Abstract

Recent studies suggest that alterations of the arginine metabolome and a dysregulation of nitric oxide (NO) homeostasis play a role in the pathogenesis of asthma. L-Arginine, a semi-essential amino acid, is a common substrate for both the arginases and NO synthase (NOS) enzyme families. NO is an important vasodilator of the bronchial circulation, with both bronchodilatory and anti-inflammatory properties, and is synthesized from oxidation of its obligate substrate L-arginine, which is catalyzed by a family of NOS enzymes. Arginase is an essential enzyme in the urea cycle, responsible for the conversion of arginine to ornithine and urea. The NOS and arginase enzymes can be expressed simultaneously under a wide variety of inflammatory conditions, resulting in competition for their common substrate. Although much attention has been directed towards measurements of exhaled NO in asthma, accumulating data show that low bioavailability of L-arginine also contributes to inflammation, hyperresponsiveness and remodeling of the asthmatic airway. Aberrant arginine catabolism represents a novel asthma paradigm that involves excess arginase activity, elevated levels of asymmetric dimethyl arginine, altered intracellular arginine transport, and NOS dysfunction. Addressing the alterations in arginine metabolism may result in new strategies for treatment of asthma.

Introduction

Asthma is a common pulmonary condition that involves heightened bronchial hyperresponsiveness and reversible bronchoconstriction together with acute-on-chronic inflammation that leads to airway remodeling [1]. Over 22 million people in the United States have asthma including more than 6.5 million chil-
dren, and as many as 250 million worldwide are affected. Mechanisms that contributed to asthma are complex and multi-factorial, influenced by genetic polymorphisms as well as environmental and infectious triggers. In susceptible individuals, this inflammation causes recurrent episodes of wheezing, breathlessness, chest tightness, and coughing, particularly at night or in the early morning. These episodes are usually associated with widespread but variable airflow obstruction that is often reversible either spontaneously or with treatment. The inflammation also causes an associated increase in the existing bronchial hyper-responsiveness to a variety of stimuli [2].

‘Asthma’ is a clinical diagnosis based on a constellation of symptoms described above, yet asthma is not one disease. Different patients have biochemically distinct phenotypes despite a similar clinical manifestation [3]. Examples include decreased activity of superoxide dismutases, increased activity of eosinophil peroxidase, S-nitrosoglutathione reductase, decreased airway pH, and finally alterations of the arginine metabolome. Low plasma arginine concentration together with increased activity of the arginase enzymes, elevated levels of asymmetric dimethylarginine (ADMA), altered intracellular arginine transport, and nitric oxide synthase (NOS) dysfunction, including endogenous NOS inhibitors and uncoupled NOS can contribute to arginine dysregulation in asthma and is the focus of this discussion.

Asthma: Global Disruption of the Arginine-Nitric Oxide Pathway

Altered Nitric Oxide Homeostasis

Nitric oxide (NO) has been well described in the literature as an important signaling molecule involved in the regulation of many mammalian physiologic and pathophysiologic processes, particularly in the lung [4]. NO plays a role in regulation of both pulmonary vascular tone as well as airway bronchomotor tone through effects on relaxation of smooth muscle. In addition, NO participates in inflammation and host defense against infection via alterations in vascular permeability, changes in epithelial barrier function and repair, cytotoxicity, upregulation of ciliary motility, altered mucus secretion, and inflammatory cell infiltration [5]. These multiple functions of NO have been implicated in the pathogenesis of chronic inflammatory airway diseases such as asthma.

NO is produced by a family of NOS enzymes that metabolize L-arginine through the intermediate N-hydroxy-L-arginine (NOHA) to form NO and L-citrulline using oxygen and NADPH as cosubstrates. Three NOS mammalian isoenzymes have been identified with varying distributions and production of
Neuronal (nNOS or NOS I) and endothelial (eNOS or NOS III) NOS are constitutively expressed (cNOS) in airway epithelium, inhibitory nonadrenergic noncholinergic (iNANC) neurons, and airway vasculature endothelial cells. Their activity is regulated by intracellular calcium, with rapid onset of activity and production of small amounts of NO on the order of picomolar concentrations. Inducible NOS (iNOS or NOS II) is transcriptionally regulated by proinflammatory stimuli, with the ability to produce large amounts (nanomolar concentrations) of NO over hours [5].

iNOS is known to be upregulated in asthmatic lungs, and increased levels of exhaled NO are well described in asthma patients. In both human and experimental animal models of asthma, increased NO production occurs in the airways related to upregulation of NOS II (iNOS) by proinflammatory cytokines after allergen challenge and during the late asthmatic reaction [4]. This upregulation of NOS II in airway epithelial cells and inflammatory cells is associated with airway eosinophilia, airway hyperresponsiveness (AHR), and increased NO in exhaled air [6]. Although initially assumed to contribute to asthma, the increased production of NO itself may not be responsible for AHR as NO also seems to have a protective effect on bronchial muscle tone. It is believed that the AHR after the late asthmatic reaction is caused by increased formation of peroxynitrite [7] that occurs due to reduced availability of L-arginine for NOS II, which potentially causes uncoupling of this enzyme [8]. Increased activity of the arginase enzyme, which competes with NOS for the substrate L-arginine seems to be, at least in part, responsible for this process [9].

Mechanisms of Arginine Dysregulation

As the obligate substrate for NOS, L-arginine bioavailability plays a key role in determining NO production, and is dependent on pathways of biosynthesis, cellular uptake, and catabolism by several distinct enzymes (fig. 1), including those from the NOS and arginase enzyme families. Biosynthesis of the semi-essential amino acid occurs in a stepwise fashion in what is called the ‘intestinal-renal axis’. L-Glutamine and L-proline are absorbed from the small intestine and converted to L-ornithine. L-Citrulline is then synthesized from L-ornithine by ornithine carbamoyltransferase and carbamoylphosphate synthetase 1 in hepatocytes as part of the urea cycle, as well as in the intestine. L-Arginine is produced from L-citrulline by cytosolic enzymes argininosuccinate synthetase 1 and argininosuccinate lyase. When L-arginine is subsequently metabolized to NO via NOS, L-citrulline is again produced and can be used for recycling back to L-ar-
arginine, which may be an important source of L-arginine during prolonged NO synthesis by iNOS [10]. Low arginine bioavailability develops abruptly during acute asthma exacerbations and normalizes with clinical recovery [11]. In severe asthma, however, low arginine bioavailability at baseline is strongly associated with airflow abnormalities [12]. Overlapping mechanism that contribute to arginine depletion in asthma are summarized below.

**Increased Arginase Concentration and Activity**

Arginase is an essential enzyme in the urea cycle, responsible for the conversion of arginine to ornithine and urea. The NOS and arginase enzymes can be expressed simultaneously under a wide variety of inflammatory conditions, resulting in competition for their common substrate [9]. Two forms of arginase have been identified, type 1, a cytosolic enzyme highly expressed in the liver, and type 2, a mitochondrial enzyme found predominantly in the kidney, prostate, testis, and small intestine [13]. Both forms are expressed in human airways. Arginase-1 is also present in human red blood cells, which has significant implications for hemolytic disorders. Of particular interest is the high prevalence of asthma in sickle cell disease [14], a hemolytic anemia also associated with an altered arginine metabolome (fig. 2) [15, 16].
Altered arginine metabolism in hemolysis. A path to pulmonary dysfunction. Dietary glutamine serves as a precursor for the de novo production of arginine through the citrulline-arginine pathway. Arginine is synthesized endogenously from citrulline primarily via the intestinal-renal axis. Arginase and NOS compete for arginine, their common substrate. In sickle cell disease (SCD) and thalassemia, bioavailability of arginine and NO are decreased by several mechanisms linked to hemolysis. The release of erythrocyte arginase during hemolysis increases plasma arginase levels and shifts arginine metabolism towards ornithine production, limiting the amount of substrate available for NO production. The bioavailability of arginine is further diminished by increased ornithine levels because ornithine and arginine compete for the same transporter system for cellular uptake. Despite an increase in NOS, NO bioavailability is low due to low substrate availability, NO scavenging by cell-free hemoglobin released during hemolysis, and through reactions with free radicals such as superoxide and other reactive NO species. Superoxide is elevated in SCD due to low superoxide dismutase activity, high xanthine oxidase activity and potentially as a result of uncoupled NOS in an environment of low arginine and/or tetrahydrobiopterin concentration or insufficient NADPH. Endothelial dysfunction resulting from NO depletion and increased levels of the downstream products of ornithine metabolism (polyamines and proline) likely contribute to the pathogenesis of lung injury, pulmonary hypertension and asthma in SCD. This model has implications for all hemolytic processes as well as pulmonary diseases associated with excess arginase production. This novel disease paradigm is now recognized as an important mechanism in the pathophysiology of SCD and thalassemia. Abnormal arginase activity emerges as a recurrent theme in the pathogenesis of a growing number of diverse pulmonary disorders. Regardless of the initiating trigger, excess arginase activity represents a common pathway in the pathogenesis of asthma and pulmonary hypertension. Reproduced with permission from the American Society of Hematology [16].
While the affinity (Km) of L-arginine for arginase is in the low micromolar range compared to the low millimolar range for NOS, substrate competition does occur between arginase and NOS because the V_max of arginase is 1,000-fold higher [13]. As arginase plays a role in regulating bioavailability of L-arginine for NOS by competitive consumption of the substrate, increased arginase activity may be responsible in part, for the AHR in asthma. In allergen-challenged mice, arginase activity is increased in the airways at the same time as L-arginine and L-citrulline levels are decreased [17]. Specific arginase inhibitor N-hydroxy-nor-L-arginine (nor-NOHA) has been shown to attenuate methacholine-induced constriction of guinea pig trachea and to increase iNANC-mediated relaxation of tracheal smooth muscle preparations, which is consistent with increased NO production through NOS under conditions of arginase inhibition [18, 19]. This effect was prevented by coincubation with NOS inhibitor N^G^-nitro-L-arginine methyl ester (L-NAME), indicating that arginase leads to AHR by decreasing cNOS-derived NO production [20]. iNANC nerve-mediated NO production and smooth muscle relaxation are also restored after the EAR by treatment with nor-NOHA to a similar level also seen with L-arginine supplementation [21]. Another specific arginase inhibitor [2(S)-amino-6-boronohexanoic acid or ABH] not only reverses AHR after both the early and late asthmatic reaction following histamine challenge in a guinea pig model of acute allergic asthma, but also prevents AHR when delivered 30 min prior to the histamine challenge, most likely related to increased NO production [22]. Similarly, intraperitoneal treatment with nor-NOHA prior to repeated allergen challenge reduced AHR to methacholine in mice [23]. In contrast, another study found that in mice sensitized to ovalbumin, arginase inhibitor S-(2-boronoethyl)-L-cysteine increased peribronchiolar and perivascular inflammation associated with increased S-nitrosothiols and 3-nitrotyrosine, but did not change allergen-induced increases in differential cell counts or cytokine levels in bronchoalveolar lavage (BAL) samples [24]. Unfortunately, the role of low arginine bioavailability and NOS uncoupling as a plausible contributing factor to excess superoxide production in this model is unknown. Finally, in chimeric mice with arginase I–/– bone marrow, no change was seen in basal or allergen-induced inflammatory cell infiltration or BAL differential cell counts, indicating that at least bone-marrow derived arginase I is not required for development of lung inflammation in this mouse model [25].

It is evident that the arginine metabolome involves a complex system of checks and balances to maintain homeostasis. NOS itself can inhibit arginase activity through accumulation of NOHA, the intermediate in NO synthesis [26]. The arginase product L-ornithine may also play a role in regulating availability of L-arginine to NOS through competitive inhibition of arginase [27] as well as
Arginine inhibition of L-arginine intracellular transport. L-Ornithine also serves as a substrate for ornithine decarboxylase, which synthesizes polyamines involved in promotion of cell growth and repair, and for ornithine aminotransferase, leading to formation L-proline which is required for collagen synthesis.

The balance of iNOS versus arginase activity and level of NO production in the airway may be related to the balance of TH1/TH2 cytokines during the inflammatory cascade. Experimental models of asthma have demonstrated that iNOS is induced by proinflammatory TH1 cytokines released from mast cells immediately after allergen challenge and during the late asthma reaction, an intense IgE-mediated inflammatory response that begins several hours after an allergen challenge [4, 28, 29]. Arginase activity is induced (and iNOS suppressed) by TH2 cytokines IL-4 and IL-10 in murine macrophages, although IL-4 does not induce arginase in human macrophages unless combined with agents that increase cAMP [30]. Arginase activity increases following challenge with allergens in guinea pig tracheal preparations, and in mouse and rat models of allergic asthma [9, 31–34]. Inducing loss of function of arginase 1 specifically in the lung of an allergic asthma mouse model using RNA interference abolished the development of TH2 cytokine IL-13-induced AHR [35]. Gene expression studies have also shown induction of arginase 1 more than arginase 2 gene expression in mouse models of allergen-challenged lungs and TH2 cytokine-mediated lung inflammation [35–38]. However, pollutant particles were recently shown to induce arginase 2 in human bronchial epithelial cells [39]. Interestingly, DNA methylation of arginase 2 and to a lesser extent, arginase 1 is significantly associated with FeNO in both asthmatic and nonasthmatic children, whereas DNA methylation of NOS genes is surprisingly not associated with FeNO [40]. These findings highlight the complexity of interactions between arginase and NOS on NO production.

Studies in human asthma confirm the importance of arginase in the pathogenesis of experimental asthma. While increased arginase activity in the sputum of asthmatic patients was documented as early as 1980 [41], its role in the pathophysiology of asthma was not further elucidated until decades later. Increased arginase I activity, mRNA and protein expression have been demonstrated in inflammatory cells and airway epithelium from bronchial biopsies as well as BAL samples from asthmatic patients [31, 42]. Single nucleotide polymorphisms (SNPs) in both arginase I and arginase II have been associated with atopy, while SNPs in arginase II were associated with increased risk of childhood asthma [43]. Increased arginase activity has also been demonstrated in the serum of asthmatic children experiencing an exacerbation, while plasma L-arginine levels and the arginine/ornithine ratio (a biomarker that inversely correlates with arginase activity) [11, 15, 44] were simultaneously reduced [11]. Clinical improve-
ment in asthma symptoms corresponded temporally with reduction of arginase activity and increase in plasma L-arginine levels and the arginine/ornithine ratio [11]. The lung function of severe asthmatics correlates directly with L-arginine bioavailability, and inversely with serum arginase activity [12]. Arginase may also play a role in the development of chronic airway remodeling through formation of L-ornithine with downstream production of polyamines and L-proline, which are involved in processes of cellular proliferation and collagen deposition [45].

**Intracellular Arginine Transport: Role of Cationic Amino Acid Transporter**

The primary source of L-arginine for most cells is cellular uptake via the Na-independent cationic amino acid transporter (CAT) proteins of the y⁺-system. In particular, upregulation of CAT-2B has been associated with increased L-arginine uptake under conditions of iNOS induction stimulated by proinflammatory mediators [46–48]. This suggests that, at least in some cells, increased uptake may offset reductions in circulating arginine levels. Ablation of the CAT-2 gene is associated with impaired iNOS-mediated NO synthesis in macrophages and astrocytes, which implies an important role of CAT-2 in uptake of L-arginine substrate for iNOS [49, 50].

L-Arginine uptake via the y⁺-system can be inhibited by other amino acids such as L-ornithine and L-lysine, as well as by polycations such as eosinophil-derived major basic protein (MBP) and poly-L-arginine [46, 51]. MBP inhibition of L-arginine uptake was associated with decreased NO synthesis in rat alveolar macrophages and tracheal epithelial cells, most likely related to reduced L-arginine availability [48]. In addition, AHR to methacholine has been shown to increase in rats and guinea pigs after treatment with poly-L-arginine, related to attenuation of epithelial NO production. Treatment with combined poly-L-arginine and the antagonist polyanion heparin restored L-arginine uptake and NO production, and reversed AHR [52, 53].

**Uncoupled Nitric Oxide Synthase**

Airway inflammation in asthma may not be the result of increased NO production itself, but rather due to the formation of the proinflammatory oxidant peroxynitrite from reaction of NO with superoxide anions in the airway. Peroxynitrite activates eosinophils, increases microvascular permeability, induces airway epithelial damage, and augments airway smooth muscle contraction [54, 55].
Airway epithelial cells and inflammatory cells from bronchial biopsies of asthmatics as well as allergen-challenged guinea pigs demonstrate increased nitrotyrosine immunostaining (a marker for peroxynitrite nitration of protein tyrosine), which is also correlated with increased exhaled NO, iNOS expression, AHR, and eosinophilic inflammation [56]. The AHR observed after allergen challenge and the late asthmatic reaction may be the result of increased peroxynitrite formation [54, 57]. Further evidence for this relationship comes from the lungs of allergen-challenged mice which demonstrate increased nitrotyrosine staining and concomitant increased expression of arginase and iNOS [34].

In contrast to the increased NO production seen during the late asthmatic reaction, the increased AHR seen after the early asthmatic reaction may paradoxically involve NO deficiency within the airways related to reduced bioavailability of L-arginine to both cNOS and iNOS. Low L-arginine conditions may also lead to increased production of peroxynitrite by uncoupling iNOS, allowing it to produce superoxide anions via its reductase domain, which react with NO to form peroxynitrite [58]. Increasing L-arginine availability increases NO production and decreases superoxide and peroxynitrite production in macrophages. This model helps to explain the paradox of reduced NO bioavailability in the face of increased expression of iNOS in asthma.

**Endogenous Nitric Oxide Synthase Inhibitors**

ADMA is an endogenous NOS inhibitor that competes with L-arginine for binding to NOS. Well established as a biomarker of cardiovascular disease and endothelial dysfunction [16], it may also contribute to inflammation, collagen deposition, nitrosative stress and abnormal lung function in asthma. High levels of ADMA together with symmetric dimethylarginine (SDMA) were found in a mouse model of allergic asthma as well as in human lung and sputum samples. Endogenous administration of nebulized inhaled ADMA to naive control mice at doses consistent with levels observed in the allergic inflamed lungs of the mouse model resulted in augmentation of AHR in response to metacholine [59]. In contrast, Riccioni et al. [60] recently reported low levels of ADMA and SDMA together with low L-arginine in the plasma of children with a history of mild allergic asthma compared to healthy subjects. Although this may appear incongruent, these studies are evaluating concentrations compartmentalized in the lung and sputum versus plasma. Extracellular plasma levels may not necessarily reflect intracellular concentrations in different cell types, organs, compartments and species. Arginine metabolism in asthma is also quite different at baseline compared to an acute exacerbation when inflammatory mediators are upregulated.
In addition, inflammatory mechanisms contributing to asthma symptoms may vary not only from animal model to human, but also from patient to patient, particularly in those with mild versus severe asthma. Patients with ‘asthma’ are often studied in trials as if asthma was just one homogeneous disease, potentially explaining discrepant observations in these and other asthma models.

**The Arginine Metabolome: A Novel Therapeutic Target for Asthma**

Increased understanding of the role of arginase in the pathogenesis of asthma naturally leads to consideration of novel therapeutic targets for treatment. As noted above, animal models of specific arginase inhibition have demonstrated prevention or reversal of AHR associated with allergen challenge. Further development and study of inhaled arginase inhibitors may be a promising area of research.

Restoration of L-arginine bioavailability to NOS through exogenous supplementation of L-arginine is another potential therapeutic target, although a great deal of orally administered L-arginine is metabolized to urea in the liver. Supplemental oral or inhaled L-arginine increases exhaled NO in both normal and asthmatic subjects, indicating that the bioavailability of L-arginine for NOS determines NO production within the airways [61]. In guinea pig tracheal preparations, L-arginine has been shown to inhibit AHR to methacholine and to increase iNANC nerve-mediated airway smooth muscle relaxation via increased production of NOS-derived NO [18, 62]. Conversely, inhibition of NOS-derived NO by L-NAME amplifies bronchoconstriction in guinea pigs [63]. L-Arginine administration also reduces peroxynitrite formation, AHR, airway injury and mitochondrial dysfunction in murine allergic airway inflammation [64, 65].

A single inhalation of nebulized L-arginine results in a significant increase of airway NO formation and improved pulmonary function in patients with cystic fibrosis [66], prompting results that have not been documented in human asthma to date. Chambers and Ayres [67] found that nebulized L-arginine paradoxically induced bronchospasm in asthmatic adults; however, a similar effect was found with the use of 2% saline in the control arm. Other studies using murine asthma models found that arginine supplementation amplified the asthmatic inflammatory response [68, 69] while a small study of 14 patients with asthma found that pretreatment with oral L-arginine (50 mg/kg) had no significant influence on AHR to histamine [70]. The same dose given twice a day for 3 months to 15 patients completing a randomized placebo-controlled trial had no impact on number of exacerbations, exhaled NO levels or lung function compared to placebo in patients studied with moderate to severe persistent asthma [71].
The few human studies that evaluate arginine therapy for asthma are all limited by small sample size and preliminary results are disappointing. The utility of arginine therapy in human asthma may be limited by excess arginase found in plasma [11], skewing metabolism away from NO towards ornithine and its downstream metabolites, proline and polyamines. Combination therapy utilizing L-arginine with an arginase inhibitor may circumvent this issue. Alternatively, L-citrulline or L-glutamine (converted to citrulline by the enterocytes) could be administered as a prodrug for L-arginine, as citrulline is converted in the kidney to arginine through the ‘intestinal-renal axis’ [10], thus bypassing liver metabolism by arginase. Interestingly, citrulline supplementation in a pilot phase II clinical trial in sickle cell patients resulted in an increase in plasma L-arginine levels [72]. Preliminary results of pharmacokinetic studies using oral L-glutamine have also demonstrated improvement in global arginine bioavailability in patients with sickle cell disease and pulmonary hypertension risk [73].

Contradictory studies of arginase inhibition also exist, reporting enhancement, attenuation, and no effect on inflammation in animal models [22–25]. This may reflect issues specific to animal models of asthma in general that often limit our understanding and treatment of asthma [74]. Since chronic asthma is a disease unique to humans, the fact that mice do not have asthma may contribute to the conflicting reports that make the mechanistic translation to human disease more of a challenge. Further studies are needed to clarify these effects and their implications in man. Understanding the biochemistry behind mechanistic variants of asthma may help identify unique subpopulations of patients with asthma and lead to novel therapies that target these pathways. The arginine metabolome represents one such pathway in asthma that holds promise for future drug development.

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Disclosure Statement

Claudia R. Morris, MD, declares no conflicts of interest, but discloses that she is the inventor or coinventor of several patents or pending patents owned by Children’s Hospital & Research Center Oakland, some which involve therapies and biomarkers of cardiovascular disease that target global arginine bioavailability, has served on scientific advisory committees for Merck and Icagen, received an educational stipend from INO Therapeutics, and has been a consultant for Biomarin, Gilead Sciences Inc., and the Clinical Advisors Independent Consulting Group.
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