaxation experiments

After equilibration, the responsiveness and stability of individual rings were checked by successive administration of submaximally effective concentration of PE (10⁻⁷ M). The integrity of the vascular endothelium was assessed pharmacologically by acetylcholine (10⁻⁷ M) to produce relaxation of PE pre-contracted rings. Aortic rings were then washed several times with Krebs-Henseleit solution, and allowed to equilibrate for 30 min before the experimental protocol began. In experiments where the involvement of endothelium was investigated, endothelium was removed mechanically prior to mounting in the bath, using a thin stainless steel wire to scrape the lumen of the aorta.

Experimental protocol

The concentration-response curves (CRCs) for NECA (10^{-11} - 10^{-5} M), CGS 21860 (10^{-11} - 10^{-5} M) and BAY 60-6583 (10^{-11} - 10^{-5} M) were run in parallel in aortic rings from A_{2A} WT and KO mice. In all cases, the drug was added to yield the next higher concentration only when the response to the earlier dose reached a steady state. In experiments where the effects of an antagonist were measured, it was added 30 min before contraction of the tissue with PE, and was present throughout the experiments. Contraction/relaxation responses were expressed as a percentage increase/decrease in the contraction with respect to PE (alone) in response to each concentration of agonist used.

Real time PCR: A_1 , A_{2A} , A_{2B} , and A_3 AR genes expression

The aortic tissues were processed for total RNA isolation using the TRIzol reagent from Life Technologies/Invitrogen followed by DNase treatment to eliminate potential genomic DNA contamination as described recently by us (Ansari et al., 2007b). This was followed by conversion of 0.5 μ g of total RNA into cDNA using High Capacity cDNA archive kit (Applied Biosystems, Foster City, CA) in a total volume of 100 μ L. Real-time PCR was then performed using an ABI PRISM 7300 Detection System (Applied Biosystems) using Taqman Universal Mastermix (Applied Biosystems, Branchburg, New Jersey) according to the instructions of the manufacturer. The reaction volume (25 μ L) consisted of 12.5 μ L of 2X Taqman Universal Mastermix, 1 μ L of cDNA, and 1.25 μ L of 20X FAM-labeled Taqman gene expression assay master mix solution. For the real-time PCR of the concerned genes (A_1 AR, A_{2A} AR, A_{2B} AR and A_3 AR), the Taqman inventoried

assays-on-demand gene expression products (Assay IDs for A₃AR, A_{2B}AR, A_{2A}AR, and A₁AR respectively are Mm00802076_m1; Mm00839292_m1; Mm00802075_m1 and Mm01308023_m1) were purchased from Applied Biosystems (Foster City, CA). 18S rRNA (Ribosomal RNA) was used as an endogenous control. The fold difference in expression of target cDNA was determined using the comparative C_T method as described earlier (Livak and Schmittgen, 2001).

Drugs used

Acetylcholine, adenosine and phenylephrine were dissolved in distilled water. 1,3-Dipropyl-8-cyclopentylxanthine (DPCPX), 2-*p*-(2-carboxyethyl)phenethylamino-5' N-ethylcarboxamido adenosine hydrochloride (CGS 21680), SCH 58261, BAY 60–6583 and 2, 4-dioxobenzo[g]pteridine (alloxazine) were dissolved in 100% DMSO as a 10 mM stock solution. The final concentration of DMSO in organ bath (10 mL) had no effect by itself on the aortic rings (Ansari et al., 2007b). Unless stated otherwise, all chemicals were of the highest grade available and were purchased from Sigma Chemicals (St. Louis, MO).

Statistical Analysis

The data were expressed as mean±SEM. Comparisons among different groups were analyzed by ANOVA (analysis of variance) followed by Tukey's multiple comparison test/Bonferroni's selected pair test. Comparison between two groups was assessed by unpaired t-test. A *p* value of less than 0.05 was considered as the level of significance for

all statistical tests. All the statistical analyses were performed using Graph Pad Prism statistical package.

Results

Response to NECA, CGS-21680 and BAY 60–6583

The aortic relaxation/contraction responses to NECA, CGS-21680 and BAY 60-6583 were studied in A_{2A} WT and KO mice. Organ bath data showed a concentration-dependent contraction response to NECA, a non-selective adenosine analog, in A_{2A} KO aorta while a biphasic concentration response was observed in A_{2A} WT aorta (Fig. 4.1). The maximum contraction produced by NECA (10^{-6} M) was 45% in KO and maximum relaxation in WT was 25% (10^{-5} M). CGS-21680, a selective A_{2A} agonist, produced a concentration-dependent relaxation response in A_{2A} WT aorta (30% at 10^{-5} M; Fig 4.2) while BAY 60-6583, a selective A_{2B} agonist, produced relaxation in both A_{2A} WT and KO aorta (38% and 33% respectively at 10^{-5} M; Fig 4.3). These data show that A_{2A} WT and KO mice had different adenosine-mediated vascular-responses, with the A_{2A} and A_{2B} receptors eliciting relaxation.

Endothelial function in A_{2A} WT and KO aorta

A_{2A} KO mice had significantly lower (~50% relaxation) aortic endothelial response to acetylcholine (Fig. 4.4 A) compared to the respective WT (~75% relaxation in controls) group.

To determine the contribution of the endothelium to A_{2A} mediated relaxation, we next evaluated the CGS 21860 concentration-response relationship in A_{2A} WT aortic tissue in which the endothelium had been scraped off (-E), as described in the method

section. Removal of the endothelium abolished CGS-21860 induced relaxation (Fig 4.4 B), suggesting the A_{2A} receptor-mediated relaxation involved an endothelium dependent mechanism.

Adenosine receptors gene expression in A_{2A}WT and KO mice

To gain further insight into the pattern of adenosine receptors (AR) expression and their involvement in vasorelaxation observed in A_{2A}WT and KO groups, we measured aortic gene expression of all four ARs. Real time PCR data showed that there was no difference in receptor expression between A_{2A}WT and KO mice (Fig 4.5). These data suggest that the absence of the A_{2A} receptor contributed to lower NECA-mediated relaxation in A_{2A}KO mice.

Effect of specific A₁ AR (DPCPX), A_{2A} AR (SCH 58261) and A_{2B} AR (alloxazine) antagonists on vascular reactivity in A_{2A} WT and KO mice

Treatment of aorta with the A₁ AR antagonist, DPCPX (10⁻⁵ M; Fig. 4.6) prior to concentration response curve (CRC) with NECA resulted in concentration-dependent vasorelaxation in A_{2A}WT aorta with maximum relaxation being 50% at 10⁻⁵M NECA. In A_{2A}KO aorta, DPCPX lowered the magnitude of contraction response, however no relaxation response was observed. The selective A_{2A} antagonist, SCH 58261 (10⁻⁶ M) abolished NECA- and CGS 21860-mediated relaxation, respectively, in A_{2A}WT aorta (Fig 4.7 A and Fig 4.7 B) while it had no effect in A_{2A}KO tissue (data not shown).

We expected inhibition of vasorelaxation by blocking of A_{2B} AR as it is considered to be involved in relaxation of aorta from mice (Grbovic and Radenkovic,

2003; Ansari et al., 2007b). Pre-treatment of aorta with the A_{2B} AR antagonist alloxazine (10⁻⁵ M) prior to NECA concentration-response resulted in inhibition of vasorelaxation observed in both A_{2A}WT and KO aorta (Fig 4.8 and Fig 4.9).

Figure 4.1 Concentration-response curves for NECA-mediated relaxation/contraction in A_{2A} WT and A_{2A} KO mice. Values are expressed as mean $\pm$ SEM ($n=8$). On the ordinate, positive and negative values indicate relaxation and contraction, respectively. * $P<0.05$

Figure 4.2 Concentration-response curves for CGS 21860-mediated relaxation/contraction in A_{2A}WT and A_{2A}KO mice. Values are expressed as mean $\pm$ SEM ($n=8$). On the ordinate, positive and negative values indicate relaxation and contraction, respectively. * $p < 0.05$

Figure 4.3 Concentration-response curves for BAY60-6583-mediated relaxation/contraction in A_{2A} WT and A_{2A} KO mice. Values are expressed as mean $\pm$ SEM ($n=4$). On the ordinate, positive and negative values indicate relaxation and contraction, respectively.

Figure 4.4 **A)** Endothelial response in A_{2A} WT (~70%) and A_{2A} KO (~50%) mice to acetylcholine. Values are expressed as mean $\pm$ SEM ($n=8$). * $P<0.05$ **B)** CGS 21860 response in A_{2A} WT endothelium denuded (-E) aorta ($n=4-8$)

(A)

(B)

Figure 4.5 Adenosine receptor gene expression in A_{2A} WT and A_{2A} KO aorta. Values are expressed as mean $\pm$ SEM ($n=8$). There was no difference in the receptor expression levels between KO and WT mice, with the exception of the A_{2A} receptor expression (* $P<0.05$)

Figure 4.6 Effect of the selective A_1 antagonist DPCPX on NECA-mediated relaxation/contraction in A_{2A} WT and A_{2A} KO mice. Values are expressed as mean $\pm$ SEM ($n=8$). On the ordinate, positive and negative values indicate relaxation and contraction, respectively. Use of DPCPX produced significantly more relaxation in A_{2A} WT aorta (* $P<0.05$) compared to the response in untreated WT tissues. In the A_{2A} KO tissues, DPCPX attenuated the contractile response (# $P<0.05$) compared to untreated KO tissues.

Figure 4.7 Effect of the selective A_{2A} antagonist SCH 58261 on (A) NECA- and (B) CGS 21860-mediated relaxation/contraction in A_{2A} WT aorta. Values are expressed as mean $\pm$ SEM ($n=8$). On the ordinate, positive and negative values indicate relaxation and contraction, respectively. * $p<0.05$

(A)

(B)

Figure 4.8 Effect of the selective A_{2B} antagonist alloxazine (10^{-5} M) on NECA-mediated relaxation/contraction in A_{2A} WT mice. Values are expressed as mean $\pm$ SEM ($n=8$). On the ordinate, positive and negative values indicate relaxation and contraction, respectively. Use of alloxazine produced significantly less relaxation in A_{2A} WT aorta (* $P<0.05$) compared to the response in untreated WT tissues.

Figure 4.9 Effect of the selective A_{2B} antagonist alloxazine (10^{-5} M) on NECA-mediated relaxation/contraction in A_{2A} KO mice. Values are expressed as mean $\pm$ SEM ($n=8$). On the ordinate, positive and negative values indicate relaxation and contraction, respectively. Use of alloxazine produced more contraction in A_{2A} KO aorta compared to the response in untreated KO * $P<0.05$

Discussion

The primary objective of this study was to determine whether A_{2A} adenosine receptors play any role in aortic vasorelaxation in mice. Our principal finding is that A_{2A} KO mice had lower aortic vasorelaxation and endothelium relaxation response compared to WT counterparts. The data indicates a role for endothelium-dependent A_{2A} receptor mediated vasorelaxation in mouse aorta with the A_{2B} receptor also contributing to aortic vascular relaxation.

The A_2 receptors are involved in adenosine-dependent vasorelaxation in almost all vascular beds studied, with the A_{2A} and A_{2B} contributing to the responses (Mustafa and Askar, 1985; Ramgopal MV et al., 1988; Abebe et al., 1994; Mustafa and Abebe, 1996; Ralevic and Burnstock, 1998). In mouse aorta, high affinity A_{2A} receptors are present but their role in aortic vasorelaxation has not been established, while low-affinity A_{2B} receptors are involved in relaxation (Talukder et al., 2002a; Ansari et al., 2007b). We found that NECA, a non-selective adenosine analog, produced a concentration-dependent contraction response in A_{2A} KO aorta. Biphasic concentration response was observed in the A_{2A} WT counterpart, suggesting the activation of more than one receptor. This data indicated the relaxation response produced by NECA partly involved activation of A_{2A} receptors as in the absence of these receptors, only contraction was observed.

We next used CGS 21680, which is a selective A_{2A} AR agonist, and found that A_{2A} WT aorta displayed concentration-dependent relaxation to CGS 21680, while the A_{2A} KO tissue did not respond. SCH 58360, a selective A_{2A} AR antagonist was used to confirm the effects of CGS 21680 in activating the A_{2A} receptors. Relaxations to CGS 21680 and NECA in A_{2A} WT aorta were abolished with the use of the SCH compound,

further confirming that the A_{2A} receptor was involved in aortic relaxation. It should be noted that while NECA is capable of activating both A_{2A} and A_{2B} receptors, the A_{2B} receptor is low affinity and may be activated only at higher concentrations of NECA (Hussain and Mustafa, 1995; Hansen et al., 2003). The role of the A_{2B} receptor thus could not be ruled out. The involvement of A_{2B} receptors was supported by the inability of NECA to produce relaxation in the presence of alloxazine, a selective A_{2B} antagonist, in A_{2A} WT aorta while greater contraction was observed in the A_{2A} KO aorta. Interestingly, use of SCH 58360 in the NECA response produced steep concentration-dependent contraction in A_{2A} WT aorta, similar to NECA alone in A_{2A} KO tissue. The A_{2B} antagonist, alloxazine, produced contraction of a lower magnitude in A_{2A} WT aorta compared to KO. To further explore the possibility of the A_{2B} receptor contributing to relaxation, concentration-response to a new and selective A_{2B} agonist BAY 60-6583 was obtained. At lower concentrations, no response was seen in either A_{2A} WT or KO aorta while at higher concentrations (10^{-6} and 10^{-5} M) relaxation was observed in both A_{2A} WT and KO tissues.

The A_1 receptor is believed to mediate contraction in the mouse aorta (Prentice et al., 2001; Tawfik et al., 2005). NECA has also been reported to cause contraction in isolated carotid rings from both A_{2A} receptor-knockout and wild-type mice and this effect was eliminated by the selective A_1 receptor antagonist DPCPX (Prentice et al., 2002). We found that incubation of tissues with DPCPX prior to obtaining NECA concentration-response produced a concentration-dependent relaxation response in A_{2A} WT aorta and abolished the biphasic response seen with NECA alone. DPCPX also lowered the magnitude of NECA-mediated contraction observed in A_{2A} KO. DPCPX is a

highly selective A_1 receptor antagonist with a K_i of 3.9 nM compared with 130 for A_{2A} , 1,000 for A_{2B} , and 4,000 nM for A_3 (Klotz et al., 1998). Our data thus confirms that the A_1 receptor mediated contraction in the A_{2A} WT and KO aorta.

The endothelium has been shown to play an important role in adenosine mediated relaxation as well as contraction and various studies have reported effects that are either completely endothelium-dependent/independent, or partially endothelium-dependent in different isolated vessels (Rubanyi and Vanhoutte, 1985; Newman et al., 1988; Yen et al., 1988; Ansari et al., 2007b; Ansari et al., 2007a). Gauging the endothelial response in A_{2A} WT and KO aorta was thus important. We found the endothelial response to acetylcholine was significantly lower in A_{2A} KO aorta than WT. This prompted us to further study the effect of the endothelium in the adenosine-mediated relaxation that was observed. Mechanical removal of the endothelium totally abolished the endothelium-dependent relaxation response to acetylcholine and completely attenuated CGS 21860-induced relaxation. This data provides evidence for the first time that A_{2A} mediated aortic vasorelaxation is endothelium dependent as CGS 21860 is a selective A_{2A} agonist.

Studies have reported the up-regulation of the A_{2B} receptor in coronary arteries of A_{2A} KO mice as a possible compensatory mechanism (Teng et al., 2008). To determine the effect of knocking out the A_{2A} receptor on the expression of the other adenosine receptors in the aorta, we studied the adenosine receptor gene expression profile in isolated aorta. These studies showed that there was no difference in the aortic expression of the four adenosine receptors with the exception of the A_{2A} receptor which was absent in the A_{2A} KO as expected. Additionally, this supports our functional data in which we

found the selective A_{2B} receptor agonist BAY 60-6583 produced relaxation in both A_{2A} WT and KO aorta that was not different.

In conclusion, the data provided here indicate a role for A_{2A} receptors in mediating vascular relaxation in mouse aorta. The A_{2B} receptors also contribute to aortic relaxation, while the A_1 receptor mediates contraction. Finally, A_{2A} mediated relaxation appears to be completely endothelium-dependent. The exact mechanism and signaling pathways of this endothelium-dependent response remains to be elucidated.

CHAPTER FIVE

EVIDENCE FOR THE ROLE OF THE A₁ ADENOSINE RECEPTOR IN ALTERED VASCULAR RESPONSES AND INFLAMMATION IN ALLERGIC MICE

Abstract

Poor lung function and respiratory disorders such as asthma have a positive correlation with the development of adverse cardiovascular events. We hypothesize that asthmatic lung inflammation has a systemic component and alters vascular responses where adenosine (AD) plays an important role through A₁ adenosine receptors (A₁AR). This study used a murine model of allergic asthma to investigate the effects of aerosolized AD to elevate lung AD levels, on vascular reactivity and inflammation in genetically modified mice, in which the A₁AR was deleted (A₁KO) and corresponding wild type mice (A₁WT). Both A₁WT and A₁KO mice were divided into 3 experimental groups: Control (CON), allergen sensitized and challenged (SEN), and SEN+ aerosolized AD (SEN+AD). Animals were sensitized with ragweed (200 µg, i.p.) on days 1 and 6, followed by 1% ragweed aerosol challenges through days 11 to 13. On day 14, SEN+AD groups received a single AD aerosol challenge (6 mg/ml) for 2 min, followed by collection of aorta and plasma on day 15. Organ bath experiments showed concentration-dependent aortic relaxation to NECA, a non-selective adenosine analog in A₁WT CON, which was impaired in A₁WT SEN and A₁WT SEN+AD. All 3 groups of A₁KO mice showed concentration dependent relaxation to NECA, with no difference observed

between A₁KO CON, A₁KO SEN and A₁KO SEN+AD. Allergen challenge caused systemic inflammation in allergic A₁WT mice with AD aerosol further enhancing it as determined by inflammatory cytokines profile in plasma whereas inflammation was absent in all 3 groups of A₁KO mice. A₁WT allergic mice also had significantly higher airway hyper-responsiveness ($P < 0.05$, compared to WT CON and all A₁KO groups) to NECA, measured as Penh (enhanced pause). In conclusion, allergic A₁WT mice showed altered vascular reactivity, increased airway responsiveness and systemic inflammation, with AD aerosol further exacerbating all these effects with A₁KO mice lacking these effects. These data suggest that A₁ AR plays an important pro-inflammatory role systemically and is responsible for altered vasoreactivity in this model of asthma.

Introduction

Asthma, a chronic lung disease, is characterized by airway hyperreactivity and inflammation which result in bronchoconstriction and poor ventilation. Evidence has increasingly implicated adenosine in the pathophysiology of asthma (Spicuzza et al., 2006). Increases in adenosine levels correspond to increased airway inflammation and tissue damage (Driver et al., 1993). A hallmark study done in the early nineteen-eighties showed inhaled adenosine to be a potent bronchoconstrictor in asthmatics as opposed to normal subjects who showed no response (Cushley et al., 1984). Genetically modified mice in which adenosine deaminase (ADA), the enzyme that breaks down adenosine, was knocked out could not survive without exogenous administration of ADA and had severe pulmonary inflammation, airway hyper-reactivity and airway remodeling (Chunn et al.,

2001; Blackburn and Kellems, 2005), indicating a strong correlation between chronic elevation of adenosine levels and increased lung inflammation.

Impaired lung function is a risk factor for cardiovascular disease (Tockman et al., 1995) and many epidemiological studies have reported that people suffering from chronic respiratory diseases, including asthma, have a higher incidence of cardiovascular diseases (Drislane et al., 1987; Toren and Lindholm, 1996; Iribarren et al., 2004; Knoflach et al., 2005; Schanen et al., 2005). There is an association between atherosclerosis and stroke with reactive airway diseases. Adult-onset asthma is associated with increased carotid atherosclerosis in women (Onufrak et al., 2007) and subjects who had bronchial hyperresponsiveness to methacholine demonstrated increased carotid intima-media thickness (Zureik et al., 2004). Systemic inflammation in these patients, believed to be a consequence of airway inflammation (Jousilahti et al., 2002; Nadeem et al., 2003), may be one of the reasons for altered cardiovascular parameters observed (Gan et al., 2004). Studies done in animal models of allergic asthma have also reported enhanced systemic inflammation, myocardial ischemia-reperfusion injury and neutrophil recruitment to the myocardium (Hazarika et al., 2004, 2007). A recent study from our lab (Ponnoth et al., 2008) has shown that allergic mice have poor vascular responses and systemic inflammation, with adenosine aerosol exacerbating these effects by activating A₁ adenosine receptors.

The four adenosine receptor subtypes, i.e., the A₁, A_{2A}, A_{2B} and A₃, have different modulatory roles in asthma, and the cardiovascular system (Belardinelli et al., 1989; Mubagwa et al., 1996; Shryock and Belardinelli, 1997). Many reports suggest that the A₁ adenosine receptor is involved in direct bronchoconstrictor effects of adenosine. A₁

receptor expression was found to be elevated in a rabbit model of asthma and the use of an A_1 antisense nucleotide to inhibit this upregulation resulted in blunted bronchoconstriction to adenosine (Nyce and Metzger, 1997; Abebe and Mustafa, 1998). Use of a selective A_1 antagonist produced significant reduction of airway hyper-responsiveness in allergic rabbits by blocking these receptors (Obiefuna et al., 2005). A very recent study reported that the expression of A_1 receptor is significantly elevated in airway smooth muscle in asthmatic patient (Brown et al., 2008). Other than the effects on airway smooth muscle, the A_1 receptor has been implicated in increased mucin production in human tracheal cells (McNamara et al., 2004) and in mediating monocyte phagocytosis (Salmon et al., 1993). All these studies suggest a strong role for the A_1 receptor in asthma, both in airway obstruction and inflammation

A_1 receptors also have systemic effects and are involved in blood pressure regulation (Brown et al., 2006) and vasoconstriction (Shepherd et al., 1996; Talukder et al., 2002b; Hansen et al., 2003; Tawfik et al., 2005). However, the role of the A_1 AR in the control of vascular tone and systemic inflammation in an allergic model of asthma has not been characterized. In addition, the vascular effects of A_1 receptors in response to exogenous adenosine challenge as an added insult in a murine model of asthma have not been studied previously. Therefore, this study was undertaken to investigate the effects of allergen challenge and inhaled adenosine on vascular reactivity and systemic inflammation using our murine model of asthma in A_1 WT and KO mice. We also determined the role of the A_1 AR in airway responsiveness by whole body plethysmography. Our data suggest that asthmatic A_1 WT mice have altered peripheral vascular reactivity, systemic inflammation and increased bronchoconstriction with

inhaled adenosine further exacerbating these effects, compared to A₁KO mice, in which these responses were not elicited.

Materials and Methods

Animals: Balb/c mice (A₁WT) 8-10 weeks old males were obtained from Harlan Sprague Dawley (Indianapolis, IN) and A₁KO mice were obtained from Stephen Tilley, Pulmonary and Critical Care, School of Medicine, University of North Carolina, Chapel Hill. The animals were maintained on a ragweed-free diet. All experimental animals used in this study were under a protocol approved by the Institutional Animal Care and Use Committee of West Virginia University.

Animal sensitization: Sensitization was performed according to the protocol described earlier from this laboratory (Fan and Mustafa, 2002; Fan et al., 2003; Oldenburg and Mustafa, 2005; Fan and Mustafa, 2006; Mustafa et al., 2007). This model of allergic asthma has been shown to develop airway inflammation and airway hyper-reactivity to methacholine. The study comprised of six groups of animals: a) A₁WT Control (A₁WT CON); b) A₁WT Allergen sensitized and challenged (A₁WT SEN); c) A₁WT Allergen sensitized and challenged further with 6 mg/ml of adenosine aerosol for 2 min on day 14 (A₁WT SEN+AD); d) A₁KO Control (A₁KO CON); e) A₁KO Allergen sensitized and challenged (A₁KO SEN); f) A₁KO Allergen sensitized and challenged further challenged with 6 mg/ml of adenosine aerosol for 2 min on day 14 (A₁KO SEN+AD). Experiments carried out previously with A₁WT CON mice subject to adenosine challenge (A₁WT CON+AD) showed that this group was not different from A₁WT CON and, hence, this

group was not included for further experimentation. Mice were sensitized on days 1 and 6 with i.p. injections of ragweed allergen (Greer Laboratories, Lenoir, NC), 200 µg per dose with 200 µL Imject Alum (Pierce Laboratories, Rockford, IL). Non-sensitized control animals received only the Imject alum with the same volumes. 10 days after sensitization, the mice were placed in a Plexiglas chamber and challenged with 1% aerosolized ragweed or with 0.9% saline as a control, using an ultrasonic nebulizer (DeVilbiss Somerset, PA) for 20 minutes both in the morning and afternoon for three consecutive days. The aerosolization of allergen was performed at a flow rate of 2 mL/min, and the aerosol particles had a median aerodynamic diameter of less 4 µm (De Vilbiss).

Acute elevations in lung adenosine levels were produced experimentally by adenosine inhalation (6 mg/ml for 2 min on day 14) to SEN+AD group to further enhance allergen-induced effects. This dose was chosen based on our previous studies in this model (Fan and Mustafa, 2002; Fan and Jamal Mustafa, 2006). Adenosine inhalation in this model has been shown to enhance allergen-induced airway inflammation and airway hyper-reactivity to adenosine (or its analog NECA) (Fan and Mustafa, 2002; Fan et al., 2003; Fan and Mustafa, 2006). ADA-deficient mice having sustained and chronic elevations in lung adenosine levels have also been shown to have similar features (Chunn et al., 2001). We chose 24 hr post-adenosine inhalation for our current studies based on an earlier study from our lab which showed maximum eosinophilic inflammation in airways after 24 hr of adenosine inhalation (Fan and Mustafa, 2006), which is a hallmark of asthmatic inflammation. CON and SEN groups received only saline on day 14.

Twenty-four hours after the last challenge, animals were sacrificed for collection of aorta and blood for further experiments.

Preparation of isolated mouse aorta and isometric force measurement

Mice were sacrificed by anesthesia with pentobarbitol sodium (65 mg/kg, i.p.) followed by thoracotomy and removal of aorta that was then cut transversely into 3-4 mm rings. The rings were mounted vertically between two stainless steel wire hooks and then suspended in 10 mL organ baths containing Krebs-Henseleit buffer. The Krebs-Henseleit buffer (pH 7.4) containing 118 mM NaCl, 4.8 mM KCl, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 25 mM NaHCO₃, 11 mM glucose and 2.5 mM CaCl₂ was maintained at 37°C with continuous bubbling of 95% O₂ and 5% CO₂. For measurement of isometric force response, aortic rings were equilibrated for 90 min with a resting force of 1g that has been used earlier in our laboratory (Tawfik et al., 2005; Ansari et al., 2007b) and change of the bathing solution at 15 min interval. At the end of equilibration period, tissues were contracted with 50 mM KCl to check the contractility of individual aortic rings twice which were washed out with Krebs-Henseleit buffer. Aortic rings were then constricted with phenylephrine (PE, 10⁻⁷ M) to obtain a steady contraction and changes in tension were monitored continuously with fixed range precision force transducer (TSD, 125 C, BIOPAC system) connected to the differential amplifier (DA 100B, BIOPAC system). The data were recorded using MP100, BIOPAC digital acquisition system and analyzed using Acknowledge 3.5.7 software (BIOPAC system).

Contraction/relaxation experiments

After equilibration, the responsiveness and stability of individual rings were checked by successive administration of submaximally effective concentration of PE (10^{-7} M). The integrity of the vascular endothelium was assessed pharmacologically by acetylcholine (10^{-7} M) to produce relaxation of PE pre-contracted rings. Aortic rings were then washed several times with Krebs-Henseleit solution, and allowed to equilibrate for 30 min before the experimental protocol began.

Experimental protocol

The concentration-response curves (CRCs) for NECA (10^{-9} - 10^{-5} M) were run parallel in aortic rings from all the groups. In all cases, NECA was added to yield the next higher concentration only when the response to the earlier dose reached a steady state. Contraction/relaxation responses were expressed as a percentage increase/decrease in the contraction with respect to PE (alone) in response to each concentration of agonist used.

Assessment of systemic inflammation

Mice were euthanized by pentobarbitone sodium (65 mg/mL, i.p.), followed by collection of blood by cardiac puncture in heparinized syringes. The blood collected from different groups was centrifuged at 800 g for 10 min at 4° C and the resulting plasma was used for cytokine assays.

Multiplex cytokine assay: Cytokine assay of IL-5, TNF α and IL-13 in the plasma was measured by a commercial kit from Linco Research (St. Charles, Missouri) using the Luminex 200 system (Luminex Corp., Austin, Tx), which is a multianalyte bioassay

detection system capable of performing up to 100 assays simultaneously in a single microtiter plate well. This system uses polystyrene microspheres internally dyed with red and infrared fluorophores that can be individually identified. The fluorescent microspheres were coated with capturing antibodies specific for different chemokines. The chemokine-captured beads after incubation with the sample were then mixed with phycoerythrin-conjugated detection antibodies to form immune complexes. Following incubation, washing and acquisition of fluorescence data, the concentration results were generated in graphical format using the standard curve generated for each cytokine. Results were expressed in pg/mL.

Airway responsiveness to Methacholine (MCh) and NECA

The airway responsiveness was assessed by whole body plethysmography using the chambers obtained from Buxco Inc. (Max II; Buxco Systems, Wilmington, NC). This system enables the measurement of airway obstruction using a dimensionless parameter called enhanced pause or Penh. Higher the Penh values, greater is the airway obstruction. Penh has been shown to correlate with airway resistance and dynamic compliance (Justice et al., 2001). Unrestrained mice were placed in individual Plexiglas chambers twenty four hours after the last allergen challenge and subjected to increasing concentrations of MCh (1.5-48.0 mg/ml) to obtain concentration-responses. In case of SEN+AD groups, this was 24 hours post adenosine aerosolization. Concentration-responses to NECA were performed 24 hours post the MCh experiment. After obtaining baseline and vehicle Penh values, mice were exposed to increasing concentrations of NECA (23.44-750 µg/ml) via the Buxco aerosol delivery system (version 1.5; Buxco,

Wilmington, NC). Each concentration of NECA was aerosolized for 2 minutes and the Penh readings were recorded for 5 minutes to establish a dose-response relationship. The next dose was not administered until the mice had returned to baseline Penh levels. Airway responsiveness was normalized to the vehicle control (normal saline) and expressed as a percentage increase in Penh.

Drugs used

Acetylcholine, adenosine and phenylephrine were dissolved in distilled water. 5'-N-ethylcarboxamidoadenosine (NECA) was dissolved in 100% DMSO as a 10 mM stock solution for vascular reactivity experiments. The final concentration of DMSO in organ bath (bath volume: 10 mL) had no effect by itself on the aortic rings (Ansari et al., 2007b). For Penh responses, MCh was dissolved in 0.9% saline and NECA was dissolved in 100% ethanol to create a stock solution from which subsequent dilutions were made with 0.9% saline. Unless stated otherwise, all chemicals were of the highest grade available and were purchased from Sigma Chemicals (St. Louis, MO).

Statistical Analysis

The data were expressed as mean $\pm$ SEM. Comparisons among different groups were analyzed by ANOVA (analysis of variance) followed by Tukey's multiple comparison test/ Bonferroni's selected pair test. Comparison between two groups was assessed by unpaired t-test. A p value of less than 0.05 was considered as the level of significance. All statistical analyses were performed using the GraphPad Prism statistical package.

Results

Systemic inflammation in control, allergic and adenosine aerosolized allergic mice

Table 5.1 shows the multiplex cytokine profile in all the groups. Allergen challenge significantly increased plasma levels of inflammatory cytokines IL-5 and IL-13 in A₁WT SEN as compared to A₁WT Con and all groups of A₁KO mice. Allergen sensitized and challenged A₁WT mice exposed to adenosine aerosol (SEN+AD) had the highest level of the pro-inflammatory cytokines compared to other groups. Other cytokines in the panel were either undetectable or not different among the groups (data not shown). These data suggest activation of the A₁ receptor has pro-inflammatory effects.

Vascular reactivity in control, allergic and allergic + adenosine groups

The aortic relaxation/contraction responses to NECA were studied in all 6 groups. Organ bath data showed a concentration dependent relaxation response to NECA in A₁WT CON, while aortic relaxations were impaired in A₁WT SEN aorta with a maximum relaxation of 7% (Fig 5.1 A). NECA failed to elicit any relaxation response in SEN+AD; it produced a concentration dependent contractile response, with a maximum contraction of 35% at 10⁻⁶ M (Fig 5.1 A). In contrast, all 3 groups of KO mice showed concentration-dependent relaxation and there was no difference in the responses between the groups (Fig 5.1 B). These data show that allergen sensitization and challenge altered the vascular reactivity with adenosine aerosol further exacerbating it in allergic A₁WT mice, indicating that the A₁ receptor was responsible for the low adenosine-mediated vascular relaxation observed. Comparison between respective groups of SEN and SEN+AD WT and KO groups are shown in figures 5.2 and 5.3.

Endothelial function in control, allergic and allergic + adenosine groups

Figure 5.4 shows the endothelial response to ACh in all experimental groups. A₁WT SEN (~39%) and SEN+AD (~38%) groups had significantly lower ($P<0.01$) endothelial response to acetylcholine compared to WT controls (~65% relaxation). There was no difference in endothelial response between any group of A₁KO mice and between the KO groups and A₁WT CON; however A₁KO SEN and A₁KO SEN+ AD had significantly higher endothelial relaxation than A₁WT SEN and A₁WT SEN+ AD groups ($P<0.05$). These data show that allergen challenge probably led to endothelial dysfunction in allergic A₁WT mice.

Airway responsiveness to Methacholine (MCh) and NECA

The airway responsiveness to MCh (Fig 5.5) was assessed and there was no difference between control A₁WT and KO mice. All groups of sensitized mice WT and KO had significantly increased airway response to MCh compared to respective controls but there was no difference between sensitized A₁WT and KO mice. Next, the response to NECA was assessed (Fig 5.6). Although baseline Penh values for control A₁WT (284.05 ± 65.4) were higher than A₁KO (180.36 ± 22.52), the difference was not statistically significant. However, allergic A₁WT SEN and A₁WT SEN+AD mice had significantly higher airway responsiveness, with the response being highest in the A₁WT SEN+AD group. In contrast, A₁KO SEN and SEN+AD groups had comparable response to A₁KO CON group. These data indicate that adenosine mediated hyper-responsiveness is mediated through the A₁ receptor.

Table 5.1: Systemic inflammation as assessed by levels of inflammatory cytokines in plasma of A₁WT and A₁KO control (CON), allergic (SEN) and adenosine aerosolized allergic (SEN+AD) mice; Values are expressed as Mean $\pm$ SEM (n=4-8). *P<0.05 compared to A₁WT CON; #P<0.05 compared to A₁WT SEN.

INFLAMMATORY MARKER (pg/ml)	CON		SEN		SEN+AD	
	A ₁ WT	A ₁ KO	A ₁ WT	A ₁ KO	A ₁ WT	A ₁ KO
IL-5	3.35 $\pm$ 0.14	3.98 $\pm$ 0.93	8.22 $\pm$ 1.65*	4.078 $\pm$ 0.57	11.12 $\pm$ 1.5*	3.88 $\pm$ 1.50
IL13	27.44 $\pm$ 5.57	UNDETECTED	45.41 $\pm$ 9.91*	UNDETECTED	170 $\pm$ 49.97*#	UNDETECTED
TNF- α	6.66 $\pm$ 1.03	UNDETECTED	6.25 $\pm$ 0.23	UNDETECTED	14.22 $\pm$ 3.60*#	UNDETECTED

Figure 5.1 Concentration-response curves for NECA-mediated relaxation/contraction in A_1 WT (A) and A_1 KO (B) with control (CON), allergic (SEN) and adenosine aerosolized allergic (SEN+AD) groups. Values are expressed as mean $\pm$ SEM ($n=4$). On the ordinate, positive and negative values indicate relaxation and contraction, respectively. * $P<0.05$ as compared to CON; \$ $P<0.05$ as compared to SEN.

Figure 5.2 Comparison between A₁KO and A₁WT sensitized mice. Values are expressed as mean $\pm$ SEM ($n=4$). On the ordinate, positive and negative values indicate relaxation and contraction, respectively. * $P<0.05$

Figure 5.3 Comparison between A₁KO and A₁WT SEN+AD mice. Values are expressed as mean $\pm$ SEM ($n=4$). On the ordinate, positive and negative values indicate relaxation and contraction, respectively. * $P<0.05$

Fig 5.4 Endothelial response to ACh in CON, SEN and SEN+AD A₁WT and A₁KO mice. A₁WT SEN and SEN+AD groups were significantly different from A₁WT CON

*P<0.01 and A₁KO SEN, SEN+AD #P<0.05

Figure 5.5 Airway responsiveness to MCh in A₁KO and A₁WT mice. Values are expressed as mean $\pm$ SEM ($n=4-8$). On the ordinate, airway response is expressed as % of the vehicle. * $P<0.05$ A₁WT SEN and SEN+AD compared to A₁WT CON; # $P<0.05$ A₁KO SEN and SEN+AD compared to A₁KO CON

Figure 5.6 Airway responsiveness to NECA in A₁KO and A₁WT mice. Values are expressed as mean $\pm$ SEM ($n=4-8$). On the ordinate, airway response is expressed as % of the vehicle. * $P<0.01$ compared to A₁KO CON; \$ $P<0.01$ compared to A₁KO SEN; # $P<0.05$ compared to A₁KO SEN+AD; % $P<0.05$ compared to A₁WT SEN, @ $P<0.05$ compared to A₁WT CON.

Discussion

The principal finding of the present study is that allergic A₁WT mice had systemic inflammation, increased airway responsiveness and impaired vasorelaxation to NECA, a non-selective adenosine analog. These outcomes were further aggravated after acute adenosine aerosol challenge in these mice. Responses in allergic A₁KO mice were not different from non-allergic wild type controls, indicating that the A₁ adenosine receptor was responsible for alterations in vascular reactivity, airway hyper-responsiveness and increased inflammation. Administration of NECA caused increased airway responsiveness in allergic A₁WT mice which was absent in corresponding A₁KO mice. Endothelium was found to be dysfunctional in allergic A₁WT mice compared to non-allergic A₁WT controls, while both allergic and non-allergic A₁KO mice had no difference in endothelial responses.

Adenosine is chronically elevated in asthmatic lungs (Mann et al., 1986b; Mann et al., 1986a; Driver et al., 1993). Evidence indicates adenosine may be involved in the release of inflammatory mediators in the lung and systemic circulation by acting on its receptors present on different cells including mast cells, eosinophils, neutrophils and other inflammatory cells (Driver et al., 1991; Fan and Jamal Mustafa, 2006; Nadeem and Mustafa, 2006; Spicuzza et al., 2006) and can thus contribute to the severity of lung inflammation. Adenosine is a known bronchoprovocant (Cushley et al., 1983) and experimentally induced temporary elevations in lung adenosine levels through inhalation of adenosine (resulting from breakdown of adenosine 5'-monophosphate) cause an increase in infiltration of eosinophils in patients with asthma (van den Berge et al., 2004). A recent study from our lab has also shown increased release of inflammatory cell

markers in the lung and plasma after inhalation of adenosine aerosol in allergic mice (Fan and Mustafa, 2002). However, the systemic effects of such an inhalation with respect to involvement of specific adenosine receptors have not been studied previously. Adenosine aerosol in human asthmatics causes increased release of neutrophil chemotactic factor in serum (Driver et al., 1991). These studies suggest that adenosine enhances the release of systemic inflammatory mediators from inflammatory cells

Systemic inflammation has been reported previously in human asthmatics and animal models of asthma (Jousilahti et al., 2002; Hazarika et al., 2004). Neutrophils and eosinophils are increased in bronchoalveolar lavage and blood in a murine model of asthma (Hazarika et al., 2007). Systemic inflammatory markers have also been shown to be elevated in patients with asthma and in allergic mouse model of asthma after adenosine aerosol challenge. (Driver et al., 1991; Fan and Jamal Mustafa, 2006). Based on the findings of these studies, we investigated the cytokine profile in systemic circulation in allergic A₁WT and A₁KO mice. We found that while allergic A₁WT mice had significantly elevated levels of pro-inflammatory cytokines, allergic A₁WT mice exposed to acute adenosine aerosol had further exacerbation of systemic inflammation as evidenced by the finding that several pro-inflammatory cytokines levels (IL-13, MCP-1, and TNF- α) were increased most in this group. In A₁KO mice, both allergic and control groups either had undetected or non-significant levels of inflammatory markers, suggesting the absence of the A₁ receptor was related to lower inflammation. From the mediator profile, IL-13 was significantly higher in the plasma of A₁WT SEN mice, with adenosine challenge exacerbating it even further. IL-13 is an important mediator in asthma and has been implicated in lung inflammation, airway remodeling and airway

hyperreactivity (Grunig et al., 1998; Lowry et al., 2008). Adenosine and IL-13 interact positively, with each increasing the levels of the other in studies done with asthmatic mice (Blackburn et al., 2003). IL-13 and adenosine thus are important in lung inflammation but their role in systemic inflammation is not known. Our data suggest a pro-inflammatory role for IL-13 and adenosine mediated systemic inflammation via the A₁ receptors.

Asthma has been associated with an increased incidence of negative cardiovascular outcomes (Drislane et al., 1987; Toren and Lindholm, 1996; Iribarren et al., 2004; Knoflach et al., 2005). While asthmatic inflammation is believed to responsible for the development of these cardiovascular effects, there is little information linking the two, especially in relation to the vascular effects of specific adenosine receptors. In the present study, NECA, a non-selective adenosine analog produced a concentration-dependent vasorelaxation in control A₁WT mice, which was impaired in allergic A₁WT mice. Vascular reactivity to NECA was further altered in aorta of allergic A₁WT mice that had received aerosolized adenosine. In contrast, NECA produced a concentration-dependent vasorelaxation response in allergic and non-allergic A₁KO groups. These findings strongly suggest that the A₁ receptor indeed is responsible for poor vascular responses in allergic mice.

In a recent study from this laboratory, we found that allergic mice had lower endothelial relaxation suggesting that allergen exposure and development of allergic asthma somehow resulted in endothelial dysfunction (Ponnoth et al., 2008). In the present study, we observed that there was no difference in the endothelial response between control and allergic A₁KO mice as opposed to significantly lower endothelial relaxation

in allergic A₁WT mice compared to A₁WT controls. The exact cause for this dysfunction is not clear but it could possibly be due to the A₁ receptor-mediated increase in systemic inflammatory cytokines. These inflammatory cytokines are known to suppress eNOS and nitric oxide production (Cardaropoli et al., 2003; Li et al., 2004). For the first time, the A₁ receptor specifically has been shown to affect the endothelium functionality in allergic mice. Further studies are required to delineate the exact mechanism for the role of the A₁AR in alteration of endothelial function especially the signaling pathways that may be involved.

Whole body plethysmography has been used previously to determine the extent of airway responsiveness and as an index of airway obstruction (Drazen et al., 1999; Fan and Mustafa, 2002; Fan et al., 2003; Oldenburg and Mustafa, 2005). We used this method to determine the differences between A₁WT and KO responses to MCh and NECA. MCh produced higher airway responsiveness in both A₁WT and KO sensitized animals compared to controls. No difference was observed between A₁WT and KO mice. However, the response to the non-selective adenosine analog NECA was different between A₁KO and WT mice. There was no difference in NECA-mediated airway response in all three groups of A₁KO mice, while A₁WT SEN mice had significantly higher responses. The highest response was observed in A₁WT SEN+AD, which was significantly different from all other study groups. The A₁ receptor has been implicated in the direct adenosine-mediated bronchoconstrictor response (Nyce and Metzger, 1997; Abebe and Mustafa, 1998) and a selective A₁ antagonist produced significant reduction of airway hyper-responsiveness in allergic rabbits by blocking these receptors (Obiefuna

et al., 2005). Our data supports a role for A_1 receptor-mediated airway hyperresponsiveness and also shows that adenosine challenge exacerbates the response.

In conclusion, our findings provide evidence for the first time that the A_1 adenosine receptor mediates alterations in systemic parameters (inflammation, lower endothelial response and impaired vasorelaxation) and airway hyper-responsiveness as a result of allergen exposure and adenosine aerosol challenge in sensitized mice. Understanding the exact contribution of adenosine and adenosine A_1 receptor mediated-systemic effects with respect to mechanisms and signal transduction would provide an important tool in the prevention of systemic inflammation and harmful systemic side effects in designing therapies for asthma. Ultimately, this could be beneficial in lowering cardiovascular events in asthmatics.

CHAPTER SIX

CONCLUSION

The work presented in this dissertation establishes that asthma has detrimental effects beyond lungs and airways in allergic mice. Systemic inflammation, endothelial dysfunction and altered vascular reactivity appear to be associated with asthma. Adenosine plays an important role in these effects and exogenously administered adenosine to increase the lung levels experimentally beyond the allergen exposure exacerbates observed outcomes.

Specific adenosine receptors are involved in the vascular responses seen, with different receptors modulating beneficial and detrimental effects. Vascular A_{2A} and A_{2B} receptors are down-regulated in allergic mice, and this was confirmed with the use of the specific receptor antagonists. To get a better insight into the role of A_{2A} receptors on aortic tone, vascular responses to various adenosine analogs were studied in A_{2A} receptor knock-out and wild type mice that were non-allergic.

In vitro aortic relaxation in A_{2A}WT mice was a biphasic response to NECA, a non-selective adenosine analog, while the response in A_{2A}KO tissue was concentration-dependent contraction. This indicated the possibility that the absence of A_{2A} receptor as being responsible for contraction. A_{2A}KO mice also had lower endothelial relaxation than their wild-type counterparts, which suggested that relaxation through A_{2A} receptors occurred via an endothelial dependent mechanism. Use of the A₁ antagonist (DPCPX) increased the relaxation in A_{2A}WT mice while the contraction observed in A_{2A}KO mice

was reduced although relaxation was still absent. This again underscored a role for the A_{2A} receptor in relaxation while confirming that the A_1 receptor was responsible for adenosine-mediated contraction. These studies determined that the A_{2A} receptor mediates vasorelaxation in the aorta in conjunction with the A_{2B} receptor.

The next aim of the study was to determine the exact role of the A_1 receptor in the allergic model. The reason for focusing on the A_1 receptor came about from the initial study when it was found that the use of DPCPX (selective A_1 receptor antagonist) restored aortic relaxation in allergic mice comparable to non-allergic controls. The A_1 receptor was also found to be up-regulated in the aorta from allergic mice further subjected to adenosine aerosol.

Genetic knock-out mice were used to study and confirm the involvement of A_1 receptor. Data showed that the allergic A_1 WT mice had lower adenosine-mediated aortic relaxation, significantly higher levels of inflammatory markers in the plasma and increased airway responsiveness. These outcomes were exacerbated in allergic A_1 WT mice that had received exogenous adenosine as a single aerosol inhalation. In contrast, no impairment in adenosine-mediated vascular responses were observed in A_1 KO (allergic and non-allergic controls) aortic tissue. There were significantly lower (or undetectable) levels of inflammatory markers and lower airway responsiveness to NECA in A_1 KO mice. In fact, there was no difference between allergic A_1 KO and control A_1 KO mice. These data implicate the A_1 receptor as having deleterious systemic and airway effects in allergic mice. The systemic effects appear to be the result of inflammation. However, the exact signaling mechanism(s) of the A_1 involved in translation of airway inflammation to the observed systemic outcomes is not known and remains to be studied.

Future Direction

The next step in this model of asthma would be to define the adenosine receptor signaling mechanisms involved. Our preliminary data show that asthmatic mice have both lung as well as systemic inflammation. It is important to have a better understanding of this correlation and the possible signaling pathways involved. Based on the data in this dissertation that shows highest levels of IL-13 in allergic mice, and review of literature which has established a close relationship between IL-13 and adenosine in lung inflammation, the next focus could be the potential role for this inflammatory cytokine as an intermediary signaling molecule, both in asthma and cardiovascular disease.

IL-13 is an important mediator in asthma and has been implicated in lung inflammation and airway remodeling. It is a product of mast cell degranulation and is also released from T-helper 2 (Th2) cells. Adenosine and IL-13 interact with each other in a positive feedback in studies done with asthmatic mice (Blackburn et al., 2003). These authors found that IL-13 caused a progressive increase in adenosine accumulation, inhibited the activity of adenosine deaminase, and augmented the expression of the A₁, A_{2B}, and A₃ adenosine receptors. Their findings suggest that adenosine signaling contributes to and influences the severity of IL-13 induced tissue responses.

IL-13 signaling occurs through the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway. This cytokine acts on its receptor (via JAK-1) and leads to phosphorylation of STAT6, which induces the transcription of several genes including 5-lipoxygenase (LO). 5-LO, via the arachidonic acid pathway, produces inflammatory leukotrienes (LTs) which are very important mediators of IL-13 induced lung injury. Recent studies have also suggested a pro-atherosclerotic role for LTs with

LTB₄ acting as a potent leukocyte chemoattractant that amplifies inflammation at atherosclerotic sites (M.Back et al., 2005).

Other than LTs, studies in asthma have shown that IL-13 also induces vascular endothelial growth factor (VEGF) from human airway smooth muscle cells, mast cells, epithelial cells and macrophages (Faffe et al., 2006). In fact, IL-13 and VEGF act on each other in positive feedback loops. VEGF and VEGF receptor inhibitors reduce lung inflammation and airway hyper-reactivity in asthma (Faffe et al., 2006). This study also found that over expression of VEGF enhances Th2-mediated sensitization and inflammation. Studies involving human atherosclerosis have noted that VEGF is thought to play a pro-atherosclerotic role. VEGF is an angiogenic factor. It induces migration and proliferation of endothelial cells and enhances vascular permeability. It has been reported that human coronary atherosclerotic plaques were positive for VEGF mRNA but not so in normal arterial tissues (Inoue et al., 1998).

IL-13 and IL-4 act synergistically with TNF- α to increase eotaxin levels in the lungs. Eotaxin increases lung eosinophilia in asthma and is very important in amplification of eosinophilic inflammation. Though eosinophilia is considered to be a characteristic hallmark of asthmatic inflammation, eosinophils are not found in any significant amounts in atherosclerotic lesions. However, eotaxin may have a novel role in atherosclerosis that does not involve eosinophils. Haley et al. have reported that eotaxin and its receptor, CCR3, were overexpressed in the inflammatory infiltrate of human atheroma (Haley et al., 2000). CCR3 was predominantly expressed on macrophages and they suggest eotaxin may participate in mast cell activation or recruitment.

Data in this dissertation show that systemic levels of IL-13 are increased significantly in allergic A₁WT mice compared to non-asthmatic controls. Interestingly, allergic A₁KO did not have any increase in IL-13, suggesting a role for the A₁ receptor. The next step would be to determine if IL-13 does, in fact, have a role in vascular inflammation and the signaling mechanism involved. Ultimately, understanding the signaling pathway (s) involved would lead to uncovering of the translational mechanism (s) of lung inflammation to systemic effects in human asthma.

Figure 6.1: Proposed pathway for IL-13 signaling effects in asthma

PUBLICATIONS AND ABSTRACTS

MANUSCRIPTS

Ponnoth DS, Nadeem A, Mustafa SJ “*Adenosine-mediated alteration of vascular reactivity and inflammation in a murine model of asthma.*” Am J Physiol Heart Circ Physiol. 2008 May;294(5):H2158-65.

Nayeem MA, Poloyac SM, Falck JR, Zeldin DC, Ledent C, **Ponnoth DS**, Ansari HR, Mustafa SJ “*Role of CYP epoxygenases in A2AAR-mediated relaxation using A2AAR-null and wild-type mice.*” Am J Physiol Heart Circ Physiol. 2008 Nov;295(5):H2068-78.

ABSTRACTS

“*Adenosine mediated systemic effects in a murine model of allergic asthma.*”

Dovenia Ponnoth, Ahmed Nadeem, R. Ray Morrison, S. Jamal Mustafa.

- Presented at the **West Virginia University Health Sciences Center Research Day April, 2006**

“*Adenosine causes impaired vasorelaxation and systemic inflammation in a murine model of allergic asthma.*” **Dovenia Ponnoth**, Ahmed Nadeem, R. Ray Morrison, S. Jamal Mustafa.

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“*The Role of Adenosine in Inflammation and Vascular Reactivity in a Murine model of Allergic Asthma.*” **Dovenia Ponnoth**, Ahmed Nadeem and S. Jamal Mustafa.

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“*Effects of adenosine on vascular reactivity and inflammation in a murine model of allergic asthma.*” Dovenia Ponnoth, Ahmed Nadeem, R. Ray Morrison, S. Jamal Mustafa.

- Presented at **Experimental Biology, Washington DC, April 2007**

“*Absence of adenosine-induced relaxation in adenosine-KO mouse aorta: role of CYP 2C and CYP 4A.*” Mohammed A. Nayeem, John R. Falck, C. Ledent, **Dovenia S. Ponnoth**, Habib R. Ansari, Shilpa P. Sakhalkar, S Jamal Mustafa.

- Presented at **Experimental Biology, Washington DC, April 2007**

“Adenosine A_{2A} receptor knock-out mice have impaired vasorelaxation and endothelial function.” **Dovenia Ponnoth**, C. Ledent and S. Jamal. Mustafa.

- Presented at **Experimental Biology, San Diego CA, April 2008**

“A₁ Adenosine Receptor Causes Inflammation and Impaired Vasorelaxation in a Murine Model of Asthma.” **D. Ponnoth**, A. Nadeem, S.L. Tilley and S.Jamal.

- Presented at **ATS (the American Thoracic Society), 2008-Toronto, Canada, May 2008.**

“Decreased tracheal relaxation via NADPH oxidase activation in A_{2A} AR deficient allergic mice.” Nadeem A, **Dovenia PS**, Batchelor T, Dey R, Ledent C, Mustafa SJ.

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APPENDIX A

The relaxation response to NECA was calculated at two different concentrations of phenylephrine (PE) used to pre-contract tissues. This was done to determine the concentration of PE to be used for experiments. There was no difference in NECA mediated relaxation in tissues precontracted with 0.1 μ M and 1 μ M of PE respectively.

The relaxation response to adenosine was calculated at two different concentrations phenylephrine used to pre-contract tissues. This was done to determine the concentration of PE to be used for experiments. There was no difference in adenosine mediated relaxation in tissues precontracted with 0.1 μ M and 1 μ M of PE respectively.

The endothelial response to acetylcholine was determined at two different concentrations phenylephrine (PE) used to pre-contract tissues. This was done to determine the concentration of PE to be used for experiments. There was no difference in adenosine mediated relaxation in tissues precontracted with 0.1 μ M and 1 μ M of PE respectively.

APPENDIX B

Preliminary experiments (airway responsiveness and vascular reactivity) were carried out with ovalbumin-sensitized and challenged A_{2A} WT and A_{2A} KO mice that had also received aerosolized adenosine challenge. A_{2A} KO mice had increased airway responsiveness as seen from the Penh data and reduced aortic relaxation to NECA compared to wild-type counterparts. However, adenosine mediated aortic vascular responses in naïve (non-allergic) mice were not known, which led to studying of vascular reactivity in A_{2A} WT and A_{2A} KO mice (Chapter 4)

Airway responsiveness in A_{2A} WT and A_{2A} KO SEN + AD mice

Aortic relaxation to NECA in A_{2A} WT and A_{2A} KO SEN+AD mice

APPENDIX C

Level of inflammatory cytokines in bronchoalveolar lavage fluid was measured in control (CON), ragweed sensitized (SEN) and ragweed sensitized +aerosolized adenosine Balb/c mice to determine the difference in lung inflammation. Sensitized mice that had received the additional adenosine aerosol challenge had the highest level of inflammatory cytokines.

***P<0.05 compared to CON; #P<0.05 compared to SEN**

**IL-5 levels in BAL fluid from
Balb/c mice**

***P<0.05 compared to CON; #P<0.05 compared to SEN**

*P<0.05 compared to CON; #P<0.05 compared to SEN

*P<0.05 compared to CON; #P<0.05 compared to SEN

Biographical Sketch

NAME (Last, First, Middle)		POSITION TITLE	
Ponnoth Dovenia S		ORISE Fellow (National Institute for Occupational Safety and Health)	
EDUCATION/TRAINING			
INSTITUTION AND LOCATION	DEGREE <i>(if applicable)</i>	YEAR(s)	FIELD OF STUDY
Bharati Vidyapeeth’s College of Pharmacy, Mumbai University India	BS	1998-2002	Pharmaceutical Sciences
East Carolina University (ECU),Greenville NC, USA		2003-2005	Pharmacology & Toxicology
West Virginia University (WVU), Morgantown WV,USA (transferred from ECU along with mentor)	Ph. D.	2005-2008	Pharmaceutical and Pharmacological Sciences

A. Positions and Honors

Graduate student ECU/WVU: August 2003-June 2006

Ph. D. candidate, WVU, Morgantown, WV: June 2006-August 2008

ORISE Fellow, NIOSH Morgantown, WV: September 2008-Present

Awards

Graduate Student Travel Award: Awarded by the American Society for Pharmacology and Experimental Therapeutics (ASPET) at Experimental Biology, Washington DC, April 2007

The Sathu Somani **Junior Scientist in Physiology Award:** Awarded by the Association of Scientists of Indian origin in America (ASIOA) at its 25th Annual Meeting, Washington DC, April 2007

Graduate Student Travel Award: Awarded by ASPET at Experimental Biology, San Diego, April 2008

Professional Memberships

Student Member of ASPET: August 2006-Present
Student Member of ASIOA: January 2007-Present

Teaching experience

Facilitator: Medical Pharmacology small group conferences (active learning exercise) for medical students conducted by the Department of Physiology and Pharmacology, West Virginia University (March 2006-Present).

B. Publications

1. **Ponnoth DS**, Nadeem A, Mustafa SJ “*Adenosine-mediated alteration of vascular reactivity and inflammation in a murine model of asthma.*” Am J Physiol Heart Circ Physiol. 2008 May;294(5):H2158-65.

2. Nayeem MA, Poloyac SM, Falck JR, Zeldin DC, Ledent C, **Ponnoth DS**, Ansari HR, Mustafa SJ “*Role of CYP epoxygenases in A2AAR-mediated relaxation using A2AAR-null and wild-type mice.*” Am J Physiol Heart Circ Physiol. 2008 Nov;295(5):H2068-78.

C. Manuscripts under review

High salt diet enhances mouse aortic relaxation through adenosine A_{2A} receptor via CYP epoxygenases

Mohammed A. Nayeem, **Dovenia S. Ponnoth**, Matthew A. Boegehold, Darryl C. Zeldin, John R. Falck and S. Jamal Mustafa. [Submitted to the American Journal of Physiology (AJP) September 2008; reviewed and under revision]

D. Manuscripts in preparation

“*Role of A_{2A} adenosine receptors in the regulation of vascular tone.*”

-**Dovenia S. Ponnoth**, Catherine Ledent, Maryam Sharifi, Kevin Roush, SJ Mustafa

“*Evidence for the role of A₁ adenosine receptors in inflammation and altered vascular reactivity in allergic mice.*”

- **Dovenia S. Ponnoth**, Ahmed Nadeem, Stephen L. Tilley, SJ Mustafa

E. ABSTRACTS

“Adenosine mediated systemic effects in a murine model of allergic asthma.”

Dovenia Ponnoth, Ahmed Nadeem, R. Ray Morrison, S. Jamal Mustafa.

- Presented at the **West Virginia University Health Sciences Center Research Day April, 2006**

“Adenosine causes impaired vasorelaxation and systemic inflammation in a murine model of allergic asthma.” **Dovenia Ponnoth**, Ahmed Nadeem, R. Ray Morrison, S. Jamal Mustafa.

- Presented at the **U.S EPA/NHLBI/NIEHS sponsored meeting-The Role of Air Pollutants in Cardiovascular Disease, October 2006.**

“The Role of Adenosine in Inflammation and Vascular Reactivity in a Murine model of Allergic Asthma.” **Dovenia Ponnoth**, Ahmed Nadeem and S. Jamal Mustafa.

- Presented at the **West Virginia University Health Sciences Center Research Day April, 2007**

“Effects of adenosine on vascular reactivity and inflammation in a murine model of allergic asthma.” Dovenia Ponnoth, Ahmed Nadeem, R. Ray Morrison, S. Jamal Mustafa.

- Presented at **Experimental Biology, Washington DC, April 2007**

“Absence of adenosine-induced relaxation in adenosine-KO mouse aorta: role of CYP 2C and CYP 4A.” Mohammed A. Nayeem, John R. Falck, C. Ledent, **Dovenia S. Ponnoth**, Habib R. Ansari, Shilpa P. Sakhalkar, S Jamal Mustafa.

- Presented at **Experimental Biology, Washington DC, April 2007**

“Adenosine A_{2A} receptor knock-out mice have impaired vasorelaxation and endothelial function.” **Dovenia Ponnoth**, C. Ledent and S. Jamal. Mustafa.

- Presented at **Experimental Biology, San Diego CA, April 2008**

“A₁ Adenosine Receptor Causes Inflammation and Impaired Vasorelaxation in a Murine Model of Asthma.” **D. Ponnoth**, A. Nadeem, S.L. Tilley and S.Jamal.

- Presented at **ATS (the American Thoracic Society), 2008-Toronto, Canada, May 2008.**

“Decreased tracheal relaxation via NADPH oxidase activation in A_{2A} AR deficient allergic mice.” Nadeem A, **Dovenia PS**, Batchelor T, Dey R, Ledent C, Mustafa SJ.

- Presented at **ATS (the American Thoracic Society), 2008-Toronto, Canada May 2008.**

ABSTRACTS (to be presented)

“Enhanced vascular relaxation through epoxygenase depends on ATP-sensitive K⁺ channels via adenosine A_{2A} receptor: Role of high salt diet”

Mohammed A. Nayeem, Matthew A. Boegehold, **Dovenia S. Ponnoth**, Darryl C. Zeldin, John R. Falck and S. Jamal Mustafa. *Experimental Biology 2009, New Orleans*.