

Mouse Models of Asthma: Can They Give Us Mechanistic Insights into the Role of Nitric oxide?

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Abstract: New clinical practice guidelines for patients with asthma include the recommendation to monitor exhaled breath nitric oxide (NO) levels. NO concentrations in exhaled breath are increased in asthmatics and increased NO levels correlate with worsening airway inflammation and asthma symptoms. The multiple roles of NO in the lung have not been delineated clearly. Clinical trials are being performed presently that test the apparently conflicting hypotheses that either donors or inhibitors of NO in the lung are effective strategies for treating asthma. These strategies evolved, in part, from results of pre-clinical studies performed in mice and other animal models.

This review evaluates the existing literature with regard to mouse models of asthma and explores the often conflicting data on the role of NO, the nitric oxide synthase (NOS) enzymes, and the arginase enzymes in allergic airway inflammation. While we will emphasize the ovalbumin exposure mouse model, we will also examine other models. Where inconsistencies are identified among the studies, we attempt to determine whether such inconsistencies arise from methodological differences or alternative mechanisms. Ultimately, we address whether the allergen-exposed mouse is a suitable model for identifying promising new drugs for the treatment of human asthma. While a consensus is building that NO is beneficial or protective in subsets of asthmatics, results from studies using mouse models to investigate the individual roles of NO and the NOS enzymes in airway inflammation are often contradictory. Further research efforts with this model will allow us to distinguish which asthma patients may benefit best from NO donors and which may benefit from NO inhibitors.

INTRODUCTION

Interest in the potential monitoring and therapeutic roles of NO in patients with asthma has increased significantly among both researchers and clinicians. High levels of NO are detected in the exhaled breath of asthmatics and increasing NO levels correlate with worsening symptoms. There has been considerable debate regarding the pro- and anti-inflammatory effects of endogenously produced NO in the airway, and this has spurred differing hypotheses about the role of NO in asthma pathogenesis. Pre-clinical studies that attempt to either block or enhance the effects of nitric oxide in the lung have shown seemingly contradictory effects on airway inflammation and airway physiology. Interpretation of these results has been difficult and translation of the results to large clinical trials is arguably premature.

In this review, we have identified more than 50 animal studies that attempt to delineate the roles of NO, the three NOS enzymes, and the arginase enzymes in the pathogenesis of airway inflammation and hyper-responsiveness. We address the limitations of the animal models and attempt to answer the question of how these collective studies have furthered our understanding of the role of NO in the airway. Finally, we address the potential biochemical targets in the nitric oxide pathway that might lead to a better pharmacological approach to treating asthma. In doing so, we present new results from our laboratory utilizing the ovalbumin model in mice, including experiments that test the efficacy of compounds such as N-hydroxy-L-arginine, thalidomide, and aminoguanidine.

THE ASTHMA PROBLEM AND A CLINICAL ROLE FOR NO

Asthma is a progressive, inflammatory airways disease that leads to debilitating symptoms in many patients and structural airway changes in most. Its prevalence has increased

dramatically and it now affects an estimated 26 million people in the United States. For adults, asthma accounts for 13.9 million office visits, 500,000 hospitalizations and 2 million emergency room visits per year. Four to five thousand Americans die from asthma annually; the risk of death is highest among African Americans, women and the elderly. The annual cost for asthma is nearly \$15 billion in the U.S., and eighty percent of the direct costs of asthma come from 20% of these patients who have difficult-to-control asthma (National Center for Health Statistics, 2002). Monitoring of patients with difficult-to-control asthma is notoriously difficult. An easily measurable, non-invasive biomarker that correlates with patient outcomes is needed. Exhaled NO represents the best candidate biomarker to become available in the past thirty years.

Numerous studies have shown that the concentration of NO in exhaled gas correlates with airway wall inflammation [1,2], sputum eosinophil counts, and airway responsiveness to methacholine [3,4]. NO is produced when L-arginine is converted to L-citrulline by the nitric oxide synthases. Production of NO may be regulated by the amounts of nitric oxide synthase available and by the availability of arginine. (Fig. 1) In the lung, the inducible isoform of nitric oxide synthase (iNOS, NOS2) has been of particular interest in models of allergic lung inflammation due to its rapid induction shortly after insult and its ability to generate and sustain nanomolar concentrations of NO in the airways for hours to days [5]. Alveolar type 2 epithelial cells, fibroblasts, smooth muscle cells, mast cells, endothelial cells, macrophages and neutrophils are among the cells types reported to express iNOS in the respiratory tract [6]. Neuronal nitric oxide synthase (nNOS, NOS1) and endothelial nitric oxide synthase (eNOS, NOS3) are the two constitutive isoforms of NOS (cNOS). The eNOS isoform is found in bronchial and large pulmonary blood vessels, alveolar capillaries, and in the pulmonary epithelium. The nNOS isoform is found in the non-adrenergic non-cholinergic nerves (NANC) and the pulmonary epithelium. It is not certain whether the activity of a specific NOS isozyme or the presence of nitric oxide itself contributes to the pathology of airway disease. Advances in the understanding of the multiple roles of NO and the NOS enzymes in the lung have, as with most mediators and receptors, come from studies in animal models. The

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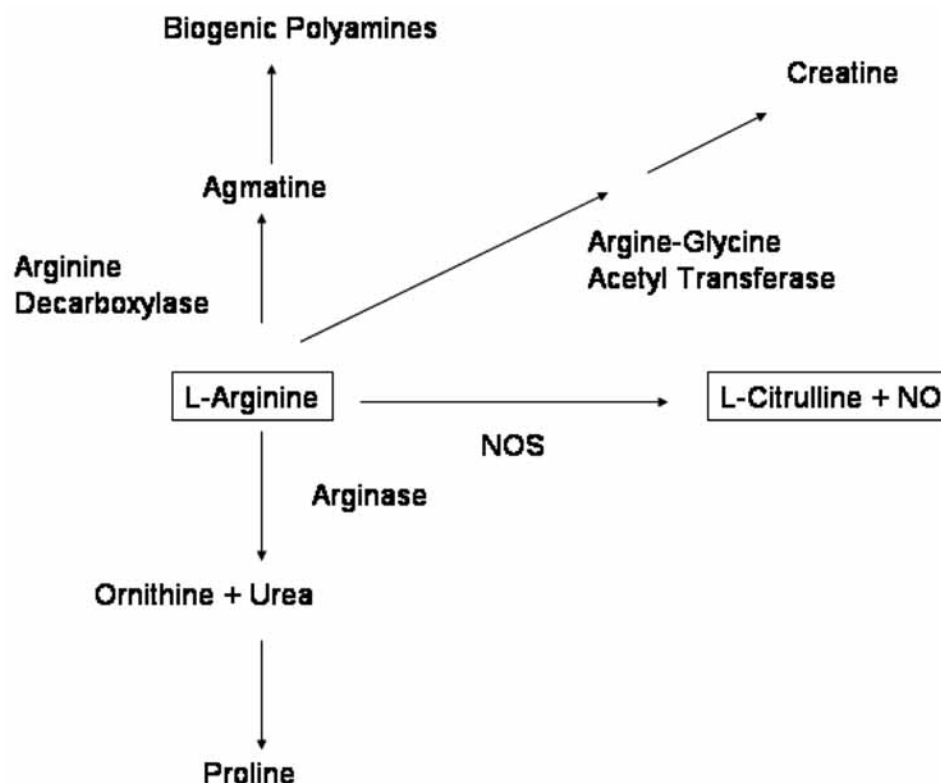


Fig. (1). The primary metabolic pathways of L-arginine.

contribution of each of the three NOS isoforms has been studied in the ovalbumin exposure mouse model, either by testing specific NOS inhibitors or using knockout strains, or both.

INDUCIBLE NITRIC OXIDE SYNTHASE-THE PRIMARY CULPRIT IN THE AIRWAY

The ovalbumin sensitization and exposure model is the most common mouse model of allergic airway inflammation. Protocols used vary widely, including variations in the sensitization adjuvant and timing, the ovalbumin dose, the number of exposures, the route of administration (aerosol or nasal), and the timing of exposures to ovalbumin aerosol [7,8]. Despite the variations, bronchoalveolar lavage fluid from mice exposed to ovalbumin consistently contains a large number of eosinophils and lymphocytes, as well as elevated levels of different Th2 cytokines. These mice also exhibit airways hyperresponsiveness (AHR) and reversible airflow obstruction, two prominent features of human allergic asthma [9,10]. Between 4 to 8 weeks of ovalbumin exposure, airway remodeling is present and this may reflect the structural airway changes in human asthma over a much longer time scale [9,11]. The BALB/c mouse strain is used frequently in these studies due to the strain's disposition towards an IgE- and Th2-mediated immune response to antigen challenge. Gene knockout and overexpression experiments, however, are often performed in less responsive mouse strains, e.g. C57BL/6, and significant variation exists in the inflammatory response, AHR, and airway remodeling among mouse strains.

Temelkovski *et al.* and our lab have demonstrated several rapid changes in the lungs of sensitized BALB/c mice exposed to 1% ovalbumin aerosols [9,10]. In BALB/c mice, we observe peak inflammation in the lungs following 1-2 weeks (3-6 exposures) of ovalbumin challenge, where the vast majority of these infiltrated leukocytes are eosinophils. We find airway

submucosal fibrosis and significantly increased airway collagen content after 4 weeks (12 exposures) of ovalbumin challenge in this susceptible mouse strain. Mucous cell hyperplasia, increased exhaled nitric oxide, and increased airway reactivity upon challenge with methacholine are also observed [12]. Several laboratories demonstrated induction of iNOS in mice exposed to ovalbumin (Table 1). Koarai *et al.* have demonstrated increased iNOS protein in ovalbumin-challenged male BALB/c mice [13]. Increased iNOS was found also in the airway epithelia and inflammatory leukocytes in challenged BALB/c mice and A/J mice [14,15]. Feder and colleagues, however, did not find more iNOS in the airways of B6D2F1/J mice exposed for 24 hours after two 1-hour periods of ovalbumin-challenge [16]. The Feder study, however, did report ovalbumin-induced eosinophilia in the lungs of challenged mice, which was attenuated by treatment with L-NAME (N-Nitro-L-arginine methyl ester), aminoguanidine, and NMMA (NG-monomethyl-L-arginine). L-arginine abolished the anti-inflammatory effect of these NOS inhibitors. In another study, L-NIL (L-N-(1-iminoethyl)lysine), a specific iNOS inhibitor, had no effect on airway eosinophilia in ovalbumin exposed mice, though its prodrug SC-51 did attenuate lung eosinophil and CD4⁺ T cell counts in sensitized Brown Norway rats [17]. A number of investigators have found that L-NAME (nonselective) and aminoguanidine (only moderately selective for iNOS) reduce inflammatory cell counts in the airways of mice, regardless of strain [15-18].

It is difficult to interpret studies of NOS inhibition when non-specific agents are used. The observation of very high structural homology and stereochemical orientation between the haem domains of iNOS and eNOS raised doubts over whether selective inhibition of either isoform was feasible [19]. Early inhibitors of NOS were designed to be analogs of the substrate L-arginine (e.g. L-NAME, L-NIO (N-(1-imino-3-butenyl)-L-orinithine), L-NIL). L-NIO and L-NIL (acetamidine-containing

Table 1. Recent Animal Model Studies that Investigate the Role of the NO and the NOS Enzymes in the Lung (2000-2006)

Author	Year	NOS Enzyme	Mouse strain	Non-Mouse	KO vs NOS Inhibitor	Conclusions
Iijima H <i>et al.</i>	2005	nNOS, iNOS	C57BL/6J	x	nNOS KO, <i>ex vivo</i>	(1) nNOS is associated w/the development of allergic airway inflammation following specific antigen challenge. (2) nNOS like iNOS is inducible in murine airways. (3) Mice deficient in nNOS had less BAL eosinophils and less NO production.
Steudel <i>et al.</i>	2000	nNOS, iNOS, eNOS	B6129/F1	x	KO & L-NAME (non-selective inhibitor)	(1) The absence of nNOS or eNOS increased baseline exhaled NO suggesting the up-regulation of a iNOS-isoform. (2) iNOS is responsible for NO exhalation in a healthy state. (3) nNOS and eNOS are not responsible for baseline NO production in iNOS KO mice.
Feletou <i>et al.</i>	2001	eNOS	CD-1, C57BL/6	x	eNOS KO	(1) In murine lungs, the 3 isoforms are constitutively expressed. (2) The origin of endogenous NO production is unlikely to be iNOS. (3) eNOS KO induces airway hyperresponsiveness, suggesting that NO derived from eNOS is protective.
Hasaneen <i>et al.</i>	2003	nNOS	C57BL/6	x	nNOS KO, <i>ex vivo</i>	(1) Endogenous NO produced by nNOS is an important transmitter of the iNANC (i.e. relaxant) system. (2) nNOS contributes to the normal relaxant state of airway smooth muscle.
Ten Broeke <i>et al.</i>	2006	eNOS	FVB, C57BL/6	x	Transgenic mice that overexpress eNOS	(1) eNOS plays a crucial role in both AHR and airway inflammation. (2) NO derived from eNOS might be involved in the attenuated production of both Th1 & Th2 cytokines, resulting in diminished airway inflammation and AHR.
Kobayashi <i>et al.</i>	2006	eNOS	C57BL/6J (Th1 strain)	x	Transgenic mice that overexpress eNOS	(1) Overexpressed eNOS-derived NO directly suppressed adhesion molecule expression & reduced the inflammatory changes in transgenic OVA mice. (2) eNOS overexpression inhibits OVA induced eosinophil inflammation by downregulating adhesion factors.
Takano <i>et al.</i>	1999	cNOS	ICR mice	x	L-NAME & aminoguanidine	(1) NO from iNOS may participate in the pathogenesis of DEP-induced airway inflammation. (2) cNOS derived NO may play a protective role in DEP-induced airway inflammation. (3) The pro-inflammatory of iNOS-derived NO overcame the cNOS-derived NO protective effect
Greene <i>et al.</i>	2005	nNOS, iNOS, eNOS	Female BALBc mice (BALB/cAnN Hsd)	x	N/A	(1) OVA-induced asthma leads to increased expression of Arg1 and Arg2, decreased NO production, & increased ornithine. (2) OVA & TMA present different gene expressions although their biological phenotypes are similar.
Eynott <i>et al.</i>	2002	nNOS, iNOS, eNOS	x	Brown-Norway rats	iNOS inhibitor SC-51 (given orally)	(1) Exhaled NO from allergen-provoked rats is derived from iNOS. (2) iNOS derived NO is important in modulating bronchial hyperresponsiveness without an effect on eosinophilic inflammation. (3) nNOS/eNOS derived NO at low levels may be acting as a functional antagonist of MCh-induced bronchoconstriction.
Chiba <i>et al.</i>	2000	cNOS	x	male Wistar rats	L-NMMA (applied separately to tissue preparations)	(1) Total NOS activities are mainly derived from the epithelial &/or endothelial eNOS. (2) The reduction of NO release by downregulation of cNOS (eNOS & nNOS) might contribute to the enhanced contractile response of AHR. (3) Induction of iNOS may account for the elevated total NOS activities and nitrite concentration in BAL in antigen-induced AHR.
Tulic <i>et al.</i>	2000	eNOS, nNOS, iNOS	x	male PVG rats	L-NMMA, aminoguanidine, L-NAME, S-MTC given subcutaneously (SC).	(1) nNOS inhibits AHR to MCh. (2) nNOS is constitutively expressed but not substrate limited. The NO produced may play a role in limiting parenchymal responses to MCh. (3) NO-mediated iNANC neurotransmission exaggerates bronchoconstriction, and that nNOS becomes substrate-limited.

(Table 1). contd.....

Author	Year	NOS Enzyme	Mouse strain	Non-Mouse	KO vs NOS Inhibitor	Conclusions
Birrell <i>et al.</i>	2003	cNOS, iNOS	x	male Sprague-Dawley rats, male BN rats, & male Wistar rats	(1) L-NAME & 1400W (given IP): in SD rats w/Sephadex, in BN rats with OVA. (2) 1400W in Wistar rats with LPS.	(1) 1400W failed to impact on either Sephadex- or antigen-induced airway eosinophilia, suggesting that activation of cNOS is responsible for the airway inflammatory responses mediated by NO. (2) cNOS (not iNOS) is the isoform involved in airway eosinophilia. (3) Activation of cNOS isoforms play a role in Sephadex- and OVA-induced airway inflammation.
De Boer <i>et al.</i>	2001	cNOS	x	Charles River SAVO guinea pigs	L-NAME (<i>in vitro</i>)	(1) NO is produced by iNOS, (2) cNOS-derived NO deficiency is a major determinant of AHR
Samb <i>et al.</i>	2001	nNOS	x	male Hartley guinea pigs	N/A	(1) nNOS activity was reduced after OVA challenge, (2) There is the absence of significant iNOS induction in this model. (3) nNOS is likely involved in the pathophysiology of asthma.
Prado <i>et al.</i>	2005	N/A	x	male Hartley guinea pigs	Chronic & Acute L-NAME treatment	(1) Chronic L-NAME treatment decreased mononuclear cell infiltration (2) NO may have a protective role in the airway remodeling process. (3) NO inhibition upregulates collagen deposition in the airway wall in this model of chronic allergic pulmonary inflammation.
Prado <i>et al.</i>	2006	iNOS, nNOS	x	male Hartley guinea pigs	(1) Chronic L-NAME treatment. (2) 1400W treatment.	(1) iNOS-derived NO increases AHR, inflammation, & collagen & elastic fiber content. (2) cNOS-derived NO bronchodilates airways & reduces airway collagen, suggesting a bronchoprotective effect.
Meurs <i>et al.</i>	2000	cNOS, Arginase	x	Dunkin Hartley guinea pigs	L-NAME	(1) cNOS-derived NO reduces AHR. (2) L-arginine is a limiting factor for NO production & antagonism of cholinergic airway tone by NO is governed by competition between arginase & cNOS for L-arginine. (3) Endogenous arginase activity potentiates cholinergic airway constriction by inhibition of NO production
Meurs <i>et al.</i>	2002	cNOS, Arginase	x	Harlan, Heathfield guinea pigs	L-NAME	(1) Absence of MCh-induced NO results in AHR. (2) Arginase activity contributes to the deficiency of NO & subsequent AHR. (3) Arginase could be involved in asthmatic airway remodeling. (4) Enhanced arginase activity may be involved in allergen-induced NO deficiency & AHR
Maarsingh <i>et al.</i>	2005	nNOS, Arginase	x	HsdPoc:Dunkin Hartley guinea pigs	Non-selective NOS inhibitor L-NNA	(1) Endogenous arginase activity restricts iNANC nerve-mediated (& the neural NO-mediated) airway smooth muscle relaxation, & it reduces generation of nNOS-derived NO. (2) nNOS-derived NO mediates iNANC tracheal relaxation. (3) Arginase activity attenuates iNANC nerve-mediated airway smooth muscle relaxation
Maarsingh <i>et al.</i>	2006	nNOS, Arginase	x	Dunkin Hartley guinea pigs	Non-selective NOS inhibitor L-NNA	(1) A deficiency of nNOS-derived NO underlies impaired iNANC relaxation. (2) Arginase activity attenuates iNANC nerve-mediated airway smooth muscle relaxation by limiting NO production. (3) Increasing L-arginine availability reversed impaired iNANC nerve-mediated airway smooth muscle relaxations
De Sanctis GT <i>et al.</i>	2005	iNOS, eNOS, nNOS	C57BL/6/J		KO	(1) iNOS is anti-inflammatory; no change in differential counts. (2) OVA-induced AHR unaffected by lack of iNOS
Duguet A, <i>et al.</i>	2001	iNOS	C57BL/6		KO	(1) iNOS is pro-inflammatory and pro-eosinophilic. (2) iNOS has no effect on tyrosine nitrosylation during inflammation
Eynott PR, <i>et al.</i>	2003	iNOS		Brown Norway Rat	SC-51 (L-NIL)	(1) iNOS proinflammatory for eosinophils and lymphocytes. (2) L-NIL prodrug decreases serum IgE
Gamba G, <i>et al.</i>	2004	iNOS	Male BALB/c		aminoguanidine	(1) iNOS pro-inflammatory, (2) iNOS suppresses humoral response to antigen
Iijima, H, <i>et al.</i>	2001	iNOS	Male A/J		1400W	(1c). iNOS is pro-eosinophilic and anti-neutrophilic (2) iNOS pro-oxidative

(Table 1). contd.....

Author	Year	NOS Enzyme	Mouse strain	Non-Mouse	KO vs NOS Inhibitor	Conclusions
Kenyon NJ, <i>et al.</i>	2003	iNOS	C57BL/6		KO	(1) iNOS anti-inflammatory and iNOS decreases AHR (2) iNOS is anti-fibrotic and anti-mucal
Koarai A. <i>et al.</i>	2000	iNOS	BALB/c		1400W	(1) iNOS pro-eosinophilic (2) iNOS increases AHR
Koarai A. <i>et al.</i>	2002	iNOS	C57Bl/6		KO	(1) iNOS has no effect on inflammation nor AHR
Komlosi ZI <i>et al.</i>	2006	iNOS	Female BALB/c		1400W	(1) iNOS has no effect on inflammation, (2) iNOS increases AHR
Landgraf RG <i>et al.</i>	2005	iNOS	C57Bl/6		aminoguanidine, KO, L-NAME	(1). iNOS inhibitor anti-inflammatory, (2) iNOS inhibitor increases AHR, (3) inhibition of endogenous iNOS decreases inflammation, total absence of iNOS by gene deletion has no effect
Muijsers RBR <i>et al.</i>	2001	iNOS	Male BALB/c		1400W	(1) iNOS increases AHR
Richard BR <i>et al.</i>	2001		BALB/c		1400W	(1) iNOS decreases AHR
Trifilieff, A <i>et al.</i>	2000	iNOS	BALB/c		AMT, EIT, L-NAME	(1) iNOS pro-inflammatory. (2) iNOS promotes Th-1 response

Definitions

AHR	=	airway hyperreactivity
BHR	=	bronchial hyperreactivity
cNOS	=	constitutive NOS (eNOS/iNOS)
FeNO	=	fraction exhaled NO
MCh	=	methacholine
ACh	=	acetylcholine
DEP	=	diesel exhaust particles
SOD	=	superoxide dismutase
Arg1, Arg2	=	arginase1 and arginase2
HM	=	hard metal
iNANC	=	inhibitory NANC (non-adrenergic non-cholinergic)
TDI	=	toluene diisocyanate
TMA	=	trimellitic anhydride
DPT	=	deoxypodophyllotoxin

analogues of arginine) have been regarded as partially selective inhibitors of iNOS versus nNOS or eNOS [20,21]. Newer inhibitors demonstrate a 50-fold increased selectivity for iNOS versus eNOS.

N-(3-(Aminomethyl)benzyl)acetamidine (1400W), identified by Garvey *et al.* [22], is a highly selective substrate-based inhibitor of iNOS over both nNOS and eNOS. It is capable of penetrating cells and tissues through competitive uptake with L-arginine *via* membrane cationic amino acid transporters. Inhibition of iNOS by 1400W is competitive with L-arginine, is NADPH-dependent and develops relatively slowly, with a likewise slow reversal indicative of a low K_d. Unfortunately, 1400W is not likely to have therapeutic use in humans due to its toxicity profile. Its use in the allergic asthma model has yielded some or no decrease in ovalbumin-induced airway inflammation and eosinophilia, but has more consistently brought about a decrease in airway hyperreactivity [13,23-25].

Aminoguanidine is a selective inhibitor of iNOS to a much lesser extent than 1400W or the acetamidine derivatives of arginine (5.5- and 11-fold over nNOS and eNOS, respectively), yet is sometimes touted in the literature as a iNOS inhibitor without regard to this caveat in the context of dose used [26]. Schuiling *et al.* considered this in their dosage of ovalbumin-challenged guinea pigs to nebulized aminoguanidine, and concluded that iNOS-derived NO can both promote and inhibit ovalbumin-induced airway hyperresponsiveness depending on the post-insult chronology [27]. Aminoguanidine binds the active site of iNOS, interacting with the conserved Glu³⁷¹ of the

murine isoform, which also interacts with the guanidino group of L-arginine [28]. Furthermore, its anti-inflammatory effect is reversed with L-arginine supplementation [16]. Finally, S-ethylisothiourea (EIT) and 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine (AMT) similarly bind the active site of iNOS with only slightly greater affinity over the other NOS isozymes; both have been found to be effective in reducing granulocytic airway inflammation and the expression of monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-2, and interferon (IFN)- γ in the ovalbumin mouse model [14,29].

In contrast to findings in BALB/c mice, we have observed that C57BL/6 mice are relatively resistant to chronic challenge with 1% ovalbumin. In turn, iNOS-deficient mice, on a C57Bl/6 background, were highly susceptible (similar in responses to the BALB/c strain) to airway inflammation and subepithelial fibrosis and methacholine-induced hyperreactivity of the airways under the same exposure protocol. Total cells, lymphocytes and eosinophils were all increased in iNOS knockout over wild-type mice after 1 week (3 exposures) of exposure to 1% ovalbumin aerosol. Our findings suggest a protective role for iNOS and for NOS-derived NO in allergen-induced airway inflammation [30]. Contrary to our findings, Xiong *et al.* have reported a pro-inflammatory role for iNOS in their knockout model (129 mouse strain background) with respect to eosinophils, lymphocyte and macrophage populations recovered by lung lavage of ovalbumin-challenged mice [31]. They attributed suppression of eosinophilia in iNOS-deficient mice to increased IFN- γ production. Duguet *et al.*

reported similar findings in their iNOS knockouts on a C57BL/6 background [32]. However, both De Sanctis *et al.* [33] and Koorai *et al.* [34] report no differences in total inflammatory cells or cell differential profiles in their ovalbumin challenged iNOS knockouts compared to wild-type C57BL/6 mice. These studies all report no significant differences between knockout and wild-type mice in methacholine-induced airway hyperreactivity subsequent to ovalbumin challenge. Landgraf *et al.* (2005) corroborate these latter findings and suggest that acute inhibition of iNOS, but not its absence, suppresses manifestations of asthma in a mouse allergic model [35].

Thus, it appears that in allergic asthma models the use of moderately to highly selective iNOS inhibitors can attenuate antigen-induced airway inflammation and methacholine-induced hyperresponsiveness. On the other hand, the airway inflammatory response of iNOS knockout mice appears to differ with the specific experimental conditions and/or strain of knockout mice used, and these mice seem to have no greater susceptibility to airway hyperresponsiveness. This may be suggestive of a homeostatic phenomenon occurring in an organism that lacks an enzyme throughout its lifetime (as opposed to a transient depletion of enzyme activity). Pharmacological blockade of NOS isozymes in human sepsis trials has shown limited success [36-38].

CONSTITUTIVE NITRIC OXIDE SYNTHASES--WHAT IS THEIR CONTRIBUTION IN THE AIRWAY?

Gene knockout experiments by de Sanctis *et al.* utilized iNOS and eNOS knockouts to investigate the role of NO in an established model of allergic asthma using mice [33,39]. The principal findings were that allergen-induced airway hyperreactivity (AHR) is fully expressed in the absence of iNOS, that iNOS is not important in the genesis of airway inflammation, and nNOS is the isoform principally responsible for the ovalbumin-induced enhancement of airway responsiveness *via* direct effects on airway smooth muscle tone [39]. A non-inflammatory link between the nNOS gene and AHR was proposed.

Hasaneen *et al.* using tracheal segments from nNOS knockout mice, tracheal and electrical field stimulation, suggested that endogenous NO produced by nNOS is a major transmitter of the inhibitory NANC system and that nitric oxide and vasointestinal peptide act as co-neurotransmitters mediating relaxation of tracheal smooth muscles [40]. This result contrasts sharply with earlier work by de Sanctis *et al.* Using nNOS knockout mice, they found that nNOS contributes approximately 40% of the total fraction of exhaled NO, and that nNOS-derived NO contributes in a pro-contraction manner to methacholine (MCh) stimulation of the airways [33]. Several mechanistic explanations were suggested by de Sanctis *et al.* including changes in reflex homeostasis (integrative cardiopulmonary response) of the nNOS knockout mice resulting in diminished MCh airway response and differences in the functional importance of nNOS- and eNOS-derived NO, and that NANC may be pro-contraction in the mouse. Other studies using animal models and isolated human airways agree more with Hasaneen *et al.* and suggest that NO appears to be a mediator of NANC neural bronchodilation [41-43].

Iijima *et al.* investigated the role of nNOS on airway inflammation in ovalbumin sensitized mice. They found half as many alveolar lavage eosinophils and less NO production in nNOS-deficient animals compared to the control mice. They surmised that nNOS deficiency blocks the upregulation of iNOS in the airway epithelium [44]. The possible interaction between nNOS and iNOS contradicts de Sanctis *et al.* [39], who concluded that nNOS is the primary isoform responsible for the

ovalbumin-induced AHR. The contradictory findings in these two studies may be partly attributable to differences in the ovalbumin exposure protocols (7 day exposure – de Sanctis *et al.*, and 3 day exposure – Iijima *et al.*). To our knowledge, no other animal studies have reported clear interactions between nNOS and iNOS after allergen exposure and this question remains an active research area.

Feletou *et al.* used eNOS (eNOS) knockout mice and various NOS inhibitors to study differences in airway responsiveness after MCh challenge [45]. The administration of the non-specific NOS inhibitor L-NAME triggered increased AHR and this was associated with an inhibition of eNOS expression. Furthermore, eNOS knockout mice were markedly more hyperresponsive to inhaled MCh than control mice, suggesting that NO derived from eNOS can ameliorate AHR. Ten Broeke *et al.* (2006) and Kobayashi *et al.* (2006) used transgenic mice that overexpress eNOS to study the effects of NO after ovalbumin sensitization and challenge [46,47]. Ten Broeke *et al.* showed that AHR to MCh was abolished in eNOS overexpressing mice, with a 50% reduction in the number of lymphocytes and eosinophils in the lung lavage fluid of the transgenic mice. They also found less cytokine production (IFN- γ , IL-5, and IL-10) and twice the amount of NOx production in bronchoalveolar lavage (BAL) fluid. Nitric oxide derived from eNOS reduced the allergic response, modulated eotaxin and reduced eosinophil cell migration, and suppressed adhesion molecule expression in vascular endothelial cells (ICAM-1 and VCAM-1). These two reports and others agree that eNOS appears to produce NO that is beneficial to the lung in response to antigen challenge [45].

Steudel *et al.* studied gene knockouts of all three NOS isoforms along with L-NAME inhibition and L-arginine treatments [48]. The absence of nNOS or eNOS increased baseline exhaled NO production rates compared to NOS WT or iNOS knockout mice, suggesting the up-regulation of a NOS isoform responsible for the NO production. Intraperitoneal L-arginine injection stimulated NO production in nNOS and eNOS gene knockouts, whereas intraperitoneal L-NAME injection significantly diminished levels of exhaled NO. This suggests enhanced expression or activity of a NOS isoform which is present in both nNOS knockout and eNOS knockout mice that might be responsible for the increased exhaled quantities of NO. In the de Sanctis study, all upper airway and nasal sources of NO were excluded, whereas in the Steudel study, exhaled NO was collection from the upper airway and skin.

Lim *et al.* investigated the role of superoxide in diesel exhaust particle (DEP)-induced airway hyperresponsiveness by analyzing the activity of P-450 reductase and superoxide dismutase (SOD) [49]. P-450 reductase produces superoxide, a reactive oxygen species and mediator of airway inflammation in asthma. Superoxide dismutase, an enzyme regulating oxidative stress, converts superoxide to H₂O₂. P-450 reductase was increased and SOD decreased in ICR strain mice challenged with intratracheal DEP. Also, DEP instillation caused a 7-fold increase in the mean number of eosinophils, a 2-fold increase in exhaled NO, and increased airway hyperresponsiveness. Airway inflammation and AHR were partially reversed by the administration of SOD. DEP exposure also increased the levels of eNOS in the airway epithelium and iNOS in macrophages. The nonspecific NOS inhibitor, L-NMMA, significantly reduced the airway hyperresponsiveness induced by DEP. Lim *et al.* concluded that both superoxide and NO contribute to the airway hyperresponsiveness and inflammation. Superoxide and NO can react to produce the highly reactive peroxynitrite, and the imbalance between superoxide production and scavenging can lead to cell damage and inflammation *via* oxidative stress. While SOD may be the primary enzymatic pathway for degrading superoxide, other antioxidant pathways exist. Other

antioxidant mechanisms in the lung include the catalases, glutathione peroxidases, and thioredoxin peroxidases [5,50].

CONCLUSIONS FROM ANIMAL STUDIES

Despite differences in experimental protocols, in the mice strains used, and in the assay methods used, some conclusions can be made. In the transgenic mouse experiments, NOS inhibitors may have actually diminished the protective effects of NO. This effect has been most strongly linked to nNOS and eNOS [45,47,51]. There is some indication that nNOS may be responsible for ovalbumin-induced increased AHR [33], and nNOS knockout mice (by not making NO) have markedly reduced eosinophil production (AHR was not measured) [44]. On the other hand, nNOS is a neurotransmitter in the NANC system resulting in tracheal smooth muscle relaxation [40]. Even in the absence of ovalbumin sensitization and challenge, NO produced *via* nNOS reduces bronchial hyperreactivity [40, 51]. If nNOS is important in the NANC system--at least 60% of the bronchodilatory effect according to Hasaneen *et al.*--then nNOS-derived NO would act as a bronchodilator and protect against AHR. eNOS-derived NO appears to be protective by reducing AHR, cytokine production, cellular infiltration, and vascular endothelial adhesion molecule expression. A careful review of the literature leads us to no firm conclusions on the proper allocation of sources for baseline production of NO between the three NOS isoforms.

NOVEL PHARMACOTHERAPEUTICS: HOW DO THE NEWER STUDIES IN MICE DEFINE NOVEL TARGETS FOR PHARMACOLOGICAL INTERVENTION?

We have published studies in iNOS knockout mice demonstrating their increased susceptibility to ovalbumin-induced airway inflammation and fibrosis [30]. Using the iNOS ovalbumin exposure model and others, we can begin to address a logical sequence of therapeutic targets for pharmacological intervention that target either the inflammatory or fibrotic endpoints or both. The airway fibrotic events include structural and functional responses of the lung to ongoing inflammation, which are often irreversible, including airway submucosal fibrosis and airway hyperreactivity.

Conventional clinical treatment of asthma invariably targets the "intermediate" events, and uses anti-inflammatory agents such as inhaled corticosteroids, or agents that inhibit contraction of airway smooth muscle such as the beta-agonists terbutaline and albuterol. Other classes of drugs used for asthma control include theophylline, cromolyn, and various leukotriene modifiers such as montelukast and zileuton. Little attention has been paid to drugs that target the later airway fibrotic events, even though these largely irreversible events are contributory to progressive morbidity and eventual mortality in patients with asthma. This relative lack of attention is due in large part to the lack of safe and effective drugs available for systemic treatment of fibrotic disease. This is an area of drug testing and design that potentially offers a major opportunity

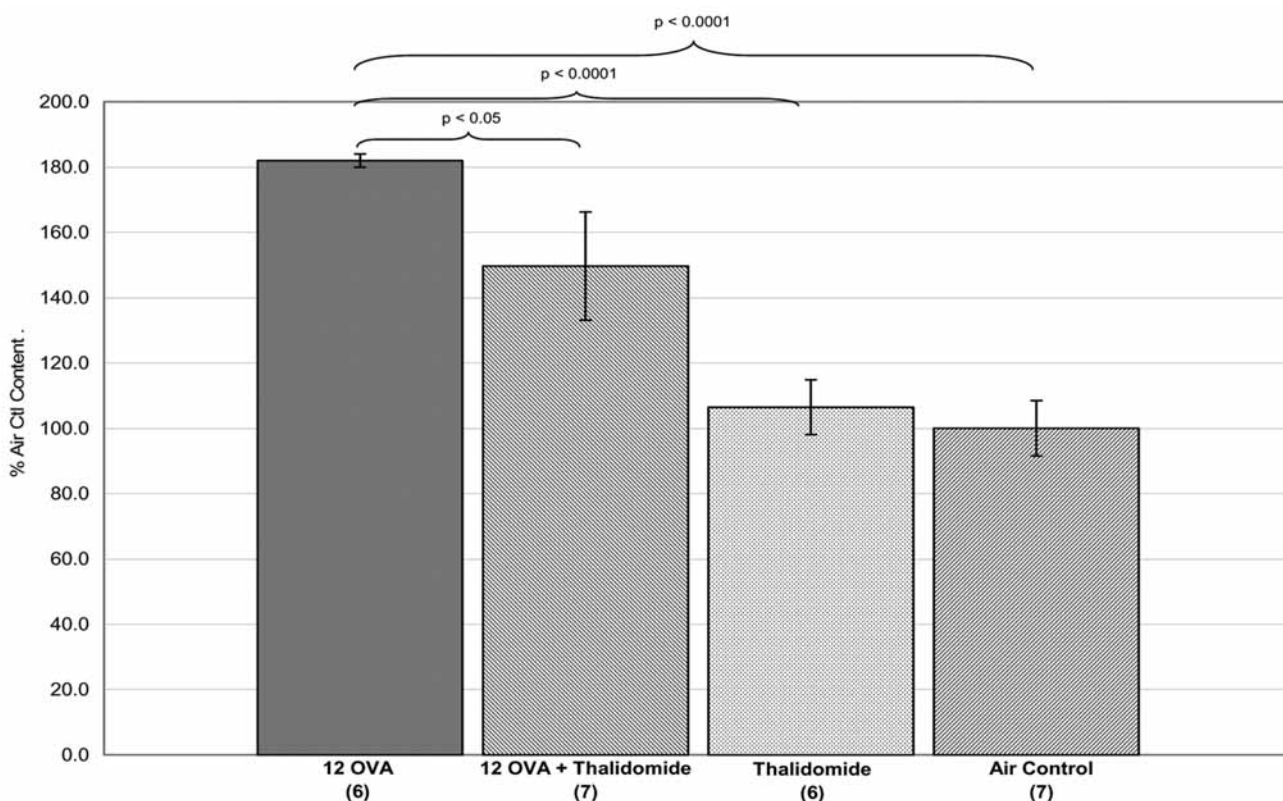


Fig. (2). 4-Hydroxyproline content of microdissected airways of ovalbumin-challenged and thalidomide-treated mice, expressed as percentage (± 1 SE) of mean air control value (with n, the number of mice per group, indicated in parentheses; V.C. Mathrani, unpublished data). To measure hydroxyproline content of airways, lobar bronchi and daughter airways were dissected from either the right lung or both lungs under a dissecting microscope. Lungs were bluntly dissected using two sets of forceps to obtain a preparation of the larger airways separated from adhering parenchyma and attached to the remaining stub of trachea after cannulation. The dissected airways were snap frozen on dry ice and stored at -80°C . The airway preparation was homogenized in water with a Branson sonifier 250 (VWR Scientific, San Francisco, CA, duty cycle 40, output control 4) until the tissue was completely homogenized. The colorimetric method of Woessner was used to assay for 4-hydroxyproline content of the lung homogenate hydrolysate [53]. Standard curves were linear between 0 and 32 μg of hydroxyproline.

for the medicinal chemist. In the following section we will present results from mouse models, including unpublished data from our laboratory, which suggest potential therapeutic approaches to treating airway fibrosis in asthma. We will also discuss challenges to the medicinal chemist to modify the drugs tested to improve their safety and/or efficacy. Such an approach to the development of pharmacological agents can be easily applied to the iNOS ovalbumin model and others to examine more mechanistically the role of NO and NOS in airway fibrosis, and we will address this.

One such anti-fibrotic drug we tested in the ovalbumin model of asthma in the mouse is thalidomide (α -phthalidoglutarimide) [52]. Interestingly, thalidomide seemed to inhibit the increase in airway collagen that we observed in sensitized BALB/c mice exposed to ovalbumin aerosol for 4 weeks. Fig. (2) Ovalbumin-challenged mice had a significant increase in airway hydroxyproline content over air control mice (82%; $p < 0.0001$). Ovalbumin-challenged mice treated with thalidomide showed a significant ($p < 0.05$) decrease in hydroxyproline content as compared to ovalbumin-exposed, untreated animals (50% above air controls). No significant difference was observed between thalidomide-treated and untreated air controls. Airway hydroxyproline content for the air control group was $5.5 \pm 1.9 \mu\text{g}$. We conclude that thalidomide can inhibit allergen-induced airway collagen deposition (airway fibrosis) as measured by 4-hydroxyproline content.

Thalidomide (I) is a known teratogen in humans, which severely limits its usefulness as a systemic agent, especially in women of childbearing age. Fig. (3) A challenge for the medicinal chemist is to identify the components of thalidomide's structure that are responsible for its anti-fibrotic activity, and to separate these active regions from those responsible for its teratogenic activity in utero. There have been suggestions that the anti-fibrotic activity of thalidomide is linked to its known inhibition of TNF- α [54,55], and the synthesis of more effective, possibly less teratogenic, analogues has been reported [55,56].

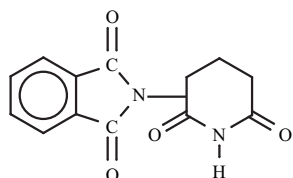


Fig. (3). Chemical structure of Thalidomide (I)

We have tested several other anti-inflammatory or anti-fibrotic drugs in the mouse ovalbumin model, and have observed significant anti-fibrotic activity with relaxin [10] and significant anti-inflammatory and anti-fibrotic activity with dexamethasone (our unpublished data; also Campos, 2006) [57]. Another very interesting drug with significant anti-inflammatory activity in this model is the active oxygen species scavenger, dimethylthiourea (DMTU) [58,59]. Based on

preliminary data from our lab, we suspect that DMTU has anti-fibrotic properties as well.

Perfenidone (5 methyl-1-phenyl-2-(1H)-pyridine-2-one), which has been reported to have anti-fibrotic activity in mouse models of lung fibrosis (and is also known to inhibit TNF- α), is another prime candidate for testing in the mouse ovalbumin model as an inhibitor of airway fibrosis; preliminary experiments in our laboratory have been negative, but dose and optimal timing of administration were not explored systematically in these experiments. We have tested various other drugs, including pentoxifylline, leflunomide, and simvastatin, and have found little or no anti-inflammatory or anti-fibrotic activity with the dosage regimens used. Again, dose and optimal timing of administration were not explored systematically in these experiments.

There are data in the literature suggesting that there would be potential anti-inflammatory and anti-fibrotic benefits were we able to specifically inhibit either nNOS or eNOS. Thus, the question arises as to the availability of relatively nontoxic drugs that could be used systemically to specifically inhibit one or the other of the three various isoforms of nitric oxide synthase. Whether pharmacological intervention to decrease the production of NO in the inflamed lung would be beneficial or deleterious is not clear, despite the fact that there are ongoing clinical trials to test the effects of NOS inhibitors on different lung diseases. Several drugs of various degrees of specificity for the different NOS isoforms have been used to inhibit NOS activity *in vivo*. It is important to emphasize that reported drug "specificity" for various NOS isoforms is relative rather than absolute, and is usually dose-dependent. Among the older drugs that have been used for this purpose, L-NAME is a broad-spectrum inhibitor of both constitutive and inducible isoforms, while aminoguanidine preferentially inhibits iNOS, the inducible isoform, especially with respect to eNOS (Boer *et al.* 2000). Newer drugs have been developed with greater (relative) specificity for NOS isoforms, including several that have been used for studies *in vivo*. Table 2 lists recent mouse studies that used NOS inhibitors. Other NOS inhibitors have been used: iNOS----L-NIL [60], SC-51 (an L-NIL prodrug) [17]; nNOS----SMTC (S-methylthiocitrulline) [61], v-NIO [62], L-NPA (N-propyl-L-arginine) [62]; eNOS----L-NIO [63].

If NO arising from the action of iNOS on arginine is "good", in the sense of protecting the lung from injury as suggested by our experiments with iNOS knockout mice, then drugs that can increase the availability of substrate arginine for iNOS might have a significant anti-fibrotic effect. It is widely accepted that there is competition between iNOS and arginase for available arginine; this suggests that a non-toxic inhibitor of arginase with a relatively long biological half-life might be an effective anti-fibrotic agent in a murine model of asthma. Several arginase inhibitors have been described; two of these that have been used successfully *in vivo* are L-NOHA (N-hydroxy-L-arginine) [64] and DFMO (difluoromethylornithine) [65]. Another challenge for the medicinal chemist would be the

Table 2. Studies with NOS Inhibitors and Route of Administration

Inhibitor	Specificity	Half-life	Dose, route, species	Reference
L-NAME (N-nitro-L-arginine methyl ester)	Non-selective	Pro-drug of L-NNA; 17-30 hr	300 mg/Kg, ip, mouse	Trifilieff <i>et al.</i> 2000
1400W (N-3-aminomethyl-benzyl-acetamidine-dihydrochloride)	iNOS	Pro-drug Nitro-L-Arg; 17-30 hr	30-100 mg/Kg, ip, rat	Birrell <i>et al.</i> 2003
7-NI (7-nitroimidazole)	nNOS	About 1 hr in peanut oil, ip	25 mg/Kg, ip, rat	Naassila <i>et al.</i> 2003
Aminoguanidine	iNOS	4 hours	200 mg/Kg, ip, rat	White <i>et al.</i> 2003

design of better drug candidates for use as arginase inhibitors with which to test the hypothesis that increasing the availability of arginine as a substrate for iNOS will be beneficial for asthma treatment. The ideal drug would exhibit specificity with regard to the two known arginase isoforms, arginases I and II, would be effective orally, and would somehow target the lung (for example, by aerosol administration). At present, it is not known which of these arginase isoforms is the appropriate therapeutic target; however, arginase I appears to be prevalent in inflammatory cells and macrophages, while arginase II appears to be the major species in the airway epithelium. The mouse ovalbumin model would appear to be an almost ideal screening system for testing drug candidates with the appropriate properties.

Further research efforts with mouse models will provide even more knowledge about the role of NO and the NOS enzymes in airway inflammation. While results from these studies are not expected to agree completely, a general consensus will build about the beneficial and detrimental levels of lung NO and the individual contributions of each NOS enzyme. Until that time, therapeutic efforts to target specific enzymes and metabolites in the NO pathways in the lung will remain more exploratory than definitive.

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LIST OF ABBREVIATIONS

1400W	=	N-(3-(aminomethyl)benzyl)acetamidine
AHR	=	Airway hyperresponsiveness
DEP	=	Diesel exhaust particles
DMTU	=	Dimethylthiourea
IFN- γ	=	Interferon- γ
cNOS	=	Constitutive nitric oxide synthase
eNOS	=	Endothelial nitric oxide synthase
iNOS	=	Inducible nitric oxide synthase
L-NAME	=	N-Nitro-L-arginine methyl ester
L-NIL	=	L-N-(1-iminoethyl)lysine
L-NIO	=	N-(1-imino-3-butenyl)-L-orinithine
L-NMMA	=	NG-monomethyl-L-arginine
MCh	=	Methacholine
NANC	=	Non-adrenergic non-cholinergic nerves
nNOS	=	Neuronal nitric oxide synthase
NOS	=	Nitric oxide synthase
SMTC	=	S-methylthiocitrulline
SOD	=	Superoxide dismutase
TNF α	=	Tumor necrosis factor- α

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