L-arginine metabolism regulates airways responsiveness in asthma
and exacerbation by air pollution

by

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A thesis submitted in conformity with the requirements
for the degree of Doctor of Philosophy

Institute of Medical Sciences

University of Toronto

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Doctor of Philosophy
Institute of Medical Sciences
University of Toronto
2011

Abstract
Asthma is a chronic respiratory disease with a high prevalence in Western countries, including Canada, and increased exacerbations have been associated with ambient air pollution. The maintenance of airways tone is critically dependent on the endogenous bronchodilator, nitric oxide (NO). The nitric oxide synthase (NOS) isoenzymes produce NO from the amino acid, L-arginine, and competition for substrate with the arginase isoenzymes can limit NO production. Imbalances between these pathways have been implicated in the airways hyperresponsiveness (AHR) of asthma. The overall objective of this work was to determine whether arginase and downstream polyamine metabolites are functionally involved in airways responsiveness in animal models of asthma and the adverse responses of allergic animals to air pollution. To this purpose, the expression profiles of proteins involved in L-arginine metabolism were determined in lung tissues from human asthmatics and murine models of ovalbumin (OVA)-induced airways inflammation. Expression of arginase 1 was increased in human asthma and animal models. Competitive inhibition of arginase attenuated AHR in vivo. The roles of the downstream metabolites of arginase, the polyamines (putrescine, spermidine and spermine) were examined by administering them via inhalation to anaesthetized mice. It was demonstrated
that spermine increases methacholine responsiveness in normal and allergic mice. Additionally, inhibition of polyamine synthesis improved AHR in a murine model. Thus, arginase and downstream polyamine metabolites contribute to AHR in asthma. Finally, the potential role of arginase in the exacerbation of asthma by air pollution was investigated. For this purpose, murine sub-acute and chronic murine models of allergic airways inflammation were employed, which exhibit inflammatory cell influx and remodeling/AHR, respectively, to determine the role of arginase in the response to concentrated ambient fine particles plus ozone. Allergic mice that were exposed to air pollution exhibited increased arginase activity and expression, compared to filtered air-exposed controls. Furthermore, inhibition of arginase attenuated the air pollution-induced AHR. Thus, the studies of the arginase pathway and downstream metabolites described in this thesis indicate that arginase inhibition may be a therapeutic target in asthma and may also protect susceptible populations against the adverse health effects of air pollution.
Acknowledgements

These studies were funded by the Ontario Thoracic Society/GlaxoSmithKline, AllerGen N.C.E., the National Sanitarium Association, the Keenan Research Centre of the Li Ka Shing Knowledge Institute of St. Michael’s Hospital, and the Heart and Stroke Foundation of Canada. Stipend support was provided by the Dr. Goran Enhorning Award in Pulmonary Physiology, Ontario Graduate Scholarship in Science and Technology, an Ontario Thoracic Society Doctoral Fellowship, and a Canadian Institutes of Health Research Frederick Banting and Charles Best Doctoral Award.

Portions of this thesis have been published by the American Physiological Society, the American Chemical Society and Bentham Press. Permissions to reproduce materials are included in Appendix A.

I would like to thank Dr. Jeremy A. Scott, who has been my supervisor since I began the Masters program in the Institute to Medical Sciences, and Dr. Frances Silverman, who joined as a co-supervisor upon my transfer to the PhD program. Dr. Scott’s expertise with animal modeling and direction of the molecular components of my studies was indispensible and very much appreciated. Dr. Silverman’s extensive experience in the field of air pollution and health was a tremendous help. I would like to thank both supervisors for their support and encouragement throughout my graduate program.

I would like to thank my Program Advisory Committee members, Dr. Hartmut Grasemann and Dr. Mingyao Liu. In particular I would like to thank Dr. Grasemann for helpful discussions and collaboration on studies of L-arginine metabolism, and members of his laboratory, Dr. Darakshanda Shehnaz and Dr. Hailu Huang. I would like to thank Dr. Liu for thoughtful input and inspiring enthusiasm.
I would like to thank the members of Dr. Scott’s and Dr. Silverman’s Laboratories, who have assisted in this work in many ways. In particular I would like to acknowledge Dr. Nivedita Khanna, whose specific contributions include assistance with pulmonary function testing of mice. I would like to thank Ms. Hajera Amatullah, who performed macrophage counts and image capture. Assistance with air pollution exposures from Dr. Bruce Urch and Mike Fila in Dr. Silverman’s laboratory is also greatly appreciated.

I am also grateful for the opportunity to work with collaborators from other laboratories, including Dr. David Christianson and Monica Ilies from the University of Pennsylvania, Dr. Mark Inman and Franco DiGiovanni from McMaster University, Dr. Jane Batt, Dr. Pamela Plant, Dr. Mike Ward, Dr. Philip Marsden, Brent Steer, and Dr. Chung-Wai Chow from the University of Toronto.

Last but not least I would like to thank my wonderful family, especially my parents, grandparents and husband for their patience and support.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>A1P</td>
<td>2-(S)-amino-5-(2-aminoimidazol-1-yl)pentanoic acid</td>
</tr>
<tr>
<td>aaRS</td>
<td>aminoacyl tRNA synthetase</td>
</tr>
<tr>
<td>ABH</td>
<td>2(S)-amino-6-boronohexanoic acid</td>
</tr>
<tr>
<td>ADMA</td>
<td>asymmetric dimethylarginine</td>
</tr>
<tr>
<td>AHR</td>
<td>airways hyperresponsiveness</td>
</tr>
<tr>
<td>Alum</td>
<td>aluminum hydroxide</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>ASC</td>
<td>apoptosis-associated speck-like protein</td>
</tr>
<tr>
<td>BAL</td>
<td>bronchoalveolar lavage</td>
</tr>
<tr>
<td>BCR</td>
<td>B cell receptor</td>
</tr>
<tr>
<td>BEC</td>
<td>S-(2-boronoethyl)-L-cysteine</td>
</tr>
<tr>
<td>CAP</td>
<td>concentrated ambient particulate matter</td>
</tr>
<tr>
<td>CAT/SLC7A</td>
<td>cationic amino acid transporter</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>COPD</td>
<td>chronic obstructive pulmonary disease</td>
</tr>
<tr>
<td>Cst</td>
<td>quasi-static compliance</td>
</tr>
<tr>
<td>DDAH</td>
<td>dimethylarginine dimethylaminohydrolases</td>
</tr>
<tr>
<td>DFMO</td>
<td>difluoromethylornithine</td>
</tr>
<tr>
<td>EAR</td>
<td>early asthmatic response</td>
</tr>
<tr>
<td>EPA</td>
<td>Environmental Protection Agency</td>
</tr>
<tr>
<td>FA</td>
<td>filtered air</td>
</tr>
<tr>
<td>FEV1</td>
<td>forced expiratory volume in one second</td>
</tr>
</tbody>
</table>
G tissue damping
GINA Global Initiative for Asthma
H tissue elastance
HDM house dust mite
Ig immunoglobulin
IL interleukin
i.p. intraperitoneal
LAR late asthmatic response
LCMS Liquid chromatography mass spectrometry
MHC major histocompatibility complex
MMA monomethylarginine
Myd88 myeloid differentiation gene 88
NANC non-adrenergic non-cholinergic
NADPH Nicotinamide adenine dinucleotide phosphate
NLRP3 NOD-like receptor pyrin domain containing 3
NO nitric oxide
NOS nitric oxide synthase
NOHA N⁶-hydroxy-L-arginine
O₃ ozone
OAT ornithine aminotransferase
ODC ornithine decarboxylase
OVA ovalbumin
PBS phosphate buffered saline
PEEP positive end expiratory pressure
PM$_{0.1}$  ultrafine particles with an aerodynamic diameter of less than 0.2 µm
PM$_{0.1-2.5}$  the fine fraction excluding ultrafines with an aerodynamic diameter between 0.1 µm and 2.5 µm
PM$_{2.5}$  fine particles with an aerodynamic diameter less than 2.5 µm
PM$_{2.5-10}$  the coarse fraction excluding fine particles with an aerodynamic diameter between 2.5 µm and 10 µm
PM$_{10}$  coarse particles with an aerodynamic diameter of less than 10 µm
PRMT  protein arginine methyltransferases
R  resistance of the respiratory system
R$_{N}$  resistance of the central airways
SDMA  symmetric dimethylarginine
SSAT  spermidine/sperimine N$^{1}$-acetyltransferase
TCR  T cell receptor
TGFβ  transforming growth factor beta
Th  T-helper
TLR  toll-like receptor
Treg  T regulatory cell
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Appendix A: Arginase in asthma – Recent developments in animal and human studies…….183
Chapter 1: Literature Review

This thesis examines the role of nitric oxide, arginase and downstream polyamine metabolites in murine models of asthma and exposure to urban air pollution. The literature review is divided into five sections. It will begin with a brief overview of human asthma and animal models, followed by introductions to nitric oxide, arginase, and polyamines. The final section of the literature review will discuss air pollution and its effects on asthma.

1.1. Asthma

1.1.1. Asthma characteristics and prevalence

Asthma is a chronic disease of the airways characterized by airflow obstruction, bronchial hyperresponsiveness and an underlying inflammation [1]. In affected individuals, symptoms include recurrent episodes of wheezing, chest tightness, breathlessness and coughing [1]. The airflow obstruction experienced during these episodes is often reversible either spontaneously or with treatment [2]. Asthma has many well-established links to allergy, as atopy to common aeroallergens is the strongest known predisposing factor to asthma [1].

Over the past 4 decades there has been a 50% increase in the global prevalence of asthma every 10 years [3]. Asthma now affects approximately 300 million people worldwide [4]. In Canada, approximately 8.4% of the population aged 12 or older self-reported having doctor-diagnosed asthma on the 2008 Canadian Community Health Survey [5]. However, the prevalence among Canadian children may be higher. In a recent multiethnic, population-based study of Toronto children in grades 1 and 2, the prevalence of current asthma was 11.3%, defined by parental report of asthma and wheeze or asthma medication use in the previous 12 months [6]. Thus, asthma poses a significant economic burden in Canada and worldwide, both due to direct medical costs such as hospital admissions, and indirect costs, such as time away
from work or school [4]. Globally, the economic costs of asthma have exceed those of HIV/AIDS and tuberculosis combined [3].

1.1.2. Inflammation in asthma

Persistent inflammation plays a defining role in asthma [1, 2, 7]. One of the central goals of the current National Institutes of Health asthma guidelines, and the Global Initiative for Asthma (GINA) guidelines is to minimize inflammation, through the use of corticosteroids [1, 2, 7]. Inflammation can affect the airways through the release of mediators such as cationic proteins, peroxidases, metalloproteases and histamine, enhancing susceptibility to bronchospasm [1, 8]. Many different types of inflammatory cells play a role in asthma, such as T lymphocytes, B lymphocytes, mast cells, eosinophils, neutrophils and macrophages [2].

There is considerable evidence pointing to T lymphocyte, or T cell, involvement in asthma [9]. T cells originate from the bone marrow and mature in the thymus [10]. During maturation T cells undergo positive selection, to ensure they interact with major histocompatibility complex (MHC) molecules, and negative selection, to ensure they are not specific for “self” proteins [11]. T cells can mature into several different subtypes, including T helper (Th) cells, cytotoxic T cells, memory T cells and regulatory T cells (Treg) [12]. T cells can be differentiated from other lymphocytes by the presence of the T cell receptor (TCR) [13]. The TCR locus contains V (variable), D (diversity) and J (joining) segments, which are randomly spliced together to produce TCRs with unique specificities, capable of recognizing a wide variety of antigens [13].

T cells must be activated by an antigen presenting cell (APC) bearing their specific antigen [12]. The most potent APC is the dendritic cell, which is present in most tissues, and highly concentrated in the lymph nodes [14]. Antigens are presented to T cells using the MHCI
class when the antigen originates from inside the cell, or the MHCII class when the antigen originates from outside cell [14]. Antigens presented on MHC I molecules are recognized by cytotoxic T cells expressing cluster of differentiation 8 (CD8) [14]. Th cells expressing CD4 recognize antigens presented on MHCII molecules [14].

Immediately after stimulation, Th cells begin to produce IL-2 and are designated Th0 [13]. As the Th0 cells continue to respond, they differentiate towards mature Th1, Th2, or Treg cells, depending on the cytokines present at the site of activation [13]. IL-12 promotes differentiation into Th1 cells, IL-4 promotes differentiation into Th2 cells, and the combination of TGF-β and IL-12 promotes differentiation into Treg cells [13]. When a T cell has been activated to mount a Th1 or Th2 response, it undergoes clonal expansion to generate more cells with the same antigen specificity, and some of these cells will be long lived and become memory T cells [14].

Th1 cells are important for activating effector cells, such as macrophages, to fight infection, through the production of cytokines such as IFN-γ and TNF-α [9]. Treg cells suppress immune responses and promote tolerance through the production of the anti-inflammatory cytokines transforming growth factor beta (TGF-β) and IL-10 [15]. Treg numbers have been shown to be reduced in the bronchoalveolar lavage fluid of asthmatics, suggesting that their ability to suppress the immune response to allergens may be impaired [16].

In allergic asthma, there is an increase in T helper type-2 (Th2) cells in the airways [17]. Th2 lymphocytes release cytokines including IL-4, IL-5 and IL-13 [9]. Th2 derived cytokines help to activate B cells to produce antibodies [9]. In particular, IL-4 and IL-13 are essential for antibody isotype switching towards the production of IgE [18]. Th2 cells also promote eosinophil activation through production of IL-5 [9]. Th2 activation has been associated with
measures of asthma severity and bronchial eosinophilia [9]. Thus, Th2 lymphocytes play an
important role in the inflammatory cascade in asthma [1].

The vast majority of asthmatics are sensitized to aeroallergens, and a central mediator in
atopic asthma is the IgE antibody, which is synthesized by antigen-specific B lymphocytes [17].
B cells play a central role in the humoral immune response, and much like T cells, B cells
undergo VDJ recombination to produce a unique variations in antigen specificity [19]. B cells
begin their development in the bone marrow are released at an immature stage [13]. In most
cases activation requires the co-operation of T cells [13]. B cells normally require two signals to
become activated by their specific antigen. The first signal involves cross-linking of the antigen
with B cell receptors (BCRs) on the cell surface, and the second signal comes from co-
stimulation by a T cell [19]. The production of IL-4 and IL-13 by T cells stimulates a “class
switch” from production of IgM antibodies to IgG or IgE [13]. IL-4 and IL-13 also stimulate
differentiation of B cells into plasma cells with expanded endoplasmic reticulum for efficient
antibody synthesis [13]. After stimulation, B cells undergo clonal expansion to generate more B
cells with the same antigen specificity to aid in the humoral immune response, and long-lived
memory cells which are able to respond swiftly in the event of a second exposure to the same
antigen [14].

Mast cells are also known to play a major role in asthma [20]. Mucosal mast cells
predominate in the human respiratory tract, and are present in greater numbers in the lungs of
asthmatics [21]. Mast cells are found in the lamina propria adjacent to blood vessels in normal
human airways, but in asthma they are also observed in the epithelium, mucous glands, and
embedded in the smooth muscle [17, 22]. Mast cells express a high-affinity receptor (FceRI),
which binds the Fc region of IgE immunoglobulins almost irreversibly, coating the mast cells
[22]. Activation of mast cells, through cross-linking of these IgE receptors induces
degranulation of the mast cell, releasing mediators such as histamine, proteases, serotonin, prostaglandins and leukotrienes [22].

Infiltration of eosinophils into the airways plays an important role in a large proportion of asthma cases [23, 24]. Eosinophils are readily recognized by their eosinophilic cytoplasmic granules, which contain toxic molecules such as histamine, eosinophil peroxidase, ribonuclease, lipase, and major basic protein, which are important in fighting helminths and other parasites, but can also be destructive to host tissues [14]. These mediators have been shown to contribute to epithelial damage and airflow obstruction in asthma [25]. Eosinophils can also secrete a wide range of cytokines, including IL-4, IL-5, IL-10, IL-12, IL-13 and TGFβ [26]. Eosinophils originate from the bone marrow, and allergen challenge in atopic asthmatic subjects increases the levels of CD34+ progenitor cells in the blood, which can undergo eosinophilopoiesis upon stimulation with IL-5 [27]. Eosinophils have played an important role in our understanding of allergic asthma, and they are still considered to be a key cell type, as treatment with corticosteroids has been shown to reduce airway eosinophilia in parallel with clinical improvement [1].

Neutrophils play a key role in the innate immune response and protection against microorganisms [28]. In asthma, neutrophils are the first inflammatory cells to accumulate in the airways following an allergen challenge [29, 30]. Neutrophils release cytokines, lipid mediators, proteases and reactive oxygen species, leading to airway damage, mucous secretion and bronchoconstriction [25]. Although neutrophils have not been traditionally thought to play a key role in asthma, the role of these cells has recently generated increasing interest, as increased sputum neutrophil counts have been associated with asthma exacerbation, and subtypes of asthma have been identified with predominant neutrophilic inflammation [24, 31, 32].
**Macrophages** are large phagocytic cells that differentiate from circulating monocytes, and can become resident in the lung [33]. Macrophages are the most numerous leukocyte resident in the airways and alveolar spaces under normal conditions [34]. Macrophages exhibit many different activation states that have been broadly classified into M1 (classical) and M2 (alternative) activation [33]. Alternatively activated macrophages have been shown to express proteins relevant to the asthmatic phenotype, such as arginase [35]. Classically activated macrophages release reactive oxygen and nitrogen species, including nitric oxide, which aid in killing pathogens but also induce cell damage and promote the recruitment of additional inflammatory cells [33]. Although macrophages play a role in inflammation, they also paradoxically have suppressive effects on lung T cells and IgE production [36].

For many individuals with asthma, exposure to allergen induces a two-phase response. Within 15-30 min of exposure allergic asthmatics experience acute bronchoconstriction, lasting 1-3 hr, termed the early asthmatic response (EAR) [37]. Some individuals also experience a late asthmatic response (LAR), which occurs 3 to 8 hours after allergen exposure [37]. It has been shown that while the EAR is dependent on histamine and release of other mediators, the LAR correlates with IL-5 signaling and eosinophilic infiltration [38]. Individuals who develop both an EAR and a LAR following allergen exposure typically have greater increases in airway hyperresponsiveness, and more profound allergen-induced airway inflammation, particularly eosinophilia [37].

Inflammation in asthma is complex, as alterations in many different leukocyte populations have been observed, as described above. In general, minimizing inflammation through pharmacologic intervention improves asthma control [1]. Future studies of the inflammatory mechanisms in asthma will continue to identify new mediators and examine them as potential therapeutic targets.
1.1.3. Remodeling in asthma

Chronic inflammation in asthma is often associated with permanent alterations in airway structure, referred to as airway remodeling. These structural changes can include epithelial thickening, goblet cell hyperplasia, sub-epithelial collagen deposition, increased smooth muscle mass, and angiogenesis [1]. Airways remodeling includes a thickening of the epithelial and excess mucus production. Thickening of the epithelia may be related to inflammation present in the airways, and contributes to mechanical airways occlusion [39]. Goblet cell hyperplasia and overproduction of mucus are also common characteristics of asthmatic airways remodeling [40]. Increased mucus production has been associated with asthma exacerbations, and mucus plugging is often seen in fatal cases of asthma [41]. Subepithelial fibrosis, including deposition of collagen has been described in asthmatic airways [1]. Collagen deposition in asthmatic patients has been demonstrated to contribute to a thickening of the basement membrane and fibrosis in the subepithelial space surrounding both the large small airways [42]. The molecular mechanisms behind fibrotic airways remodeling are not well understood. However, one important mediator of remodeling is TGFβ, which increases the production of extracellular matrix proteins by lung fibroblasts [43]. Increased smooth muscle mass is another important feature observed in asthmatic airways remodeling, and in fatal cases of asthma [44]. TGFβ also induces smooth muscle growth, and may be involved in promoting the increase in smooth muscle mass [43, 45]. Increased smooth muscle mass may be a consequence of hyperplasia, hypertrophy, or a mixture of the two processes [46]. Chronic inflammation in the lung can also promote angiogenesis and lymphangiogenesis, and thus bronchial vascular and lymphatic remodeling have also been associated with the structural remodeling changes in asthma [47]. Despite the effectiveness of current therapies in controlling inflammation, the remodeling process continues to progress in patients treated according to the current guidelines [1, 48].
Thus, remodeling is an important feature of asthma that requires further study in humans and animal models.

1.1.4. Airways hyperresponsiveness in asthma

Airways hyperresponsiveness is the characteristic functional feature of asthma [2]. Airways hyperresponsiveness describes the increased propensity for the airways to constrict [2]. Airways hyperresponsiveness is typically tested for in the clinical setting using methacholine as a smooth muscle agonist. Methacholine is preferred over histamine because of reduced variability and lower risk of systemic side effects [49]. Methacholine testing provides a direct-acting measure of airways responsiveness, as it is a non-specific muscarinic agonist that acts on receptors located directly on smooth muscle cells [50]. Methacholine responsiveness is calculated as the dose that produces a 20% fall in the forced expiratory volume (FEV\textsubscript{1}), referred to as the provocative concentration (PC\textsubscript{20}) [49, 50]. A PC\textsubscript{20} of less than 8 mg/mL is usually taken to indicate airways hyperresponsiveness [50]. Airways hyperresponsiveness is a sensitive test for asthma, with limited specificity, as a negative test rules out that asthma, while other conditions can also be associated with a positive test [1, 49]. The biological basis for airways hyperresponsiveness is thought to be increased eosinophil numbers in the airways of asthmatics, epithelial damage, and thickening of the airways smooth muscle [50]. However, the mechanisms are not completely understood and pathways such as L-arginine metabolism, described in further detail in Chapter 1.2 and 1.3, require further examination. Animal models are often used to explore the basic mechanisms of airways hyperresponsiveness in asthma due to the availability of molecular tools, the ability to administer pharmacologic reagents and the invasiveness of some necessary measurements.
1.1.5. Animal models of asthma

i Large animal models

Model systems include both non-rodent and rodent species. Non-rodent models include horses, cats, and primates. Horses suffering from the spontaneous inflammatory disease, heaves, exhibit many of the pathophysiological characteristics of asthma, including increased Th2 cytokines, airway obstruction, airway inflammation, mucus overproduction and airways hyperresponsiveness [51, 52]. Cats can also spontaneously develop atopic disease with eosinophilic airway inflammation, airway hyperreactivity and remodeling [53]. Primates provide a good model of the lung structure of humans, including the number of airway branches, the epithelial-mesenchymal trophic unit, and the presence of respiratory bronchioles [54]. However, large animal models also have different drawbacks, such as the lack of eosinophilia in equine heaves, the differences in innervation of the airways between humans and cats, and the necessity to deliberately challenge the airways in non-human primates to evoke an inflammatory response [52, 53, 55]. Non-rodent models are not commonly used in asthma studies as they are costly and pose the technological and ethical challenges of working with large mammals.

ii Guinea pig and rat models

The guinea pig and rat were the most commonly used animal models of asthma before the explosion of molecular technologies in the mouse [56]. Guinea pig and rat models have the advantage of recapitulating both the early and late reactions to allergen [57]. However, a potentially problematic feature of the guinea pig response to allergen is its extreme nature, sometimes requiring that the animals be pre-treated with antihistamines to prevent anaphylaxis [58]. In contrast, rats typically require high doses of bronchoconstrictors to evoke a reaction [59]. The use of these species have contributed to many important advances in asthma research,
including the elucidation of the role of histamine through studies in the guinea pig, and the use of rats in important toxicological testing of asthma drugs such as steroids [56, 58, 60]. However, the mouse has now become the rodent of choice for most investigators due to the wider range of specific molecular technologies, biochemical reagents, and transgenic and inbred strains.

iii Murine models

Sensitization and challenge murine models are currently the most commonly used animal systems in asthma research [61]. Sensitization is typically induced through intraperitoneal injection, and the experimental allergen is often combined with an adjuvant such as aluminum hydroxide ( alum) [62]. Despite its long-standing clinical use, the specific mechanisms of adjuvanticity were a mystery until recently [63]. In 2007, Li et al. demonstrated that when human or murine dendritic cells are stimulated with toll-like receptor (TLR) agonists plus alum in vitro, caspase-1 is activated to cleave pro-IL-1β and release the mature pro-inflammatory cytokine [64]. The role of TLRs in the adjuvant properties of alum are also supported by the fact that deficiency in myeloid differentiation primary response gene (MyD88), the universal TLR adapter protein, reduced the immunopotentiating effect of alum [65]. It has also been shown that NOD-like receptor pyrin domain containing 3 (NLRP3) inflammasome signaling was required for an efficient antibody response to antigen and alum in vivo, as transgenic mice deficient in NLRP3, caspase-1, or the intermediary protein apoptosis-associated speck-like protein (ASC) failed to mount a response [66]. In murine models of asthma, sensitization with antigen and alum elicits a Th2 response, including production of allergen-specific IgE [62]. Subsequent exposure of the respiratory system to the allergen results in lung-specific allergic inflammation [56].
Some recognized human allergens such as house dust mite (HDM), *Aspergillus fumigatus* or ragweed extracts are used in murine sensitization and challenge models of allergic airways inflammation [58]. HDM extract contains cysteine proteases, Der p 1 and Der p 9, which can act as antigens with built-in adjuvant activity [67]. HDM also may contain small amounts of endotoxin and fungal toxins [68]. HDM is useful for both acute and chronic murine models of allergic airways inflammation and remodeling [69-71].

*Aspergillus fumigatus* is a ubiquitous filamentous fungi with cell walls containing beta-glucan [72]. The organism contains a variety of potential antigens, including ribotoxin Asp f 1, perixosome-like protein Asp f 3, superoxide dismutase Asp f 6, and allergic Asp f 2 [73]. *A. fumigatus* antigens activate innate immune cells via TLR2 and TLR4 [74]. The beta-glucan content of the *A. fumigatus* cell wall is also recognized by dectin-1, which amplifies TLR-mediated inflammatory cytokine secretion [75]. Thus, HDM and *A. fumigatus* murine models of allergic airways inflammation do not require sensitization with an adjuvant such as alum, and both sensitization and challenge can be carried out through the intranasal or inhalational route [76, 77].

Short ragweed and common ragweed (*Ambrosia artemisiifolia*, and *Ambrosia elatior*, respectively) are the ragweed species to which most allergic individuals are sensitized [78]. Ragweed pollen is composed of over 20 specific proteins divided into six major groups, of which Amb a 1 and Amb a 2 demonstrate the highest affinity for human IgE derived from sensitized individuals [79, 80]. Although certain reports have indicated that ragweed pollen may possess some adjuvant activity [81], murine ragweed models of asthma commonly employ alum as an adjuvant to establish sensitization [82, 83].

Allergens such as ovalbumin (OVA), a common experimental allergen derived from chicken egg, also require the use of an adjuvant in order for sensitization to occur, and do not
produce inflammation if given strictly through the inhalational route [61]. These represent limitations of the use of OVA as a model of allergy, which usually occurs spontaneously in humans [62]. However, there are also advantages of using OVA. It is inexpensive, non-toxic, specific molecular reagents are available, and when animals have been previously sensitized, inhalation of OVA produces robust pulmonary inflammation [58].

Although no murine model recapitulates the full range of clinical symptoms of human asthma, many do exhibit certain features of the disease, such as inflammation, airways hyperresponsiveness and remodeling [84]. Thus, it is essential to choose the appropriate murine model to study the desired human phenotype or distinct pathogenic mechanism of interest, rather than attempting to model asthma as a whole [85].

Mouse models offer several advantages including; reduced cost and different ethical considerations compared to human studies, the availability of inbred and transgenic strains, knowledge of the complete mouse genome, availability of specific reagents and experimental platforms [86]. The utility and validity of murine models have been debated heavily, likely due to their popularity and the limitations encountered in attempting to translate results into new treatments [84]. However, many potential treatments are tested as prophylactic agents in mice, not as therapeutics [86]. This reinforces the need for appropriate choice of animal models for the question being asked, and interpretation of data accordingly.

Many important strides towards understanding asthma were made using murine models, including studies crucial to our understanding of Th1/Th2 polarization, IL-4, IL-5 and IL-13 [58, 61]. While “humans remain the best model of human disease”, the use of model systems is indispensable for pre-clinical studies [87]. The following sections will discuss the modeling of three important aspects of asthmatic disease in mice; inflammation, remodeling and airways hyperresponsiveness.
iv Inflammation in murine models

Murine models play an important role in studies of allergic lung inflammation. However, the specific sensitization and challenge protocol, particularly the duration of the challenge phase plays an important role in determining the extent of inflammation produced [62]. Although different laboratories have developed many differing protocols to different experimental allergens, in general sub-acute murine models of asthma involve challenge periods of between one and three days, while acute models continue for four to nine days, and both have proven to be useful for studying inflammation [84, 88]. Generally, within the four to nine day time-frame, increasing the number of challenge days results in increasing inflammatory cell influx into the lung, and does not induce tolerance [56].

Mouse models have been used in the past to identify important inflammatory features of asthma, such as eosinophilia [61]. Thus, the ability of murine models to mimic the inflammatory phenotype of asthma provides important opportunities for research and therapeutic testing.

iii Remodeling in murine models

Research into the structural changes in asthmatic airways remodeling can also be aided by the use of murine models. While some of the signs of remodeling, such as goblet cell hyperplasia and epithelial cell hypertrophy may be observed in acute models of allergic airways inflammation, chronic murine models better mimic the full range of structural changes in human disease such as collagen deposition, increase in smooth muscle mass and angiogenesis [1, 56, 89, 90].

Hypertrophy, hyperplasia and imbalance between cell proliferation and apoptosis have been implicated in the increase in airway smooth muscle mass, but the molecular mechanisms
have not been clearly delineated [91]. Chronic murine models are important tools for dissecting the mechanisms involved in remodeling, and have contributed to our understanding of the role of TGFβ [43]. Current asthma therapies are not able to prevent or reverse airways remodeling, and chronic murine models will be indispensable in the search for new treatments [48].

iv Airways hyperresponsiveness in murine models

In animal models, the increased propensity of the airways to narrow in response to bronchoconstrictor agents is typically demonstrated by comparing an allergen challenged group, to controls [50]. Both acute and chronic murine models of allergic airways inflammation mimic the airways hyperresponsiveness feature of asthma, depending on the dose and duration of exposure to the experimental allergen [88, 92]. Increasing days of challenge in acute murine models between four and nine days generally results in increasing airways hyperresponsiveness, correlating with increasing cellular infiltration [62]. Chronic murine models of asthma exhibit a more mild and persistent airways hyperresponsiveness, and in these models challenges generally must take place less frequently or tolerance to the experimental allergen will develop [93].

Several techniques have been employed to estimate or measure airways resistance in mice, the two most widely used techniques being the whole-body plethysmograph and the small animal ventilator. The whole-body plethysmograph has the advantage of being noninvasive and repeatable on the same animals at different times. Enhanced pause, or Penh, is a term used to quantify plethysmography measurements, however, it is not a direct measure of the airways resistance [94]. Recent studies have found that Penh does not correlate as consistently with lung resistance as previously thought [85, 95].

The current gold standard for the measurement of airways responsiveness in small animals employs mechanical ventilation, and the forced oscillation technique. The flexiVent®
system is used widely to demonstrate the effects of genetic, pathological and environmental factors on respiratory mechanics and methacholine responsiveness [96-98]. One disadvantage of the forced oscillation technique is that the level of invasiveness requires that measurements are made while the mouse is anaesthetized, and therefore the measurements can not be made while the mouse is in its “natural state” [99].

Thus, murine models of allergic airways inflammation, coupled with the use of the flexiVent® to assess airways responsiveness, represent important tool for the exploration of the molecular mechanisms contributing to airways hyperresponsiveness in asthma.
1.2. Nitric oxide

Thus, asthma is a chronic respiratory disorder with a high prevalence of approximately 8% in Canada. Inflammation, airways hyperresponsiveness and remodeling are features of asthma that can be studied using the mouse as a model system, acknowledging that there are limitations to the use of animals as models for human disease. This section will begin to delve into molecular mechanisms that have been implicated in the pathophysiology of asthma. The nitric oxide synthase pathway plays an important role in the health and disease of the respiratory system [100] and is currently under investigation to elucidate its role in asthma and identify potential therapeutic targets.

1.2.1. The discovery of nitric oxide

In 1980, Furchgott et al. made the critical observation that the relaxation of blood vessels by acetylcholine is dependent on the presence of an intact endothelium [101]. They coined the term endothelial derived relaxing factor (EDRF) to describe the unknown molecule responsible for this phenomenon [101]. In 1986, Ignarro et al. were examining the role of cyclic guanosine monophosphate (cGMP) in the endothelium-dependent relaxation of blood vessels, and demonstrated direct activation of purified guanylate cyclase by EDRF [102]. Moncada et al. demonstrated the short half-life of EDRF, and showed that it could be prolonged by superoxide dismutase, suggesting it may be a free radical-like molecule [103-105]. Murad et al. had proposed back in 1979 that nitrovasodilators may generate nitric oxide in vivo, which binds to the heme moiety of guanylate cyclase and activates the enzyme [106, 107]. In 1988, both Furchgott and Ignarro proposed that NO and EDRF may be the same molecule [108, 109]. Ten years later, Furchott, Ignarro and Murad were jointly awarded the Nobel Prize in Physiology or Medicine for the discovery of NO [110].
1.2.2. Nitric oxide synthases

Nitric oxide is a relatively stable free radical that can dissolve across comparatively large distances in aqueous solutions, and freely cross biological membranes [111]. These properties make it an effective signaling molecule, requiring no specific receptors or targeted degradation [112]. Since its discovery in the cardiovascular system, it has been found to be involved in many homeostatic and pathologic processes in the respiratory system as well [100, 111].

The enzyme responsible for generating NO, Nitric Oxide Synthase (NOS), was identified in 1990 by Bredt and Snyder [113]. Three isoforms have been identified, expressed from different genes [111]. Their prefixes reflect the tissues from which they were isolated, while their official nomenclature reflects the order in which they were characterized; neuronal NOS (NOS1), inducible NOS (NOS2), and endothelial NOS (NOS3) [111]. NOS functions as a homodimer, converting L-arginine substrate into L-citrulline and NO co-products, in a two-step reaction with Nω-hydroxy-L-arginine (NOHA) as an intermediate [114]. NOS requires nicotinamide adenine dinucleotide phosphate (NADPH) and molecular oxygen co-substrates and flavin-adenine dinucleotide, flavin mononucleotide, and tetrahydrobiopterin cofactors [111, 114]. The constitutive isoforms, NOS1 and NOS3, require activation by calcium and calmodulin [114]. Calmodulin binds tightly to NOS2, and persistently activates this isoform in a calcium-independent manner [115]. NOS1 and NOS2 have been characterized as cytosolic proteins, while NOS3 is targeted to caveolae invaginations of the plasma membrane [112].

1.2.3. Nitric oxide in lung homeostasis

As it was noted that removal of the endothelium in isolated blood vessels resulted in increased vasoconstriction, it was similarly discovered that removal of the airway epithelium
increased the sensitivity to bronchoconstrictor agents such as acetylcholine [116]. Thus, the importance of an “epithelium-derived relaxing factor” in the lung was proposed [116]. It is now known that NO plays a variety of important roles in homeostasis, including regulation of blood flow, neurotransmission, cytotoxic defense and regulation of bronchiomotor tone [112]. Thus, a wide variety of cells produce and release NO, including vascular endothelial cells, airway epithelial cells, nerves and inflammatory cells [117]. NOS1 is present in the airway epithelium and non-adrenergic non-cholinergic (NANC) neurons surrounding both airways and blood vessels [117-119]. NOS2 is constitutively present in airway epithelial cells, and inducible in smooth muscle, fibroblasts, macrophages, neutrophils and mast cells [117-120]. NOS3 is constitutively present in endothelial and epithelial cells [121].

The NO produced by these cells can have two main actions, cytotoxicity and signal transduction. The constitutive NOS isozymes produce picomolar amounts of NO when activated by increased intracellular calcium levels evoked by membrane depolarization or by contractile agonists [100]. However, NOS2 produces nanomolar levels of NO, and is regulated at the transcriptional level, with activation sustained for hours to days after the protein is induced [100]. Thus, when NOS2 expression is induced by proinflammatory cytokines such as tumour necrosis factor (TNF-α), IFN-γ or IL1-β, cytotoxic levels of NO are produced [112]. High levels of NO have been shown to be effective in killing pathogens, and inhibiting tumour growth [114]. However, cytotoxic NO also has the potential to damage lung tissues.

When NO is produced by the constitutive isozymes, or by the constitutive low-level expression of NOS2 in the epithelium, the main action is signal transduction [100]. The best characterized signal transduction pathway is initiated when NO diffuses from lung epithelial cells into adjacent smooth-muscle cells, co-coordinating with the heme group of guanylyl cyclase and activating it to produce cyclic guanosine monophosphate (cGMP) from guanosine...
triphosphate [122]. Increased cGMP concentration leads to smooth-muscle relaxation through the activation of cGMP-dependent protein kinase, reducing intracellular calcium levels and decreasing the sensitivity of the contractile system to intracellular calcium [100]. NO also mediates relaxing effects that are independent of guanylyl cyclase [123]. Thiol groups on cysteine residues in proteins and biological small molecules can react with NO, producing S-nitrosothiols (SNO) [124]. S-nitrosylation of specific thiol groups on proteins induces redox-based post-translational modifications, changing the function of a wide variety of proteins [125]. The most common SNO is s-nitrosoglutathione (GSNO), which forms from the reaction of NO and glutathione and is present at higher concentrations than free NO in the normal airway, due to its increased stability [126]. GSNO and protein-SNOs act as a stable store of bioavailable NO [112]. Thus, as a bronchodilating signaling molecule, NO plays an important role in modulating airways tone.

1.2.4. Nitric oxide in asthma

Inflammation

Inflammation is an important characteristic of asthma, and many inflammatory cells that infiltrate the lung can be induced to express NOS2, including eosinophils and macrophages [1]. Eosinophils have also been shown to transcribe NOS2 mRNA and increased nitrite/nitrate levels have been shown in eosinophil cell culture [127]. Upregulation of NOS2 and production of NO or stable metabolites have now been shown in macrophages derived from subjects several diseased states [128]. NOS2 is now accepted as the hallmark of “classical M1 macrophage activation” [33].

The role of NO in inflammation can also be assessed non-invasively, as its activity can be detected through exhaled NO. Gustafsson et al. first demonstrated that endogenous NO is
present in the exhaled air of experimental animals and humans [129]. Shortly afterwards, several investigators demonstrated that exhaled NO concentrations are higher in patients with asthma, compared to controls [130-133]. It was determined through immunohistochemistry that this increase was likely caused by increased NOS2 in airway epithelia, eosinophils and other inflammatory cells in asthmatic patients [134]. Exhaled nitric oxide measurements are now recognized as a valuable tool in the management of asthma as a surrogate marker of increased airway inflammation [135, 136].

Thus, much attention has been paid to the role of NOS2 in asthma, while the contributions of NOS 1 and 3 have remained relatively more mysterious. One exception being the systematic study of the all three isozymes using targeted gene knockout mice performed by De Sanctis et al. [88]. The development of airways inflammation and production of specific IgE was not affected by the knockout of any NOS isozymes [88]. Knockout of a single NOS isozyme, or NOS1 and 3 concurrently did not affect total NOS activity compared to wild type [88]. Sensitization and challenge with OVA was associated with an increase in total NOS activity, and NOS2-derived activity, in wild-type and all knockouts except NOS2−/− [88]. However, knockout of the NOS2 gene did not affect the development of airways hyperresponsiveness [88]. Surprisingly, OVA/OVA NOS1−/− and NOS1&3−/− knockout mice were significantly less sensitive to methacholine [88]. This study demonstrated that NOS2 is not required for the development of airways inflammation and hyperresponsiveness, while the genetic knockout of NOS1 attenuated AHR [88]. Thus, this study suggested a functional link between NOS1 and airways hyperresponsiveness. Human genetic linkage studies have also demonstrated associations between polymorphisms in NOS1 and asthma [137-139]. Additionally, genetic associations have been made between NOS1 genotype and exhaled NO
Thus, the relationship between inflammation, exhaled NO, the induction of specific NOS isoymes and airways hyperresponsiveness in asthma has not been completely elucidated.

ii  **Airways tone and responsiveness**

As expired NO and NOS2 protein expression are increased due to the inflammatory phenotype in asthma, NOS inhibition was considered as a potential therapy for asthma. However, studies have not been able to prove NOS inhibition an effective therapeutic strategy in human asthma [141]. This illustrates a paradox in the field of nitric oxide and asthma, because although the concentration of exhaled NO is increased in patients with asthma, and NO is known to relax airways smooth muscle by co-coordinating with the heme group of guanylyl cyclase and activating it to produce cGMP [122], airway responsiveness is increased in asthma [100]. Therefore, the location of increased NO production vs. reduced NO production in the lung may be important. While exhaled NO is increased, this is likely due to NOS2 induction in the epithelium and inflammatory cells [134]. However, NO bioactivity in the smooth muscle cells may be limiting, leading to increased airways tone and responsiveness [57]. Resistance to bronchoconstriction in response to agonists is thought to be dependent on the action of the constitutive NOS isoymes [112]. However, the production of NO is also dependent on the availability of L-arginine to NOS, as demonstrated by the rise in exhaled NO in normal subjects following oral treatment with L-arginine [142].

In asthma, eosinophilia and increased eosinophil degranulation may result in an increase in major basic protein release, which inhibits the transport of L-arginine through the Na⁺-independent cationic amino acid transporters (CAT) of the y+ system [143]. Under conditions of substrate limitation for NOS, uncoupling of the enzyme and generation of superoxide from the reductase domain can occur [144]. NO can react with superoxide free radicals producing
peroxynitrite, a powerful oxidant [145]. This both disrupts the normal bioactivity of NO and results in the oxidation of biomolecules, including tyrosine groups on proteins [112]. Peroxynitrite can also directly evoke airways hyperresponsiveness and cause cellular damage leading to a potentiation of inflammation [146].

The NO pathway is also important in mediating nervous control relating to airways responsiveness. Early on it was determined that the actions of some neurons in the airways could not be blocked by adrenergic or cholinergic antagonists, and these were termed the nonadrenergic, noncholinergic (NANC) nerves [147]. Inhibitory (iNANC) nerves are the only known neural bronchodilator pathway in the human airways, and work to oppose cholinergic bronchoconstrictor stimuli through the release of bronchodilating NO [148]. Thus, impairment of NO production in asthma due to substrate limitation can exacerbate airways responsiveness through several mechanisms, and recent hypotheses have focused on the modulation of bioavailability by another branch of L-arginine metabolism, arginase, discussed in more detail in the next section.

1.3. Arginase

Nitric oxide, produced through the NOS pathway, is important in the normal functioning of the respiratory system and is perturbed in asthma. Another pathway that has recently been implicated in asthma is the arginase pathway. The NOS and arginase pathways are closely related, as described below, and the balance of L-arginine metabolism between these two pathways is likely important in asthma.
1.3.1. **Arginase isozymes**

While the discovery of NOS is comparatively recent, arginase activity was detected in the early 1900’s [149, 150]. In 1932, arginase was deduced to be the sixth and final enzyme in the urea cycle by Krebs and Henseleit [151]. Arginase is a hydrolytic enzyme that metabolizes L-arginine into L-ornithine and urea [152]. Two isozymes of arginase, arginase 1 and 2, are expressed from different genes [153]. Genetically, each arginase isoenzyme consists of eight exons, with similar intron-exon boundaries but non-homologous promoter regions, indicative of a gene duplication event [154]. Both arginase 1 and 2 function as homotrimers, and require manganese as a cofactor [152, 155]. Although the arginase isozymes share similar structures, they exhibit different organ-specific and intracellular expression patterns. Arginase 1 is a cytoplasmic enzyme, highly expressed in the liver and inducible in other cell types such as macrophages [152]. Arginase 2 is localized to the mitochondria and expressed in many extrahepatic cell types [150].

Deficiency of arginase 1 in humans leads to the rare disorder hyperargininemia, which can halt normal development in childhood, cause progressive neurodegeneration and potentially fatal hyperammonemia [156]. Similarly, arginase 1 knockout mice die postnatally [157]. Arginase 2 deficiency has not been reported in humans, and knockout of this gene in mice results in only subtle phenotypes, including elevated plasma L-arginine levels and reduced fertility in males [158]. Although arginase has been known to play a vital role in metabolism for some time, its role in the normal and pathological functioning of the respiratory system and regulation of nitric oxide production has only recently become an area of intense research.
1.3.2. Arginase regulates L-arginine bioavailability and NO production

Reciprocal regulation of NOS and arginase have been proposed both at the level of induction by Th1 and Th2 cytokines, respectively, and at the level of enzyme activity [159]. The production of NO can affect arginase activity through S-nitrosylation, resulting in increased stabilization of the arginase trimer and increased activity (Figure 1) [160]. However, NOS also inhibits arginase activity by releasing small amounts of its reaction intermediate, N-hydroxy-L-arginine (NOHA), a competitive arginase inhibitor (Figure 1) [161]. Cationic amino acids, including L-ornithine, produced by arginase, inhibit uptake of L-arginine through the CAT transporters (Figure 1) [162, 163]. Therefore arginase also functions reciprocally to inhibit NOS activity, by consuming its substrate and reducing transport.

Hey and Racké et al. first reported substrate competition between the NOS and arginase pathways in studies employing macrophages [164]. Although the $K_m$ of L-arginine for arginase
is 1000-fold higher than for NOS, the $V_{\text{max}}$ is also 1000-fold higher, supporting the theory that substrate competition can occur [150, 165, 166]. However, it is also important to consider where the enzymes may be expressed in the same microenvironment, or co-localized within the same cells. NOS1 has been demonstrated to localize primarily to airway epithelial and smooth muscle cells [167]. Arginase 1 has also been shown in human airway epithelial cells in bronchoscopy samples from asthmatic subjects [76, 168]. Thus, arginase and NOS1 may be in competition for intracellular L-arginine pools within airway epithelial cells and under conditions of increased arginase 1 expression this competition may be exacerbated.

1.3.2. Arginase in asthma

Genetic associations have been made between asthma/atopy and arginase isozymes. Hakonarson et al. demonstrated a link between chromosome 14q24, a genetic locus containing the arginase 2 gene, and asthma in Icelandic families [169]. In an independent study, Li et al. genotyped 4 single nucleotide polymorphisms each in arginase 1 and 2 in 433 children with asthma and their parents [170]. They demonstrated an association between arginase 1 polymorphisms and atopy, and arginase 2 polymorphisms were associated with relative risk of developing asthma [170]. Finally, a recent study by Litonjua et al. demonstrated that polymorphisms in the arginase 1 gene are associated with the response to bronchodilator drugs in four independent asthma cohorts [171]. Genetic associations between the arginase genes, atopy and asthma are suggestive of an important functional role for this pathway in human asthma.

1. Arginase and inflammation

In 1995, Corraliza and Modolell first reported the induction of arginase in murine macrophages by Th2 cytokines [159, 172]. Further studies revealed that arginase 1 is induced by
IL-4 and IL-13 through a STAT6-dependent enhancer-mediated mechanism [173, 174]. The upregulation of arginase in inflammatory cells first became of interest to asthma researchers when a study by Zimmermann et al. was published in 2003. The authors demonstrated increased arginase 1 mRNA expression in two independent murine models of allergic airways inflammation, localized to macrophages and areas of peribronchiolar inflammation [76]. Arginase-positive inflammatory cells were also demonstrated in the mucosa of human asthmatic bronchial biopsy and bronchoalveolar lavage samples [76].

Following these seminal findings, alternative activation of macrophages has been proposed to be important in murine models of allergic inflammation [35, 175]. The existence of an alternative activation state in macrophages was proposed in the early 1990’s, as a phenotype induced by IL 4 and characterized by expression of the macrophage mannose receptor [175, 176]. At present, arginase 1 is classified as one of the defining molecules of the alternative-activation state [35].

Munder et al. performed the first comparative examination of arginase expression in human and murine immune cells and showed that in contrast to the highly-inducible expression of arginase that occurs in murine macrophages and dendritic cells, human neutrophils express arginase 1 constitutively [177]. Arginase is sequestered within azurophilic granules in these cell types, and exocytosed after activation [177, 178]. The expression of arginase in neutrophils in human asthma may prove important to our understanding of the disease, as asthmatic subtypes with severe disease and predominant neutrophilic inflammation have been increasingly identified and studied [24, 179]. Further, as mentioned above, increased neutrophil cell counts in sputum have been associated with episodes of airflow obstruction and acute exacerbations [31, 32, 180]. Thus, while there are some species-specific differences in the inflammatory cell
types expressing arginase, the enzyme is highly expressed in inflammatory cells and has been
demonstrated in human asthma and murine models.

ii Remodeling

As described in section 1.1.3, remodeling in asthma is generally thought to be driven by
chronic airway inflammation. In particular, eosinophilia and secretion of TGF-β and IL-13 are
considered to play an important role [181]. Lung fibrosis studies can inform our understanding
of the role of arginase in airway remodeling, as the mechanisms underlying increased collagen
deposition in this disease likely exhibit some degree of overlap with those at work in asthma.
Interestingly, arginase activity in mouse lung tissue and fibroblasts was dose-dependently
increased by the profibrotic factor TGF-β and has been correlated to collagen content in rat lung
allografts [182]. Another group recently interrogated the genetic pathways upregulated by TGF-
β and found that arginase 1 and 2 expression were induced [183]. These data support the
hypothesis that arginase is induced by TGF-β as part of a pro-fibrotic genetic program, likely
contributing via the metabolism of L-arginine to L-ornithine, leading to increased downstream
production of proline and polyamines.

Similar to the implication regarding substrate competition, the subcellular localization of
the arginase isoenzymes may also have implications in the downstream fates of the products of
arginase and NOS. The localization of ornithine decarboxylase (ODC) in the cytosol may
preferentially steer cytosolic L-ornithine towards the production of polyamines, which are
important in cell proliferation. Ornithine aminotransferase (OAT) is localized to the
mitochondria, potentially directing the L-ornithine produced by arginase 2 towards the
production of proline and collagen synthesis [184]. Future studies of the differential roles of the
arginase isozymes in airways remodeling and potential therapeutic interventions will need to consider sub-cellular localization.

The effect of steroids on arginase expression in human asthma is currently unclear. However, a recent study by Lara et al., provides some information. In this study of severe asthmatics, moderate asthmatics and healthy controls, the severe asthmatics exhibited increased serum arginase activity which was related inversely to airflow, despite the fact that 95.2% of these patients were being treated with inhaled corticosteroids, and 38.1% with systemic corticosteroids [185]. Thus, there is significant potential for new therapies to target arginase, as current treatments do not appear to attenuate the activity of this pathway or prevent remodeling.

These findings suggest that increased arginase activity in chronic asthma could contribute to airways remodeling. Studies in chronic murine models are needed to examine the expression and functional effects of this enzyme.

iii Airways tone and responsiveness

The effects of increased arginase activity on airways responsiveness in asthma models was first demonstrated by Meurs et al., using guinea pig tracheal rings excised from ovalbumin sensitized and challenged animals [186]. Interestingly, the allergen-induced AHR was completely inhibited by nor-NOHA to the level of normoresponsive unchallenged controls [186]. The effect of nor-NOHA was completely prevented by co-incubation with an NOS inhibitor, clearly demonstrating that arginase inhibition reduces the AHR by restoring the production of cNOS-derived NO [186]. Further in vitro studies from the same group demonstrated that treatment with nor-NOHA also restored the impaired iNANC-nerve mediated NO production and airway smooth muscle relaxation [187].
The first *in vivo* demonstration of the effects of arginase inhibition was performed by Yang *et al.*, who employed RNA interference to attenuate arginase expression and activity in a murine model of IL-13 induced AHR [188]. The authors demonstrated that interfering with the upregulation of arginase 1 by IL-13 abrogated the development of AHR to methacholine, measured *in vivo* with the flexiVent® system [188]. This study provides evidence that arginase upregulation is functionally relevant to airways responsiveness *in vivo*, however it did not address whether or not pharmacologic modulation of arginase activity would affect airways responsiveness *in vivo* in asthma models.

Maarsingh *et al.* examined the effects of pharmacologic inhibition of arginase *in vivo* in a guinea pig model of acute allergic asthma and found an attenuation of AHR [189]. Distinct from the treatment-based protocol, the protective effect of arginase inhibitors on allergen-induced AHR in asthma has also been studied in both guinea pigs and mice. Remarkably, the sensitivity of the airways to inhaled allergen was reduced when inhaled arginase inhibitor was administered prior to allergen challenge [189]. This anti-allergic effect of arginase inhibition may involve increased formation of NO, which is known to inhibit mast cell activation [190]. The protective effect of arginase inhibitors on the development of allergen-induced AHR was confirmed in a mouse model of asthma, showing that intraperitoneal treatment with arginase inhibitor over two weeks significantly reduced the AHR to methacholine [191].

In conclusion, targeting increased arginase activity using inhaled arginase inhibitors may be a novel therapeutic strategy in allergic asthma, however, studies that employ a treatment-based protocol for delivery of the inhibitor may have more relevance, compared to prophylactic protocols.
1.4. Polyamines

1.4.1. Polyamine metabolism

Polyamines were first described in the 17th century, as crystals observed in human semen by the Dutch scientist Antonie van Leeuwenhoek [192]. These crystals were then demonstrated to consist of spermine in 1924 by Otto Rosenheim [193]. The polyamines (putrescine, spermidine and spermine) are now known to be low molecular weight aliphatic amines that are highly charged cations under physiological conditions [194]. Polyamines are ubiquitous, as all eukaryotic cells contain measurable concentrations [195].

In mammalian cells, the primary pathway for polyamine synthesis is initiated immediately downstream of arginase, by ornithine decarboxylase (ODC), a pyridoxal phosphate-dependent enzyme, that converts L-ornithine into putrescine (Figure 2) [195]. Spermidine synthase can then convert putrescine into spermidine, deriving the required aminopropyl group from decarboxylated S-adenosylmethionine (Figure 2) [195]. Spermine is then formed through a second aminopropyltransferase reaction, via spermine synthase (Figure 2) [196].

It was originally thought that only plants and microorganisms could synthesize polyamines from agmatine, the decarboxylation product of L-arginine [195]. However, Li et al. demonstrated the production of agmatine in the bovine brain by an endogenous arginine decarboxylase [197]. Agmatine represents an alternative pathway for the synthesis of polyamines, as it is converted into putrescine and urea by agmatinase, a member of the arginase superfamily (Figure 2) [198].
Figure 2: Polyamine Synthetic Pathways

Putrescine, the first polyamine in this pathway, is synthesized from L-ornithine, the product of arginase, by ornithine decarboxylase (ODC). Spermidine and spermine are synthesized by spermidine synthase (SpdS) and spermine synthase (SpS), respectively.

As polyamines must be maintained at relatively stable concentrations in order to promote cell growth and survival, the activity of ODC is regulated at many levels. Suppression can occur through antizyme, which binds to ODC, inhibiting its activity and targeting it for degradation (Figure 2) [199]. The cell maintains a pool of mRNA coding for antizyme, which is rapidly transcribed into protein following a programmed +1 ribosomal frameshift that responds to increased cellular polyamine levels [200]. Interestingly, agmatine is also capable of inducing antizyme, and thus paradoxically reduces cellular polyamine content and proliferation (Figure 2) [201]. It is postulated that the activity of ODC is highly controlled because decreased polyamine levels are detrimental to cell survival, while an excess is toxic [202].

Catabolism of polyamines also modulates cellular levels of these endogenous small molecules, and the inducible enzyme spermidine/spermine N\(^1\)-acyetyltransferase plays an
important role [203]. However, the catabolism of higher order polyamines back to spermidine or putrescine generates hydrogen peroxide, contributing to oxidative stress and cellular damage [204]. Igarashi et al. propose that under normal conditions the concentration of free polyamines is low, as they normally exist in complex with macromolecules, and normal levels of polyamine breakdown does not result in appreciable oxidative stress [205]. However, under conditions of inflammation or tissue damage, excessive polyamine breakdown can contribute to cellular injury [205]. Thus, the metabolism of polyamines is a highly complex and cyclical pathway, and its importance in cell proliferation is discussed in the following section.

1.4.2. Polyamines and cellular proliferation

The essential role of polyamines in cell growth and differentiation is well known, however the specific mechanisms are still under active investigation. The positive charges on polyamines are distributed along the carbon chain, making their electrostatic charge structure different from the point charges of ions, and allowing them to interact in unique ways with polyanionic macromolecules [196]. Spermidine and spermine are capable of interacting with phosphate groups on DNA, bridging the gap between the major and minor grooves, and bringing together distant sites on the same strand [196]. Polyamines also promote B to Z conversion of DNA, a phenomenon that promotes transcription, and may be involved in the activation of promoters of growth-related genes [205].

1.4.3. Polyamines in lung biology and disease

Polyamines likely play an important role in lung biology, as they are selectively transported and concentrated in the lung to levels much higher than those found in the blood serum [206]. Accumulation of polyamines in the lung was originally characterized due to the
unfortunate effects of the herbicide paraquat, a selective lung toxin [207]. The pulmonary toxicity of paraquat has been attributed to uptake through the same polyamine transport mechanism that concentrates endogenous polyamines in the lung, and this discovery formed the basis for further studies demonstrating the active transport of spermidine and spermine [208]. Although the polyamine transport system has been well characterized kinetically and pharmacologically, to date no one has been able to clone and characterize a specific polyamine transporter [209]. Uemura et al. recently described the role of caveolae-dependent endocytosis in polyamine uptake, and demonstrated that the polyamine export protein, solute carrier 3A2 can also mediate the uptake of polyamines under certain conditions [210]. In addition to the roles polyamines play in cell growth, described above, they have also been shown to participate in certain lung pathologies.

Polyamines have been implicated in the pathology of several serious lung diseases, such as cystic fibrosis, pulmonary arterial hypertension and lung cancer. Rennert et al. first reported elevated blood levels of polyamines in cystic fibrosis patients [211]. Subsequently, another group determined that levels of polyamines in the urine of children with cystic fibrosis were also elevated compared to age-matched controls [212]. Additionally, the level of spermine in the urine correlated with the degree of disease severity [212].

A role for polyamines has also been proposed in pulmonary arterial hypertension. Olson et al. demonstrated a significant increase in polyamines in the lungs of rats injected with monocrotaline, and that treatment with an ornithine decarboxylase inhibitor, difluoromethylornithine (DFMO) attenuated the increase in pulmonary arterial pressure [213]. This group has subsequently shown that increased polyamine transport is involved in a rat model of hypoxic pulmonary hypertension, and have suggested that polyamine depletion with DFMO should be considered for clinical trials in pulmonary arterial hypertension patients [214].
Finally, likely due to their important role in cell growth, polyamines have also been shown to play a role in cancer [215]. Cancer therapies based on the inhibition of ODC activity and reduction of polyamine levels are currently under intense investigation [215].

Interestingly, increased arginase activity and expression have also been demonstrated in cystic fibrosis, pulmonary hypertension, and lung cancer [216-219]. However, as ODC is generally considered to be the rate-limiting enzyme in polyamine synthesis, the link between increased arginase activity in these lung diseases and increased polyamine concentrations has not been well described.

1.4.4. Polyamines in asthma

i. Inflammation

Polyamines are involved in several cellular pathways that are relevant to inflammation, including the activation of T cells, B cells and mast cells. It has been demonstrated that lymphocyte stimulation is accompanied by a rise in ODC activity and polyamine synthesis [220]. Polyamines have been shown to play a role in mast cell activation and the release of histamine. Kurosawa et al. demonstrated that the release of histamine by mast cells was rapidly initiated by 10 mM spermine or spermidine and that 0.1 mM spermine was capable of enhancing the anti-IgE-induced histamine release [221]. Thus, polyamines may play a role in allergic reactions.

Shortly after polyamines were demonstrated to promote histamine release from mast cells, increased polyamine levels were demonstrated in humans during spontaneous mild asthma attacks [222]. Murine models also support the potential for increased polyamine levels in the asthmatic lung, as Zimmermann et al. have demonstrated increased putrescine levels [76].
However, the role of increased polyamine levels in airways hyperresponsiveness of asthma is not known.

ii Remodeling

Polyamines likely contribute to airway remodeling in asthma by promoting cell proliferation. In addition to the mechanisms described in Section 1.4.2, by which polyamines are known to promote proliferation, they may also be involved in airways remodeling through the modulation of smooth muscle phenotype.

Increased airway smooth muscle mass is a characteristic feature of airways remodeling. Polyamines have been reported to play a role in both cell growth and DNA replication and thus may play a role. In has been observed that the expression of ODC is low in freshly isolated contractile smooth muscle cells, but increases in cultured cells, concurrent with the change from contractile to proliferative phenotype [223]. Reduction of polyamine content in culture media and DFMO treatment have been shown to inhibit this transition [223]. It was also demonstrated that DFMO-induced polyamine depletion arrests vascular smooth muscle cells in S-phase without affecting cell viability [224]. Recently, the effects of the anti-inflammatory traditional medicine, black seed oil, were assessed in a rat model of ovalbumin induced airways inflammation and remodeling [225]. Administration of black seed oil concurrent with intranasal ovalbumin challenges for seven days suppressed inflammation, collagen deposition, arginase activity, ODC activity and polyamine levels [225]. Thus, polyamines likely play a role in airways remodeling, however their functional role(s) in the asthmatic airways is not understood.

iii Airways tone and responsiveness
Polyamines may also be involved in the regulation of airways tone through effects on the NOS pathway. Polyamines have been reported to affect the production of NO by the NOS isoenzymes in various cell types. Hu et al. examined the inhibitory effects of polyamines on the conversion of $[^3]$H-L-arginine to $[^3]$H-L-citrulline in rat cerebellar lysates containing NOS1 [226]. The authors determined the following order of potency; spermine > spermidine > putrescine, with an IC$_{50}$ value of 56 µM for spermine [226]. The authors demonstrated that changing concentrations of L-arginine affected the ability of polyamines to inhibit NOS1, and thus suggest a competitive mechanism [226]. Blachier et al. examined NOS2 inhibition in lysates from endotoxemic rat liver and likewise found that spermine was the strongest inhibitor, reporting an IC$_{50}$ of 500 µM [227]. The ten-fold difference in IC$_{50}$ between the Hu and Blanchier papers may be due to different susceptibilities between the isozymes to inhibition by spermine, or could be confounded because both groups employed cell lysates in their studies, rather than pure enzyme preparations. Thus polyamines, particularly spermine, may promote increased airways responsiveness through the attenuation of nitric oxide production. In summary, polyamines may be important in asthma, however, their role has not been adequately explored.
1.5. Air Pollution

The balance between the NOS and arginase pathways has been demonstrated to be important in the maintenance of airways tone and airways responsiveness. Additionally, polyamines may play a role in asthma. However, the relationship between these basic mechanisms of asthma and precipitating factors of asthma exacerbations, such as air pollution, have not been explored.

1.5.1. Urban particulate matter

i Regulation, size fractions and sources

Air pollution is an important issue that impacts on the respiratory health of all Canadians. However, asthmatics likely represent a vulnerable group that may be more sensitive to adverse effects. Thus, it is important to assess the potential for air pollution to exacerbate asthma.

Following the “London Fog” incident in Great Britain in the 1950’s, discussed in more detail below, a greater public awareness of the health effects of air pollution spurred governments in the Western world to begin regulating air pollution and particulate matter. In Canada, the National Air Pollution Surveillance Network was established in 1969 to monitor air quality [228]. In the United States, the Environmental Protection Agency (EPA) was formed in 1970 to carry out research, monitoring, and enforcement related to the environment [229]. Canada passed the Clean Air Act in 1971, and the Department of the Environment (now called Environment Canada) was founded, and charged with establishing national ambient air quality objectives [230].

Urban air pollution is a complex mixture of particles and gases. Particulate air pollution is composed of solids or liquids suspended in the air [231]. Early air pollution monitoring and research primarily measured total suspended particles to estimate particulate air pollution levels [232]. However, as different sized particles are able to penetrate to different depths in the lung,
examining different particulate size fractions became of interest to both researchers and regulators [233]. In 1987 the EPA instituted air quality standards based on PM$_{10}$, or particles with an aerodynamic diameter equal to or less than 10 µm, as these particles are capable of penetrating into the thoracic region [234]. The Harvard Six Cities Study (discussed in more detail below) was the first to employ dichotomous samplers to measure PM$_{2.5}$ (particles of an aerodynamic diameter of 2.5 µm or less), beginning in 1978 [235]. The findings of the Six Cities Study began to be published in the late 1980’s, and the EPA instituted PM$_{2.5}$ air quality standards in 1997 [236]. Canada employed total suspended particles to regulate particulates until 1998, when the Canada Wide Standards were established [237]. Currently the Canada Wide Standard target for PM$_{2.5}$ is a 24 hour average of 30 µg/m$^3$ [238].

Ultrafine particles (PM$_{0.1}$), or particulate matter with an aerodynamic diameter of 100 nm or less, have also recently been recognized as an important size fraction [239]. Thus far, there are no Canadian ambient air quality standards pertaining to ultrafine particles. However, there are concerns that due to the small size of these particles, they may be able to translocate from the lungs to the circulation and lead to systemic effects [240]. In summary, the current conventions for specific size fractions of particulate matter include coarse, fine and ultrafine, or PM$_{10}$, PM$_{2.5}$, and PM$_{0.1}$, respectively. Additional size fractions that are recognized are the coarse fraction of PM$_{10}$ (PM$_{2.5-10}$), and the fine fraction that excludes ultrafines (PM$_{0.1-2.5}$).

Sources of ambient particulate matter include both anthropogenic and non-anthropogenic processes. Natural processes that can result in the generation of particulate matter include wildfires, volcanic eruptions, wind-blown dust and salt, pollen and microbial products [231]. Anthropogenic sources of particulate matter include industry, construction, transportation, biomass burning, farming, heating and cooking [241]. Primary particles are those produced directly from the pollutant source, such as a smokestack or combustion engine, while secondary
particles are formed in the air through processes such as condensation or chemical transformation [231].

Constituents of particulate matter vary widely due to both the source of the primary particle and the secondary transformations it may have undergone. A large proportion of urban particles in the fine and ultrafine size ranges are produced by traffic and combustion [242]. Thus, constituents commonly include elemental carbon, organic carbon, metals and inorganic ions [242, 243]. Urban coarse particulate matter is derived from processes such as abrasion of vehicle brakes, construction, dispersion of road dust, crustal materials and bioaerosols [244].

In North America and Europe, increased legislative controls have been effective in ensuring a lower annual average, reducing overall exposures in recent years [245]. However, in Asia and other parts of the developing world, PM$_{10}$ levels reach 200 µg/m$^3$ to 600 µg/m$^3$, and are increasing [246, 247]. In addition, occupational activities and domestic cooking can result in elevated exposure levels for many [248-250]. Thus it is important to understand the health effects of exposure to particulate matter.

ii Epidemiological evidence of harmful effects

The first incident that drew the attention of the world to the adverse effects of air pollution on human health was the “London Fog”, which occurred in England from December 5$^{th}$-9$^{th}$, 1952 [251]. In the 1950’s, the mean annual PM$_{10}$ levels were approximately 300 µg/m$^3$, and during the incident they rose to approximately 3000 µg/m$^3$ [252]. Evidence suggests that approximately 12,000 people may have died as a result of the exceptionally-high suspended particulate levels during the London fog [251]. While the events that befell those in the greater London area were tragic, they helped to inspire public awareness of the adverse effects of air
pollution and a great expansion of research in the field of environmental health in the half-century since [251, 252].

Indoor and outdoor environments both present the potential for inhalational exposure to air pollutants. Indoor air pollution is a major issue, as almost half of the world’s population relies on solid fuels for everyday household energy needs [253]. A high level of evidence is available to link indoor air pollution to acute lower respiratory infections, chronic obstructive pulmonary disease (COPD), and lung cancer [254]. On average, Canadians spend 90% of their time indoors [255, 256]. However, indoor air pollution levels in Canada are on average much lower than in the developing world, as the use of biomass burning indoors is relatively uncommon [255, 256]. Thus, while indoor air pollution still remains an issue of public health importance in Canada, the focus of the research presented in this thesis is outdoor air pollution.

Strong associations between increased levels of outdoor particulate air pollution and mortality began to be widely recognized with the publications resulting from the Harvard six cities prospective cohort study [257]. In these epidemiological studies the authors demonstrated statistically significant associations between air pollution and mortality, with an odds ratio of 1.26 for the most polluted city (Steubenville, Ohio) vs. the least polluted city (Portage, Wisconsin) for death from lung and cardiovascular disease, but not from other causes [257].

Recently, the World Health Organization estimated that outdoor air pollution annually contributes to 2 million premature deaths worldwide [258]. The Canadian Medical Association also recently estimated that approximately 20,000 Canadians die each year from the effects of outdoor air pollution [259]. While most of these deaths are the result of chronic exposures, over 10% are associated with acute effects [259]. As air pollution costs the Canadian economy over $8 billion per year, this issue remains an important public health and economic concern, and more studies are needed to understand and attempt to mitigate the effects [259].
Schwartz et al. determined that outdoor particulate matter with aerodynamic diameter of less than 2.5 µm (PM$_{2.5}$) is specifically associated with daily mortality, exhibiting a stronger association than the coarse fraction [260]. More recently, Zanobetti et al. examined mortality and air pollution levels in 112 U.S. cities and also found that the association between PM$_{2.5}$ and respiratory deaths was the most dramatic, indicating that fine particles may pose the greatest public health risk [261]. Thus, the effects of urban PM$_{2.5}$ are of particular interest to researchers today attempting to delineate the biological mechanisms behind adverse effects on asthma.

Epidemiological studies have described a strong relationship between ambient levels of air pollution, and many important indicators of asthma exacerbation. The negative effects of air pollution on asthma have now been demonstrated in studies from all across the world. A study encompassing 10 European countries demonstrated a positive association between short-term increases in PM$_{10}$ levels and increases in daily admissions to hospital for asthma [262]. Ko et al. examined hospital admissions for asthma and air pollution levels in Hong Kong, and demonstrated an association across all age groups [263]. Epidemiological studies in North America have also yielded similar results. A study examining inner city communities in the U.S. demonstrated an association between PM$_{2.5}$ and asthma-related absenteeism from school, at levels that were generally below the National Ambient Air Quality Standards [264]. In Canada, a study examining emergency department visits across seven cities demonstrated significant effects of both PM$_{10}$ and PM$_{2.5}$ on visits for asthma, particularly during the warm season [265]. Therefore, it has been established through epidemiological studies that air pollution negatively impacts upon asthma symptoms.

### iii Adverse Effects on Asthma – Controlled Exposure Studies
Investigations of the health impact of air pollution using controlled human exposures have demonstrated acute cardiopulmonary effects in both healthy subjects and asthmatics [266-268]. Diesel exhaust is an important component of anthropogenic air pollution, and several investigators have employed this pollutant in controlled exposure studies. Nordenhall et al. exposed atopic asthmatics to 300 µg/m³ diesel PM₁₀ and demonstrated an increase in methacholine responsiveness compared to filtered air exposures in the same subjects [269]. Holgate et al. performed controlled exposures using 100 µg/m³ diesel particles and demonstrated that both control subjects and those with asthma experienced a statistically significant increase in airway resistance following exposure [270]. Stenfors et al. exposed subjects with mild asthma and non-atopic controls to diesel exhaust or filtered air for 2 hours [271]. Similar to the other studies, Stenfors demonstrated that diesel exhaust increased airway resistance in both groups [271]. The authors also found an increase in epithelial staining for IL-10 in asthmatics, but not controls, after exposure [271]. Thus, exposure to diesel exhaust, an important component of urban particulate matter, increases airways resistance and methacholine responsiveness in asthmatic subjects.

Exposures to concentrated ambient urban particles have also been performed in healthy controls and asthmatic subjects. Gong et al. exposed asthmatics and healthy controls to an average of 174 µg/m³ concentrated ambient PM₂.₅ from Los Angeles for 2 hours [267]. However, this study did not find any significant changes in respiratory symptoms in either group [267]. Recently, Urch et al. exposed mild asthmatics and healthy controls to concentrated ambient PM₂.₅ from Toronto, over a range of levels (48 µg/m³ – 199 µg/m³) for 2 hours [272]. The authors did not observe any effects on pulmonary function, but demonstrated increased systemic IL-6 levels in both controls and asthmatics [272].
In summary, diesel exposure studies provide evidence of increased airways responsiveness following exposure in asthmatic subjects, while studies of concentrated urban particles demonstrate more limited effects of exposure. The lack of increased pulmonary responsiveness to particulate matter in asthmatics in controlled exposure studies are surprising given that epidemiological studies consistently show that increased exposures are associated with asthma exacerbation (discussed in further detail below). Perhaps this is due to medication use, as medication has been shown to be a confounding factor in assessing the effects of particulate matter exposures in asthmatics [273]. Additionally, it has been suggested that patients with pre-existing disease may have impaired baseline values, and therefore have decreased response to PM [274].

1.5.2. Ground-level ozone and respiratory effects

Chemical structure, sources and levels

Ozone is an extremely reactive chemical composed of triatomic oxygen (O$_3$). Ozone is created naturally by lightning and ultraviolet light interacting with oxygen [275]. The ozone layer is an important component of the lower stratosphere, which helps to block harmful ultraviolet radiation from space [275]. However, at ground level, ozone is a powerful oxidant and a major component of urban “smog”. Ozone is a secondary air pollutant, as it is not directly emitted from air pollution sources, but is formed through photochemical reactions [276]. Volatile organic chemicals can initiate free radical reactions in the atmosphere leading to NO$_2$ formation and the formation of ozone [277]. Thus, vehicle emissions in urban centres can contribute to elevated ground-level ozone.

As ozone is not emitted directly, it can be difficult to identify the source pollutants responsible for elevated levels. Peak ozone levels typically occur in rural areas downwind of
cities, as the ozone accumulates after VOCs and nitrogen oxides have been exposed to sunlight for some time [278]. In Canada, border regions with the U.S. have been a concern for regulators and policy makers, as long range and cross-border transport of ozone regularly takes place [238, 279]. Ground-level ozone has increased by approximately 13% in Canada since 1990, and the current Canada Wide Standard is 65 ppb [238, 280]. Unfortunately a recent border air quality study in southwestern Ontario indicated that most communities in the area continue to experience levels exceeding the recommended standards, particularly during the summer season [281]. Thus ambient ozone levels are a concern and elucidating the effects of this powerful oxidant in combination with other pollutants, such as urban PM$_{2.5}$, is of public health importance.

ii  Adverse effects of ozone

Some of the first episodes of photochemical air pollution that received public attention occurred in the Los Angeles area in the 1960’s, when excessive rubber cracking, crop injury and eye irritation were noted [282]. It was determined early on that ozone exposure may have serious health effects due to its powerful oxidant properties. Ozone exposure results in headache and irritation of the throat at low doses, “blistering” of the respiratory tract at approximately 1 ppm, and pulmonary edema at high doses [283].

The Harvard six cities study examined ozone levels and demonstrated decrements in lung function associated with ozone exposure, at levels well below the National Ambient Air Quality Standards of the time [284]. Controlled exposure studies have also demonstrated effects of ozone on lung function. Bates et al. demonstrated that a 2 hour exposure to 0.75 ppm O$_3$ resulted in respiratory symptoms that were aggravated if the exposure was performed with intermittent exercise [285]. Defining the effects of ozone on a population level has been
challenging, due to the presence of co-pollutants and complex mixtures. However, long-term ozone exposure has recently been linked to mortality from respiratory causes [286]. Thus, further studies are important to understand the effects of ozone on sensitive populations, such as asthmatics, and in combination with other agents.

iii Adverse Effects on asthma

Controlled exposure studies have been instrumental in shedding light on the effects of ozone exposure in asthmatics. A clear relationship was established early on between the dose of \( O_3 \) and decline in forced expiratory volume in normal subjects [287]. In controlled studies, some asthmatics have been found to be more responsive to inhaled allergen after exposure to low levels of ozone, however some did not respond [288, 289]. When asthmatics are exposed to moderate to high levels of ozone, or exposure is combined with exercise, asthmatics have been shown to respond as a group with a significant increase in responsiveness to non-specific bronchoconstrictors and allergen [290, 291]. Thus, ozone increases airways responsiveness in asthmatics.

Epidemiological studies also demonstrate an association between ozone exposure and asthma exacerbation. Levels of ambient ozone have been associated with respiratory admissions to hospitals in Ontario, and in the City of Toronto [292, 293]. White et al. demonstrated a 37% increase in exacerbations of childhood asthma on days following high ozone levels (>0.11 ppm) in Atlanta [294]. A study in California demonstrated increased risk of hospitalization of children with asthma was associated with ozone levels over an 18 year period [295]. Another recent study examined severe asthma attacks, resulting in admission to intensive care units in New York [296]. The authors found that for each 22 ppb increase in ambient ozone, there was a 20% increase in I.C.U. admissions for asthma in children ages 6 to 18 years [296]. Mechanisms of
exacerbation of asthma by ozone are not well defined. However, ozone has been demonstrated to cause an influx of neutrophils into the lung, coupled with increased airways responsiveness to methacholine [297]. In summary, the harmful effects of ozone on asthma are well recognized, but the specific mechanisms behind the adverse effects are not well defined.

1.5.3. Animal models of asthma and exacerbation by air pollution

Animal models represent valuable tools for understanding the mechanisms behind the adverse effects of air pollution. Studies in animal models have demonstrated respiratory effects of particulate matter, ozone and combined exposures. Goldsmith et al. examined the effects of exposure to concentrated ambient particles (CAP) vs. residual oil fly ash in mice who were sensitized and challenged with ovalbumin (OVA/OVA) and controls [298]. While they did not find an effect of CAP, residual oil fly ash exposure resulted in augmentation of the airways responsiveness to methacholine in the OVA/OVA mice, measured by Penh [298]. Harkema et al. employed a rat model of hypersecretory airways to study the effects of concentrated particulate matter from Detroit, a community with a high incidence of childhood asthma [299]. The authors demonstrated that particulate matter was preferentially retained in rats with hypersecretory airways, and that pulmonary function responses were highly dependent on CAP composition [299]. Hamada et al. focused on young mice to assess the effects of air pollution exposure on exacerbation of a “juvenile” asthma model [300]. The authors showed that juvenile allergic mice demonstrated AHR following exposure to residual oil fly ash, while the air pollutant had no effect on airway responses in non-sensitized juvenile mice [300]. In a subsequent study, Hamada et al. also showed that in adult mice, airways hyperresponsiveness could be induced by an aerosolized mixture of the major metal constituents of residual oil fly ash, but not by any individual metal alone [301]. Thus, while effects of particulate matter may
be highly dependent on constituents, animal models of asthma have demonstrated an increase in airways responsiveness following particulate exposure.

Animal models have also been employed to study the effects of ozone on the airways. Gordon et al. demonstrated an increase in airways responsiveness to histamine in guinea pigs which was not abolished by severing the vagal nerve, demonstrating that ozone exerts damage to the airways that directly promotes increased responsiveness [302]. O’Byrne et al. demonstrated that ozone increased neutrophil influx into the airways in dogs, similar to findings in controlled human exposure studies [303]. When neutrophils were depleted with hydroxyurea, the authors demonstrated that airways hyperresponsiveness in response to ozone exposure was attenuated [303]. In murine models of allergic airways inflammation, mice previously challenged with allergen exhibit an increase in airways responsiveness following ozone exposure [304]. Larsen et al. demonstrated that even a single 3 hour exposure to 100 ppb of ozone exacerbates airways responsiveness to methacholine in mice that had previously been sensitized and challenged with ovalbumin [304]. Similar to particulate matter, ozone studies have revealed that this component of urban air pollution is capable of inducing increased reactivity of the airways.

Understanding the effects of combined exposures is very important, as persons with asthma living in urban communities are likely exposed to CAP and ozone concurrently [241]. Kobzik and Goldsmith et al. examined the effects of single or combined 5 hour exposures to 0.3 ppm ozone plus CAP, vs. filtered air on a murine model of OVA-induced airways inflammation [305, 306]. The authors found a small but significant increase in Penh following CAP or CAP+O₃ exposure in both PBS and OVA challenged mice [305, 306]. However, as Penh is not a direct measure of airways resistance, additional studies utilizing a similar protocol with invasive measures of lung function may be able to better characterize the response.
Studies employing repeated exposures have also been undertaken. Recently Farraj et al. exposed mice in an OVA model of asthma once a week for four weeks to the PM$_{2.5}$ fraction of diesel exhaust particles, ozone (0.5 ppm), combined exposures, or filtered air [307]. The authors measured lung function and responsiveness using the FlexiVent®, and found that diesel exhaust or O$_3$ exposure alone did not induce AHR, but the combined exposure produced a significant increase [307]. However, the pollution-induced AHR was not associated with an increase in inflammation, suggesting that other mechanisms may be at work [307]. Therefore, animal models of asthma employing combined exposures to particulates and ozone support the theory that complex mixtures of pollutants may be more harmful, however the basic mechanisms of pollution-induced airways hyperresponsiveness are not fully understood.

1.5.4. Arginase and air pollution

Arginase expression and activity is upregulated in asthma and has been shown to be functionally important in airways responsiveness. Although upregulation of arginase in response to air pollution has not been investigated, there have been some studies that indicate that arginase expression and activity may be environmentally responsive. Bergeron et al. examined arginase expression by immunostaining in asthmatic airways [168]. This study revealed increased arginase expression in asthmatics who smoke, localized to epithelia and the peribronchiolar region [168]. This study suggests a role for arginase in injury and repair initiated by cigarette smoke insult [168]. A role for arginase has also been demonstrated in the aberrant injury and repair leading to accelerated intimal hyperplasia following intravenous exposure to cigarette smoke extract in rabbits [308]. This study demonstrated that arginase is involved in impaired NO production and vascular remodeling associated with cigarette smoke extract in rabbit carotid artery [308].
Furthermore, there is evidence to support uncoupling of the endothelial NOS in the vasculature following exposure to diesel exhaust [309], and dysfunction of endothelial-dependent vasorelaxation following exposure to second-hand tobacco smoke [310], possibly as a consequence of a reduction in the bioavailability of L-arginine for the NOS pathway.

Reactive oxygen species have also been associated with increased arginase activity. In a study examining the response of erythrocyte preparations to oxidative stress produced by the presence of cysteine-iron particles, dose-dependent increases in arginase activity were detected [311]. Additionally, both arginase 1 and 2 proteins were shown to be up-regulated in the peribronchiolar region in rats by exposure to 100% oxygen [312]. Que et al. also demonstrated increased arginase activity and a decrease in lung nitrogen oxides [312]. Slightly lower levels of hyperoxia (95% O₂), for longer periods of time (7 days) also have been demonstrated to up-regulate arginase activity and disrupt NO-mediated relaxation in lung parenchyma [313]. Hyperoxia has also been shown to increase polyamine content in rat lungs, a potential marker of increased arginase activity [314]. Hacker et al. reported increased putrescine, spermidine and spermine after 5 days of exposure to 85% O₂ [314].

Hydrogen peroxide can also be used to experimentally induce oxidative stress. In the cardiovascular system, arginase upregulation has been shown after 1 hour of exposure to hydrogen peroxide in coronary arterioles, suggesting that arginase 1 expression is responsive to oxidative stress [315]. Thengchaisri et al. also demonstrated changes in smooth muscle function and demonstrated that arginase inhibition restored the hydrogen peroxide-impaired vasodilation [315]. These studies suggest that arginase may be responsive to the environment. However, further studies of L-arginine metabolism and the role of arginase in the exacerbation of airways responsiveness by air pollution are warranted.
Chapter 2: Hypothesis and research plan

2.1. Overall hypothesis

Arginase and polyamines metabolites are functionally involved in airways responsiveness in animal models of asthma and the adverse responses to air pollution.

2.2. Research plan

2.2.1. Arginase in asthma

Rationale:
To date, a comprehensive examination of the protein expression profile for L-arginine-related enzyme pathways and transporters has not been performed in murine models of allergic airways inflammation and compared to the expression profile in human asthmatic lungs. Additionally, the ability of arginase inhibition to attenuate airways hyperresponsiveness in acute and chronic murine models (i.e., in the absence vs. presence of airways remodeling, respectively) has not been evaluated. Such investigations may be important to fully understand the causes and implications of dysregulation of L-arginine metabolism in asthma.

Objectives:

1) Determine whether the expression profiles of L-arginine-related proteins are dysregulated in human asthma and common murine models of allergic airway inflammation.

2) Determine whether arginase inhibition can attenuate airways hyperresponsiveness in acute and chronic murine models.

2.2.2. Polyamines in asthma

Rationale:
Although it has traditionally been proposed that AHR develops as a result of increased substrate competition between arginase and nitric oxide synthase (NOS), leading to reduced production of NO, it remains unknown whether the consequently increased polyamine concentrations in the allergic inflamed lung also play a functional role. Spermine and spermidine have been shown to be competitive inhibitors of NOS, but it is not known if this occurs in the airways at physiologically relevant concentrations.

**Objectives:**

1) Determine whether polyamines increase methacholine responsiveness in naïve mice and a sub-acute model of ovalbumin-induced allergic airways inflammation.

2) Determine whether blockade of the synthesis of polyamines can improve the hyperresponsiveness to methacholine in an acute murine model.

### 2.2.3. Arginase and air pollution

**Rationale:**

Arginase has been shown to be further upregulated in smoking asthmatics. Thus, it is plausible that dysregulation of L-arginine metabolism as a consequence of air pollution-induced upregulation of pulmonary arginase could contribute to the exacerbation of respiratory symptoms in susceptible asthmatics.

**Objectives:**

1) Determine whether arginase expression is augmented in response to exposures to environmental air pollutants, in sub-acute and chronic models of allergic inflammation.

2) Determine whether arginase inhibition will attenuate the air pollution-induced AHR.
3 Chapter 3: Materials and methods

3.1 Molecular methods

3.1.1. Lung homogenization and Western blotting

i Homogenization

Lysis buffer containing 0.1% Triton X-100, 0.8% glycerol, 0.7 µg/ml pepstatin A (Bioshop, Burlington, ON, Canada), 0.7 µg/ml antipain (Bioshop), 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Invitrogen, Carlsbad, CA) and 2 mM Ethylenediaminetetraacetic acid (Bioshop Canada, Burlington, ON) was prepared and pH was adjusted to 7.3. Murine or human lung tissues were combined with lysis buffer approximately 1:1 wt/vol and placed on ice. Samples were homogenized in a 15 ml culture tube using a hand-held rotor-stator (TissueRuptor, Qiagen, Valencia, CA). Tubes were centrifuged at 5,000 x g for 15 minutes, and the supernatant was pipetted into a 1.5 ml microtube. The microtube was centrifuged at 20,00 x g for 15 minutes and supernatant was aliquotted into 1.5 ml microtubes and frozen at -80°C.

ii Protein assay

The Bradford protein assay kit (Biorad, Mississauga, ON) was used to determine protein content of lung homogenates. Briefly, a six-point curve of protein standards was prepared according to manufacturer’s instructions (0 - 0.5 mg/ml). Bio-Rad reagent was prepared with ultrapure water (Millipore, Billerica, MA). Diluted reagent (0.2 ml) was added to each well of a 96-well microplate. Samples were diluted as appropriate to fall in the range of the standard curve, and 10 µl of diluted sample or standard was added to each well and mixed with the pipette tip. All standards and samples were measured in triplicate. The plate was incubated at room temperature for 5 minutes, and measured using a spectrophotometer at 595 nm. The mean
of the triplicate values of absorbance for the blank wells were subtracted from all other absorbance values and a standard curve was plotted. Concentrations of samples were calculated using the equation of the standard curve and multiplied by the dilution factor.

iii Western Blotting

The expression of proteins related to L-arginine metabolism were determined in tissue homogenates from human and murine lungs as follows. Polyacrylamide gel electrophoresis was performed on denaturing gels using the Mini-PROTEAN 3 Cell system (Bio-Rad Laboratories, Hercules CA). NOS isozymes were separated on a gel containing 12% acrylamide, CAT isozymes were separated on 10% gels, while arginase, agmatinase and ODC were separated on 12% gels (Sigma). Sample buffer contained 10% 2-mercaptoethanol, 5% glycerol, 2.5% SDS, and 0.0001% Bromophenol blue. Western blotting of 30–50 µg of tissue homogenate was performed to assess arginase 1, arginase 2, NOS2, NOS3, CAT1 (SLC7A1), and CAT2 (SLC7A2) expression, as appropriate for the expression level of the protein. NOS1 and agmatinase expression levels were assessed by immunoprecipitation (300 and 250 µg, respectively, for mouse and human samples) followed by Western blotting (ExactaCruz, Santa Cruz Biotechnologies, Santa Cruz, CA). All antibodies were purchased from Santa Cruz Biotechnologies, except CAT2 (SLC7A2), which was obtained from Orbigen (San Diego, CA). Further details of each antibody are provided in Table 1. Samples undergoing immunoprecipitation were pre-cleared of immunoglobulins with Protein G-Plus (Santa Cruz Biotechnologies, Santa Cruz, CA) and then immunoprecipitated using Exactacruz Rabbit IP matrix (Santa Cruz) bound to primary antibody. Membranes were blocked with 2% skim milk. Western blots were stripped of the primary antibodies and reprobed for actin as a loading control. Proteins were visualized using Western Lightning reagent (PerkinElmer Life Sciences,
Boston, MA), and the polyvinylidene difluoride membranes were exposed to X-Omat Blue XB-1 film (Kodak, Rochester, NY). Additionally, Western blots presented in Chapter 6 were captured using a Bio-Rad Fluor-S MultilMager with the Bio-Rad Quantity One 4.3.0 software package (Bio-Rad Laboratories, Hercules, CA). Densitometry was performed using GelEval 1.22 (Frogdance Software, University of Dundee).

Table 1: Antibodies used for Western blotting, immunoprecipitation and immunohistochemistry.

<table>
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<tr>
<th>Target</th>
<th>Epitope a</th>
<th>Isotype b</th>
<th>Catalogue Number</th>
<th>Source c</th>
</tr>
</thead>
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<td>G</td>
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<td>SCBT</td>
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<tr>
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<td>Internal (H)</td>
<td>G</td>
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<td>SCBT</td>
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<td>Internal (H)</td>
<td>G</td>
<td>sc-21516 (F-14)</td>
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</tr>
</tbody>
</table>

a H, human; R, rat  
b G, goat polyclonal IgG; R, rabbit polyclonal IgG  
c SCBT, Santa Cruz Biotechnology Inc.

3.1.2 Arginase activity measurements

Lung tissue homogenates were analyzed for arginase activity using the method of Corraliza et al. [316]. Briefly, this endpoint assay involves activating the arginase enzyme in activation buffer containing manganese cofactor, incubating with 0.5M L-arginine substrate solution for 60 minutes, followed by reaction of urea with α-isonitrosopropiophenone (Sigma)
and spectrophotometric measurement at 540 nm. A standard curve of 0-1.665 µmol urea was generated. Lung homogenate protein was reconstituted to 200 µL in lysis buffer for the assay (resultant protein concentration: 0.04-1.0 mg/mL). Dilutions were different between high and low arginase expressing groups to maintain levels of urea production within the linear range of the standard curve. Each sample was measured in triplicate. The assay was linear with respect to micrograms of liver protein added to the reaction mixture. The specificity of the arginase activity assay in lung samples was confirmed by 94% inhibition of de novo urea formation in the presence of the specific arginase inhibitor BEC (5.0 mM). Urease activity was assessed by spiking the reaction mixture with 0.25 mg/ml urea, followed by incubation under the standard assay conditions and determination of urea. The mean recovered urea was 98%, and not statistically different from 100% (Wilcoxon signed rank test, P = n.s.), therefore interference from urease in this assay does not cause significant interference. Arginase activity in experimental samples is expressed as nanomoles of urea produced per minute per microgram of lung homogenate protein.

3.1.3 Bronchoalveolar lavage, histology, and immunohistochemistry

After methacholine challenge, bronchoalveolar lavage samples were obtained by instilling and gently recovering 0.5 ml sterile PBS three times (total 1.5 ml). Total cell counts were performed by the Trypan blue dye exclusion test (Wisent, St. Bruno, PQ) on cell counting micro chamber slides. Slides of BAL samples were prepared using a Cytospin centrifuge (Shandon, Thermo Scientific, Waltham, MA). Slides were stained with DiffQuick (Dade Behring Inc., Newark, NJ). Differential cell counts were performed under a light microscope, by counting more than 300 cells per slide.
Lungs were collected for histology and immunohistochemical staining and inflated to a pressure of 20 cmH2O with 10% neutral buffered formalin (Sigma, Mississauga ON) [317]. All mounting and staining steps were performed at the Toronto Centre for Phenogenomics Pathology Core Facility. Briefly, lungs were embedded in paraffin, bisected into superior and inferior portions, cut into 5 µm sections and mounted on slides. Briefly, paraffin was removed with xylene and the sections were rehydrated in ethanol followed by PBS. Slides were stained with hematoxylin and eosin for the assessment of inflammatory changes, or endogenous hydrogen peroxidase was blocked for immunohistochemistry. Antigen retrieval was performed by incubation with 0.125% trypsin, and blocking was performed with Dako serum-free protein (Dako, Mississauga, Ontario, Canada). All tissues were incubated overnight with goat anti-arginase I or II (1:100 dilution; Santa Cruz Biotechnology), or rabbit anti-NOS1 (1:1000 dilution; Santa Cruz) at 4°C. After three 5-min washes in PBS, tissues were incubated for 1-2 hours at room temperature with horse anti-goat or goat anti-rabbit biotinylated secondary antibodies (1:200 dilution; Vector Laboratories, Burlington, Ontario). Tissues were further incubated in a 1% avidin-biotinylated-peroxidase complex (1:50 ABC; kit PK-4000, Vectastain, Vector Laboratories). Antibody labeling was revealed by 3,3-diaminobenzidine tetrahydrochloride for 10 min at room temperature, followed by washing in deionized water and mounting on slides. Slides were visualized on a Leica inverted microscope and images were captured using a micropublisher RTV 5.0 camera with QCapture image capture software (Quorum Technologies Inc., Guelph, ON).

3.2 In vivo methods

3.2.1 Murine OVA-sensitization and challenge models of allergic airways inflammation
i  **Sub-acute model**

Female BALB/c mice (6-8 weeks of age; Charles River Laboratories, Saint-Constant, PQ) were sensitized to OVA by intraperitoneal (i.p.) injection (25 µg in 0.2 ml PBS with 1 mg Al(OH)$_3$; Sigma Aldrich, Mississauga, ON) one week apart (days 0 and 7) (**Figure 3A**). On day 14, mice were randomized to repeated exposure to PBS (OVA/PBS), or 6% OVA in PBS (OVA/OVA) for 25 minutes/day (**Figure 3A**). Challenges in the sub-acute model take place on days 14-16 (3 challenge days) (**Figure 3A**).

ii  **Acute model**

Female BALB/c mice (6-8 weeks of age; Charles River) were sensitized to OVA in the same manner as in the sub-acute model, and randomized to inhalation challenges of PBS (OVA/PBS), or 6% OVA in PBS (OVA/OVA) for 25 minutes/day from days 14-20 (7 challenge days) (**Figure 3B**).

iii  **Chronic model**

Female BALB/c mice (6-8 weeks of age; Charles River) were sensitized to OVA in the same manner as above, and were challenged with PBS (OVA/PBS), or 2.5% OVA in PBS (OVA/OVA) for 25 minutes/day for two consecutive days every two weeks up to 12 weeks (**Figure 3C**). All animal protocols were approved by the University of Toronto Animal Care Committee and were conducted in accordance with the guidelines of the Canadian Council on Animal Care.
Figure 3: Murine sensitization and challenge models of allergic airways inflammation

A) The sub-acute model consists of two sensitizations, one week apart, followed by three daily challenges (25-minutes) with PBS or 6% OVA.

B) The acute model consists of two sensitizations, one week apart, followed by seven daily challenges (25-minutes) with PBS or 6% OVA.

C) The chronic model consists of two sensitizations, one week apart, followed by two daily challenges every two weeks (25-minutes) with PBS or 2.5% OVA, up to 12 weeks.
3.2.2 Respiratory function testing and methacholine challenge

Twenty-four hours after the final OVA or PBS challenge, mice were anesthetized with ketamine (i.p., 50 mg/kg, Bioniche, Belleville, ON)/xylazine (i.p., 10 mg/kg, Bayer Inc., Toronto, ON) prior to intubation with an 18G stainless steel cannula (BD Biosciences Canada, Mississauga, ON) for \textit{in vivo}, ventilator-based assessment of methacholine-responsiveness using the FlexiVent® system (Scireq Inc., Montreal, PQ). Mice were ventilated at 150 breaths per minute, with a tidal volume of 10 ml/kg and a positive end expiratory pressure (PEEP) of 3 cm H$_2$O. Snapshot sinusoidal and QuickPrime broad-band (frequency range 0.5 – 19.75 Hz) perturbations were used (Scireq). At the beginning of the experiment, two total lung capacity (Scireq) maneuvers were performed to standardize the volume history. These perturbations involve slowly inflating the lung to 30 cm H$_2$O, over 6 seconds, and then releasing the pressure. The baseline measurements were collected approximately 10 minutes after the script was initiated. Baseline measurements were repeated if necessary until a stable baseline was established (i.e. with coefficient of determination values greater than 0.95). After baseline determination of airway resistance, mice were challenged with methacholine (0-100 mg/mL in sterile PBS; Sigma) nebulized directly into the ventilatory circuit for 10 seconds, synchronized with inspiration (AeroNebLab nebulizer). Following each dose of methacholine, twelve perturbations were performed over approximately 3.6 minutes. We used two models to assess pulmonary function and airways responsiveness to methacholine; the linear first-order single compartment model, which determines the resistance of the total respiratory system, and the constant phase model, which utilizes forced oscillation to differentiate between airways Newtonian resistance ($R_N$) and peripheral tissue parameters ($G$, tissue damping and $H$, tissue elastance) to assess pulmonary function and airways responsiveness to methacholine [318, 319]. Anaesthesia was maintained throughout the experiment by repeated administration of \textasciitilde 25% the
initial dose of ketamine/xylazine every 25 minutes until completion. All data were collected using the FlexiVent software (Scireq) and analyzed off-line using Excel (Microsoft Corporation, Redmond, WA). After completion of the assessment of pulmonary function and methacholine-responsiveness, mice were euthanized with an overdose of anesthetic.

### 3.2.3 Isotopomer cocktail infusion

For in vivo studies of L-arginine metabolism, mice were intubated for pulmonary function testing as described above, and an additional jugular venous cannula was inserted. After determination of baseline total lung resistance, central airways resistance and peripheral tissue damping, a stable isotope solution ([C13]-L-arginine; Cambridge Isotopes Inc.) was infused via the jugular vein to allow determination of L-arginine flux down the NOS and arginase pathways. All mice received a primed (nmol) and constant infusion (nmol/h) of three stable isotopes: L-Arginine.HCl [U-13C6, 97-99%] (prime: 850 nmol, constant: 1700 nmol/h), L-Citrulline [5-13C, 99%; 4,4,5,5-D4, 93%] (prime: 425 nmol, constant: 850 nmol/h) and L-Ornithine.HCl [15N2, 98%] (prime: 215 nmol, constant: 430 nmol/h). The bolus was delivered in 0.25ml over 20 seconds, and followed by the stable infusion at a rate of 1 ml/h for an additional 45 minutes. After the infusion, blood samples were withdrawn via the tail vein, to obtain plasma samples. The mice were then euthanized with ketamine/xylazine and necropsy was performed to isolate the lung and trachea for subsequent mass spectrometric analysis.

### Liquid chromatography/ mass spectrometric (LCMS) analysis

**Plasma Samples:** Calibrators for liquid chromatography / mass spectrophotometric analysis (LCMS) were prepared by spiking pooled plasma with a standard curve: 0, 25, 50, 75, 100, 125, and 150 µmol/L of L-arginine, L-ornithine and L-citrulline, respectively and 0, 0.25,
0.50, 1.0, 2.0, 4.0, and 8.0 µmol/L of ADMA and SDMA, respectively. The calibrators were treated the same as the unknown plasma samples. 125µM of L-[³H7]-Arginine, L-[³H7]-Ornithine, L-[ureido ¹³C;5,5-²H₂] and 5µM of L-[³H7]-ADMA were added as internal standards. Quantitative determinations in 50 µl plasma was acquired by the addition of 20 µl of the internal standards mix. Plasma proteins were precipitated with 500 µl of methanol, and dried supernatants were derivatized with 100 µl 3M, HCl-Butanol (Regis Technologies, Morton Grove, IL) at 65°C for 20 min. The dried butylated samples were reconstituted in 250 µl, 0.1% formic acid (FA).

Lung tissues: Lung tissues were homogenized in 2.5 mL, 0.1% formic acid (FA) and 11.25 mL methanol (MeOH) per gram tissue. After centrifugation, the supernatants were stored at -20°C for mass spectrometric analysis. Calibrators were prepared by spiking pooled tissue homogenates (prepared from 2.5 mL, 0.1% FA per gm tissue) with 6 different concentrations: 0, 25, 50, 75, 100, 125, and 150 µmol/L for L-arginine, L-ornithine and L-citrulline, respectively and 0, 0.25, 0.50, 1.0, 2.0, 4.0, and 8.0 µmol/L for ADMA and SDMA, respectively. The calibrators were treated the same as the unknown tissue homogenate samples. 125µM of L-[³H7]-Arginine, L-[³H7]-Ornithine, L-[ureido ¹³C;5,5-²H₂] and 5µM of L-[³H7]-ADMA were added as internal standards mix. Quantitative determinations in 100 µl tissue homogenate was acquired by the addition of 20 µl of the internal standards mix. Tissue proteins were precipitated with 200 µl of methanol, and dried supernatants were derivatized with 100 µl 3M, HCl-Butanol (Regis Technologies, Morton Grove, IL) at 65°C for 20 min.

The dried butylated samples were reconstituted in 250 µL, 0.1% formic acid (FA). An API 4000 triple quadrupole mass spectrometer was operated as described by Urschel et al. for the determination of the concentration of L-arginine and its derivatives [320]. Prior to entering the triple quadrupole MS, the individual butylated amino acids were separated using a Dionex
Acclaim organic acid column (5µm 120A, 4.0 x 250mm; Dionex Canada, Oakville, ON, Canada).

**LCMS Calculations:** The concentrations of the amino acids were calculated against standard curves created from the same matrix as the samples of interest. The endogenous values (µmol/L) of the amino acids were calculated using the slope and the positive y-intercept of the calibrators by the Analyst NT v1.4.1 software (Applied Biosystems/MDS SCIEX). The expected ratio of analyte to corresponding internal standard ([analyte]/[internal standard]) was plotted against the observed peak area ratio of the analyte to internal standard to extract the slope and intercept.
Chapter 4:

Functionally important role for arginase 1 in the airways hyperresponsiveness of asthma

Michelle L. North, Nivedita Khanna, Philip A. Marsden, Hartmut Grasemann and Jeremy A. Scott


Contributions of Authors:

M.L.N. performed the protein expression profiling of human and murine lungs, arginase activity testing, immunohistochemistry analysis, participated in pulmonary function data analysis, and drafted the manuscript. N.K. performed sensitization and challenge of animals, pulmonary function testing, and participated in pulmonary function testing data analysis. P.A.M. facilitated the procurement of human samples, and critical revision of the manuscript for important intellectual content. H.G. participated in the conception and design of the study, and critical revision of the manuscript for important intellectual content. J.A.S. conceived of and designed the study, participated in pulmonary function data analysis, and critical revision of the manuscript for important intellectual content.
Chapter 4: Functionally important role for arginase 1 in the airways hyperresponsiveness of asthma

4.1. Abstract

L-Arginine metabolism by the arginase and nitric oxide synthase (NOS) families of enzymes is important in NO production, and imbalances between these pathways contribute to airways hyperresponsiveness (AHR) in asthma. To investigate the role of arginase isozymes in AHR we determined the protein expression of arginase 1 and arginase 2, the NOS isozymes, as well as other proteins involved in L-arginine metabolism, in lung tissues from human asthmatics and in acute (3-week) and chronic (12-week) murine models of ovalbumin (OVA)-induced airways inflammation. Expression of arginase 1 was increased in human asthma, while arginase 2, NOS isozymes, and the other L-arginine-related proteins (i.e., CAT1, CAT2, agmatinase, and ornithine decarboxylase) were unchanged. In the acute murine model of allergic airways inflammation, augmentation of arginase 1 expression was similarly the most dramatic change in protein expression. However, arginase 2, NOS1, NOS2 and agmatinase were also increased, while NOS3 expression was decreased. Arginase inhibition in vivo, with nebulized S-(2-boronoethyl)-L-cysteine (BEC), attenuated the methacholine responsiveness of the central airways in mice from the acute model. Further investigations in the chronic murine model revealed an expression profile that more closely paralleled the human asthma samples; i.e., only arginase 1 expression was significantly increased. Interestingly, in the chronic mouse model, which generates a remodeling phenotype, arginase inhibition attenuated methacholine responsiveness of both the central and peripheral airways. The similarity in arginase expression between human asthma and the chronic model, and attenuation of AHR following in vivo treatment with an arginase inhibitor suggests the potential for therapeutic modification of arginase activity in asthma.
4.2 Introduction

The semi-essential amino acid, L-arginine, is substrate for both the nitric oxide synthase (NOS) and arginase enzyme families [321]. Studies in affected patients and animal models have indicated that L-arginine metabolism is altered in asthma, through increased expression and activity of arginase [76, 168, 185, 322]. Previous studies have shown that arginase contributes to the regulation of airway smooth muscle tone likely by reducing the bioavailability of L-arginine for NOS and subsequently NO production [323-325].

Genetic association studies have suggested functionally important contributions from haplotypes/alleles of the arginase 1 and arginase 2 genes in asthma and atopy, as well as a role for arginase 1 in the responsiveness to bronchodilators [169-171]. Thus, there is a need to develop a thorough understanding of the consequences of biochemical imbalances of the L-arginine pathways in asthma, the suitability of common model systems in terms of mimicking these alterations, and the potential for arginase inhibition to attenuate airways hyperresponsiveness in multiple animal models to predict the effectiveness of these pharmacologic agents for future human asthma therapies.

To date, a comprehensive examination of the protein expression profile for L-arginine-related enzyme pathways and transporters has not been performed. L-Arginine bioavailability within the cell is directly related to its uptake by the cationic amino acid (CAT) transporters and catabolism via the arginase, NOS and arginine decarboxylase pathways. Increases in CAT2 expression have been detected in murine models of allergic inflammation using microarray, and mediators released from eosinophils can inhibit the function of CAT transporters [76, 143]. However, the expression levels of CAT2 and CAT1 have not previously been described in human asthmatic lung tissue. The nitric oxide synthase isoenzymes (NOS1, NOS2 and NOS3) are immunologically distinct proteins that are expressed from independent genes, and have been
of significant interest in asthma due to the observation that exhaled nitric oxide levels are increased in asthma [132, 133]. The expression of the NOS isozymes has been demonstrated in bronchial biopsies from asthmatic patients using immunohistochemistry [134].

Studies in animal models have also highlighted arginase as a novel therapeutic target in asthma through the use of specific inhibitors. Meurs et al. demonstrated that the increase in contractile responsiveness of isolated tracheal preparations to methacholine in ovalbumin (OVA)-sensitized and challenged guinea pigs was attenuated by treatment with an arginase inhibitor [186]. The same group recently investigated the acute and protective effects of arginase inhibition in vivo in a guinea pig model of asthma and demonstrated reversal of allergen-induced AHR when the inhibitor was administered prior to allergen challenge [189]. They further noted a reduction in sensitivity to allergen with chronic arginase inhibition [189]. In murine models of allergic airways inflammation, systemic administration of arginase inhibitor has caused increases in NO metabolite concentrations [326, 327]. However, the expression profiles of L-arginine related proteins have not been investigated in common murine models of allergic airways inflammation and compared to human asthma. Additionally, the ability of arginase inhibition to attenuate airways hyperresponsiveness in acute and chronic murine models (i.e., in the absence vs. presence of airways remodeling, respectively) has not been evaluated. Further, agmatinase, an enzyme that catabolizes the decarboxylation product of L-arginine, has not been investigated in human asthma. Such investigations may be important to fully understand the causes and implications of dysregulation of L-arginine metabolism in asthma. Based on these important insights derived from animal models and observations in human asthmatic subjects, and to test the hypothesis that augmented expression of arginase contributes to the airways hyperresponsiveness of asthma, we investigated the expression of proteins related to L-arginine metabolism, and compared the biochemical profiles of two commonly used acute and chronic
murine models of allergic inflammation to those of asthmatic human lung tissues. We also investigated and compared the immediate effects of nebulized arginase inhibitors on methacholine responsiveness in these murine models using a treatment-based protocol.

4.3 Materials and methods

**Lung homogenization, Western blotting and arginase activity**

Lung specimens from control and physician-diagnosed asthmatic human subjects (n=7 and 6 specimens, respectively) were obtained from the National Disease Research Interchange (Philadelphia, PA) and frozen at -80°C until homogenization and determination of protein expression. Details on the human lung samples are given in Table 2. Frozen tissues were thawed, macroscopically dissected, and airway tissues were isolated. Human and mouse specimens were homogenized, and Western blotting was performed as described in section 3.1.1. Arginase activity measurements were carried out as in section 3.1.2.

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<th>Age range (years)</th>
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<tr>
<td>Control</td>
<td>19 – 70</td>
<td>6 / 1</td>
</tr>
<tr>
<td>Asthma*</td>
<td>29 – 68</td>
<td>4 / 2</td>
</tr>
</tbody>
</table>

*One asthmatic subject had a prior history of smoking, and there was no indication of steroid use among any of the subjects.
**Immunohistochemistry assessment**

Immunohistochemistry for arginase 1, arginase 2 and NOS1 was performed using the same antibodies as for Western blotting, as described in section 3.1.3.

**Inhibition of arginase in-vivo**

To investigate the role of arginase in the airways hyperresponsiveness, we used both acute (3-week) and chronic (12-week) OVA-sensitization and –challenge murine models. These protocols were approved by the University of Toronto Faculty Advisory Committee on Animal Services, and were conducted in accordance with the guidelines of the Canadian Council on Animal Care. The sensitization and challenge protocols were carried out as in section 3.2.1. On day 21, pulmonary function testing and methacholine challenge was performed as in section 2.2.2. After determination of baseline pulmonary mechanics parameters in the OVA-sensitized and OVA or PBS-challenged mice, BEC (Alexis Biochemicals, San Diego, CA) (40 µg/g body weight) was delivered via nebulization directly into the ventilatory circuit 15 minutes prior to reassessment of pulmonary mechanics and initiation of the methacholine dose-response curve.

**Statistical analyses**

Statistical analyses were performed using the statistical software package in GraphPad Prism 4.0b (GraphPad Software, Inc., San Diego, CA). Data are presented as the mean ± SEM, except where noted. Mann-Whitney t-tests or one-way ANOVA with Kruskall-Wallis test and Dunn’s post-hoc test were performed to compare between groups. Differences were considered to be significant when p<0.05. All Western blot and immunoprecipitation findings were derived from at least 3 independent experiments, based on samples from 6-14 mice/group. Immunohistochemical staining was conducted on at least 4 mice per treatment group.
Pulmonary function testing and methacholine-responsiveness was conducted on 8-14 mice per group. Sample sizes are indicated in the figure legends.

4.4 Results

Lung arginase 1 expression and activity is increased in human asthma

We examined the expression of several proteins related to L-arginine metabolism in human asthmatic lung samples and controls obtained from the National Disease Research Interchange. Expression of arginase 1 was increased 4.4 ± 1.0-fold in lung tissue homogenates from human asthmatic subjects compared with controls (P<0.01, Figure 4). A significant elevation in arginase activity in the asthma samples was also detected (35.7 ± 4.6 vs. 62.2 ± 6.0 pmol urea/min/µg protein for control vs. asthma, respectively; P = 0.0087). However, no significant differences in arginase 2, NOS1, NOS2, or NOS3 protein expression were detected between human asthma and controls by either Western blot or immunoprecipitation (Figure 4). Additionally, no alterations in the expression of CAT1 (SLC7A1), CAT2 (SLC7A2) or ODC were observed in human asthma (Figure 4). Thus, augmented ARG1 expression was solely responsible for the increased arginase activity in human asthma.
Figure 4

Figure 4: Expression of proteins related to L-arginine metabolism in control and asthmatic human lungs.

Protein expression determined in lung homogenates from non-asthmatic (open) and asthmatic human subjects (filled). All results are expressed relative to the control lung samples to assess the fold change in human asthma. ARG1 expression was significantly increased in asthmatics compared to control subjects. Expression of ARG2, NOS1, NOS2, NOS3, CAT1, CAT2, agmatinase and ODC was not significantly different between groups (**P<0.01; n = 6 7/group). Data in the inset panel are expressed as the median and interquartile range.

Protein expression, localization, and arginase inhibition in the acute murine model of allergic airways inflammation

After examining the protein expression profile in human asthma, we used an acute murine model of allergic airways inflammation to determine the functional relevance of the augmentation of arginase 1 protein expression. This is one of the most common animal models employed in the study of allergic asthma, and we employed this system to determine the effects of arginase inhibition on AHR in vivo. First we confirmed that the augmentation of arginase 1 was modeled in this system. Indeed, OVA/OVA lung homogenates exhibited significantly up-regulated arginase1, 11.3-fold, compared with the OVA/PBS controls (P<0.001; Figure 5), and
Expression of L-arginine related proteins in acute model lung homogenates. All results are expressed relative to the OVA/PBS group. Western blot for ARG1 and ARG2 demonstrated significant up regulation in OVA/OVA mice from the acute model (P<0.001, Figure 5). NOS1 and NOS2 expression were up-regulated 3.4-fold and 3.0-fold in the OVA/OVA lung homogenates (P<0.001 and P<0.01, respectively, Figure 5), while expression of NOS3 was decreased -2.1-fold (P<0.01, Figure 5). Expression of agmatinase was increased 1.65-fold, compared to OVA/PBS (P<0.05, Figure 5). Although the expression of many proteins involved in L-arginine metabolism was altered in this model, the increase in arginase 1 expression was the largest in magnitude, similar to the human asthma samples.

Figure 5: Expression of arginine related proteins in the acute murine model.
Expression of L-arginine related proteins in acute model lung homogenates. All results are expressed relative to the OVA/PBS group. Western blot for ARG1 and ARG2 demonstrated significant up regulation in OVA/OVA mice from the acute model (filled) compared with controls (open). NOS1, NOS2 and agmatinase were also significantly increased. NOS3 expression was significantly attenuated in the OVA/OVA mice (***P<0.001, **P<0.01, and *P<0.05; n=6 9/group).
Immunohistochemistry was performed to assess the potential for increased expression of arginase to lead to substrate limitation for NOS, by co-expression in the same cells or tissues within the lungs. Arginase 1 was visibly increased throughout the lungs and strong-positive staining was observed in infiltrating inflammatory cells, bronchiolar and alveolar macrophages and in the peribronchiolar regions in the OVA/OVA mice (Figure 6A). Increased arginase 1 expression was also detected at the apical surface of ciliated airway epithelial cells compared to the respective OVA/PBS controls (Figure 6A). Diffuse arginase 2 staining was detected in lung tissues from both OVA/OVA and OVA/PBS mice in the acute model, with more prominent staining in areas of inflammation in the lungs from the OVA/OVA mice (Figure 6B).

Immunohistochemical staining for NOS1 revealed expression in airway epithelial cells (Figure 6C). The results of immunohistochemical staining in the acute model revealed that arginase 1 and NOS1 are co-expressed in airway epithelial cells in this model, indicating the potential for increased arginase 1 expression to result in substrate deficiency for NOS in the same cell populations within the lung.

Finally, we assessed the effect of the nebulized arginase inhibitor, BEC, on airways responsiveness to methacholine in this model, to predict the functional consequences of the increased arginase 1 expression observed in the human asthma samples. Interestingly, responsiveness of the central airways to methacholine (indicated by the Newtonian resistance; R\textsubscript{N, Max}) was significantly attenuated following treatment with nebulized BEC (P<0.05, Figure 7A). Meanwhile, the maximum peripheral tissue damping/resistance (G\textsubscript{Max}) was unaffected by BEC treatment in the acute OVA model (Figure 7B). The decreased AHR in the central airways in this model supports that increased arginase expression and activity in asthma is directly relevant to airway function.
Figure 6: Immunohistochemistry in the acute model

Immunohistochemistry demonstrated increased ARG1 staining in inflammatory cells, epithelial cells and the peribronchiolar region in the acute OVA/OVA mice, relative to controls (A). Immunohistochemistry also revealed increased ARG2 staining (B) in areas of inflammation and increased epithelial NOS1 staining (C) in the OVA/OVA group compared with controls. Photomicrographs of representative sections from n = 4 mice/group (magnification; 200×).
Protein expression, localization, and arginase inhibition in the chronic murine model of allergic airways inflammation

While the acute murine model exhibited similarity with the protein expression profile of the human asthma, in that arginase 1 was the protein with the largest observed alteration in expression, the acute model also exhibited some changes in protein expression that were not detected in the human specimens. Specifically, the increased arginase 2, NOS1 and NOS2 expression, and decreased NOS3 expression, might accentuate the functional relevance of increased arginase 1 expression in human asthma. Thus, we employed a chronic murine model of allergic airways inflammation to determine whether a less severe but prolonged challenge phase would result in an expression profile of L-arginine related proteins that was more similar
to human asthma. This chronic model system was then used to test the functional relevance of the increased arginase 1 expression to AHR.

Similar to the specimens from human asthma, arginase 1 expression was increased 26-fold in the chronic model (P<0.001) (Figure 8). Total arginase activity was increased significantly (5.3 ± 1.1 vs. 405.1 ± 63.3 pmol urea/min/µg protein for OVA/PBS vs. OVA/OVA, respectively; P<0.0001). However, no significant alterations of arginase 2, NOS2, or NOS3 expression were detected by Western blotting (Figure 8). No differences in NOS1 or agmatinase expression were detected by immunoprecipitation. Furthermore, there were no significant changes in the expression of CAT1, CAT2 or ODC between OVA/OVA and OVA/PBS mice in the chronic murine model. The sole augmentation of arginase 1 in the expression profile of L-arginine related proteins in the chronic murine model was very similar to that observed for human asthma, suggesting that this model would be suitable for examining the functional relevance of increased arginase 1 expression.

Immunohistochemical staining demonstrated increased arginase 1 expression in chronic model OVA/OVA mice at the apical surface of ciliated airway epithelial cells compared to their respective OVA/PBS controls, similar to the acute model (Figure 9A). Immunohistochemical staining for NOS1 also revealed expression in airway epithelial cells (Figure 9B), which was not modified by OVA-challenge. Thus, arginase 1 augmentation was the only significant alteration in protein expression detected in the chronic model.

After determining that the chronic murine model of allergic airways inflammation closely mimicked the protein expression profile of human asthma, we investigated the functional relevance of the augmented arginase 1 expression by administering an arginase inhibitor in vivo. Responsiveness to methacholine was significantly attenuated following treatment with nebulized BEC, as determined by decreased peak response of the central airways
Newtonian resistance ($R_{\text{NMax}}$; Figure 10A), and maximum peripheral tissue damping/resistance ($G_{\text{Max}}$; Figure 10B) ($P<0.01$ and 0.05, respectively). Thus, in this chronic model, a model system that effectively mimics the expression of L-arginine related proteins in the human asthma lung samples, inhibition of arginase resulted in reduction of both the central and peripheral airways responsiveness to methacholine.

**Figure 8: L-Arginine related protein expression in the chronic murine model**

Expression of L-Arginine related proteins in murine lung homogenates from the chronic model. All results are expressed relative to the OVA/PBS group to assess fold change in the OVA/OVA mice. Expression of ARG1 was significantly increased in OVA/OVA mice (filled) compared to OVA/PBS controls (open). ARG2, NOS1, NOS2, NOS3, CAT1, CAT2, agmatinase and ODC expression were not significantly different between groups (***$P<0.001$, $n = 6$ 14/group).
Figure 9: Immunohistochemistry in the chronic OVA-model.

A) ARG1 positivity was detected in inflammatory cells, epithelial cells and the peribronchiolar region in OVA/OVA treated mice. B) NOS1 staining was also detected in the airway epithelia (B). Representative of n = 4 mice/group (magnification; 200×).

Figure 10: Functional improvement in airways responsiveness with BEC treatment in the chronic model of allergic airways inflammation.

Maximum central airways resistance (A), and peripheral tissue damping (B) in OVA/OVA mice (filled) were attenuated with BEC treatment, relative to PBS (open) (*P<0.05, **P<0.01, ***P<0.001 to OVA/PBS, with the same drug treatment/control; #P<0.05, ###P<0.01, ####P<0.001 to untreated control, same challenge group, n = 8-14).
4.5 Discussion

Summary

In this study, we examined the expression of the arginase and NOS isozymes, and other proteins related to L-arginine uptake and metabolism in human lung specimens from asthmatic patients and non asthmatic controls. We found that arginase 1 alone was significantly up regulated in lungs from asthma patients. To determine the functional relevance of dysregulation of L-arginine metabolism due to increased expression of arginase 1 on airways hyperresponsiveness, we then investigated the effects of arginase inhibition in two murine models of OVA-induced allergic airways inflammation. In the acute murine model, in which expression of all arginase and NOS isozymes, as well as agmatinase, were significantly altered, arginase inhibition resulted in reduced airways hyperresponsiveness to methacholine in the central, but not peripheral, airways. In the chronic murine model the expression profile of L-arginine related proteins more closely mimicked those observed in human asthma, with only arginase 1 being significantly up-regulated. Nebulized arginase inhibitor in this model resulted in attenuation of both central and peripheral airways hyperresponsiveness to methacholine. These findings support the potential for pharmacologic inhibition of arginase, specifically inhibition of arginase 1, to reduce airways hyperresponsiveness in asthma.

Expression of proteins relevant to L-arginine metabolism in asthma

While genetic linkage and association studies have provided compelling evidence suggesting role(s) for the NOS and arginase isozymes in asthma [137, 138, 140, 169, 170], the specific contributions of imbalances in L-arginine metabolism have only recently become appreciated [171, 185, 322, 328]. Morris et al. reported decreased L-arginine bioavailability and increased serum arginase activity in pediatric asthma patients [322]. In a sub-group of patients
who were admitted to hospital with respiratory distress and status asthmaticus, these investigators also reported increased L-arginine bioavailability and reduced serum arginase activity at discharge [322]. Furthermore, Lara et al. recently described increased bioavailability in plasma, increased L-arginine catabolism and a direct relationship between L-arginine bioavailability and airflow obstruction in severe asthma [185]. Thus, these reports support the delicate balance in L-arginine metabolism in asthma. The data reported herein build upon these previous findings and provide new information regarding the expression of a panel of proteins related to L-arginine metabolism, in lung tissue from human asthma patients.

The absence of a significant increase in arginase 2 in human tissues in this study also demonstrated, for the first time, that the increased arginase activity in human asthma was solely attributable to changes in arginase 1 expression. Consistent with previous studies [134], we observed a trend towards increased NOS2 expression in our asthma samples; however, this increase was not significant (P = 0.1). One caveat in the interpretation of the protein expression relates to the fact that the severity of asthma in these patients was unknown, since detailed clinical information regarding the asthma subjects was not available, and the sample size was limited. This may explain the lack of a significant increase in NOS2. These findings reinforce that further investigation of the expression of proteins related to the L-arginine metabolome in a properly characterized patient population is warranted.

Although the cationic amino acid transporter 2 (CAT2) is induced in certain murine models of asthma, and mediators released from eosinophils can inhibit the function of CAT transporters [76, 143], the expression of CAT2 and CAT1 proteins has not previously been examined in human asthma. Uptake of L-arginine by the CAT transporters is critical to L-arginine bioavailability within the cell. As expression of arginase 1 was significantly up-regulated in the human asthmatic lung specimens compared with the non-asthmatic controls, in
the absence of augmented expression of either CAT1 or CAT2, our results support the potential for decreased intracellular bioavailability of L-arginine for NOS in asthmatic airways.

**L-Arginine metabolism and arginase inhibition in an acute murine model of allergic airways inflammation**

Mouse models can be useful to investigate specific mechanisms related to disease pathology [329]. However, one of the limitations in their utility is that mice do not develop asthma [85, 330]. Thus, allergen-induced airways inflammation to otherwise innocuous agents (i.e., OVA) has been used to elicit augmented AHR, increased IgE levels, goblet cell metaplasia, mucous production and airways remodeling, as surrogates of the asthma phenotype [85, 331, 332]. Furthermore, these models can be either sub-acute, acute or chronic, to elicit different specific asthma phenotypes (i.e., greater AHR, inflammatory cell infiltration/activity or remodeling) [333]. To investigate the functional importance of the increase in arginase 1 protein expression detected in the human samples, we employed a commonly used acute murine model of OVA-induced allergic airways inflammation as a first step.

In the acute (3 week) OVA model, arginase 1 protein expression was increased, consistent with previous studies in mice [76, 326, 334, 335]. The concomitant expression of NOS1 and arginase in airway epithelial cells supports the hypothesis that augmented arginase activity has the potential to decrease the local bioavailability of L-arginine for NOS1 in these cell types [327, 335]. Indeed, previous studies in guinea pigs have suggested that arginase activity can affect airway tone through increased competition with NOS1 in homeostasis and under conditions of allergic inflammation [189, 324, 325]. Expression of arginase 1 in the airway epithelium is also consistent with studies in humans, as increased arginase 1 mRNA expression has previously been described in these cell types and in areas of inflammation in
human asthmatics [76]. Arginase 1 protein has also been demonstrated in the airway epithelia in human asthma by immunohistochemistry [168]. However, the in vivo effects of arginase inhibitors on pulmonary function have only recently been described in guinea pigs by Maarsingh et al., who demonstrated that the arginase inhibitor, 2(S)-amino-6-boronohexanoic acid (ABH), reduced AHR following the early and late allergic response [189]. Decreased responsiveness to methacholine has also been observed following arginase inhibition in a murine model [191, 327]. In this study, we inhibited arginase using a treatment based administration protocol to examine the functional relevance of increased arginase 1 protein expression in asthma, using the mouse as a model system. We found that acute arginase inhibition reduced central airways responsiveness to methacholine in the acute murine model, supporting that increased arginase 1 expression and arginase activity likely play a functional role in AHR.

Our observation of augmented arginase 2 expression in the acute murine model is consistent with previous findings obtained in similarly acute models using gene expression microarray [76] and Western blotting [326]. However, arginase 2 protein expression was not up-regulated in the chronic murine model or the human asthma specimens. While genetic studies have demonstrated association of polymorphisms of arginase 2 with the diagnosis and severity of asthma in children [170], and have identified chromosome 14q24 as a major susceptibility gene for asthma [169], the current findings do not support a functional role for arginase 2 in the AHR that develops in the chronic model of asthma, and do not support a role for arginase 2 in human asthma. While Zimmerman et al. reported upregulation of arginase 1 in human asthma, they did not report arginase 2 expression in their patient population [76]. Thus, we speculate that the augmented arginase 2 expression is related to the acute inflammatory response in the acute murine model and does not play a role in chronic allergic airways inflammation.
Many of the proteins examined were found to be up or down regulated in the acute model, likely indicative of the severity of the inflammation induced by repeated exposure to OVA for 7 consecutive days. One interesting alteration was the increase in agmatinase in the OVA/OVA lungs from this model. Agmatine is synthesized from L-arginine by arginine decarboxylase, and is subsequently catabolized by agmatinase, which is part of the arginase superfamily [336]. Agmatinase has been implicated in the inflammatory response, and may be involved as a temporal switch between the generation of NO and the induction of arginase [199]. Thus, the alterations in protein expression that were observed only in the acute model might be related to the time course of the acute inflammatory response.

**L-Arginine metabolism and arginase inhibition in a chronic murine model of allergic airways inflammation**

Interestingly, in the chronic murine model of experimental asthma, arginase 1 was the sole significantly up regulated isoyme; which was more similar to the expression profile of the human asthma samples. The chronic model is typically used for investigations of airway remodeling [333, 337]. However, we also found it useful for modeling the more subtle functional changes in L-arginine metabolism associated with chronic disease.

Localization of arginase 1 in the peribronchiolar regions was consistent with the features of remodeling, such as collagen deposition, that are typical of this model [333, 337, 338]. Additionally, co-expression of arginase 1 and NOS1 in the airway epithelia supports the hypothesis that competition for L-arginine substrate is increased in allergic asthma.

The finding that the responsiveness of the peripheral lung and airways to methacholine was attenuated in the chronic model, but not the acute model of allergic airways inflammation is intriguing. This difference may be due to the severity of the acute model and the altered
expression of L-arginine related proteins described above. Chronic murine models of allergic airways inflammation, similar to the one described herein, have been shown to exhibit features of remodeling and sustained airway dysfunction [333, 337, 338]. Despite the presence of airway remodeling, arginase inhibition was effective in significantly reducing both central and peripheral airways hyperresponsiveness to methacholine in the chronic model. These findings from the chronic model provide strong support for the importance of arginase1 up-regulation in asthma. As arginase 2 was not increased in this model, the effects of arginase inhibition on central and peripheral airways responsiveness in this study were due solely to inhibition of arginase 1. The reduction in methacholine responsiveness following in vivo treatment with BEC in both murine models supports the importance of L-arginine bioavailability in asthma. Furthermore, the concordance between the findings of augmented arginase 1 expression in human asthmatics, and in the murine and guinea pig models of asthma, supports the potential for arginase as a therapeutic target in human asthma.

4.6 Conclusions

L-Arginine metabolism contributes to the maintenance of homeostasis in the respiratory system and imbalances can contribute to disease. This study demonstrated altered expression of arginase 1 in human asthmatic lung samples and up regulation of arginase 1 was consistently observed in both the acute and chronic murine models of allergic airways inflammation. The expression of other proteins related to L-arginine metabolism (i.e., NOS isozymes, transporters, etc.) was also determined. Colocalization of arginase1 and NOS1 was demonstrated in the airway epithelia in both murine models and supports the direct competition for substrate in these cell types. Finally, arginase inhibition in vivo attenuated airways responsiveness to methacholine in both acute and chronic murine models of allergen-induced airways inflammation. These
results support the therapeutic potential of specific arginase inhibitors as a novel treatment for asthma, and suggest that arginase 1 should be targeted specifically.
5. Chapter 5:

Polyamines play a functional role in airways responsiveness in murine models of allergic airways inflammation.

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In preparation

Contributions of Authors:
M.L.N. performed the polyamine administration studies, histology analysis, protein expression, arginase activity testing, nitrate/nitrite analysis and drafted the manuscript. N.K. and M.L.N collaboratively performed the ornithine decarboxylase inhibition studies. H.G. participated in the conception and design of the study, and critical revision of the manuscript for important intellectual content. J.A.S. conceived of and supervised the study, and critically revised the manuscript for important intellectual content.
5.1 Abstract

**Background:** Arginase is upregulated in asthma and plays a functional role in airways hyperresponsiveness (AHR) in murine models of allergic airways inflammation. The product of arginase, L-ornithine, can be metabolized via the polyamine or proline pathways, which are important in cellular proliferation and collagen deposition, respectively. L-Ornithine is converted into polyamines beginning with putrescine production by ornithine decarboxylase (ODC), considered to be the rate-limiting enzyme in this pathway, and further to spermidine and spermine. Increased levels of polyamines have been detected in human asthmatics, and demonstrated in murine models. However, the direct effects of polyamines on airways responsiveness are not well understood. Therefore, we tested the hypothesis that increased levels of polyamines contribute to augmented responsiveness to methacholine in normal and allergically inflamed airways. The objectives of this study were to 1) investigate the effect of polyamines on methacholine responsiveness in naïve mice and a sub-acute model of ovalbumin-induced allergic airways inflammation, and then 2) to determine whether blockade of the synthesis of polyamines would improve the hyperresponsiveness to methacholine in an acute murine model.

**Methods:** Putrescine, spermidine, spermine (0-10 mM), or PBS vehicle were nebulized directly into the airways of naïve mice and methacholine responsiveness was determined using the flexiVent system. The effects of exogenous spermine on airways responsiveness were examined further in a sub-acute murine ovalbumin (OVA) sensitization and –challenge model. Finally, an acute model of airways inflammation and hyperresponsiveness (AHR) was employed to determine the effects of inhibition of polyamine synthesis by difluoromethylornithine (DFMO). DFMO was administered at 1 mg/ml, *ad libitum*, in the drinking water concurrent with the
challenge phase. Methacholine responsiveness was determined in vivo 24 hours after the final challenge. Lungs were harvested for histological assessment of inflammation, protein analyses and Greiss assay.

**Results:** Spermine administration, but not putrescine or spermidine, significantly augmented maximum respiratory and central airways responsiveness to methacholine in naïve mice. Spermine administration in OVA-sensitized and –challenged (OVA/OVA) mice in the sub-acute model also resulted in a significant augmentation of methacholine response. In the acute model, up-regulation of arginase 1 and histologic scoring of inflammation were not affected by DFMO treatment. Treatment with DFMO significantly attenuated the respiratory responsiveness and abrogated the central airways responsiveness to methacholine, compared to untreated controls.

**Conclusions:** Spermine increases airways responsiveness to methacholine. Pharmacologic blockade of polyamine synthesis through ODC, concurrent with OVA challenge, attenuates airways hyperresponsiveness in this murine model of allergic airways inflammation. These findings further support that imbalances in L-arginine metabolism down-stream of arginase are important in asthma.
5.2 Introduction

Metabolism of the amino acid, L-arginine, plays an important role in the maintenance of homeostasis in the respiratory system, and imbalances can contribute to disease [218, 321, 339]. Two pathways of L-arginine metabolism are important in asthma; the nitric oxide synthase (NOS) pathway, whereby L-arginine is converted into nitric oxide (NO) and L-citrulline, and the arginase pathway, which produces urea and L-ornithine (Figure 11). We, and others, have shown that arginase activity and expression are increased in human asthma and animal models, likely leading to increased competition between these pathways for their common substrate, L-arginine [76, 322, 340]. Pharmacologic inhibition of arginase attenuates airways responsiveness to methacholine in both murine and guinea pig models of asthma [71, 189, 191, 340]. However, the impact of the polyamines, that are produced downstream of arginase, has not been explored in asthma.

As a product of the metabolism of L-arginine through the arginase pathway, L-ornithine is converted into the polyamine, putrescine, by ornithine decarboxylase (ODC). Putrescine is then further metabolized into the polyamines spermidine and spermine, by spermidine synthase and spermine synthase, respectively [150, 218] (Figure 11). Polyamines are known to accumulate in the lung, particularly in the epithelium, via active transport mechanisms [341]. Increased levels of polyamines have been detected in human asthmatics with active symptoms, and in murine models [76, 222]. Polyamines are also likely involved in airways remodeling, as they promote cell growth [321]. Recently, the effects of the traditional medicine, black seed oil, were assessed in a rat model of ovalbumin induced airways inflammation and remodeling [225]. Administration of black seed oil concurrent with intranasal ovalbumin challenges for seven days suppressed inflammation, collagen deposition, arginase activity, ODC activity and lowered polyamine levels [225].
Although it has traditionally been proposed that asthmatic airways hyperresponsiveness (AHR) develops as a result of increased substrate competition between arginase and nitric oxide synthase (NOS), leading to reduced production NO [218, 321, 339], it remains unknown whether the consequently increased polyamine concentrations in the allergic inflamed lung also play a functional role.

Spermine and spermidine have been shown to be competitive inhibitors of NOS, but it is not known if this occurs in the airways at physiologically relevant concentrations [226, 227]. Therefore, we tested the hypothesis that increased levels of polyamines in the airways play a functional role in contributing to airways responsiveness to methacholine. We show that exogenous spermine increases airways responsiveness in naïve mice and in mice with pre-existing allergic inflammation. We also demonstrate that treatment with difluoromethylornithine (DFMO), an irreversible ornithine decarboxylase inhibitor, significantly attenuates airways responsiveness in mice in a model of ovalbumin (OVA)-induced allergic inflammation.
Figure 11: Polyamine metabolic pathway.

The polyamine, putrescine is synthesized by ornithine decarboxylase (ODC) from L-ornithine, the product of arginase. Spermidine and spermine are synthesized by spermidine synthase and spermine synthase, respectively.

5.3 Materials and Methods

Administration of polyamines to naïve mice

To investigate the effects of polyamines on airways responsiveness, mice were anesthetized for pulmonary function testing as in section 3.2.2. After baseline determination of airway resistance, dose-response curves to polyamines were performed. Solutions of 0-10 mM of putrescine, spermidine and spermine (adjusted to pH 7) were delivered by nebulization directly into the ventilatory circuit for 10 seconds per dose (AeroNebLab nebulizer),
synchronized with inspiration (n = 12 per polyamine group and n = 10 for vehicle control). The cumulative dose of each polyamine administered was 12 ± 0.1 nmol/g body weight. In the control group, equivalent volumes of PBS were nebulized to match each dose of polyamine. Following polyamine administration, mice were challenged with methacholine (0-100 mg/mL; Sigma), as described in section 3.2.2.

Administration of spermine to OVA-sensitized and -challenged mice

To investigate whether exogenous spermine would augment airways responsiveness to methacholine in mice with allergically inflamed lungs, we used OVA/OVA mice from a sub-acute model of OVA-induced allergic inflammation, as described in section 3.2.1. The sub-acute model was used in this study, as the OVA/OVA mice in this model do not exhibit significant AHR and thus allow for the detection of increased airways responsiveness following spermine administration. Twenty-four hours after the final OVA challenge, mice were anaesthetized for pulmonary function testing, as described in section 3.2.2. Spermine (10 ± 0.2 nmol/g body weight) or hypertonic PBS of equal ionic strength were nebulized for 10 seconds, synchronized with inspiration, 15 minutes prior to determination of methacholine responsiveness.

Inhibition of ornithine decarboxylase in the acute model of allergic airways inflammation

To investigate the effects of pharmacologic ablation of polyamine synthesis in the development of airways hyperresponsiveness in a murine model of allergic airways inflammation we employed the acute model, which has been described previously [340]. The irreversible ornithine decarboxylase (ODC) inhibitor, difluoromethylornithine (DFMO), was administered ad libitum in the drinking water, beginning on day 14, to pharmacologically block
polyamine synthesis. On day 21, 24 hours after the final PBS or OVA exposure, mice were anaesthetized and methacholine responsiveness was assessed as described in section 3.2.2. After the completion of pulmonary function testing, lungs were harvested for histological staining, Western blotting or nitrate/nitrite measurement.

Semi-quantitative assessment of inflammation

The degree of peribronchial inflammation was assessed from captured images of hematoxylin and eosin stained slides, centered on airways, at 200x magnification. Ten images from each mouse were semi-quantitatively assessed for peribronchiolar inflammation according to the following scoring system: 0, normal; 1, few cells; 2, a ring of inflammatory cells 1 cell layer deep; 3, a ring of inflammatory cells 2–4 cells deep; 4, a ring of inflammatory cells of >4 cells deep [342].

Nitrate/nitrite measurement and Western blotting

To determine the effects of DFMO treatment on stable end products of NO, measurement of total nitrates + nitrites was carried out using the Greiss assay according to manufacturer’s directions (Cayman Chemical Company, Ann Arbor, MI). Western blotting for arginase 1 was performed as described in section 3.1.1.

Statistics

Dose-response curves were compared using the F-test, with the null hypothesis that the data from all groups could be modeled by the same curve, and using two-way ANOVA with Bonferroni’s post-hoc test. Western blotting densitometry data were analyzed using one-way ANOVA with Bonferroni’s multiple comparison post-hoc test. P-values < 0.05 were considered
significant. All statistical analyses were performed using GraphPad Prism 4.0c (GraphPad Software, Inc, LaJolla, CA).

5.4 Results

Administration of polyamines to naïve mice

Administration of polyamines alone did not directly alter respiratory (R) or airways tone (R_{N}) (data not shown). However, spermine significantly augmented the response to subsequent methacholine challenge (Figure 12). The maximum total lung resistance (R_{Max}) and central airways resistance (R_{N Max}) were significantly increased in naïve mice that were treated with a 12 ± 0.1 nmol/g body weight cumulative dose of spermine prior to methacholine challenge (Figure 12 A & B). Neither spermidine nor putrescine affected the maximum responsiveness to methacholine (Figure 12).

Administration of spermine to OVA-challenged mice

After demonstrating an increase in methacholine responsiveness following spermine administration in naïve mice, we examined the effects of spermine on mice with pre-existing allergic airways inflammation. Compared to OVA/OVA mice treated with nebulized PBS of equal ionic strength, OVA/OVA mice treated with 10 mM nebulized spermine (10 ± 0.2 nmol/g body weight) exhibited significantly increased responsiveness of the respiratory system (R) to methacholine and augmented R_{Max} (Figure 13A & B). Mice that were treated with nebulized spermine also demonstrated significantly increased responsiveness of the central airways to methacholine, and R_{N Max} (Figure 13C & D). Thus, spermine augmented methacholine responsiveness in allergically inflamed airways.
Figure 12: Effects of polyamines on airways responsiveness in naïve mice

A) Effects of polyamine administration on maximum total respiratory system resistance ($R_{\text{Max}}$) in naïve mice. B) Effects of polyamine administration on maximum central airways resistance ($R_{\text{N Max}}$) in naïve mice (*$P<0.05$; $n = 10-12$).

Figure 13: Effects of spermine on methacholine responsiveness in mice from the sub-acute model of allergic airways inflammation

Dose-response relationships for the increase in $R$ (A) and $R_{\text{N}}$ (C) to methacholine in OVA/OVA mice from the sub-acute model administered spermine or ionic strength–matched PBS. Effects of polyamine administration on $R_{\text{Max}}$ (B) and $R_{\text{N Max}}$ (D) (*$P<0.05$, **$P<0.01$, ***$P<0.001$; $n = 12$ per group).
Inhibition of ornithine decarboxylase in an acute model of allergic airways inflammation

To determine the effects of blockade of polyamine synthesis, downstream of arginase, on the development of airways inflammation and responsiveness, we employed an acute murine model and provided a sub-group of randomly selected OVA/PBS and OVA/OVA mice with the ornithine decarboxylase inhibitor DFMO (1 mg/ml) in drinking water. DFMO administration did not affect \( H_2O \) intake, food intake or body weight, and a mean dose of \( 0.16 \pm 0.04 \) g/kg/day was consumed by the treated mice (Table 3). Assessment of histological sections stained with hematoxylin and eosin (Figure 14) and semi-quantitative scoring revealed significant inflammation in the OVA/OVA mice, compared to OVA/PBS (\( P<0.0001 \)), but no affect of DFMO (n.s. DFMO treated to untreated). Additionally, no effect of DFMO on the upregulation of arginase 1 expression in the acute model was detected by Western blotting (data not shown). Total nitrates + nitrites were significantly reduced in OVA/OVA mice compared to OVA/PBS mice (Figure 15). DFMO significantly increased nitrate + nitrite levels in the treated OVA/OVA mice, but did not affect levels in treated OVA/PBS controls (Figure 15).

Assessment of respiratory function revealed no effect of DFMO on basic respiratory mechanics, such as lung volume (\( V_{end} \)), quasi-static compliance (\( C_{st} \)), or the Salazar-Knowles parameters (Table 4). However, determination of methacholine responsiveness revealed a significant attenuation of responsiveness of the total respiratory system (\( R_{Max} \)) in OVA/OVA mice that received DFMO, compared to controls provided with normal drinking water (Figure 16 A & B). Significant attenuation of methacholine responsiveness was also demonstrated in the central airways of OVA/OVA mice treated with DFMO, compared to controls (Figure 16 C & D). Thus, pharmacologic blockade of polyamine synthesis through ODC significantly attenuated airways hyperresponsiveness in this model.
Figure 14: Hematoxylin and eosin staining of DFMO treated/untreated mice
A) Representative pictomicrographs of hematoxylin and eosin stained histological sections from DFMO treated and untreated OVA/PBS and OVA/OVA mice (representative of n = 8 per group, bar indicates 100 µm, 200x magnification).

Figure 15: Effects of ornithine decarboxylase inhibition on nitrate + nitrite levels in acute model mice.
Results of Greiss assay in lung homogenates from mice treated with DFMO or vehicle control in the acute model, expressed per µg of lung protein (*P<0.05, n = 11-12 per group).
Table 3: Body weight and food/liquid intake and of H₂O and DFMO treated mice.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>DFMO</th>
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<tbody>
<tr>
<td></td>
<td>OVA/PBS</td>
<td>OVA/OVA</td>
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<tr>
<td>Body Weight (g) *</td>
<td>18.28 ± 0.25</td>
<td>18.97 ± 0.29</td>
</tr>
<tr>
<td>Food Intake (g/day)</td>
<td>2.85 ± 0.15</td>
<td>3.07 ± 0.15</td>
</tr>
<tr>
<td>Liquid Intake (ml/day)</td>
<td>3.36 ± 0.24</td>
<td>3.21 ± 0.16</td>
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</table>

*Standard error of the mean is given (n = 10-13 per group). DFMO treatment did not affect body weight and food or water consumption (P = n.s.).

Table 4: Pulmonary function and Salazar-Knowles parameters for DFMO-treated and control mice.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>DFMO</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>OVA/PBS</td>
<td>OVA/OVA</td>
</tr>
<tr>
<td>Vend (ml)</td>
<td>1.0 ± 0.02</td>
<td>0.86 ± 0.04*</td>
</tr>
<tr>
<td>Vend/wt</td>
<td>50.63 ± 1.34</td>
<td>42.73 ± 2.07*</td>
</tr>
<tr>
<td>Cₜ (ml/cmH₂O)</td>
<td>0.091 ± 0.008</td>
<td>0.062 ± 0.007*</td>
</tr>
<tr>
<td>Eₜ (cmH₂O/ml)</td>
<td>11.4 ± 1.0</td>
<td>15.0 ± 0.9*</td>
</tr>
<tr>
<td>A (ml)</td>
<td>0.61 ± 0.04</td>
<td>0.60 ± 0.02</td>
</tr>
<tr>
<td>B (ml)</td>
<td>2.0 ± 0.8</td>
<td>0.96 ± 0.04*</td>
</tr>
<tr>
<td>K (/cm H₂O)</td>
<td>0.21 ± 0.04</td>
<td>0.23 ± 0.08</td>
</tr>
</tbody>
</table>

*P<0.05 to OVA/PBS, same drug treatment. DFMO treatment did not affect pulmonary function or Salazar-Knowles parameters in OVA/PBS or OVA/OVA groups, relative to untreated controls (P = n.s.). Vend; lung volume at 30 cmH₂O, Cₜ; quasi-static compliance, Eₜ; quasi-static elastance, A, B, K; Salazar-Knowles parameters. Standard error of the mean is given (n = 10-13 per group).
Figure 16: Effects of ornithine decarboxylase inhibition on methacholine responsiveness in mice from the acute model treated with DFMO or H₂O control.

Dose-response relationships for R (A) and R₉ (C) to methacholine in OVA/PBS (open) and OVA/OVA (filled) mice from the acute model administered DFMO (1 mg/ml) in the drinking water or vehicle (H₂O). Effects of DFMO on R₉₉₉ (B) and R₉₉₉₉ (D) in treated and untreated acute model mice (*P<0.05, **P<0.01, ***P<0.001; n = 10-12 per group).
5.5 Discussion

Summary

In this study, we investigated whether the polyamines, which are synthesized downstream of arginase contribute to the airways hyperresponsiveness of asthma. We demonstrated that spermine administration, but not putrescine or spermidine, significantly augmented maximum respiratory and central airways responsiveness to methacholine in naïve mice. We also showed that methacholine responsiveness was augmented by spermine in mice with allergically inflamed airways. In an acute model of allergic airways inflammation, blockade of polyamine synthesis with DFMO significantly attenuated the respiratory responsiveness and abrogated the airways hyperresponsiveness to methacholine, compared to untreated controls, with no alterations in control (OVA/PBS) mice. These findings further support that imbalances in L-arginine metabolism down-stream of arginase are important in murine models of asthma.

Upregulation of arginase and polyamines in asthma

The metabolism of L-arginine plays an important homeostatic role in the airways, through regulation of the bioavailability of the substrate for the synthesis of the bronchodilating molecule, nitric oxide (NO), by the nitric oxide synthase (NOS) isozymes [100]. The arginase isozymes 1 and 2, convert L-arginine into L-ornithine and urea, and thus compete with the NOS isozymes for substrate [321]. Zimmermann et al. showed that arginase 1 mRNA was upregulated in bronchial biopsy and bronchoalveolar lavage samples from human asthmatics [76]. We have further shown upregulation of arginase 1 protein expression and activity in human asthmatic lungs [340]. The arginase isozymes have also been demonstrated to play a functional role in the airways hyperresponsiveness (AHR) in animal models of asthma,
employing different species and allergens [71, 76, 189, 326, 334, 339, 340]. Furthermore, elevated arginase activity has been demonstrated in asthmatic subjects during exacerbation and L-arginine bioavailability has been correlated to airflow obstruction in severe asthma [185, 322]. Thus, while arginase is known to be upregulated in asthma, the effects of increased arginase activity on downstream metabolites and the role of the polyamine pathway in the pathogenesis and symptomology of asthma remain undefined.

Although ornithine decarboxylase activity is generally considered to be the rate-limiting step in the synthesis of polyamines, increased production of the substrate L-ornithine by arginase can also promote polyamine synthesis. In the seminal paper by Zimmermann et al., examining the arginase pathway, the authors also demonstrated increased polyamine levels in murine models of allergic airways inflammation [76]. Similarly, increased polyamine levels have been demonstrated in humans during spontaneous mild asthma attacks [222]. Kurosawa et al. also demonstrated that in one patient who was admitted to hospital for a severe exacerbation, serial polyamine measurements revealed an elevation at admission, followed by a decrease to normal levels in parallel with clinical improvement [222]. This demonstrates similarities to the time-course of increased arginase activity during asthma exacerbation reported by Morris et al. [322].

Polyamines have also been implicated in the pathology of other serious lung diseases, such as cystic fibrosis, pulmonary arterial hypertension and lung cancer [211-215, 341]. Interestingly, increased arginase activity and expression have also been demonstrated in patients with these conditions [216-219]. However, the link between increased arginase activity in these lung diseases and the increased polyamine concentrations has not been well described. Herein, we demonstrate that polyamines likely also play a role in the increased airways responsiveness that occurs in asthma.
Effects of polyamines on airways responsiveness

Although the specific mechanisms by which polyamines increase airways responsiveness were not investigated in this study, some interesting effects that may be involved are the activation of mast cells, promotion of oxidative stress, and effects on the NOS pathway. Polyamines have been shown to play a role in the induction of Th2 responsive genes, mast cell activation and the release of histamine. Alternatively activated macrophages play an important role in the murine Th2 response in helminth infection and allergic inflammation models. Recently, increased polyamine production through the arginase 1/ODC pathway have been shown to participate in the induction of E-cadherin important for inflammatory cell interactions [343]. Kurosawa et al. demonstrated that the release of histamine by mast cells in vitro was rapidly initiated by 10 mM spermine and that 0.1 mM spermine was capable of enhancing the anti-IgE-induced histamine release [221]. Increased levels of polyamines due to upregulated arginase activity may also increase the catabolism of polyamines. However, the conversion of higher order polyamines back to spermidine or putrescine generates hydrogen peroxide and 3-acetoaminopropanal, which contribute to oxidative stress and cellular damage [204]. Oxidative stress and $H_2O_2$ have been shown to induce direct contractions of airway smooth muscle, and to play a role in the pathogenesis of asthma [344, 345]. Thus, increased histamine release and oxidative stress are two plausible mechanisms by which polyamines may contribute to increased airways responsiveness.

Polyamines have also been reported to affect the production of NO by the NOS isoenzymes in various cell types. Hu et al. examined the inhibitory effects of polyamines on the conversion of $[^3]H$L-arginine to $[^3]H$L-citrulline in rat cerebellar lysates containing NOS1 [226]. The authors determined the following order of potency; spermine > spermidine > putrescine,
with an IC<sub>50</sub> value of 56 µM for spermine [226]. The authors demonstrated that changing concentrations of L-arginine affected the ability of polyamines to inhibit NOS1, and thus suggest a competitive mechanism [226]. Blachier et al. employed a similar design, but examined NOS2 inhibition in lysates from endotoxemic rat liver and employed L-[Guanido-14C]arginine as substrate [227]. The authors also found that spermine was the strongest inhibitor, and reported an IC<sub>50</sub> of 500 µM [227]. The single log-order difference in IC<sub>50</sub> between the Hu and Blanchier reports may be due to different susceptibilities between the isozymes to inhibition by spermine, or could be confounded because of the use of cell lysates, rather than pure enzyme preparations. Thus polyamines, particularly spermine, may promote increased airways responsiveness through the inhibition of nitric oxide production. The inhibition of NOS by spermine is consistent with our finding that nitrate/nitrite concentrations in lung homogenates were normalized in the mice with allergic airways inflammation that were treated with DFMO.

One alternative explanation for this effect could be the inhibition of arginase by DFMO treatment, resulting in increased bioavailability of L-arginine for the NOS pathway and increased nitrate/nitrite accumulation in these tissues. However, the K<sub>i</sub> of DFMO for reversible inhibition of arginase is several orders of magnitude greater than the K<sub>i</sub> for the irreversible inhibition of ODC [346, 347].

5.6 Conclusions

This study demonstrates that the polyamine, spermine, increases airways responsiveness to methacholine. Pharmacologic blockade of polyamine synthesis through ODC, concurrent with OVA challenge, attenuates airways hyperresponsiveness in this murine model of allergic airways inflammation. These findings further support that imbalances in L-arginine metabolism down-stream of arginase are important in asthma.
Chapter 6:

Augmentation of arginase 1 expression by exposure to air pollution exacerbates the airways responsiveness in murine models of asthma

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Published: *Respiratory Research*. 2011, 12:19.

Contributions of Authors:
M.L.N. performed the animal modeling, protein expression, arginase activity testing, immunohistochemistry analysis and drafted the manuscript. H.A. performed the macrophage analysis. N.K. performed the differential cell counts. B.U. participated in the design of the exposure system and methodology. H.G. participated in the conception and design of the study, and critical revision of the manuscript for important intellectual content. F.S. participated in the conception and design of the study, and critical revision of the manuscript for important intellectual content. J.A.S. participated in the conception and design of the study, and critical revision of the manuscript for important intellectual content.
Abstract

Background: Arginase overexpression contributes to airways hyperresponsiveness (AHR) in asthma. Arginase expression is further augmented in cigarette smoking asthmatics, suggesting that it may be upregulated by environmental pollution. Thus, we hypothesize that arginase contributes to the exacerbation of respiratory symptoms following exposure to air pollution, and that pharmacologic inhibition of arginase would abrogate the pollution-induced AHR.

Methods: To investigate the role of arginase in the air pollution-induced exacerbation of airways responsiveness, we employed two murine models of allergic airways inflammation. Mice were sensitized to ovalbumin (OVA) and challenged with nebulized PBS (OVA/PBS) or OVA (OVA/OVA) for three consecutive days (sub-acute model) or 12 weeks (chronic model), which exhibit inflammatory cell influx and remodeling/AHR, respectively. Twenty-four hours after the final challenge, mice were exposed to concentrated ambient fine particles plus ozone (CAP+O₃), or HEPA-filtered air (FA), for 4 hours. After the CAP+O₃ exposures, mice underwent tracheal cannulation and were treated with an aerosolized arginase inhibitor (S-boronoethyl-L-cysteine; BEC) or vehicle, immediately before determination of respiratory function and methacholine-responsiveness using the flexiVent®. Lungs were then collected for comparison of arginase activity, protein expression, and immunohistochemical localization.

Results: Compared to FA, arginase activity was significantly augmented in the lungs of CAP+O₃-exposed OVA/OVA mice in both the sub-acute and chronic models. Western blotting and immunohistochemical staining revealed that the increased activity was due to arginase 1 expression in the area surrounding the airways in both models. Arginase inhibition significantly reduced the CAP+O₃-induced increase in AHR in both models.

Conclusions: This study demonstrates that arginase is upregulated following environmental exposures in murine models of asthma, and contributes to the pollution-induced exacerbation of
airways responsiveness. Thus arginase may be a therapeutic target to protect susceptible populations against the adverse health effects of air pollution, such as fine particles and ozone, which are two of the major contributors to smog.
6.2 Introduction

Epidemiological studies have described a relationship between ambient levels of air pollution, and respiratory admissions to hospitals [292, 348]. It has become increasingly imperative to determine the biological effects of urban air pollutants, as they pose a serious risk to public health and continue to present an enormous and increasing health and economic burden [259, 349]. Investigations of the health impact of air pollution using controlled human exposures have demonstrated acute cardiopulmonary effects in both healthy subjects and asthmatics [266-268]. Fine particulate matter, with an aerodynamic diameter of less than 2.5 µm, has been specifically associated with increased mortality, pulmonary inflammation and oxidative stress [261, 350, 351]. Ozone (O₃) exposure has also been associated with asthma-related hospital visits [352]. Fine particulate matter and O₃ typically occur together in urban settings [268]. Therefore, it is important to understand the combined effects of these criteria air pollutants on cardiopulmonary disease. In particular, the role of these pollutants in asthma exacerbations remains to be fully understood.

Studies of gene-environment interactions have focused on the role of oxidative stress-responsive genes and air pollution exposures in asthma [353, 354]. However, the mechanism(s) linking exposure to air pollution and asthma exacerbation remains unclear. The metabolism of L-arginine plays an important homeostatic role in the airways, through synthesis of the bronchodilating molecule, nitric oxide (NO), from L-arginine, by the nitric oxide synthase (NOS) isozymes [100]. The arginase isozymes (arginases 1 and 2), convert L-arginine into L-ornithine and urea, and thus compete with the NOS isozymes for substrate [321]. We and others have shown that arginase expression is upregulated in human asthma [76, 322, 340] and that the arginase isozymes play a functional role in the airways hyperresponsiveness (AHR) in animal models of asthma, using ovalbumin (OVA) [76, 191, 334, 340], Aspergillus fumigatus [76], 106
trimellitic anhydride exposure [334], and more recently house dust mite [71]. We have previously demonstrated that the AHR in a chronic murine model of allergic airways inflammation to OVA is due to arginase 1 overexpression [340]. Furthermore, single nucleotide polymorphisms of arginase 1 have been specifically associated with responsiveness to bronchodilators, and L-arginine bioavailability can impact airflow in asthma [171, 185].

The arginase pathway has not previously been examined as a potential mechanism underlying the air pollution-induced exacerbation of asthma symptoms. However, arginase has been shown to be further upregulated in smoking asthmatics who are regularly and voluntarily exposed to high levels of particulate matter [168]. Furthermore, there is evidence to support uncoupling of the endothelial NOS in the vasculature following exposure to diesel exhaust [309], and dysfunction of endothelial-dependent vasorelaxation following exposure to second-hand tobacco smoke [310], likely as a consequence of a reduction in the bioavailability of L-arginine or tetrahydrobiopterin for the NOS pathway. Thus, it is plausible that dysregulation of L-arginine metabolism as a consequence of air pollution-induced upregulation of pulmonary arginase could contribute to the exacerbation of respiratory symptoms in susceptible asthmatics.

We tested the hypothesis that arginase expression is augmented in response to exposures to environmental air pollutants, using two independent murine models of allergic airways inflammation; sub-acute and chronic models that mimic the inflammatory response and airways remodeling/AHR, respectively [44, 333, 355]. We demonstrate further upregulation of arginase following exposure to air pollution and attenuation of the pollution-induced AHR following treatment with an arginase inhibitor in both murine models of allergic airways inflammation.
6.3 Materials and Methods

Sub-acute and chronic models of allergic airways inflammation

All protocols were approved by the University of Toronto Faculty Advisory Committee on Animal Services, and were conducted in accordance with the guidelines of the Canadian Council on Animal Care, ensuring that the animals were treated humanely. To investigate the role of arginase in the exacerbation of airways responsiveness induced by air pollution exposure, we utilized two murine models of allergic airways inflammation: the subacute (16-day) and chronic (12-week) OVA-sensitization and -challenge models, as described in section 3.2.1m which represent short-term allergic inflammatory changes and remodeling/hyperresponsiveness of the airways, respectively [333, 355]. Twenty-four hours after the final OVA or PBS challenge, mice were exposed to concentrated ambient particles plus ozone (CAP+O₃) or HEPA-filtered lab air (FA), as described below, and depicted in Figure 17.

Air pollution exposures

Combined exposures to CAP and O₃ were employed in this study. For controlled exposures to concentrated ambient fine particulate matter, we used the Harvard Ambient Particle Concentrator [356], which is a high-flow (5000 L/min) three-stage virtual impactor system that is part of the Southern Ontario Centre for Atmospheric Aerosol Research at the Gage Occupational and Environmental Health Unit. In this system, ambient air is drawn in, and real-world particles with an aerodynamic diameter 0.1-2.5 μm are concentrated approximately 40-fold (range: 196-954 μg/m³). O₃ was produced by an arc generator using medical-grade oxygen and was introduced into the transition plenum between the second and third stages of the concentrator. CAP and O₃ levels (>175 μg/m³ and 2 ppm, respectively) were selected based
upon previous inhalation exposure studies in rodents [357-359]. Mice were exposed to CAP+O₃ or FA for 4 hours at a flow rate of 2 L/min (Figure 17) using a modified inExpose nose-only inhalation system (Scireq Inc., Montréal, PQ) within a Plexiglas chamber. The O₃ levels achieved using this system were monitored on the outflow from the chamber, using a Dasibi Model 1008RS ozone analyzer (Dasibi Environmental Corp, Glendale CA), and particle levels were determined gravimetrically (Table 5). In a subset of exposures, the constituents of the CAP were measured and the levels of major constituents (i.e., organic and elemental carbon, NO₃⁻, SO₄²⁻, and NH₄⁺) were found to be consistent with our previous analyses of PM₂.₅ in Toronto [360] (data not shown). As our nose-only exposure system allows for the simultaneous exposure of 6 mice, CAP+O₃ and FA exposures were conducted on 3 OVA/OVA mice and 3 OVA/PBS controls at a time, to ensure comparable exposures between groups. Preliminary experiments indicated that the increase in methacholine responsiveness following exposure to CAP+O₃ was greater than that to either CAP or O₃ alone (data not shown).
Figure 17 A)

**Sub-Acute model**

*Sensitization*
i.p. OVA

**Challenge**
Nebulized OVA

Day 0 7 14 17

**Chronic model**

*Sensitization*
i.p. OVA

**Challenge**
Nebulized OVA

Week 0 1 2 4 6 8 10 12

B)

Exposure
FA vs. CAP+O₃

Baseline
PFTs
Nebulized
BEC or Saline

MCh dose-
response

End-Points
Histology
BAL
Enzyme
Western Blot

4 hours 15 minutes 45 minutes

**Figure 17: Experimental design and time-course for CAP+O₃ exposure studies**

A) Schemas of the sensitization and challenge regimens of the sub-acute and chronic murine models of allergic airways inflammation. B) Experimental design and time-course of the pollution exposure day.

**Table 5: CAP and ozone exposure levels for the sub-acute and chronic models**

<table>
<thead>
<tr>
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<th>Sub-acute</th>
<th>Chronic</th>
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<tbody>
<tr>
<td>CAP (µg/m³)</td>
<td>553 ± 79</td>
<td>456 ± 44</td>
</tr>
<tr>
<td>Ozone (ppm)</td>
<td>1.80 ± 0.07</td>
<td>1.79 ± 0.04</td>
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* Values represent the mean±standard error of n = 8-11 exposures.
† There were no significant differences between the exposure levels for the sub-acute and chronic model mice.
Pulmonary Function Testing and Arginase Inhibition

Following the CAP+O₃ or HEPA FA exposures, mice were anesthetized for measurement of in vivo airways responsiveness, as described in section 3.2.1. The arginase inhibitor, BEC (40 µg/g body weight) or PBS vehicle were nebulized directly into the airways after establishment of baseline resistance parameters, and allowed to equilibrate for 15 minutes prior to pulmonary function testing, in randomly selected mice from each model. We have previously found this dose to be effective in inhibiting arginase in acute and chronic murine models of asthma [339, 340]. Following pulmonary function testing, bronchoalveolar lavage (BAL) was performed in a subset of mice, for assessment of inflammation and 8-isoprostane as a marker of oxidative stress. All remaining lungs were harvested for protein analysis or immunohistochemical staining.

Arginase activity and isozyme expression

Total arginase activity testing and Western blotting for arginases 1 and 2 were performed as described in section 3.1.1 and 3.1.2. Semi-quantitative assessment of the Western blots was conducted using a Bio-Rad Fluor-S MultiImager with the Bio-Rad Quantity One 4.3.0 software package (Bio-Rad Laboratories, Hercules, CA). Densitometry was performed using GelEval v1.22 (Frog-Dance Software, Dundee UK).

Inflammation and assessment of immunohistochemical localization of arginase 1

Differential cell counts and immunohistochemical staining of BAL cells and histological sections were performed as in section 3.1.3. For immunohistochemical counts of arginase 1-positive macrophages, macrophages were identified based on size and morphology using a hematoxylin counterstain.
**Oxidative Stress Marker**

As a marker of oxidative stress, 8-isoprostane levels (8-iso-prostaglandin F2α) were measured in BAL fluid using an enzyme immunoassay kit (8-Isoprostane EIA Kit. Item No. 516351, Cayman Chemical Company, Ann Arbor, MI), according to the manufacturer’s instructions and standardized to protein concentration in the BAL, as determined by Bradford assay (BioRad, Hercules, CA).

**Statistics**

Statistical analyses were performed independently on the data from the sub-acute and chronic models. Specific respiratory measurements (R, R_N, G), arginase activity and Western blotting densitometry data were analyzed using one-way ANOVA with Bonferroni’s multiple comparison post-hoc test. BAL differential cell counts were analyzed using the non-parametric Kruskal-Wallis test with Dunn's Multiple Comparison post-hoc test, as some cell types were not observed in the OVA/PBS controls (i.e., eosinophils). Dose-response curves were compared using the F-test, with the null hypothesis that the data from all groups could be modeled by the same curve, and using two-way ANOVA with Bonferroni’s post-hoc test. Correlations between exposure parameters and protein expression were determined by Spearman’s test. P-values <0.05 were considered significant. All statistical analyses were performed using GraphPad Prism 4.0c.

**6.4 Results**

**Arginase activity and expression**

To investigate whether alterations in the arginase pathway were induced by exposure to air pollution we measured total arginase activity in mouse lung homogenates from FA and
CAP+O$_3$ exposed mice. FA-exposed OVA/OVA mice from both models exhibited significantly increased pulmonary arginase activity, relative to the FA-exposed OVA/PBS controls (Figure 18 A & B). In both models, OVA/OVA mice exposed to CAP+O$_3$ exhibited further significant increases in pulmonary arginase activity, compared to the FA-exposed OVA/OVA mice (1.7- and 1.6-fold, respectively). CAP+O$_3$ exposure did not affect total pulmonary arginase activity in the OVA/PBS mice.

We used Western blotting to determine the contribution of the arginase isozymes to the increased total arginase activity. Arginase 1 expression was significantly increased in lungs from FA-exposed OVA/OVA mice in both models, relative to their respective OVA/PBS controls (Figure 18 C & D). Following exposure to CAP+O$_3$, OVA/OVA mice in the sub-acute and chronic models exhibited further significant increases in pulmonary arginase 1 expression, relative to the FA exposed OVA/OVA controls (2.6- and 1.7-fold, respectively). Interestingly, in the sub-acute model, the pulmonary expression of arginase 1 correlated directly with CAP exposure levels at concentrations lower than 565 µg/m$^3$ (Spearman $\rho = 0.622$, $p = 0.013$; linear regression $r^2 = 0.32$; n = 15 mice from 11 independent exposure days) (Figure 18E), suggesting that the CAP-induced increase in expression of arginase 1 was dose-dependent. At exposure levels above 565 µg/m$^3$ we observed no further increase in arginase 1 expression, indicating a plateau in the response at higher levels. As the ozone exposures were fixed at the target concentration of 2 ppm, there was no correlation with protein expression. While pulmonary arginase 2 protein expression was increased significantly in the sub-acute model OVA/OVA mice under FA conditions, it was not further augmented by CAP+O$_3$ exposure. No significant increases in arginase 2 protein expression were observed in the chronic model mice, regardless of whether they were exposed to FA or CAP+O$_3$. 
Figure 18: Arginase activity & expression in CAP+O\textsubscript{3} and FA exposed mice

Total arginase activity in OVA/PBS (open) and OVA/OVA (filled) FA and CAP+O\textsubscript{3} exposed mice in the sub-acute (A) and chronic (B) models. Quantification of arginase 1 and actin loading controls in the sub-acute (C) and chronic (D) models (*P<0.05, **P<0.01, ***P<0.001). E) Correlation between levels of arginase 1 expression in the OVA/OVA mice in the sub-acute model and CAP exposure (Spearman ρ = 0.6219; p = 0.013, n = 11 independent exposure dates).
Localization of increased arginase 1 expression

To determine which cell types were responsible for the augmented arginase 1 expression following exposure to CAP+O₃, we investigated BAL and lung tissues, using immunohistochemical staining. We first examined the differential cell counts of the BAL samples from the sub-acute model. While there was an overall increase in the numbers of inflammatory cells in the OVA/OVA compared to OVA/PBS mice, there were no significant alterations in the differential cell counts in the CAP+O₃ compared with the FA exposure groups (Figure 19A).

As arginase 1 is known to be expressed in alternatively-activated macrophages [35], we investigated arginase 1 expression in BAL cells using immunohistochemistry. We did not observe any change in the proportion of arginase 1-positive macrophages in the immunostained BAL slides from the CAP+O₃-exposed OVA/PBS or OVA/OVA mice compared to their respective FA controls (Figure 19B). Thus, the increase in arginase 1 expression in the CAP+O₃-exposed mice was not due to an increased proportion of alternatively-activated macrophages infiltrating the lung.

We then investigated the expression of arginase 1 in airways in lung sections using immunohistochemical staining (Figure 20). Although expression was not quantifiable by these methods, staining was localized to the peribronchiolar region in both the sub-acute and chronic models.
Figure 19: Bronchoalveolar lavage differential cell counts and macrophage expression of arginase 1

A) Differential cell counts from BAL samples in the sub-acute model OVA/PBS (open) and OVA/OVA (filled) mice exposed to FA or CAP+O₃ (*P<0.05). B) Images of arginase 1 immunostained slides of BAL samples and quantification of the percentage of positive macrophages (400x magnification; bar = 100µm; brown colour indicates positivity; representative images of n = 5-6/group; *P<0.05, **P<0.01).
Figure 20: Immunohistochemical staining of arginase 1 in CAP+O$_3$ and FA exposed mice

Arginase 1 immunostained lung tissues from OVA/PBS, OVA/OVA mice from the sub-acute (A) and chronic (B) models exposed to filtered air or CAP+O$_3$ (200x magnification; bar = 100 µm; representative images of n = 4-5 per group, brown colour indicates positivity, arrows highlight positive areas, key positive areas inset).
**Effects of air pollution on methacholine responsiveness**

After demonstrating augmentation of arginase 1 protein expression in OVA/OVA mice exposed to CAP+O₃, we initially examined the functional effects of air pollution exposure on methacholine responsiveness *in vivo* in the sub-acute model. Total lung resistance (R) to methacholine was not significantly augmented in the OVA/OVA mice compared to OVA/PBS controls under FA conditions (**Figure 21 A & B**), making this model suitable to investigate the development of AHR induced specifically by CAP+O₃ exposure. Exposure to CAP+O₃ did not evoke any significant change in the methacholine responsiveness of the total lung in OVA/PBS mice (**Figure 21A**). However, significant augmentation of the methacholine dose-response curve was observed in the CAP+O₃-exposed OVA/OVA mice, with a two-fold increase in the maximum resistance to methacholine, compared with the FA-exposed OVA/OVA controls (F-test and 2-way ANOVA, *P*<0.001, **Figure 21B & C**). In the chronic model, FA-exposed OVA/OVA mice exhibited a moderate increase in methacholine responsiveness compared with the OVA/PBS, FA-exposed controls (*P* = 0.0418), which was further augmented by 1.6-fold in CAP+O₃ exposed OVA/OVA mice (*P* = 0.0071)(**Figure 21D**).

**Arginase inhibition abrogates the CAP+O₃-induced AHR**

After determining that exposure to CAP+O₃ resulted in exacerbation of methacholine responsiveness in mice with pre-existing allergic airways inflammation, paralleling the up-regulation of pulmonary arginase 1, we administered the arginase inhibitor, BEC, or vehicle control (PBS) to randomly selected sub-groups of mice following the CAP+O₃ exposures in both the sub-acute and chronic models. The maximum total respiratory resistance (R$_{\text{Max}}$) was significantly increased in OVA/OVA mice vs. OVA/PBS from both models after the CAP+O₃ exposure (**Figure 21C & D**). After treatment with BEC, the R$_{\text{Max}}$ values in the CAP+O₃-
exposed OVA/OVA mice was significantly attenuated compared with the PBS-treated controls (i.e., CAP+O₃-exposed OVA/OVA mice), and were indistinguishable from the Rₘₐₓ for the OVA/PBS controls. Thus, treatment with the arginase inhibitor completely reversed the CAP+O₃-induced exacerbation of symptoms in the OVA/OVA mice.

To confirm that the exacerbation of symptoms was due to effects on the airways, we assessed the contribution of airways resistance (Rₙ Max) and peripheral tissue damping (Gₘₐₓ) to the total response of the lung. In the sub-acute model, Rₙ Max was not altered significantly following CAP+O₃ exposure, or by BEC treatment (Figure 22A). Interestingly, Gₘₐₓ was increased significantly following exposure to CAP+O₃ in the sub-acute OVA/OVA mice, and was attenuated to control levels by arginase inhibition with BEC (Figure 22C). Meanwhile, in the chronic model OVA/OVA mice, Rₙ Max was significantly augmented by CAP+O₃, and significantly reversed by treatment with BEC (Figure 22B). A significant increase in Gₘₐₓ was also observed in the chronic model OVA/OVA mice following CAP+O₃ exposure, however this was not attenuated by BEC treatment (Figure 22D). Exposure to CAP+O₃ or administration of BEC did not affect any of the responsiveness parameters in the OVA/PBS mice in either model (Figures 21 and 22).
Figure 21: Functional effects of CAP+O\textsubscript{3} exposure on airways responsiveness to methacholine and attenuation by arginase inhibition

Dose-response relationships for the increase in total lung resistance (R) to methacholine in OVA/PBS (A) and OVA/OVA (B) mice from the sub-acute model exposed to FA or CAP+O\textsubscript{3}. Effects of arginase inhibitor (BEC) vs. vehicle control (PBS) on maximum total lung resistance (R\textsubscript{Max}) in OVA/PBS (open) and OVA/OVA (filled) mice following CAP+O\textsubscript{3} exposures in the sub-acute (C) and chronic (D) models (*P<0.05, ** P<0.01, *** P<0.001; n = 9-14/group).
Figure 22: Arginase inhibition in CAP+O$_3$ exposed mice

Effect of treatment with arginase inhibitor (BEC) vs. vehicle control (PBS) on central airways Newtonian resistance ($R_{NMax}$; A and B) and peripheral tissue damping ($G_{Max}$; C and D) in OVA/PBS (open) and OVA/OVA (filled) mice from the sub-acute (A and C) and chronic (B and D) models following CAP+O$_3$ exposures (*P<0.05, **P<0.01, n = 9-14/group).
Oxidative Stress Due to CAP+O₃ Exposures

To assess the level of oxidative stress induced by exposure to CAP+O₃, we determined levels of 8-prostaglandin F₂α (8-isoprostane) in BAL supernatants from both the sub-acute and chronic models (Table 6). In the subacute model, the levels of 8-isoprostane were 7.9 ± 3.6 and 9.7 ± 4.1 pg/mg of BAL protein in the OVA/PBS and OVA/OVA FA groups, respectively (P = n.s.). OVA/PBS and OVA/OVA mice exposed to CAP+O₃ exhibited 5.4- and 7.0-fold increases compared to the FA groups (P < 0.05 to FA). In the chronic model, BAL levels of 8-isoprostane in the OVA/OVA FA-exposed mice were 1.9-fold greater than those in the OVA/PBS FA-exposed mice (P = 0.017). OVA/PBS and OVA/OVA mice exposed to CAP+O₃ exhibited 3.5- and 2.3-fold increases in 8-isoprostane levels compared to their respective FA controls (P < 0.05). There was no significant difference in BAL 8-isoprostane levels between the OVA/PBS and OVA/OVA CAP+O₃-exposed groups.

Table 6: 8-isoprostane levels in BAL samples from the sub-acute and chronic OVA-model mice exposed to filtered air or CAP+O₃

<table>
<thead>
<tr>
<th></th>
<th>Filtered Air ⁴</th>
<th>CAP+O₃</th>
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<tr>
<td></td>
<td>OVA/PBS</td>
<td>OVA/OVA</td>
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<tr>
<td>Sub-acute</td>
<td>7.9 ± 3.6</td>
<td>9.7 ± 4.1</td>
</tr>
<tr>
<td>Chronic</td>
<td>19.0 ± 3.3</td>
<td>36.8 ± 5.0*</td>
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⁴ Values are expressed as pg 8-isoprostane/mg BAL protein and represent the mean ± standard error of n = 5-6 samples.

* P < 0.05 to OVA/PBS, same exposure, # P < 0.05 to Filtered air, same treatment.
Discussion

Summary

This study demonstrated that the increased arginase activity in the lungs of mice from both sub-acute and chronic models of allergic airways inflammation was further augmented by exposure to CAP+O₃, and that this was primarily driven by arginase 1. We also determined that the up-regulation of arginase 1 in the lung was not related to increased influx of macrophages. Finally, we demonstrated that induction of AHR by CAP+O₃ was specific to the mice with pre-existing allergic airways inflammation, and that local delivery of an arginase inhibitor after exposure, significantly reduced the CAP+O₃-induced AHR in both models; thus providing further support for the potential of targeting this pathway therapeutically in asthma.

Arginase induction by CAP+O₃

There is increasing evidence to support the role of arginase in the pathophysiology of asthma, and that further up-regulation of arginase likely results in worsening of asthma symptoms [76, 189, 321, 322, 340]. The sub-acute model mice in the present study, challenged with ovalbumin daily for three days, exhibited significantly lower arginase 1 expression and airways responsiveness, compared to the acute OVA-model mice reported in our previous study, in which we employed seven consecutive daily challenges [340]. Thus, it is likely that increased arginase 1 expression is directly associated with the increasing airways responsiveness in these murine models. We speculate that there is a critical threshold of arginase induction, at which the increased arginase activity exhibits physiological effects. Air pollution is known to contribute to asthma exacerbations [361-363]. Increased levels of particulate matter and ozone have been associated with increased oxidative stress and decreased pulmonary function in children with asthma [364]. Increased arginase protein expression has been observed in smokers
with asthma [168], but it is not known whether arginase plays a role in air pollution-induced exacerbations of respiratory symptoms. In this study we demonstrated further augmentation of arginase activity and arginase 1 expression in the airways of our OVA-sensitized and – challenged mice following exposure to CAP+O₃.

Arginase 1 protein expression in blood serum has recently been associated with markers of oxidative stress in a healthy human population [365], and augmented arginase activity correlates with indices of oxidative stress (i.e., malondialdehyde and protein carbonylation levels) in platelets and plasma from patients with chronic obstructive pulmonary disease exposed to wood smoke [366]. Arginase 1 has also been reported to be induced following one hour of ex vivo exposure to hydrogen peroxide-induced oxidative stress [315]. Thengchaisri et al. also demonstrated changes in smooth muscle function in response to increased oxidative stress, and demonstrated that arginase inhibition restored the hydrogen peroxide-impaired vasodilation [315]. However, in our model we only observed an increase in arginase 1 in mice that were previously sensitized and challenged with ovalbumin. This may be due to differences in experimental procedures, as Thengchaisri et al. exposed isolated coronary arterioles to hydrogen peroxide ex vivo [315], while we examined the whole lung following in vivo acute CAP+O₃ exposure. Additionally, while CAP+O₃ are known to induce oxidative stress under many conditions [367, 368], we cannot confirm an association between arginase 1 augmentation and oxidative stress in our study, as we did not directly examine markers of oxidative stress in our mice. Alternatively, toll-like receptors, the hallmark regulators of the innate immune response to bacterial, viral, and parasitic components, have recently been shown to upregulate arginase 1 via an alternative promoter region [369]. It is highly likely that some of these biologic exposures are relevant to our CAP exposures. Further work is necessary to elucidate the
mechanisms underlying the upregulation of arginase 1 in response to environmental stimuli in the presence of an inflammatory response.

While we did not investigate the effects of long-term pollution exposure in these models, our findings raise the interesting question of whether continued exposures would result in chronic upregulation of arginase, and lead to remodeling of the airways. Increased airway wall remodeling has been observed by Dai et al., following exposure of rat tracheal explants to Ottawa urban air particles [370]. Diesel exhaust particles can also potentiate airways remodeling in a house dust mite murine model of allergic airways inflammation [71] that is known to exhibit augmented arginase expression [71]. Thus, arginase may be induced as part of the host response to cell damage by air pollution, as it is known to be involved in cell growth and wound healing [371]. As the metabolic pathways downstream of arginase are related to cellular proliferation and collagen biosynthesis, it is likely that augmented arginase expression contributes to airways remodeling in asthma [321]. The role of L-arginine metabolism in the effects of chronic air pollution exposure, and the effects of concomitant inhibition of arginase represent future avenues for investigation.

**Functional improvement of airways hyperresponsiveness with arginase inhibition**

Although the arginase pathway has been shown to be functionally involved in the development of AHR *in vivo* following allergen challenges with ovalbumin [71, 330, 334, 340] and house dust mite [71], it was not clear whether this pathway would be functionally important in the exacerbation of AHR induced by air pollution. In this investigation we used two mouse models of asthma, which exhibit the inflammatory changes, remodeling and mild AHR as symptoms of allergic airways disease; while the OVA/OVA mice in the sub-acute model did not exhibit AHR to methacholine, those in the chronic model did exhibit moderate AHR, which was consistent with our previous report [340]. We further demonstrated exacerbation of the AHR in
the chronic model and the development of AHR in the sub-acute model after exposure to air pollution. In both murine models we demonstrated an increase in the maximum total lung resistance following air pollution exposure, and that inhibition of arginase, post-exposure, blocked this effect. We also examined the contribution of the airways and peripheral tissue to the net response. Using a sub-acute model of allergic airways inflammation, Tomioka et al. previously demonstrated that the effects of allergic inflammation in this model were more pronounced in the lung periphery and thus affected peripheral lung mechanics more strongly than conducting airways mechanics [318]. However, it was not known how the added challenge of a CAP+O$_3$ exposure would affect peripheral lung mechanics in the sub-acute model. Our data suggest that CAP+O$_3$ exposure specifically aggravated peripheral lung responsiveness to methacholine in the OVA/OVA mice in this sub-acute model. Arginase inhibition with BEC completely abrogated the augmented G$_{Max}$, strongly suggesting a role for arginase in the functional exacerbation of peripheral AHR by air pollution in the sub-acute model.

The particles we employed were derived from the ambient air in Toronto, Ontario. Particles within the 0.1-2.5 µm size range were concentrated, representing real-world fine particles. We examined the effects of concentrated ambient particles and ozone, as these pollutants have been shown to be associated with increased asthma exacerbations in humans, and because concomitant exposures have been shown to increase respiratory resistance in mice, thus allowing our study to examine the biological mechanisms responsible for these effects [292, 307, 348, 352]. Murine models present several limitations, as no animal model exhibits all of the clinical features of human asthma [85, 330, 355, 372]. However, our sub-acute model exhibits airways inflammation and our chronic model recapitulates airways remodeling and mild hyperresponsiveness, all of which are important features of human asthma [44, 50], and mice in both models exhibited an even greater degree of airways hyperresponsiveness following air
pollution exposures. Asthmatics as a group represent a potential susceptible population that would be more significantly affected by air pollution than those who do not have underlying respiratory disease. Our results support this idea, as we did not observe increased airways responsiveness in the control mice, but demonstrated an increase in mice with pre-existing allergic airways inflammation.

While the doses of particulate matter and ozone employed in this study are high, similar doses have been shown to be useful for studying acute mechanisms of air pollution induced AHR in the setting of allergic airways inflammation and healthy controls [357-359]. Exposure to high levels of ozone can induce pulmonary edema and lung injury [359, 373]. While OVA/PBS control mice that were exposed concurrent with the OVA/OVA mice in our study did not exhibit any alterations in inflammatory cell counts or profiles in bronchoalveolar lavage samples obtained immediately after CAP+O\textsubscript{3} exposures, the possibility remains that our findings could be due solely to the high-level exposures. Interestingly, the OVA/PBS mice exposed to CAP+O\textsubscript{3} exhibited a slight increase in methacholine responsiveness compared with FA. Similarly, the OVA/OVA mice exposed to CAP+O\textsubscript{3} that were treated with BEC exhibited a similar increase of methacholine responsiveness, suggesting that pathways unrelated to arginase induction, such as edema, could contribute to this non-significant increase. Thus, further investigations will be necessary to determine whether the pathways activated by acute high-level exposures are similar to those activated after chronic exposures to lower levels of particulate matter and ozone.

The chronic model offers the ability to study exacerbation of established disease, in a model that recapitulates more of the features of chronic human asthma, including remodeling, collagen deposition, smooth muscle hypertrophy, and mild AHR [44, 333, 337, 340, 374]. Furthermore, we have previously shown that the chronic model exhibits alterations in the profile
of L-arginine-related protein expression that are most similar to those of human asthma [340]. In the chronic model OVA/OVA mice, we found that both central airways resistance and peripheral tissue damping contributed to the pulmonary response to CAP+O₃ exposure. However only central airways resistance was attenuated by BEC, suggesting that arginase-independent effects, such as lung edema, may also be induced by exposure to air pollution and contribute to AHR in the lung periphery. We observed increased arginase 1 staining in the central airways in OVA/OVA mice exposed to CAP+O₃, suggesting a functional role for arginase in the airways narrowing following CAP+O₃ exposure in the chronic model. While we observed a correlation between CAP exposures and arginase induction in OVA-sensitized and –challenged mice, future studies should assess the dose-effects of air pollutants and corroborate the findings with additional model allergens.

6.6 Conclusions

Arginase activity and arginase 1 expression are upregulated following environmental exposures in both sub-acute and chronic murine models of allergic asthma. Pollution-induced AHR is attenuated by arginase inhibition in both models. Thus, induction of arginase 1 is likely a key step in the short-term response to air pollution and inhibition may represent a therapeutic target to treat or prevent environmental pollution-induced exacerbations of allergic airways disease.
Chapter 7: General Discussion

Summary

This thesis focused on the chronic respiratory disease, asthma, an inflammatory disorder of the airways that affects many Canadians, and the negative effects of air pollution. Imbalances in the metabolism of the amino acid, L-arginine, between the NOS and arginase pathways have been implicated in the pathogenesis of asthma. In this thesis, the effects of increased arginase activity, polyamine downstream metabolites and environmental air pollution on airways responsiveness were investigated.

First, the expression profiles of L-arginine related proteins were determined in human asthmatic lung samples and two murine models of allergic airways inflammation. It was demonstrated that arginase 1 is upregulated in human asthma. Similarly, the expression of arginase 1 was the largest significant change observed in both the acute and chronic murine models of ovalbumin-induced airway inflammation. Arginase inhibition in vivo was shown to reduce airways hyperresponsiveness in both models, in the absence and presence of airways remodeling, respectively.

Metabolites downstream of arginase, specifically the polyamines, were then examined. Increased levels of polyamines have been detected in human asthmatics, and demonstrated in murine models [76, 222]. In Chapter 5, spermine was shown to increase methacholine responsiveness in naïve mice and mice with pre-existing allergic airways inflammation. Treatment with an ornithine decarboxylase inhibitor to block the synthesis of polyamines attenuated the hyperresponsiveness to methacholine in an acute murine model. Thus, arginase activity and downstream metabolites of this pathway contribute to airways hyperresponsiveness (AHR) in asthma.
Finally, the effects of the additional insult of environmental air pollution on airways responsiveness was examined in murine models of allergic airways inflammation. The role of arginase in the exacerbation of asthma by air pollution was explored in mice exposed to concentrated ambient fine particles plus ozone (CAP+O\textsubscript{3}). Arginase 1 was found to be significantly upregulated in allergic mice exposed to CAP+O\textsubscript{3}, compared to filtered air. Inhibition of arginase \textit{in vivo} attenuated the CAP+O\textsubscript{3}-induced increase in airways responsiveness to methacholine. Therefore, the work presented in this thesis supports the hypothesis that arginase and downstream polyamine metabolites are functionally involved in airways responsiveness in animal models and in the exacerbation of airways responsiveness induced by air pollution.

\textit{Arginase and asthma}

Dysregulation of L-arginine metabolism has been shown to be important in asthma and in other respiratory diseases [218]. The study in Chapter 4 demonstrated that arginase 1 was significantly upregulated in lungs from asthma patients [340]. As samples of human lung tissue are relatively difficult to obtain, there have been only a few studies, with limited sample sizes, that have employed immunostaining [76, 168], and no other studies that have employed Western blotting to quantify arginase 1 expression in the human lung. The results presented in Chapter 4 suggest a role for upregulation of arginase 1 in the dysregulation of L-arginine metabolism in asthma, demonstrated by others in more readily available samples, such as blood serum [185, 322].

Since the data in Chapter 4 was published, important research has continued in human studies of arginase in asthma, including further genetic associations between arginase, asthma severity, and bronchodilator response [375]. Vonk \textit{et al.} recently employed a longitudinal cohort
of 200 asthma patients and demonstrated that polymorphisms in the arginase 2 gene were associated with asthma and a more severe airways obstruction phenotype [375]. Arginase 1 and 2 polymorphisms were also associated with increased airway hyperresponsiveness and reduced β2 agonist bronchodilator response [375]. Additionally, an arginase 1 polymorphism was associated with diminished response to corticosteroid treatment in terms of the prevention of decline in FEV₁ [375]. Thus, there is mounting evidence of a functional role for arginase in human asthma.

**Arginase inhibition and airways responsiveness**

Competition between NOS and arginase are evidenced by the multiple *in vivo* and *ex vivo* studies demonstrating that arginase inhibition reduces airways responsiveness. Recently, Maarsingh *et al.*, demonstrated that the arginase inhibitor, 2(S)-amino-6-boronohexanoic acid (ABH), reduced AHR following the early and late allergic response [189]. Decreased responsiveness to methacholine has also been observed following arginase inhibition in murine models [191, 327]. In chapter 4, data was presented demonstrating that arginase inhibition using a treatment based administration protocol attenuated airways responsiveness in acute and chronic murine models of allergic airways inflammation [340]. The efficacy of arginase inhibition using a treatment-based protocol is important, as potential human medications must demonstrate efficacy in people who have already developed symptoms. Additionally, the study described in Chapter 4 demonstrated attenuation of airways hyperresponsiveness in a chronic murine model of allergic airways inflammation [340]. The chronic model is typically used for investigations of airway remodeling [333, 337], and arginase inhibition had not previously been employed in a chronic model. The protein expression profile in this model revealed that arginase 1 was the sole significantly up regulated isozyme, similar to the expression profile of the human
asthma samples [340]. Despite the presence of airways remodeling, arginase inhibition was effective in significantly reducing both central and peripheral airways hyperresponsiveness to methacholine in the chronic model [340]. After the publication of the work in Chapter 4, Maarsingh et al. examined the role of arginase in airway remodeling using a chronic sensitization and challenge model in guinea pigs [376]. The authors treated the guinea pigs with ABH or PBS via inhalation before each allergen or saline-challenge and observed attenuated pulmonary hydroxyproline content, reduced putrescine content, reduced airway eosinophilia and abrogated allergen-induced hyperresponsiveness of tracheal rings [376]. Therefore, arginase is likely important both in the airways hyperresponsiveness of asthma and in chronic airways remodeling.

Subsequent to the publication of the data in Chapter 4, collaborative studies between our group and the laboratory of Dr. David Christianson at the University of Pennsylvania have demonstrated the efficacy of a newly-synthesized 2-aminimidazole amino acid inhibitor of arginase [377]. The inhibitor, 2-(S)-amino-5-(2-aminimidazol-1-yl)pentanoic acid (A1P) was administered in a treatment-based protocol to mice in the acute model of allergic airways inflammation (Figure 23) [377].
Figure 23: Impact of arginase inhibition by A1P in the acute model of allergic airways inflammation.

A) A1P significantly attenuated the maximum total lung resistance (R) evoked by methacholine challenge in OVA/OVA mice, compared to untreated controls. B) A1P also significantly attenuated the central airways responsiveness (R_N) in the same model.

Thus, Figure 23, reproduced with permission from the American Chemical Society, demonstrated that A1P significantly attenuated methacholine responsiveness of the total respiratory system (R_{Max}) and the central airways (R_{N\,Max}) in the acute murine model (Figure 23) [377]. These new data are important, as there are concerns with the potential toxicity of boron-containing inhibitors, such as BEC and ABH. Therefore A1P may be of therapeutic interest, as it employs an aminoimidazole heterocycle, containing only carbon, nitrogen and oxygen, to coordinate with the manganese cofactor in the active site of arginase [377].

This study also adds new information to the collection of studies that have thus far investigated the role of arginase in airways responsiveness by pharmacologic inhibition. Our group recently published a review paper in collaboration with the laboratory of Dr. Herman Meurs at the University of Groningen [339]. In this review paper, reproduced with permission from Bentham Press in Appendix B, we summarized the available studies employing different arginase inhibitors, with different treatment protocols [339]. From our summary of the literature,
we concluded that arginase inhibition attenuates airways hyperresponsiveness across species and in several different models of allergic airways inflammation [339].

**Arginase inhibition and airways inflammation**

To further elucidate the role of arginase in allergic inflammation, recent studies have explored arginase inhibition in animal models, with somewhat controversial results. Ckless et al. have suggested that arginase inhibition enhances allergic inflammation [327]. Niese et al. recently reported no significant effect of arginase inhibition on inflammation [378]. Additionally, three recent studies have shown decreased inflammation upon arginase inhibitor treatment [71, 189, 191].

Ckless et al. demonstrated an increase in semi-quantitative histological inflammatory scores in a murine model of ovalbumin-induced allergic inflammation following oropharyngeal aspiration of the arginase inhibitor (S)-(2-Boronoethyl)-l-cysteine (BEC) [327]. Thus, suggesting that arginase inhibition may lead to increased inflammation in a murine model of asthma. Niese et al. employed a model of chimeric mice with bone marrow derived from arginase 1−/− pups and demonstrated that arginase expressed in inflammatory cells derived from the bone marrow is the primary source of arginase in allergic airways inflammation but is not required [378]. This suggests that arginase inhibition has no effect on inflammation, which is likewise what we have observed (Chapter 4 and 6). However, the data of Niese et al.[378] do not preclude the possibility that arginase expression in structural, epithelial or smooth muscle cells play an important role, and as our own studies [340, 379] have been treatment-based, there may have not been sufficient time to observe either a pro- or anti-inflammatory effect.

Two independent groups employing murine models have now reported anti-inflammatory effects of arginase inhibition. Bratt et al. demonstrated that administration of the
arginase inhibitor nor-NOHA prior to each ovalbumin challenge reduced the total cell count in BAL fluid by approximately 65% [191]. The same group recently demonstrated that increased expression of arginase in isolated airways correlate with levels of lung inflammation [326]. Interestingly, an increased inflammatory response to ovalbumin was observed in NOS2 knockout mice, supporting the anti-inflammatory effects of NO [191]. More recently, Takahashi et al., employed a house dust mite-induced mouse model of asthma and arginase inhibition with nor-NOHA, administered intranasally [71]. The authors similarly observed decreased numbers of eosinophils in BAL fluid as well as decreased goblet cell proliferation in mice that received the inhibitor [71].

Recent studies in guinea pig models of allergen-induced airway inflammation also support the anti-inflammatory potential for arginase inhibition [189]. Maarsingh et al. administered ABH by inhalation 30 minutes before and 8 hours after allergen challenge [189]. The allergen-induced increases in the numbers of BAL cells, including eosinophils, macrophages and total inflammatory cell infiltration were all inhibited by approximately 50% by ABH [189]. The same group also recently reported reduced eosinophilia in a chronic guinea pig model with ABH treatment [376].

Arginase inhibitors may reduce airway inflammation by attenuating the formation of proinflammatory peroxynitrite via restoration of the L-arginine availability to NOS2, thereby preventing the uncoupling of this enzyme [380]. Additionally, arginase inhibition may promote an increase in NO production by cNOS, and reduce NF-kB activation [189, 327].

**Competition between NOS and arginase in asthma**

Although many studies have suggested competition between the NOS and arginase isoenzymes for L-arginine substrate, and arginase inhibition has been demonstrated to reduce airways responsiveness, direct competition between these pathways is difficult to demonstrate in
We recently performed stable isotope infusion studies in the acute murine model of allergic airways inflammation (methods are described in Chapter 3.2.3). We demonstrated that both NOS and arginase activity were elevated in this model (Figure 24). These results demonstrated that it is possible to measure L-arginine metabolism in vivo, and that the in vitro/ex vivo measurements of NOS and arginase activity made previously [340], are reflective of the in vivo activity.

Additionally, the bioavailability of L-arginine in lungs from the acute model mice, calculated as the ratio of L-arginine:L-ornithine, is significantly reduced, by approximately 90% (Figure 25). Finally, we measured L-arginine bioavailability in human lung homogenates, and demonstrated that they also exhibit significantly reduced ratios compared to control lungs (Figure 25). This demonstrated that there is indeed reduced L-arginine bioavailability for synthesis of NO, under conditions of allergic airways inflammation and in human asthma, likely due to increased arginase activity.

**Figure 24: Measurement of in vivo NOS and arginase activity**
Mass spectrometric measurement of NOS (A) and arginase (B) activities in lung samples from the OVA model. (*P<0.05 compared to OVA/PBS controls, n = 6-8).
**Polyamines and asthma**

Endogenous NOS inhibitors, spermidine and spermine, also have the potential to participate in modulating the delicate balance between the NOS and arginase pathways in asthma. While ornithine decarboxylase activity is generally considered to be the rate-limiting step in the synthesis of polyamines, increased production of the substrate L-ornithine by arginase can also promote polyamine synthesis [76]. As arginase is known to be upregulated in asthma, this suggests the potential for increased polyamine production. Indeed, putrescine levels have been shown to be increased in murine and guinea pig models of allergic airways inflammation [76, 376]. Increased polyamine levels have also been demonstrated in humans during spontaneous mild asthma attacks [222]. Serial polyamine measurements in one patient who was admitted to hospital for an asthma exacerbation, revealed an elevation at admission, followed by a decrease to normal levels in parallel with clinical improvement [222]. This time-course demonstrates similarities to serial arginase activity measurements during asthma exacerbations reported by Morris et al. [322].
Interestingly, increased arginase activity and polyamine concentrations have been independently associated with lung diseases such as asthma, cystic fibrosis, pulmonary arterial hypertension and cancer [216-219]. However, the link between increased arginase activity (in these lung diseases) and the increased polyamine concentrations has not been well described. In Chapter 5 increased airways responsiveness following spermine administration was demonstrated in naïve mice, and in mice with preexisting allergic airways inflammation. Additionally, in an acute model of allergic airways inflammation, blockade of polyamine synthesis with DFMO significantly attenuated methacholine responsiveness (Chapter 5). These findings support a role for polyamines in the dysregulation of L-arginine metabolism in asthma.

**Endogenous NOS inhibition and L-arginine metabolism**

There is new evidence suggesting that in addition to the upregulation of arginase and polyamines in asthma, other endogenous NOS inhibitors may also play a role in the dysregulation of L-arginine metabolism. L-arginine can be metabolized by aminoacyl tRNA synthetase (aaRS) into arginine-tRNA and then become incorporated into protein (Figure 26). Protein arginine methyltransferases (PRMTs) methylate L-arginine residues in proteins, and upon their breakdown release monomethylarginine (MMA), asymmetric dimethylarginine (ADMA), and symmetric dimethylarginine (SDMA) (Figure 26) [381]. MMA and ADMA are endogenous inhibitors of NOS and promote uncoupling of the enzyme [382]. SDMA competes with L-arginine transport through the CAT transporters and therefore may reduce L-arginine bioavailability [383, 384]. MMA and ADMA can be degraded to L-citrulline and methylamines by dimethylarginine dimethylaminohydrolases (DDAH) [381]. Thus, a delicate balance exists in L-arginine metabolism with many levels of reciprocal regulation at work.
Figure 26: ADMA pathway

L-arginine is incorporated into protein, through aminoacyl tRNA synthetase (aaRS) and the ribosome (R). L-arginine can be methylated by protein arginine methyltransferases (PRMT). Upon breakdown, monomethylarginine (MMA) asymmetric dimethylarginine (ADMA), and symmetric dimethylarginine (SDMA) are produced.

The inhibition of NOS by ADMA is potentially important in regulating NO production and oxidative stress in the lung. Wells et al. demonstrated that exogenous ADMA administration induces uncoupling of NOS, resulting in the formation of superoxide in lung epithelial cells [385]. The same group subsequently investigated the effects of ADMA administration in vivo, and demonstrated that arginase activity and collagen deposition were increased after two weeks of ADMA infusion [386]. Additionally, they showed increased lung resistance in response to methacholine challenge in ADMA-infused mice [386]. This suggests that ADMA might take part in the reciprocal regulation of NOS and arginase.

Endogenous ADMA production has recently been shown to be upregulated in a murine model of asthma. Ahmad et al. demonstrated increased ADMA content in lungs of allergic mice and increased expression of protein-arginine methyltransferase-2, an ADMA-synthesizing enzyme [387]. Administration of L-arginine reduced ADMA in OVA/OVA mice and attenuated
peroxynitrite production [387]. Thus, ADMA likely participates in the delicate balance between the NOS and arginase pathways, and in the dysregulation of L-arginine metabolism in asthma.

As part of a collaborative metabolic study with Dr. Grasemann at The Hospital for Sick Children, we recently tested the hypothesis that ADMA is important in asthma and contributes functionally to airways hyperreactivity. Using sputum samples obtained from a group of pediatric asthma patients, lung specimens from adult asthmatics and a murine model of allergic airways inflammation, we assessed the alterations of L-arginine, ADMA and SDMA using LC-tandem mass spectrometry. ADMA and SDMA concentrations were increased in the acute murine model (Figure 27 A and B). A trend towards increased ADMA levels was observed in human asthma lung homogenates, and SDMA was significantly increased (Figure 27 C and D).

Exogenous administration of ADMA to naïve mice, at doses consistent with the levels observed in the allergically-inflamed lungs, resulted in augmentation of the airways responsiveness to methacholine (Figure 28). Additionally, ADMA levels in pediatric sputum
significantly and inversely correlated with FeNO, consistent with our hypothesis that ADMA affects airways NOS activity (Spearman ρ value = 0.4943; p = 0.037). These data demonstrate dysregulation of the L-arginine, ADMA and SDMA levels, suggesting that NOS activities are likely affected by ADMA in the lungs from the mouse model and human asthma.

**Figure 28**

![Graph showing effect of ADMA on methacholine responsiveness in naïve mice.](image)

**Figure 28: Effect of ADMA on methacholine responsiveness in naïve mice.**

Respiratory system resistance ($R_{Max}$; A), and central airways resistance ($R_{NMax}$; B) were increased in mice treated with ADMA (* P<0.05, n = 10-12).

Thus, these studies suggest a new way of conceptualizing L-arginine metabolism, where downstream metabolites of arginase, such as polyamines, and modified L-arginine based compounds, such as ADMA are also important in regulating NO production (**Figure 29**).
Figure 29: L-arginine metabolic pathways
L-arginine enters the cell via the cationic amino acid transporter (CAT). Increased arginase activity leads to increased competition between the nitric oxide synthase (NOS) and arginase (ARG) pathways. Endogenous NOS inhibitors, ADMA and spermine also limit NO production.

Air pollution and asthma

Investigations into the molecular mechanisms underlying air pollution-induced exacerbation of asthma are of paramount importance, as the public health and economic costs of air pollution continue to rise. Arginase has been shown to be further upregulated by cigarette smoke in asthma [168], and L-arginine metabolism is intimately associated with airways tone and responsiveness. Thus, the examination of the effects of air pollution on this pathway represented a logical next step.

In Chapter 6 it was demonstrated that arginase activity in the lungs of mice from both sub-acute and chronic models of allergic airways inflammation were further augmented by exposure to CAP+O₃, and that this was primarily driven by arginase 1 [379]. It was also determined that the up-regulation of arginase 1 was localized to the peribronchiolar region.
Finally, the air pollution-induced AHR was attenuated by treatment with arginase inhibitor after exposure (Chapter 6). This strongly supports an intimate link between perturbation of L-arginine metabolism and the worsening of airways responsiveness.

Air pollution is known to contribute to asthma exacerbations [361-363]. Increased arginase protein expression has been observed in smokers with asthma [168], but it was not known whether arginase would play a role in air pollution-induced exacerbations of respiratory symptoms. It was initially surprising that a 4-hour in vivo exposure to CAP+O₃ was sufficient to upregulate arginase 1 in the lung. However, in porcine coronary arterioles, arginase was induced after only 1 hour of exposure to hydrogen peroxide [315], suggesting that arginase 1 is part of the oxidative stress response. This rapid response is intriguing, and suggests that arginase 1 participates in mounting the host response to environmental insults.

The particles we employed were derived from the ambient air in Toronto, Ontario, and particles within a specific size range (0.1-2.5 µm) were concentrated, representing real-world particles. Thus, this study is able to provide links between in vivo health effects and pollution that Canadians are exposed to every day. We examined the effects of combined exposures because both ambient particles and ozone have been shown to be associated with increased asthma exacerbations in humans, and these pollutants typically occur as a mixture in urban areas.

Asthmatics as a group represent a potential susceptible population that would be more significantly affected by air pollution than those who do not have underlying respiratory disease. Our results support this, as we did not observe increased airways responsiveness in the control mice, but demonstrated an increase in mice with pre-existing allergic airways inflammation. The results presented herein are suggestive that some of the increased vulnerability to air pollution
observed in the asthmatic population may due to the fact that perturbation of L-arginine metabolism in the presence of pre-existing dysregulation, likely has more profound effects.

Some limitations of these studies include their applicability to human asthma. Although arginase expression has been shown to be upregulated in both animal models and human asthma, and inhibitor studies have shown promise in animal models, the efficacy of pharmacologic inhibition of arginase has not been demonstrated in human asthma at this time. Studies are ongoing to determine the levels of polyamines in human lung samples, naïve murine lungs and murine lungs from models of allergic airways inflammation. This will help determined the applicability of the levels of polyamines studied in Chapter 5 to the levels that are found endogenously in the lung under normal and diseased conditions. The main limitation of the study presented in Chapter 6 is the use of high levels of air pollution during a single exposure period. Although exposure studies employing high levels are important to understand toxicological mechanisms, and may have relevance to certain occupational settings, further studies employing lower levels, potentially repeated over a longer period of time, will be necessary to determine the applicability of the results to a broader range of real-world settings.

**Integrated cellular model of L-arginine metabolism in asthma**

Arginase and NOS are expressed in a wide variety of cell types in the normal lung and expression levels are altered under conditions of allergic inflammation. Additionally, downstream metabolites of arginase and other endogenous, small molecules can also play a role in modulating NOS activity. Increased arginase expression in asthma is also exacerbated in murine models by exposure to air pollution [379]. In Chapter 4, concomitant expression of NOS1 and arginase was demonstrated in airway epithelial cells [340] (Figure 30). The expression of ARG1 in the airway epithelium in the chronic murine model is consistent with
studies in humans, as increased ARG1 mRNA expression has previously been described in the epithelium and in inflammatory cells from human asthmatics [76, 168]. NOS1 has been demonstrated by ourselves and others to localize primarily to airway epithelial and smooth muscle cells [167, 340]. Thus, arginase and NOS may be in competition for intracellular L-arginine pools within the same cells, and under conditions of allergic inflammation this competition may be exacerbated, resulting in decreased intracellular NO production and increased airway smooth muscle tone. Additionally, infiltrating inflammatory cells have been shown to express high levels of both iNOS and arginase [100, 340, 379], contributing to the increased expired levels of NO in asthma [132, 133]. The presence of increased inflammatory cells may therefore also be involved in further decreasing the extracellular L-arginine bioavailability for transport into smooth muscle cells and epithelia (Figure 30). Polyamines are also known to accumulate in the epithelial cells via active transport mechanisms, however, the specific transporter(s) responsible have not been completely elucidated [208-210]. NO signaling from the epithelia has been shown to be critical for the regulation of smooth muscle tone, therefore the increased expression of arginase and increased polyamine content likely plays a role in increased smooth muscle constriction. ADMA is also increased in the lung in asthma, however, the specific cellular sources and localization of the increased ADMA are not clear at this point.
Arginase and NOS are overexpressed in the epithelia, inflammatory cells and smooth muscle in asthma. Concomitant expression of NOS and arginase has been demonstrated in airway epithelial cells, thus arginase and NOS likely compete for intracellular L-arginine pools. Inflammatory cells are known to express both NOS and arginase and therefore are likely involved in decreasing the extracellular L-arginine bioavailability. Polyamines are known to accumulate in the epithelial cells due to active transport. NO signaling from the epithelia is important for the regulation of smooth muscle tone. ADMA is also increased in the lung in asthma.
Chapter 8: General Conclusions

The studies presented in this thesis demonstrate the importance of dysregulation of L-arginine metabolism in asthma. Increased expression of arginase 1 was demonstrated in human asthmatic lung samples and in acute and chronic murine models of allergic airways inflammation. Co-expression of arginase 1 and NOS1 was demonstrated in the airway epithelia in both murine models, supporting the potential for direct competition. Arginase inhibition in vivo attenuated airways responsiveness to methacholine in both acute and chronic murine models. Additionally, polyamine downstream metabolites, specifically spermine, also increased airways responsiveness to methacholine in murine models. Pharmacologic blockade of polyamine synthesis through ODC, concurrent with OVA challenge, attenuated airways hyperresponsiveness. Finally, other insults to the respiratory system that are known to exacerbate asthma, such as air pollution exposure, also perturb L-arginine metabolism in murine asthma models. Arginase activity and arginase 1 expression were demonstrated to be upregulated following environmental exposures in both sub-acute and chronic murine models of allergic asthma. Pollution-induced AHR is attenuated by arginase inhibition in both models. Thus, imbalances in L-arginine metabolism by increased arginase activity and downstream production of polyamines are important in asthma, and arginase may represent a therapeutic target in asthma and for exacerbations of allergic airways disease related to pollution exposure.
Chapter 9: Future Directions

Arginase and Airways remodeling

It has been suggested that arginase is involved in airways remodeling through the production of L-ornithine for downstream metabolism via the polyamine and proline synthetic pathways [321, 339]. However, this has not been directly demonstrated using chronic pharmacologic inhibition of arginase in a chronic model of asthma. The development of A1P opens up new possibilities for this type of study, as this inhibitor has the potential for decreased toxicity in vivo that would be useful for a long-term study. Administration of arginase inhibitors via inhalation prior to each OVA challenge in the chronic model would help to determine if arginase is involved in the development of increased smooth muscle mass and collagen deposition exhibited in this model.

Polyamines in asthma

Chronic administration of ADMA, another endogenous NOS inhibitor, via implanted minipump results in collagen deposition and increased arginase activity [386]. Further murine studies should examine the effects of chronic administration of spermine, either by inhalation or infusion. The effects of chronically increased polyamines on airway structure and responsiveness in the absence of airways inflammation are currently unknown. Additionally, studies of polyamine levels in normal human and asthmatic lungs would be helpful to improve the level of evidence for increased polyamines in asthma, and to determine if levels correlate to less invasive and more readily available samples such as blood serum or sputum.

Arginase and air pollution
Arginase has been shown to be upregulated acutely in a murine model of asthma by a single exposure to air pollution. However, the chronic effects of pollution exposure on this pathway remain unknown. Studies should focus both on normal mice and those with concomitant allergic inflammation exposed chronically to air pollution. It would be interesting to determine if chronic exposures to low levels of pollution also upregulate arginase, or if the effects observed were the result of lung injury following exposure to high levels. The effects of air pollution on the arginase pathway in humans should also be examined through controlled exposure studies, or in panel studies in biological samples that can be correlated to regional air pollution data.
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Appendix B:

Arginase in asthma – Recent developments in animal and human studies

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Published The Open Nitric Oxide Journal. Volume 2, March 2010, pgs 20-36.

Contributions of Authors:

All authors contributed equally to writing this review. J.A.S and Harm M. contributed equally as senior authors.
Arginase in Asthma – Recent Developments in Animal and Human Studies

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Abstract: The enzyme, arginase, converts L-arginine into L-ornithine and urea, and has been implicated in the pathogenesis of lung diseases, related to dysregulation of L-arginine metabolism and remodeling. Allergic asthma is a chronic condition characterized by inflammation, lung remodeling and airways hyperresponsiveness (AHR). Increased expression of arginase may contribute to AHR in asthma by reducing L-arginine bioavailability for the nitric oxide synthase (NOS) isoforms, thus, limiting the production of the endogenous bronchodilator, nitric oxide (NO). Reduction of intracellular L-arginine concentrations as a consequence of augmented arginase expression and activity may also promote NOS uncoupling, resulting in increased formation of peroxynitrite, a powerful oxidant that promotes bronchoconstriction and inflammation. In chronic asthma, increased arginase expression may also contribute to airways remodeling, through increased synthesis of L-ornithine, and hence the production of polyamines and L-proline, which are involved in cell proliferation and collagen deposition, respectively. New drugs targeting the arginase pathway could have therapeutic benefits in asthma. This review focuses on recent developments in our understanding of the role of arginase in AHR, inflammation and remodeling, highlighting studies that advance our knowledge of L-arginine dysregulation in human asthma and animal studies that explore the therapeutic potential of arginase inhibition.

Keywords: Nitric oxide, L-arginine, airways remodeling, inflammation, airway hyperresponsiveness, polyamines, fibrosis, proline, peroxynitrite.

INTRODUCTION

The metabolism of the semi-essential amino acid L-arginine has been of great interest since the Nobel Prize winning research programs of Furchgott, Ignarro and Murad, which led to the discovery of nitric oxide (NO) as a bioactive molecule. For many years, the catabolism of L-arginine to NO and L-citrulline was considered the most important biologically active pathway. In recent years, however, metabolism of L-arginine to urea and ornithine via the arginase family of isoymes has emerged as an important pathway in numerous physiological and pathophysiological conditions, including lung disease.

SUMMARY OF REVIEWS TO DATE

Reflecting the increased interest in arginase, there have been several recent reviews describing the role in lung disease and immunology. Maarsingh et al. recently described the therapeutic potential of arginase inhibition in asthma [1]. A review by Meurs et al. focused on the airways hyperresponsiveness of asthma both in vitro and in vivo in animal models of variable and chronic AHR [2]. The role of arginase in the regulation of airway smooth muscle tone by the nervous system was also described, along with the role of arginase in the chronic structural changes and remodeling of airway smooth muscle in AHR [2]. Maarsingh et al. reviewed the current knowledge of arginase induction in a variety of pulmonary diseases including asthma, COPD, cystic fibrosis, pulmonary hypertension, and remodeling and fibrotic disorders [3]. The unifying role of arginase in these diseases of the lung was the regulation of the synthesis of NO, polyamines and L-proline [3], contributing to smooth muscle reactivity, tissue hypertrophy/hyperplasia and collagen deposition, respectively. In another review from 2008, Maarsingh et al. reviewed the importance of altered L-arginine metabolism in AHR, inflammation and remodeling in allergic asthma, focusing on the three mechanisms that regulate L-arginine bioavailability within the cell: uptake, catabolism and recycling from L-citrulline [4]. In 2007, Peranzoni et al. reviewed the potential roles of L-arginine metabolism in the early and late immune response to inflammatory insults and in the pathology of a variety of neoplastic and autoimmune diseases [5]. Mori reviewed the regulation of apoptosis by the balance of nitric oxide synthesis through arginase and L-arginine recycling through L-citrulline [6]. Morris also reviewed the current state of knowledge about L-arginine metabolism in 2007, including transport into the cell and mitochondria and its...
interconversion from and into different amino acids as well as the multiple products of different pathways of L-arginine catabolism [7]. The interested reader is directed to these comprehensive reviews. The aim of the current review is to unify the recent advances in our knowledge of arginase in asthma, specifically focusing on the in vivo inhibitory studies, and reports that translate L-arginine metabolism via arginase to human asthma.

**NITRIC OXIDE HOMEOSTASIS AND AIRWAY FUNCTION**

NO is synthesized from L-arginine in a 2-step reaction with N$^\omega$-hydroxy-L-arginine (NOHA) as an intermediate by a family of NO-synthases that utilize oxygen and NAPDH as cosubstrates and yield L-citrulline as a coproduct [8,9]. Three NOS isoforms that are expressed from three distinct gene products have been identified: neuronal NOS (nNOS or NOS I) and endothelial NOS (eNOS or NOS III), which are constitutively expressed, and inducible NOS (iNOS or NOS II). In the airways, the constitutive NOS (cNOS) isozymes are mainly expressed in airway epithelium (NOS1 and NOS3), inhibitory nonadrenergic noncholinergic (iNANC) neurons (NOS1), and in endothelial cells from the airway vasculature (NOS3). cNOS isoforms produce relatively low levels of NO in response to increases in intracellular Ca$^{2+}$, evoked by membrane depolarization or by contractile agonists [10]. The activity of NOS2, which produces large amounts of NO, is regulated at the expressional level, e.g. by proinflammatory cytokines during airway inflammation [10-12].

NO is an important endogenous bronchodilator, inducing airway smooth muscle relaxation via mechanisms that are both dependent and independent of cGMP, generated through activation of soluble guanylyl cyclase by NO, collectively leading to decreased cytosolic Ca$^{2+}$-concentrations, decreased Ca$^{2+}$-sensitivity, and membrane hyperpolarization of the smooth muscle cell [10]. The bioavailability of L-arginine for the NOS isozymes determines the formation of NO in the airways, as illustrated by the finding that in healthy subjects the levels of exhaled NO are increased by oral or inhalational administration of L-arginine [13-15]. In guinea pig airway preparations in vitro, L-arginine diminishes airway constriction by methacholine [16], and increases iNANC nerve-mediated smooth muscle relaxation [17], as a consequence of increased formation of cNOS-derived NO.

In many cells, the availability of L-arginine for the NOS isoforms depends on L-arginine uptake by the cell through specific cationic amino acid transporters (CATs), recycling of L-citrulline to L-arginine, and on the activity of arginase which competes with NOS for the common substrate [4]. Uptake of L-arginine via the Na$^+$-independent CATs of the y$^+$ system (Fig. 1), the major transcellular L-arginine transporter [18], can be inhibited by other cationic amino acids such as L-ornithine and L-lysine, as well as by polycations, including major basic protein (MBP) from eosinophils [19]. Thus, MBP and its analogue poly-L-arginine attenuates L-arginine uptake and NO-synthesis in alveolar macrophages and tracheal epithelial cells [19], and increases airway responsiveness of rats and guinea pigs to methacholine, in vivo and in vitro [20,21] by inducing a deficiency of NO [21]. The L-arginine availability may also be regulated by recycling of the NOS-product L-citrulline, through the L-citrulline/L-arginine cycle [22,23]. This cycle consists of the enzymes L-argininosuccinate synthase and L-argininosuccinate-lyase (ASS and ASL, respectively), which, in various tissues, are colocalized and coinduced with NOS isoforms [23-27]. Although recycling of L-citrulline does not play a role in iNANC NO-mediated relaxations of human and guinea pig airway smooth muscle under basal

**Fig. (1).** Interactions between L-arginine metabolic pathways.

ADMA, asymmetric (N$^\gamma$,N$^\delta$) dimethyl arginine; CAT, cationic amino acid transporters; NO, nitric oxide; NOHA, N$^\omega$-hydroxy-L-arginine; NOS, nitric oxide synthase; OAT, ornithine aminotransferase; ODC, ornithine decarboxylase; PRMT, protein arginine methyl transferase; SDMA, symmetric (N$^\gamma$,N$^\delta$) dimethyl arginine.
conditions, the inhibitory effect of the NOS-inhibitor N\textsuperscript{\textdegree}-nitro-L-arginine was reversed by L-citrulline [28,29], which was prevented by ASS and ASL inhibitors, indicating that the L-citrulline/L-arginine cycle is effective under conditions of low L-arginine utilization by NOS1 [29]. For a detailed review on the role of L-arginine transport and L-citrulline recycling in regulating airway function in health and disease see: [4].

The third and most studied mechanism that regulates the L-arginine availability to NOS is substrate competition by arginase, which converts the amino acid into L-ornithine and urea [4,30], and will be the focus of the current review. Although the \( K_m \) of L-arginine for arginase is up to 1000-fold higher than for NOS, substrate competition can occur, because the \( V_{\text{max}} \) is also 1000-fold higher [30-32]. Two isozymes, arginase 1 and arginase 2, encoded by different genes, have been identified [30,33,34]. Arginase 1 is the major isozyme in the liver and is involved in the urea cycle, whereas arginase 2 is predominantly expressed extrahepatically [30,35,36]. In the airways, both isozymes are constitutively present in structural cells, including the epithelium, endothelium, (myo)fibroblasts and airway smooth muscle cells [37-40], as well as in inflammatory cells, such as alveolar macrophages and neutrophils [36,37,41]. It is becoming increasingly appreciated that the cellular and sub-cellular localization of these two isozyme families likely contributes to their potential roles in homeostasis and disease pathology (this will be discussed later in this review).

The significance of arginase in regulating airway function is clearly demonstrated by the effects of the potent and specific arginase inhibitor N\textsuperscript{\textdegree}-hydroxy-nor-L-arginine (nor-NOHA) on epithelial-derived and nerve-derived NO-generation, even in non-diseased airways. Thus, nor-NOHA attenuated methacholine-induced relaxations of isolated tracheal smooth muscle preparations [17,42]. These effects of nor-NOHA were quantitatively similar to those of L-arginine, indicating that substrate competition between NOS and arginase is indeed involved [16,17,42].

However, in the non-inflamed mouse airways, inhalation of arginase-specific inhibitors (i.e., BEC [43] and nor-NOHA) did not affect basal central airways or total lung resistance, whereas the nonspecific arginase inhibitor NOHA, which also acts as substrate for the NOS isozymes and thus increase NO levels directly, reduced baseline tone (North et al., unpublished observation). The ineffectiveness of specific arginase inhibitors is likely a consequence of the low-levels of arginases expressed in normal, non-diseased lungs [43]. Furthermore, in unrestrained guinea pigs, permanently instrumented with a pulmonary balloon catheter for on-line measurement of pleural pressure, inhalation of the potent and isozyme-nonsensitive arginase inhibitor 2(S)-amino-6-boronohexanoic acid (ABH) or L-arginine did not decrease the basal responsiveness of the airways to histamine, measured as a 100% increase of pleural pressure, either [44]. Thus, it would appear that under normal homeostatic conditions in the airways \textit{in vivo}, arginase and NOS do not compete significantly for L-arginine as substrate.

In alveolar macrophages, arginase was found to regulate NO-production by NOS, and \textit{vice versa}. Thus, NOS was able to inhibit arginase activity through NOHA, the intermediate product in NO synthesis [45]. The bioavailability of L-arginine to NOS could also be regulated by the arginase product L-ornithine, which competitively inhibits the uptake of L-arginine via specific CATs of the y\textsuperscript{\textdegree}-system [46-48]. Consistent with this finding, incubation with L-ornithine increased airway responsiveness in perfused guinea pig tracheal preparations by inducing a deficiency of cNOS-derived NO [49]. Although L-ornithine also competitively inhibits arginase activity [32,49,50], the increase in airway responsiveness shows that the inhibitory effect on L-arginine uptake is larger than that on arginase activity [49]. Thus, mutual interactions between NOS and arginase regulate L-arginine homeostasis and NO-synthesis in the airways (Fig. 1) and changes in this balance may underlie the pathophysiology of obstructive airways diseases, including asthma.

**ARGINASE IN ASTHMA**

Allergic asthma is a chronic inflammatory disorder of the airways that is characterized by reversible airways obstruction. Characteristic features of the disease are allergen-induced, IgE-mediated early (EAR) and late (LAR) asthmatic reactions, infiltration of inflammatory cells - particularly eosinophils and Th2 lymphocytes - into the airways, and inflammation-induced acute and transient airway hyperresponsiveness (AHR) that is present both after the EAR and LAR [51,52]. Chronic airway inflammation may induce airway remodeling, characterized by structural changes in the airway wall, including subepithelial fibrosis and increased airway smooth muscle mass [51,53,54]. Airway remodeling may contribute to a progressive decline in lung function, as well as to the development of persistent AHR [51,52]. The mechanisms underlying AHR are only partially understood. However, recent evidence suggests that alterations in L-arginine metabolism induced by arginase may be importantly involved in both acute and chronic AHR, via altered NO metabolism and synthesis of polyamines and L-proline, respectively [2,4,55].

**Role of Altered NO Metabolism in Acute Allergen-Induced AHR**

In asthmatic patients, the production of NO in the airways is considerably increased due to marked upregulation of NOS2 by pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-\(\alpha\) and IL-1\(\beta\) [10]. The increased NO production by NOS2 is associated with increased concentrations of NO in the exhaled air [14]. In experimental asthma, NOS2 is induced during the allergen-induced LAR, similarly leading to enhanced levels of NO in the exhaled air [56,57]. Significant correlations between exhaled NO, NOS2 expression in airway epithelial and inflammatory cells, airway eosinophilia and AHR have been observed in asthmatics, whereas all are reduced after glucocorticosteroid treatment [58-60]. Therefore, the NO concentration in exhaled air is considered as a sensitive marker of airway inflammation in asthma [61].

However, many studies have indicated that airway inflammation is not primarily caused by high concentrations of NOS2-derived NO by itself, but rather by increased
formation of peroxynitrite (ONOO\textsuperscript{-}), a highly reactive oxidant synthesized by the reaction of NO with superoxide anions (O\textsubscript{2}\textsuperscript{-}) in the inflamed airways [58,62]. Thus, whereas NO causes bronchodilation and has anti-inflammatory actions by inhibiting the activation of NF-κB [10,11,63-65], peroxynitrite has shown to induce pro-inflammatory effects associated with epithelial damage, eosinophil activation, MUC5AC expression, and vascular hyperpermeability [66-69]. In addition, peroxynitrite promotes airway smooth muscle contraction [12,70]. Accordingly, increased nitrotyrosine immunostaining, indicative of protein tyrosine nitration by peroxynitrite, has been observed in airway epithelial and inflammatory cells of bronchial biopsies from asthmatic patients as well as in airways of allergen-challenged guinea pigs [58,67]. Moreover, increased levels of exhaled NO correlate with increased nitrotyrosine concentrations in exhaled breath condensate of asthmatic patients [71], while nitrotyrosine staining in bronchial biopsies of these patients was shown to correlate with NOS2 expression, eosinophilic airway inflammation and AHR [58]. In line with these observations, animal studies have indicated that increased formation of peroxynitrite may importantly contribute to the development of allergen-induced AHR after the LAR in acute and chronic asthma [12,62,66,70,72].

Remarkably, in transgenic mice overexpressing NOS2 in the airways, increased levels of exhaled NO were associated with reduced airway resistance and airway hyporesponsiveness, with no signs of airway inflammation and increased nitrotyrosine staining, indicating that increased production of NO per se is not detrimental, but rather protective [73]. Moreover, allergen-induced airway inflammation and AHR were more pronounced in NOS2 knock-out mice than in wild type animals [74], although unchanged AHR [75] and inflammation [75,76] as well as reduced inflammation [77,78] have also been reported (for a comprehensive review on the role of NOS isoforms in asthma, see Mathrani et al [79]).

Despite the increased concentration of NO in exhaled air of asthmatics, various studies in animal models and in asthmatic patients have now indicated that acute allergen-induced AHR is paradoxically due to a deficiency of bronchodilating and anti-inflammatory NO in the airway wall, which may be caused by a reduced L-arginine availability to both cNOS and NOS2 [3,4,55,80]. The first evidence for a role of reduced production of cNOS-derived NO in allergen-induced AHR came from studies in guinea pig models of acute allergic asthma. In these models, NOS2 is not detected before the LAR [57], making it possible to investigate the distinct role of cNOS-derived NO in the AHR immediately after the EAR. In an initial study, it was demonstrated that NO in the exhaled air drops below baseline during the allergen-induced EAR, which might contribute to the obstructive reaction as well as to the ensuing AHR [81]. Indeed, both in vivo and ex vivo studies using a guinea pig model of acute allergic asthma indicated that a deficiency of contractile agonist-induced cNOS-derived NO is an important determinant of the AHR after the EAR [82-84]. A deficiency of cNOS activity and endogenous bronchodilating NO causing AHR was also demonstrated after repeated allergen challenges of sensitized guinea pigs [85]. Interestingly, a deficiency of iNANC nerve-derived NO was also found after single or repeated allergen challenges [86,87]. Ricciardolo and coworkers importantly demonstrated that a reduction in cNOS-derived NO also contributed to the AHR in patients with severe asthma treated with glucocorticosteroids, that inhibit the expression of NOS2 [88]. Moreover, as in guinea pigs, the deficiency of cNOS-derived NO could be induced by allergen challenge [89]. Interestingly, reduced cNOS-(presumably iNANC nerve)- derived NO could also be involved in attenuated deep inspiration-induced bronchoprotection in asthmatic patients [90-92].

Animal studies further indicated that reduced L-arginine availability to cNOS importantly accounts for the NO deficiency after the allergen-induced EAR (for review see [4]). Thus, exogenous L-arginine reduced the AHR to methacholine in perfused tracheal preparations of allergen-challenged guinea pigs ex vivo [16], and increased iNANC nerve-mediated airway smooth muscle relaxation in tracheal open-ring preparations from these animals [86]. Moreover, it was recently demonstrated that inhalation of L-arginine attenuates the allergen-induced AHR to histamine after the EAR in guinea pigs in vivo [44].

Since it has been shown that (particularly NOS3-derived) NO inhibits airway inflammation by suppression of NF-κB activity, thereby inhibiting the expression of pro-inflammatory cytokines as well as of NOS2 [63-65,93], the deficiency of cNOS-derived NO after the EAR could also contribute to the inflammatory response during the LAR. Accordingly, in asthmatic patients significant correlations were found between allergen-induced AHR after the LAR and reduced NOS3 expression as well as increased NOS2 expression in bronchial biopsies [89]. Moreover, allergen-induced airway inflammation was markedly reduced in NOS3-overexpressing mice as compared with wild-types [93,94]. Furthermore, in the acute ovalbumin sensitization and challenge model in mice, pulmonary NOS2 expression is up-regulated and NOS3 expression is down-regulated [43], likely further contributing to peribronchiolar inflammation in this model.

The enhanced production of peroxynitrite observed after the LAR could be caused by reduced L-arginine availability to NOS2. Thus, studies in macrophages have indicated that under conditions of low L-arginine availability NOS2 not only produces NO by its oxygenase moiety, but also synthesizes superoxide anions by the reductase moiety, leading to extremely efficient formation of peroxynitrite [95]. Increasing the L-arginine concentration in these cells stimulates NO production, while reducing the production of superoxide anions, and hence peroxynitrite [96]. The potential importance of this mechanism in the AHR after the LAR was recently indicated by observations in a guinea pig model of asthma, demonstrating that administration of exogenous L-arginine inhibits the allergen-induced AHR after the LAR both ex vivo and in vivo [44,97].

Since the bioavailability of L-arginine to NOS isoymes is regulated by arginase, increased consumption of the amino acid by arginase could explain the L-arginine limitation and subsequent AHR after the EAR and LAR in allergic asthma. In support of this hypothesis, the arginase activity in tracheal homogenates obtained from ovalbumin-challenged guinea pigs after the EAR was increased almost 4-fold compared to controls [98], whereas a 2-fold increase was observed after
the LAR [97]. Ovalbumin also strongly increased lung arginase activity in mouse [43,99,100] and rat [101] models of acute allergic asthma. The arginase activity in mouse lung was also increased after challenge with Dermatophagoides farinae [102], Aspergillus fumigatus [99] and trimellitic anhydride [100], suggesting that arginase is a key mediator in the response to allergic and occupational stimuli.

A number of studies using different mouse models of allergic asthma have investigated mRNA or protein expression of arginase 1 and 2 after allergen challenge (for review see [3]). Both isoforms are induced in lungs of mice challenged with either ovalbumin [43,99], D. farinae [102], A. fumigatus [99], trimellitic anhydride [100] or S. mansoni eggs [103], the induction of arginase 1 being the most prominent. Microarray analysis of gene expression in mouse lung after challenge with either ovalbumin or A. fumigatus showed that among the 291 genes that were commonly induced by these allergens, the genes encoding for arginase 1 and 2 were among the most predominantly overexpressed [99]. Th2 cytokines, which are elevated in allergic asthma, seem to play an important role in the induction of arginase 1 [99,103-105] and – to a lesser extent – arginase 2 [99]. In addition, arginase 1 was among the 26 commonly expressed transcripts that were (strongly) increased in lung inflammation in 5 different mouse models of Th2 cytokine-mediated inflammation [106]. The cytokine-induced expression of arginase 1 is under important control of the transcription factors STAT6 and CCAAT-enhancer binding protein [99,104,107-109], whereas the induction of arginase 2 is largely STAT6 independent [99].

**Role of Arginase in Allergen-Induced NO Deficiency and Acute AHR Ex vivo**

To study whether increased arginase activity indeed contributes to the reduced bioavailability of L-arginine and subsequent NO deficiency and AHR after the EAR, the effect of the specific arginase inhibitor nor-NOHA was studied in perfused tracheal preparations obtained from allergen-challenged guinea pigs [98]. Interestingly, the allergen-induced AHR after the EAR was completely inhibited by nor-NOHA to the level of normoreponsive unchallenged controls [98]. The effect of nor-NOHA was completely prevented by coinubcation with the NOS inhibitor L-NAME, clearly demonstrating that arginase inhibition reduces the AHR by restoring the production of cNOS-derived NO [98]. Treatment with nor-NOHA also restored the impaired iNANC-nerve mediated NO production and airway smooth muscle relaxation after the EAR [86]. The effects of nor-NOHA were quantitively similar to the effect of treatment with L-arginine [86,98]. The importance of increased arginase in limiting the L-arginine bioavailability and NO production is supported by the observation that levels of L-arginine and L-citrulline are reduced in the airways of allergen-challenged mice, whereas the arginase activity is increased [110]. In conclusion, increased arginase activity underlies the deficiency of neuronal and nonneuronal NO and subsequent AHR after the EAR by limiting the substrate availability to cNOS isoforms. Of note, since L-arginine transport via the y-system is inhibited by L-ornithine [46-48] leading to a deficiency of NO and increased airway responsiveness [49], increased formation of L-ornithine by arginase could also contribute to the L-arginine limitation and NO deficiency in allergic asthma.

As indicated above, the AHR after the LAR is caused by increased formation of peroxynitrite [12], due to reduced bioavailability of L-arginine for NOS2, which causes uncoupling of this enzyme [95,97]. Since the arginase activity in the airways of these animals is increased [97], increased consumption of L-arginine by arginase could by involved in this process. Indeed, inhibition of arginase reduced the AHR by restoring the production of bronchodilating NO, presumably by preventing the uncoupling of NOS2 [97]. These findings clearly indicate that the AHR after the LAR is caused by increased formation of peroxynitrite and not by an excess of NO. Increased arginase activity similarly underlies the peroxynitrite-mediated AHR in a guinea pig model of chronic asthma [72]. The importance of arginase in uncoupling of NOS2 and subsequent peroxynitrite formation is further supported by the observation that increased expression of arginase and NO2 are colocalized with increased nitrotyrosine staining in lungs of D. farinae-challenged mice [102].

Collectively, these functional *ex vivo* studies demonstrate that increased arginase activity contributes importantly to allergen-induced AHR after both the EAR and LAR by limiting the bioavailability of L-arginine to the NOS isozymes, leading to a deficiency of bronchodilating NO and increased formation of peroxynitrite.

**In vivo Treatment with Arginase Inhibitors**

The effectiveness of arginase inhibition in reducing *in vivo* AHR in allergic asthma has been recently studied in different animal models using a number of inhibitors (Table 1). The first report on the effect of arginase inhibitors on AHR *in vivo* was in a guinea pig model of acute allergic asthma, measuring airway responsiveness to inhaled histamine in permanently instrumented, freely moving animals. In these animals, treatment via inhalation with the specific arginase inhibitor ABH at 5.5 and 23.5 h after a single allergen challenge acutely reversed the AHR after the EAR and the LAR [44]. Using the same animal model of asthma, the AHR after the EAR was also acutely reduced after treatment with inhaled nor-NOHA (Table 1; Maarsingh *et al*., unpublished observation). Similar to the finding in guinea pigs, inhalation of nor-NOHA acutely reversed the allergen-induced AHR of the central airways to inhaled methacholine in an acute murine model of allergic airways inflammation (Table 1; North *et al*., unpublished observation). Moreover, in these mice inhalation of the specific arginase inhibitor S-(2-boronoethyl)-L-cysteine (BEC) normalized the allergen-induced AHR of the central airways to inhaled methacholine in an acute murine model of allergic airways inflammation (Table 1; North *et al*., unpublished observation). In these mice, BEC did not reduce – but rather accelerated - the peak-response of the peripheral airways to methacholine [111]. An explanation for the discrepancies is currently not at hand. In addition to the specific arginase inhibitors ABH, BEC and nor-NOHA, inhaled treatment with the non-specific arginase inhibitor, NOHA, normalized the AHR to methacholine in a mouse
Arginase in Asthma

The Open Nitric Oxide Journal, 2010, Volume 2  25

Table 1. Effectiveness of Arginase Inhibitors in Reducing (Allergen-Induced) AHR In Vivo in Various Animal Models of Asthma

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Species</th>
<th>Route</th>
<th>Dose</th>
<th>Inhibition of AHR</th>
<th>Asthma Model</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABH</td>
<td>Guinea pig</td>
<td>Inhalation</td>
<td>25 mM$^c$</td>
<td>60% (EAR); 61% (LAR); 85% (EAR); full (LAR)</td>
<td>Acute OVA</td>
<td>[44]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- acute$^a$</td>
<td>25 mM$^d$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>- protective$^b$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BEC</td>
<td>Mouse</td>
<td>Inhalation</td>
<td>40 μg/g$^a$</td>
<td>Full</td>
<td>Acute OVA</td>
<td>[43]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- acute$^c$</td>
<td>40 μg/g$^a$</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Oropharyngal aspiration$^d$</td>
<td>0.3 mM$^e$</td>
<td>No inhibition</td>
<td>Acute OVA</td>
<td>[111]</td>
</tr>
<tr>
<td>Nor-NOHA</td>
<td>Guinea pig</td>
<td>Inhalation</td>
<td>5 mM$^f$</td>
<td>48% (EAR)</td>
<td>Acute OVA</td>
<td>[**]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- acute$^g$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mouse</td>
<td>Inhalation</td>
<td>40 μg/g$^h$</td>
<td>43%</td>
<td>Acute OVA</td>
<td>[**]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>- protective$^i$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NOHA</td>
<td>Mouse</td>
<td>Intraperitoneal instillation$^j$</td>
<td>25 μg$^k$</td>
<td>Full</td>
<td>Acute OVA</td>
<td>[**]</td>
</tr>
<tr>
<td>Arginase 1</td>
<td>Mouse</td>
<td>Intratracheal instillation$^l$</td>
<td>25 μg$^m$</td>
<td>Full</td>
<td>Acute IL-13</td>
<td>[104]</td>
</tr>
</tbody>
</table>

$^a$ Assessment of AHR at 5 h (EAR) and 23 (LAR) after single allergen challenge, treatment 15 min before reassessment of AHR at 6 h and 24 h;
$^b$ treatment 0.5 h before and 8 h after single allergen challenge, AHR assessed at 6 h (EAR) and 24 h (LAR);
$^c$ treatment 2 h after allergen challenge, AHR assessed at 48 h;
$^d$ treatment 15 min before assessment of AHR at 24 h after the last of 6 daily allergen challenges;
$^e$ treatment 15 min before assessment of AHR at 24 h after the last of 12 allergen challenges;
$^f$ Assessment of AHR at 5 h (EAR) after single allergen challenge, treatment 15 min before reassessment of AHR at 6 h;
$^g$ treatment 0.5 h before each of 6 allergen challenges, AHR assessed 1-3 h after the last challenge;
$^h$ treatment 27 h and 24 h before IL-13 instillation, AHR assessed 12 h after IL-13 instillation;
$^i$ nebulizer concentration in an 8 L cage;
$^j$ total volume of 40 μl, equals ~3 μg BEC;
$^k$ body weight, equals ~800 μg BEC;
$^l$ in 100 μl PBS;
$^m$ 25 μg of plasmid in 50 μl ExGen500 and 5% w/v glucose;
[**] Maarsingh et al, unpublished observation;
[**] North et al, unpublished observation.

Abbreviations: ABH, 2(S)-aminoboronohepxanoic acid; AHR, airway hyperresponsiveness; BEC, S-(2-boronoethyl)-L-cysteine; EAR, early asthmatic reaction; LAR, late asthmatic reaction; OVA, ovalbumin; NOHA, N$^\text{o}$-hydroxy-L-arginine; nor-NOHA, N$^\text{o}$-hydroxy-nor-L-arginine.

model of allergic asthma (Table 1; North et al., unpublished observation). However, NOHA is also substrate for NOS. Therefore, the effect of NOHA may at least partially result from a direct stimulation of NO synthesis, in addition to increasing the L-arginine availability to NOS by inhibiting arginase.

AHR to inhaled methacholine persists in a mouse model of chronic allergic asthma, in which the animals were challenged every other week for 2 consecutive days for a period of 12 weeks [43]. Treatment with inhaled BEC 24 h after the last of 12 allergen challenges completely normalized the AHR of the central as well as the peripheral airways in these mice (Table 1), clearly indicating that increased arginase activity also contributes to the AHR in vivo in chronic asthma [43]. Furthermore, in the chronic asthma model the isozyme responsible for the AHR appears to be arginase 1, since the expression of arginase 2 is not increased in this model [43].

Distinct from the aforementioned treatment-based protocols, the protective effect of arginase inhibitors on allergen-induced AHR in asthma has also been studied in both guinea pigs and mice. Remarkably, the sensitivity of the airways to the inhaled allergen of sensitized guinea pigs was largely reduced after treatment with inhaled ABH 0.5 h prior to allergen challenge as compared to pretreatment with saline [44]. This anti-allergic effect of the arginase inhibitor may involve increased formation of NO, which is known to inhibit mast cell activation, as well as a number of mast cell-
mediated inflammatory processes [112]. In addition, NO inhibits the allergen-induced mast cell mediator release from guinea pig lung parenchymal tissue [113]. When saline- and ABH-pretreated animals were challenged to a similar bronchial obstruction, thus requiring a higher allergen dose in the ABH-treated animals, the AHR after the EAR as well as after the LAR was partially reduced in the ABH-treated animals [44]. However, almost full inhibition of the AHR after the EAR and full inhibition of the AHR after the LAR were obtained, when the ABH-treated guinea pigs were challenged with the same allergen dose that induced airway obstruction in saline-treated animals (Table 1) [44]. The initial allergen-induced bronchial obstruction and the magnitudes of the early and late asthmatic reactions were also greatly reduced by pretreatment with ABH [44]. The protective effect of arginase inhibitors on the development of allergen-induced AHR was confirmed in another mouse model of asthma, showing that treatment with intraperitoneally applied nor-NOHA 0.5 h before each of 6 allergen challenges, over the course of two weeks, significantly reduced the AHR to methacholine assessed 1-3 h after the last allergen challenge (Table 1) [74]. In addition, treatment with interference RNA against arginase 1 prevented the IL-13-induced upregulation of arginase 1 in mouse lung as well as the AHR induced by this cytokine [104]. In this mouse model, increased arginase 1 expression correlated temporally with the development, persistence, and resolution of IL-13-induced AHR [104]. All arginase inhibitors studied in the in vivo models are subtype nonselective and therefore the contribution of the two arginase isoforms to the development of allergen-induced AHR in these models is not known. However, whereas both isoforms were increased in the acute mouse model, only arginase 1 was upregulated in the chronic mouse model [43].

In conclusion, targeting increased arginase activity using (inhaled) arginase inhibitors may be a novel therapeutic strategy in allergic asthma, since these inhibitors acutely reduce allergen-induced AHR, have anti-inflammatory properties and protect against the development of allergen-induced AHR and bronchial obstructive reactions.

Role of Arginase in Human Asthma

There is increasing evidence that arginase is also important in the pathophysiology of human asthma [114]. Quite remarkably, as early as in 1980 enhanced arginase activity was found in sputum from asthmatic patients, which at that time was ascribed to disturbed liver function due to hypoxia [115]. It took, however, more than 20 years to appreciate the potential importance of this finding [55,98,99,114]. Increased arginase expression and/or activity have now also been demonstrated in airway and lung tissue, BAL cells and serum from asthmatic patients [39,43,99,114,116].

Increased protein and mRNA expression of arginase 1 in asthmatic patients was first described by Zimmerman et al. [99]. Northern blot analysis revealed increased mRNA expression of arginase 1 in inflammatory cells and airway epithelium in bronchial biopsies of these patients, while increased protein expression of the enzyme was observed in inflammatory cells, presumably macrophages, in the BAL. Accordingly, the recent study by North et al. [43] demonstrated increased arginase activity and protein expression of arginase 1, but not arginase 2, in lung tissues from asthmatic patients. Furthermore, immunohistochemical studies have indicated augmented arginase 1 protein expression in the airway epithelium and airway smooth muscle layer of smoking asthmatics compared to non-smoking patients, which might be induced by nicotine [39].

Remarkably, in asthmatic patients experiencing an exacerbation, arginase activity in serum was considerably increased, whereas L-arginine levels in the plasma were strikingly reduced [116]. Moreover, improvement of asthma symptoms in some of these patients was associated with a decline in arginase activity and increased L-arginine concentrations [116]. These findings indicate that changes in arginase expression are not solely confined to the airways and that reduced levels of circulating L-arginine could contribute to NO deficiency in the airways. In support, in patients with severe asthma serum arginase activity was inversely correlated with FEV1 and FEV1/FVC, whereas a positive correlation was found between L-arginine bioavailability and lung function [114]. Of interest is the recent finding that human neutrophils contain high levels of arginase in azurophilic granules [41], as it is known that infiltration and activation of these cells in the airways is particularly enhanced in severe asthma.

In addition to the changes in expression and activity of arginase described above, single nucleotide polymorphisms (SNPs) in the arginase 1 and 2 genes have recently also been associated with allergic asthma. Thus, a significant association between SNPs in arginase 2 and increased risk of childhood asthma has been observed, whereas in the same population of children SNPs in both arginase 1 and arginase 2 were associated with atopy [117]. Quite remarkably, pharmacogenetic screening of 844 SNPs in 111 candidate genes for association with inhaled β2-agonist-induced bronchodilation recently identified arginase 1 as a potential β2-agonist response gene [118].

ARGINASE IN INFLAMMATION

Persistent inflammation plays a defining role in asthma, and one of the central goals of current asthma therapy is to minimize this process [119,120]. Inflammation and expression of arginase has also been demonstrated in all commonly-studied animal models of allergic inflammation, including rat, guinea pig, and mouse, as well as in human asthma [39,43,86,97-99,101,110]. While arginase expression has been found to be upregulated in epithelia, smooth muscle and the peribronchiolar region [39,43,99,102], this section will focus on new developments in our understanding of arginase expression and function in immune cells.

In 1995, Corraliza and Modolell reported the induction of arginase in murine macrophages by Th2 cytokines [121, 122]. Further studies have revealed upregulation of arginase1 by IL-4 and IL-13, through a STAT6-dependent enhancer-mediated mechanism [109,123]. Increased arginase 1 mRNA expression was demonstrated in two independent murine models of allergic airways inflammation by Zimmermann et al., localized to macrophages and areas of peribronchiolar inflammation [99]. Arginase-positive inflammatory cells were also demonstrated in the mucosa of human asthmatic bronchial biopsies and bronchoalveolar lavage samples [99].
Following these seminal findings, arginase has been found to be highly expressed in alternatively-activated macrophages [124,125], and to play an important role in allergic inflammation and asthma [43,100-102,104,110]. Relatively little information is available regarding the expression and function of arginase in human inflammatory cells, compared to murine models. This section will provide an overview of arginase in immune cells, as it is reviewed comprehensively elsewhere in this supplement.

Munder et al. [41] reported the first comparative examination of arginase expression in human and murine immune cells and showed that in contrast to the highly-inducible expression of arginase that occurs in murine macrophages and dendritic cells, human polymorphonuclear leukocytes and eosinophils express arginase 1 constitutively; with no significant inducibility in any of the human immune cell populations. Arginase is sequestered within azurophlic granules in these cell types, and exocytosed after activation [41,126]. As such, the release of arginase 1 by PMNs contributes towards the antimicrobial/fungicidal activity [41], controls L-arginine concentrations in the local microenvironment, and suppresses the activation of T-lymphocytes [127] and natural killer cells [128]. The subcellular localization of arginase in PMNs has been challenged by Jacobsen et al., who described that arginase 1 is localized to gelatinase granules of human PMNs, as opposed to the earlier-stage azurophlic granules [129]. Since considerable heterogeneity in infiltrating cell types can exist between asthmatic individuals, and since asthmatic subtypes with severe disease can exhibit predominantly neutrophilic inflammation [130-133], arginase induction within this cell population could likely contribute to arginine dysregulation in human asthma.

Murine macrophages are capable of inducing expression of arginase 1 in response to various stimuli, including Th2 cytokines and transforming growth factor (TGF) [134]. Furthermore, Erdely et al. also demonstrated that arginase 1 could be induced by IL-4 in combination with pharmacologic tools that increase cAMP levels (i.e., forskolin, which activates adenylyl cyclase, and phosphodiesterase inhibitors) in both human alveolar macrophages and murine RAW264.7 cells [134]. Thus, the potential for arginase to play a role in arginine balance/imbalance in macrophages is likely dependent upon the cell populations studied and the conditions used for induction. Understanding the complex control of arginase expression within these cells will be important to advance research in allergic asthma.

Arginase and Alternatively-Activated Macrophages

The primary function of classically-activated macrophages is the cytotoxic killing of microbes through the production of reactive oxygen and nitrogen species, including high levels of NO produced by NOS2. Classical activation of macrophages depends on cytokines derived from activated CD4+ T helper 1 cells (Th1), particularly IFN-γ [125]. Alternative activation of macrophages was proposed in the early 1990’s, as a phenotype induced by the Th2 cytokine IL-4 and characterized by suppression of IL-1 synthesis and expression of the macrophage mannose receptor [135,136]. Alternative-activation is characterized by the expression of several signature proteins, including arginase 1, chitinase-like molecules (Ym1, Ym2 and acidic mammalian chitinase; AMCase), and resistin-like molecule (RELmα, also known as FIZZ1) [124]. The cells responsible for producing IL-4 and initiating the alternative activation of macrophages include CD4+ Th2 cells, CD8+ T cells, NK cells, basophils, mast cells, and eosinophils, indicating that alternative activation can be elicited by either the innate or the acquired immune system [125]. Alternative-activation and expression of arginase 1 has been described in macrophages in a variety of diseases, including interstitial pulmonary fibrosis [137]. Arginase likely plays a detrimental role by limiting substrate for NOS2 in diseases in which a Th1 response is required. However, arginase expressed in alternatively-activated macrophages likely plays a role in T cell regulation, and the shift of L-arginine metabolism from NO production to the synthesis of polyamines and proline is likely important for the resolution of inflammation and wound healing [125,138,139]. Given that arginine metabolism via the arginine pathway leads to the production of polyamines and collagen, it is exciting to postulate that alternative activation of macrophages might contribute to the airway remodeling of asthma.

Arginase Inhibition and Allergic Inflammation

To elucidate the role of arginase in allergic inflammation, recent studies have explored arginase inhibition in animal models. These studies, involving different animal models and administration protocols, have yielded conflicting results, with one group reporting enhancement of inflammation [111], two groups reporting attenuation [44,74], and one group reporting no effect [140]. Mechanisms proposed to be important in the alteration of inflammatory status following arginase inhibition are the modulation of anti-inflammatory NO, pro-inflammatory peroxynitrite, and subsequent effects on the transcription factor, NFκ-B.

In a recent study, Ckless et al. investigated the effects of arginase inhibition in an ovalbumin sensitization and – challenge model of allergic airways inflammation employing female BALB/c mice [111]. Mice were sensitized to ovalbumin on days 0 and 7, and challenged for 30 min with aerosolized 1% ovalbumin or vehicle on days 14–16 [111]. While BEC did not affect allergen-induced increases in differential cell counts or cytokine levels in BAL samples, the authors report significant enhancement of peribronchiolar and perivascular inflammation based on semi-quantitative scoring of histological sections [111]. Increases in both S-nitrosothiols and 3-nitrotyrosine were detected, without any apparent change in nitrite/nitrate levels [111]. The authors also report increased DNA binding of the NFκ-B transcription factor, resulting in augmentation of cDNA expression for proinflammatory cytokines, neutrophil attractant protein (KC) and macrophage inflammatory protein-3α (CCL20) [111]. The same group and others have previously shown that the activation of NFκ-B is redox-sensitive, activated by tyrosine nitration, but inhibited by S-nitrosothiols [141-143]. In lung epithelial cells the cytokine-induced NFκ-B activation was decreased following...
arginase inhibition, with concomitantly increased concentrations of NO and S-nitrosothiols [144]. In another study, the cytokine-induced activation NFκB was decreased following arginase inhibition in lung epithelial cells, with concomitant increases in NO and s-nitrosothiols [144]. In addition the expression of KC and CCL20 in epithelial cells was attenuated by treatment with BEC [144]. Ckless et al. explain these apparent discrepancies by pointing to the possibility that increased NO produced following arginase inhibition may be converted into peroxynitrite [111]. While increased 3-nitrotyrosine was demonstrated in their study, S-nitrosothiols were also increased [111], making it unclear whether the balance lies in favour of reactive nitrogen species or anti-inflammatory S-nitrosothiols following arginase inhibition.

In another recently-published murine study, Bratt et al. demonstrated that administration of the arginase inhibitor, nor-NOHA (100 μg i.p.), or vehicle, prior to each ovalbumin challenge reduced the total cells in BAL fluid by approximately 65% [74]. The authors postulated that arginase inhibition shunts l-arginine substrate towards the NOS pathway, partially blocking the allergen-induced inflammation through the anti-inflammatory effects of NO [74]. The same group recently demonstrated that increased expression of arginase in isolated airways correlate with levels of lung inflammation [110]. Interestingly, an inflammatory response to ovalbumin was observed in NOS2 knockout mice, supporting the anti-inflammatory effects of NO [74].

Recent studies in guinea pig models of allergen-induced airway inflammation also support the anti-inflammatory potential for arginase inhibition [44]. The arginase inhibitor ABH was administered by inhalation 30 minutes before and 8 hours after allergen challenge [44]. The allergen-induced increases in the numbers BAL cells, including eosinophils, macrophages and total inflammatory cell infiltration were all inhibited by ABH by approximately 50% [44]. As mentioned above, arginase inhibitors may reduce airway inflammation through an increase in anti-inflammatory NO production by cNOS as well as reduced NF-kB activation [44,94,144]. In addition, inhibition of arginase may also attenuate airway inflammation by reducing the formation of proinflammatory peroxynitrite via restoring the L-arginine availability to NOS2, thereby preventing the uncoupling of this enzyme, caused by allergen-induced upregulation of arginase [97]. Remarkably, the sensitivity of the airways to the inhaled allergen was also markedly reduced in the ABH-pretreated guinea pigs compared to saline-pretreated animals [44].

A recent study investigated the role of bone marrow cell-derivated arginase 1 in the development of allergen-induced lung inflammation. To this aim, Niese et al. generated chimeric mice using irradiated CD45.1 congenic mice and bone marrow derived from arginase 1+/- pups [140]. At 7–14 weeks post-irradiation, the authors employed two independent models of experimental asthma; the ovalbumin model and the A. fumigatus model [140]. In developing the chimeric mouse, the authors found that arginase 1 deficiency did not affect bone marrow reconstitution, immune cell development or adaptive immunity, as assessed by the production of allergen-specific antibodies [140]. Mice that received arginase 1+/- bone marrow exhibited decreased arginase 1 expression by Northern blot and in situ hybridization following allergen challenge, compared to mice that received arginase 1+/- bone marrow [140]. Additionally, the arginase 1-deficient chimeras did not exhibit significantly increased arginase activity following allergen challenge, indicating that bone marrow derived arginase 1 is the main contributor to increased arginase activity in experimental asthma [140]. In support, allergen-challenged arginase 2 knockout mice experience the same upregulation of lung arginase activity as wild type mice [140]. However, the ablation of arginase 1 from bone-marrow derived cells, or the complete knockout of arginase 2, did not affect basal nor allergen-induced inflammatory cell infiltration or differential counts in BAL [140]. These data suggest that arginase 2 and bone marrow-derived arginase 1 are not required for the development of lung inflammation in murine experimental asthma [140]. However, the results from the bone marrow cell derived arginase 1 deficient mice do not preclude the possibility that arginase 1 expressed in structural cells, such as the epithelial [43,99] or smooth muscle cells [39,43], may contribute to the development of allergic airways inflammation. The drastic reduction of arginase expression and activity did not induce an exacerbated inflammatory phenotype [140] as observed in the above-mentioned study using the arginase inhibitor BEC in mice [111].

By contrast, Pesce et al. recently generated arginase-1 floxed transgenic mice crossed with two independent macrophage-specific cre-recombinase expressing strains, resulting in two strains of mice in which arginase 1 was conditionally knocked out in the macrophages [139]. Using a model of Schistosoma mansoni infection, which typically occurs through Th2 cytokines, these investigators demonstrated that deletion of arginase 1 actually augmented the hepatic fibrosis and granulomatous inflammation in diseased animals [139]. Thus, in this model, arginase 1 expression in macrophages acts to suppress Th2-dependent fibrosis and inflammation [139].

Taken together, the role of arginase in airway inflammation in experimental asthma is not completely understood as both anti-inflammatory and pro-inflammatory effects of arginase inhibition have been observed, whereas knockdown of arginase 1 in bone marrow derived cells or total knockdown of arginase 2 does not affect lung inflammation. Further studies, using conditional knockdown of arginase 1 in structural cells, will be key in understanding the complex role of altered L-arginine metabolism in allergic inflammation. While many interesting advances in our understanding of the apparently divergent roles of L-arginine metabolism in allergic inflammation have been made recently in animal models, we must consider how these mechanisms may be manifest in human asthma, and how they may potentially be modulated for future therapies.

**ARGINASE IN CHRONIC AIRWAY REMODELING**

As previously described, chronic airway inflammation may induce airway remodeling. In particular, eosinophilia and secretion of TGF-β and IL-13 are considered to play an important role [145]. However, Holgate et al. suggested an important interactive role for the structural cells of the airways in the remodeling process as put forward by in the
epithelial mesenchymal trophic unit hypothesis [146-148]. Recent advances also implicate arginase in airway remodeling, through the production of L-ornithine and subsequent synthesis of polyamines and L-proline (Fig 1). Polyamines promote cell growth and proliferation, while L-proline is essential for collagen synthesis.

Lung fibrosis studies can also inform our understanding of the role of arginase in airway remodeling, as the mechanisms underlying increased collagen deposition in this disease likely exhibit some degree of overlap with those at work in asthma. Interestingly, the arginase activity in mouse lung tissue and fibroblasts was dose-dependently increased by the profibrotic factor TGF-β [149]. Moreover, levels of TGF-β, arginase activity and collagen content are all increased in rat lung allografts, with a strong correlation between arginase activity and collagen content [149]. Kitowska et al. recently explored the role of arginase in remodeling in lung fibrosis [150]. Increased expression of arginase 1, arginase 2 and collagen I, and a decrease in L-arginine availability, without alterations in protein arginine methyltransferases or CATs, were observed in mouse models of bleomycin-induced pulmonary fibrosis [101,151]. In addition, arginase 1 and 2 mRNA were induced by TGF-β1 in murine lung fibroblasts and inhibition of arginase by the nonspecific inhibitor NOHA attenuated TGF-β1-stimulated collagen deposition in a post-transcriptional manner [101].

Another group studying lung fibrosis recently interrogated the genetic pathways upregulated by TGF-β, using transgenic mice expressing human TGF-β1 under the control of the Clara cell 10-kD (CC10) promoter [152]. After treatment with doxycycline, these animals displayed upregulation of IL-13, infiltration of alternatively-activated macrophages and increased pulmonary fibrosis [152]. Importantly, arginase 1 and 2 expression and total arginase activity in BAL were also upregulated in the transgenic mice at day 14 post TGF-β1 induction [152]. These data support the hypothesis that arginase is induced by TGF-β as part of a pro-fibrotic genetic program, likely contributing via the metabolism of L-arginine to L-ornithine, leading to increased downstream production of proline and polyamines. These studies together provide a functional link between TGF-β1, arginase and collagen deposition in lung fibrosis. As TGF-β1 is also known to contribute to lung remodeling in asthma [153,154], these studies provide strong support towards arginase playing a key role.

In murine models of allergic airways inflammation there have also been recent developments implicating arginase in airway remodeling. Recently, treatment of mice in an ovalbumin model of allergic inflammation with mepacrine, an antimalarial drug, was shown to reduce the levels of IL-4, IL-13 and TGF-β1 as well as the expression and activity of arginases in the lung [155]. In this model, involving sensitization to ovalbumin on days 0, 7 and 14, followed by challenge with ovalbumin or vehicle beginning on day 21 for 12 consecutive days, Masson trichrome staining revealed sub-epithelial fibrosis, which was also attenuated by treatment with mepacrine[155]. However, mepacrine is also known to reduce Th2 cytokines and cysteinyl leukotrienes [155], therefore this study does not provide specific evidence that reduced arginase is the sole factor contributing to the reduction of remodeling in this model. To date no data is available regarding arginase inhibition with a specific inhibitor in a chronic model of allergic airways inflammation, but this study provides some encouraging findings. However, a recent study indicates that arginase 2 and bone marrow cell-derived arginase 1 are not important for allergen-induced fibrosis [140].

Increased arginase activity could also play a role in lung fibrosis by decreasing the NO production, since NO is antifibrotic. The importance of NO in regulating lung collagen content was supported in a guinea pig model of ongoing asthma. Thus, the allergen-induced collagen deposition around non-cartilaginous airways [156], but not in the alveolar septa [157] was further increased by chronic treatment with the NOS inhibitor L-NAME. Moreover, increased deposition of collagen in the lung has been observed in allergen-challenged NOS2-/- mice compared to allergen-challenged wild types [158].

Recently, novel studies have focused on the role of asymmetric dimethylarginine (ADMA) in respiratory function and disease. ADMA is an endogenous competitive inhibitor of NOS, derived from the breakdown of proteins containing L-arginine residues previously methylated by protein-arginine methyltransferases (PRMTs; Fig 1) [159,160]. The role of ADMA in the regulation of the L-arginine/NO pathway in vivo and airway remodeling is just beginning to be elucidated. Wells et al. infused saline or ADMA (30–90 mg/kg/day) via implanted osmotic minipumps in naïve Balb/c mice for 2 weeks [161]. Increased lung hydroxyproline content – as a marker for lung fibrosis - and collagen staining on histological sections were observed in ADMA-treated mice compared with saline-treated controls with concomitant higher arginase activity, decreased nitrite concentrations and increased urea in lung homogenates [161]. Interestingly, ADMA treatment did not affect IL-13 or arginase expression [161]. Finally, it was shown that ADMA induced an increase in arginase activity and collagen fiber formation in primary mouse lung fibroblast cultures, and that this was preventable through addition of NOHA [161]. In addition, treatment with the NOS inhibitor L-NAME has been shown to further increase the IL-4-induced production of TGF-β by lung fibroblasts [162].

Another characteristic of airway remodeling is increased smooth muscle mass. In the vasculature, arginase has been shown to play a role in smooth muscle proliferation [163,164]. Elevated production of polyamines from L-ornithine as well as decreased synthesis of NO due to increased arginase activity could therefore contribute to the process of airway smooth muscle thickening. The synthesis of the polyamines (putrescine, spermidine and spermine) is initiated by ornithine decarboxylase (ODC), which converts L-ornithine into putrescine [30]. Polyamines are involved in cell proliferation [3,30,55,80,165] and induce the expression of genes involved in cell proliferation by promoting histone acetyltransferase activity and chromatin hyperacetylation [166]. Studies in the vasculature have shown that the expression/activation of arginase and ODC can be induced by growth factors, leading to increased polyamine levels [167-171] and this activation of ODC has also been observed in the airway smooth muscle [172]. Importantly, levels of polyamines in lungs of allergen-challenged mice [99] and in...
serum of asthmatic patients are increased [173]. The potential importance of arginase in this context is illustrated by the observation that arginase activity in the airways and lungs are increased in animal models of chronic allergic asthma [43,72].

In contrast to polyamines, NO inhibits mitogen-induced proliferation of cultured airway smooth muscle cells from human [174-176] and guinea pig [177] origins, by inhibiting the G_1 phase and the S phase of the cell cycle [175] This inhibitory effect of NO on airway smooth muscle proliferation is mediated by the activation of soluble guanylyl cyclase and subsequent cGMP production [174,175,177], but also involves cGMP-independent inhibition of ribonucleotide reductase [175]. The exact downstream mechanisms contributing to NO-mediated inhibition of airway smooth muscle cell proliferations are currently unknown, but studies in vascular smooth muscle cells have shown that NO represses cell cycle promoting genes and induces inhibitors of the cell cycle [178] and inhibits PDGF-induced activation of protein kinase B [179]. In addition, NO inhibits the activation of ODC via S-nitrosylation of the enzyme under normal conditions [180].

Bergeron et al. examined L-arginine related proteins by immunostaining in asthmatic airways [39]. This study revealed increased arginase expression in asthmatics who smoke, localized to epithelia and the peri-bronchiolar region. This study suggests a role for arginase in injury and repair initiated by cigarette smoke insult [39]. It also demonstrates arginase expression in steroid-naïve asthmatics in anatomical proximity to remodeling features.

Conclusively, these findings suggest that increased arginase activity in chronic asthma could also contribute to the airway smooth muscle thickening via an increased proximity to remodeling features.

This study suggests a role for arginase in injury and repair initiated by cigarette smoke insult [39]. It also demonstrates arginase expression in steroid-naïve asthmatics in anatomical proximity to remodeling features.

CELLULAR AND SUB-CELLULAR LOCALIZATION

A key level at which NOS and arginase can compete for substrate is through cellular and subcellular localization throughout the airways. Co-expression of arginase and NOS has been demonstrated in epithelial cells of the airways. The epithelial cells of the large airways are known to undergo significant changes in asthma, and their importance in AHR is beginning to be appreciated [181,182]. Damage and shedding of the epithelia has been observed in asthmas and may contribute to the loss of bronchoprotection by cNOS-derived NO [88]. Increased arginase 1 has been shown in asthmatic airway epithelial cells in bronchoscopy samples [39,99]. Recent human data and murine models of allergic airways inflammation suggest expression in the apical layer of airway epithelial cells [43,150].

Importantly, concomitant expression of NOS1 and arginase in airway epithelial cells supports the potential for arginase overexpression to reduce the local bioavailability of L-arginine for NOS1 in these cell types [102,111]. NOS1 has been demonstrated by ourselves and others to localize primarily to airway epithelial and smooth muscle cells [43,182]. As noted above, previous studies in guinea pigs have suggested that arginase activity can affect airway tone through increased competition with NOS1 in both homeostasis and under conditions of allergic inflammation [17,42,86,98]. Thus, arginase and NOS1 may be in competition for intracellular L-arginine pools within the same cells and under conditions of allergic inflammation this competition may be exacerbated by increased arginase 1 expression.

In addition to whether or not specific cell types co-express the arginase/NOS isozymes, sub-cellular localization of L-arginine-related enzymes may be critical to understanding the outcome of dysregulation of proteins related to L-arginine metabolism. NOS1 contains a PDZ domain that participates in the formation of active dimers, and interactions with other proteins [183,184]. NOS1 has been localized to the cytosol, neuronal synapses and the plasma membrane [185,186]. In contrast, NOS2 has a relatively wide-ranging distribution in various cell types, including the cytosol, perinuclear space, plasma membrane and mitochondria [187-189]. NOS3 has been demonstrated at the plasma membrane, where it has complex interactions with caveolin and CAT1 [185,190-192]. Similar to NOS2, NOS3 has also been reported to be present in mitochondria [189]. Meanwhile, arginase 1 is localized primarily in the cytosol, while arginase 2 is confined to the mitochondria. Both arginase 1 and 2 have been implicated in regulating NOS1 activity through the depletion of intracellular L-arginine [193,194]. In cultured endothelial cells arginase 2 has been demonstrated to regulate the activity of NOS3, and this regulation has been shown to take place within non-freely exchangeable L-arginine pools [195,196]. Furthermore, over-expression of arginase 1 has been demonstrated to result in impaired NO production by NOS1 in cultured 293 embryonic kidney cells stably transfected with NOS1 [194]. Ckless et al. investigated the colocalization of arginase 1 with NOS isoforms in C10 murine alveolar type II epithelial cells [144]. Through immunoprecipitation it was demonstrated that arginase 1 colocalized with NOS3, but not NOS1 or NOS2 in this cell type [144].

The subcellular localization of the arginase isoenzymes may also have implications in the downstream fates of the products of arginase and NOS, L-ornithine, and L-citrulline, respectively. The localization of ornithine decarboxylase (ODC) in the cytosol may preferentially steer cytosolic L-ornithine towards the production of polyamines, which are important in cell proliferation. Ornithine amino transferase (OAT) is localized to the mitochondria, potentially directing the L-ornithine produced by arginase 2 towards the production of proline and collagen synthesis [197]. However, transport of L-ornithine across the mitochondrial membrane is known to occur [30]. Furthermore, recycling of L-citrulline to L-arginine by ASS and ASL may be important in regulating L-arginine availability for the NOS isoenzymes. ASS produces argininosuccinate from L-citrulline and aspartate as part of the L-citrulline-NO cycle, whereas ASL cleaves argininosuccinate into L-arginine and fumarate [30]. Both ASS and ASL are localized to the cytoplasm, indicating that the full recycling of L-citrulline to L-arginine may take place within that cellular compartment [198]. Future investigations of the role(s) of L-arginine-related proteins/pathways in the pathogenesis of disease will need to consider the potential impact of sub-cellular localization of
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In a recent paper examining the effectiveness of inhaled steroids on preventing remodeling, Kai et al. recently investigated collagen synthesis [199]. The authors measured sputum levels of procollagen type I C-terminal peptide (PICP), a marker of ongoing collagen synthesis, in 25 normal volunteers and 25 steroid-naive asthmatics, who immediately began steroid treatment and were followed up one month later [199]. Asthmatics exhibited increased PICP, relative to controls, consistent with a previous study by the same authors. While treatment with inhaled corticosteroids improved the FEV1, and decreased sputum eosinophil counts, TGF-β+ cells and sputum PICP concentrations, treatment did not normalize PICP concentrations to the level of controls, suggesting ongoing airway remodeling in steroid-treated asthmatics [200].

The effect of steroids on arginase expression in human asthma is currently unclear. There is some evidence that increased arginase expression can be induced by steroids, such as in the digestive tract of piglets treated with glucocorticoids [201]. Dexamethasone has also been found to increase arginase activity when administered to rescue mice from endotoxemia [202]. Whereas, in rat airway fibroblasts, dexamethasone has been reported to inhibit the IL-4/IL-13 induced upregulation of arginase [38]. However, dexamethasone was also reported to have no effect on endotoxin/TNFα-induced upregulation of arginase in bovine pulmonary arterial endothelial cells [203]. More studies on the effects of corticosteroid treatment on arginase expression in asthma are needed, but a recent study by Lara et al., provides some information. In this study of severe asthmatics, moderate asthmatics and healthy controls, the severe asthmatics exhibited increased serum arginase activity, which was related inversely to airflow, despite the fact that 95.2% of these patients were being treated with inhaled corticosteroids, and 38.1% with systemic corticosteroids [114]. Thus, there is significant potential for new therapies to target arginase, as current treatments do not appear to attenuate the activity of this pathway or prevent remodeling.

FUTURE DIRECTIONS

Increased arginase activity importantly contributes to the development of key processes of allergic asthma, such as AHR, airway inflammation and – possibly – remodeling. Although some conflicting results have been reported, most studies investigating the effect in vivo inhibition of arginase demonstrate a beneficial effect of arginase inhibitors on AHR and airway inflammation. Therefore, arginase inhibitors may have therapeutic potential in the treatment of allergic asthma. Future experiments are warranted to elucidate the specific role the two arginase isoforms in disease pathogenesis and in homeostasis. To this aim, further investigation of new, more selective inhibitors and methods of administration that will minimize non-specific effects (i.e., inhibition of arginase in extrapulmonary tissues) as well as the use of conditional knockout animals are needed.

ACKNOWLEDGEMENTS

This work was supported by the Netherlands Asthma Foundation (grant 00.24), the Schering-Plough Research Institute, Oss, The Netherlands, the Ontario Thoracic Society and the AllerGen NCE. MN is supported by Ontario Graduate Scholarship in Science and Technology and Canadian Institutes of Health Research Frederick Banting and Charles Best Doctoral Award.

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