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Modeling gas phase nitric oxide release in lung epithelial cells

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ABSTRACT

Nitric oxide (NO) is present in exhaled breath and is generally considered to be a noninvasive marker of airway inflammation, and is thus of particular relevance to monitoring asthma. NO is produced when L-arginine is converted to L-citrulline by NO synthase (NOS); however, L-arginine is also the substrate for arginase and both enzymes are upregulated in asthma. Recent reports have speculated that enhanced expression of one or both enzymes could lead to a limitation in substrate availability, and hence impact downstream targets or markers such as exhaled NO. The non-linear nature and vastly different kinetics of the enzymes make predictions difficult, particularly over the wide range of enzyme activity between baseline and inflammation. In this study, we developed a steady state model of L-arginine transmembrane transport, NO production, diffusion, and gas phase NO release from lung epithelial cells. We validated our model with experimental results of gas phase NO release and intracellular L-arginine concentration in A549 cells, and then performed a sensitivity analysis to determine relative impact of each enzyme on NO production. Our model predicts intracellular L-arginine and gas phase NO release over a wide range of initial extracellular L-arginine concentrations following stimulation with cytomix (10 ng/ml TNF-α, IL-1β, and INF-γ). Relative sensitivity analysis demonstrates that enhanced arginase activity has little impact on L-arginine bioavailability for NOS. In addition, NOS activity is the dominant parameter which impacts gas phase NO release.

Introduction

Nitric oxide (NO) is a reactive, free radical that plays important roles in many regulatory functions, including smooth muscle relaxation, immune response and neurotransmission. The concentration of nitric oxide in the exhaled breath (eNO or F_{ENO}) is a potential noninvasive biomarker of inflammation in asthma. Inflammatory stimuli such as IL-1β, TNF-α, IFN-γ, and IL-13 can induce inducible NOS (iNOS) expression [1–3] in airway epithelial cells, which has been proposed to be the main source of exhaled NO [4]. However, the clinical application of exhaled NO as a biomarker remains limited as significant intersubject variability has been reported within clinically similar individuals [5–7].

NO is produced when L-arginine is converted to L-citrulline by NO synthase (NOS). Interestingly, L-arginine is also the substrate for arginase, which produces urea and L-ornithine. L-Ornithine is a precursor for polyamines and L-proline, which are involved in cell proliferation and collagen synthesis, respectively. Asthma is a disease characterized by both inflammation and fibrosis. Not surprisingly, recent studies have reported elevated levels of arginase in subjects with asthma [8] leading to the hypothesis that competition for L-arginine between NOS and arginase could limit substrate availability for one or both enzymes and thus impact downstream pathways or markers such as eNO.

Although arginase activity has been associated with inhibition of NO production in activated macrophages [9] and bovine pulmonary arterial endothelial cells [10], the mechanism is poorly understood and complicated by several factors. Unfortunately, the kinetics of NOS and arginase are non-linear (generally obeying Michelis–Menten kinetics), and the kinetic model parameters (i.e., K_m and V_{max}) vary by orders of magnitude between enzymes and following exposure to inflammatory cytokines. For example, NOS affinity for L-arginine ($K_m \cong 2$–20 μM) is three orders of magnitude higher than arginase ($K_m \cong 2$–20 mM); however the total enzyme activity (V_{max}) of NOS is approximately three times smaller than arginase [11]. Furthermore, the transmembrane uptake of extracellular L-arginine into the cell is via specific cationic amino acid transporters (CATs) [12], which can also be described by non-linear and saturable kinetics.

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A simple model has been previously presented to describe the competition between arginase and iNOS and predict the consequences of arginase inhibition in the presence of NOS on L-arginine availability in macrophages [13]. According to this model, arginase activity needs to exceed a critical threshold to regulate NO synthesis. However, this model did not consider transmembrane L-arginine transport, and the impact of variable kinetic parameters. In addition, no model has been developed to describe NO production, diffusion and gas phase release from lung epithelial cells.

We have previously reported an *in vitro* airway epithelial cell culture model for direct gas phase NO measurement [2,3]. In this study, our aims are to (1) develop a steady state model of gas phase NO release from lung epithelial cells; (2) validate the model results with experimental data from A549 cells; and (3) perform a sensitivity analysis of the model parameters to determine their relative importance in understanding gas phase release from lung epithelial cells.

Materials and methods

Cell culture

A549 cells were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and cultured as previously described [2]. A549 cells are a model of human alveolar Type II cells and have been used extensively to understand NO biochemistry [14,15]. In brief, A549 cells were grown on T-75 flasks (Corning, Fisher) in a 37 °C, 5% CO_2/95% air incubator in F12-K medium (ATCC, Rockville, MD) containing 10% fetal bovine serum (Mediatech, VA). When subconfluent, cells were trypsinized and seeded onto Costar polyester Transwells® inserts with 0.4 μm pore size (Corning, Fisher) at a density of 1.5×10^5 cells/well. Once the cells reached confluence, they were shifted to an air–liquid interface (medium only in the basal lateral compartment) for 2 days. L-Arginine-free media was used 24 h before exposure to cytokines. The cells were then cultured in medium with 50, 100, or 1000 μM L-arginine. The cytokine activation of cells was performed with a combination of TNF-α, IL-1β, IFN-γ (10 ng/ml each, R&D Systems, Minneapolis, MN) commonly referred to as cytomix. A series of endpoints related to NO production, NOS expression, and arginase expression were then monitored for 48 h.

Real time gas phase NO measurement

Direct gas phase NO was measured in the air–liquid interface culture system as previously described [2,3]. In brief, 12-well Transwell® plates were fitted with modified lids, and edges were sealed to form a gas tight enclosure. One of the holes on the top surface of the lid was connected to the inlet of a chemiluminescent nitric oxide analyzer (NOA 280, Sievers, Boulder, CO) and flow (Q) was controlled at 40 ml/min. The NO concentration in the gas phase over the cells reaches a plateau value, C_p (ppb), representing the steady state NO release into the gas phase after the washout of accumulated NO from the headspace. C_p was determined by fitting an exponential form to the smoothed transient response, as previously described [2,3].

Intracellular L-arginine concentration measurement

Cells were washed twice with PBS and lysed with RIPA buffer without TRIS. The analysis of L-arginine concentration was performed by the Protein Chemistry Lab (Texas A&M University, College Station, TX). The intracellular concentration of L-arginine was then estimated following correction of dilution.

Model development

Glossary

A_e	surface area of the epithelial monolayer (cm^2)
A_m	effective surface area for NO diffusion to the medium (cm^2)
Arg_{ex}	extracellular L-arginine concentration (μM, μmol l^{-1})
Arg_{in}	intracellular L-arginine concentration (μM, micromole l^{-1})
α	dimensionless number representing the ratio between the rate of NO production and the rate of NO diffusion in the epithelial cell
C_{no}^{g}	gas phase NO concentration from lung epithelium release (mole ml^{-1})
C_{no}^{e}	intracellular NO concentration (mole ml^{-1})
C_{no}^{m}	NO concentration in the medium (mole ml^{-1})
$D_{NO,e}$	diffusion coefficient of NO within the epithelium (cm^2/s)
$D_{NO,m}$	diffusion coefficient of NO in the medium (cm^2/s)
h	coefficient of proportionality between NOS activity and transmembrane L-arginine uptake
$J_{NO,L}$	diffusion flux of NO to the air at position $x = L$ (mole cm^{-2} s^{-1})
$J_{NO,0}$	diffusion flux to the medium at position $x = 0$ (mole cm^{-2} s^{-1})
k_m	mass transfer coefficient between epithelium and medium (cm s^{-1})
k_m^{arg}	Michelis–Menten affinity of arginase for L-arginine (micromole cm^{-3})
K_m^{h}	Michelis–Menten affinity of the high affinity CAT transporter for L-arginine (micromole cm^{-3})
K_m^{l}	Michelis–Menten affinity of the low affinity CAT transporter for L-arginine (micromole cm^{-3})
K_m^{NOS}	Michelis–Menten affinity of NOS for L-arginine (micromole cm^{-3})
L_e	epithelium thickness (μm)
l_m	thickness of medium in the bottom compartment (μm)
p	porosity of the 0.4 micron polyester Transwell membrane (0.005)
Q	flow of air within the headspace over the epithelium(ml min^{-1})
V_{max}^{h}	maximal high affinity CAT transport rate (μM, micromole l^{-1})
V_{max}^{l}	maximal high affinity CAT transport rate (μM, micromole l^{-1})
V_{max}^{NOS}	maximal NOS reaction velocity (micromole l^{-1} min^{-1})
V_{max}^{arg}	maximal arginase reaction velocity (micromole l^{-1} min^{-1})
$\dot{V}_{NO}$	rate of NO production from iNOS (micromole l^{-1} min^{-1})
$\dot{V}_{urea}$	rate of urea production from arginase (micromole l^{-1} min^{-1})
$\dot{V}_{trans}$	rate of transmembrane L-arginine transport (micromole l^{-1} min^{-1})
V_e	volume of epithelial monolayer (cm^3)

Fig. 1 is a schematic of L-arginine transmembrane transport, NO production from NOS, intracellular diffusion of NO, and gas phase release from an epithelial cell (Fig. 1A) and from the monolayer as a whole (Fig. 1B). Two CAT transporters of the y+ family, the high

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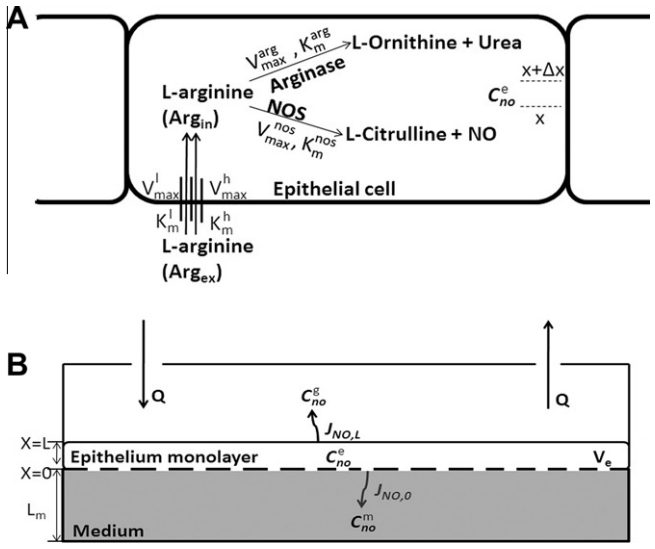

Fig. 1. Schematic of L-arginine transmembrane transport, NO production from NOS, intracellular diffusion of NO, and gas phase release. (A) Individual epithelial cell; (B) monolayer of epithelial cells. Extracellular L-arginine (Arg_{ex}) is transported into airway epithelial cells through CAT transporters on the cell membrane. Intracellular L-arginine (Arg_{in}) is the substrate for both NOS, yielding NO and L-citrulline, and arginase, yielding L-ornithine and urea. Once NO is produced, two potential fates are considered in this model: (1) diffusion as a free molecule towards the air, and (2) diffusion towards the medium and is converted into nitrite/nitrate. The latter is negligible for the conditions of the experiments in this study as shown in the Appendix.

affinity CAT1 and the low affinity CAT2B [16], model L-arginine uptake in A549 cells. Two enzymes that both use L-arginine as the substrate are included in the model: arginase and NOS. In airway epithelial cells, constitutive NOS (eNOS) contributes negligible gas phase NO release at baseline [2], thus only iNOS, following cytomix exposure, is considered as a source of NO in this model. Once intracellular NO is produced, it has three primary fates: (1) diffuses as a free molecule towards the air, (2) diffuses towards the medium and is converted into nitrite/nitrate, (3) is consumed with oxygen or superoxide in the presence of glutathione (GSH) to produce GSNO [17]. It has been previously demonstrated that the majority of free NO in exhaled NO is due to diffusion of free NO (fate #1) from the epithelial cell [17]. Thus, fate #2 (see Appendix for details) and #3 are neglected in our model for simplicity. $\dot{V}_{NO}$, $\dot{V}_{arg}$, and $\dot{V}_{trans}$ [18] are all modeled using Michaelis–Menten-type kinetics,

$$\dot{V}_{trans} = \left(\frac{Arg_{ex}}{K_m^h + Arg_{ex}}\right)\left(\frac{V_{max}^h}{1+\frac{Arg_{in}}{K_m^h}}\right) + \left(\frac{Arg_{ex}}{K_m^l + Arg_{ex}}\right)\left(\frac{V_{max}^l}{1+\frac{Arg_{in}}{K_m^l}}\right) \quad (1)$$

$$\dot{V}_{NO} = \frac{V_{max}^{NOS} Arg_{in}}{K_m^{NOS} + Arg_{in}} \quad (2)$$

$$\dot{V}_{urea} = \frac{V_{max}^{arg} Arg_{in}}{K_m^{arg} + Arg_{in}} \quad (3)$$

The rate of intracellular NO diffusion is much greater than the rate of NO production (see Appendix for details). As a result, NO concentration within the epithelial cell can be considered "well-mixed" or without spatial gradients. Furthermore, the intracellular L-arginine concentration changes slowly in time (pseudo steady state). One can then conserve intracellular L-arginine, intracellular NO, and NO in the gas phase, respectively, and write the following three mass balance equations,

$$\dot{V}_{trans} + \dot{V}_{urea} + \dot{V}_{no} = 0 \quad (4)$$

$$\frac{V_{max}^{NOS} Arg_{in}}{K_m^{NOS} + Arg_{in}} V_e - J_{NO,L} A_e = 0 \quad (5)$$

$$J_{NO,L} A_e - Q C_{NO}^g = 0 \quad (6)$$

Finally, a previous study has demonstrated that NOS activity (V_{max}^{NOS}) strongly correlates with L-arginine uptake rate $\dot{V}_{trans}$ [19] in a linear ($r^2 = 0.8$) fashion, or we can write,

$$V_{max}^{NOS} = h\dot{V}_{trans} \quad (7)$$

Inserting Eq. (7) into Eq. (5), then adding Eqs. (5) and (6), and rearranging produces the following result for the rate of NO release from the epithelial monolayer,

$$QC_{NO}^g = J_{NO,L} = \frac{h\dot{V}_{trans} Arg_{in}}{K_m^{NOS} + Arg_{in}} V_e \quad (8)$$

Eqs. (1)–(4), and (8) comprise the governing model equations for steady state intracellular L-arginine concentration and gas phase NO release from the epithelial monolayer.

Solution of governing equations

The governing model equations (Eqs. (1)–(4), and (8)) represent the steady mass balance for L-arginine transport, NO production and gas phase release. There are two dependent variables ($J_{NO,L}$ and Arg_{in}) and eight independent parameters (Table 1). These nonlinear algebraic equations are solved using Matlab (Natick, MA) on a personal computer.

Sensitivity analysis

The major purpose of the sensitivity analysis is to identify the input (independent) parameters that have the most significant impact on the output (dependent) parameters in an effort to understand the dynamics which impact gas phase NO release. We have chosen to use Latin Hypercube Sampling (LHS) to perform the sensitivity analysis as it is computationally more efficient than Monte Carlo sampling, and considers the uncertainty of all input parameters simultaneously. LHS has been successfully used previously in physiological modeling [17,20].

The first step in LHS is to estimate central (or baseline) values for the input parameters, as well as an estimate of their uncertainty. Table 1 summarizes the values for the eight input parameters and the sources from the literature. A high uncertainty value (80%) was assigned to the V_{max} of all enzymes because cytokines can potentially regulate enzyme expression and activity, and only limited data is available for V_{max} in this particular experiment setting. In contrast, K_m has a relatively low uncertainty (50%) because these parameters are less susceptible to change following cytokines exposure. Three different Arg_{ex} (50, 100, 1000 μM) were used for three different sets of simulations.

The next step is to divide each model input parameter into 100 equally probable values where the mean is the central value, and the range (minimum and maximum value) is the central value plus

Table 1
Latin hypercube sampling parameters, central value and uncertainty range.

Symbol	Central value	Uncertainty (%)	Source
K_m^h	110 μM	50	[40]
V_m^h	625 μM/min	80	[16]
K_m^l	320 μM	50	[40]
V_m^l	1300 μM	80	[16,40]
V_{max}^{arg}	1400 μM/min	80	[12]
K_m^{arg}	10,000 μM	50	[12]
h	0.01	80	[41]
K_m^{NOS}	10 μM	50	[12]

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or minus the uncertainty. The choice of 100 values for each parameter is somewhat arbitrary, but the recommended minimum is twice the number of input parameters (or 16 for our model) [20]. A large number of simulations improves the statistical significance of the results. Each model simulation then utilizes a random selection of the input parameters without replacement; thus, for each of the 100 simulations, the set of input parameters are unique. Three sets of 100 simulations were performed, one for each value of Arg_{ex}.

The sensitivity index of each parameter from LHS is the partial-rank correlation coefficient, β, for each parameter following multiple linear regression of the model simulations, defined by,

$$Y_i^k = \lambda + \beta_{i,1}X_1^k + \beta_{i,2}X_2^k + \cdots + \beta_{i,n}X_n^k \quad (9)$$

where Y is the value of the model output, λ is a constant, X is the value for the model input parameter, k is the simulation number, i is the specific model output, and n is the number of model inputs. All model outputs and inputs are normalized by the central value (Table 1), and then multiple-linear regression is used to determine the values of β. 95% confidence intervals are used to determine if the values are statistically different from zero. In essence, LHS approximates a non-linear model in linear form, and the specific linear coefficient (i.e., β) is the relative sensitivity coefficient, S, of the model output to the specific input defined as,

$$S_{i,n} = \frac{\partial Y_i}{\partial X_n} = \beta_{i,n} \quad (10)$$

Results

L-Arginine concentration in lung epithelial cells

The average experimental Arg_{in} increases from 665 μM to 920 μM and finally to 1767 μM as Arg_{ex} increases from 50 μM to 100 μM, and then to 1000 μM (Fig. 2A, $n = 3$). The mean model predicted values for Arg_{in} are 719 μM, 988 μM, and 1792 μM, for Arg_{ex} of 50 μM, 100 μM, and 1000 μM. Median and quartiles of model-predicted Arg_{in} are presented in Fig. 2A, and easily encompass the experimental values. Hence, our model predicts comparable values and a similar trend as the experimental results. Importantly, no parameter optimization was employed to achieve the simulation result.

Gas phase NO release from lung epithelial cells

Cytokine-induced experimental maximum $J_{NO,L}$ increases from 0.19 to 0.34, and finally 0.90 pl NO s^{-1} cm^{-2} (corresponding to C_{NO}^{g} of 0.32–0.57 and finally 1.5 ppb) (Fig. 2B, $n = 3$) for Arg_{ex} of 50 μM, 100 μM, and 1000 μM, respectively. Model-predicted $J_{NO,L}$ increases from 0.36 to 0.47, and finally to 0.81 pl s^{-1} cm^{-2} (corresponding to C_{NO}^{g} of 0.6–0.79 and finally 1.4 ppb). Median and quartiles of model-predicted $J_{NO,L}$ are shown in Fig. 2B. Thus, our model-predicted NO concentration and flux are similar in absolute values and trend as results obtained experimentally without the need to perform parameter optimization.

Sensitivity analysis

Fig. 3 presents the relative sensitivity coefficients as a function of Arg_{ex} for both model outputs, Arg_{in} and $J_{NO,L}$. Arg_{in} depends strongly on the parameters describing the CAT transmembrane transporters and arginase (both K_m^{arg} and V_{max}^{arg}) and the sensitivity is essentially independent of the extracellular L-arginine concentration over a range from 1 to 1000 μM. Increased CAT activity leads to an increase in Arg_{in}, and enhanced arginase activity results in a decrease of Arg_{in} (Fig. 3A). When Arg_{ex} is low (less than 5 μM), NOS activity (V_{max}^{NOS}) influences Arg_{in} (Fig. 3A). For $J_{NO,L}$, the dominate parameter is V_{max}^{NOS} over the entire concentration range of Arg_{ex}, indicating that NOS activity is the major determinant of gas phase NO release. Our model demonstrates that even when Arg_{ex} is very low (<5 μM), variation in arginase activity has a negligible impact on NO production (Fig. 3B).

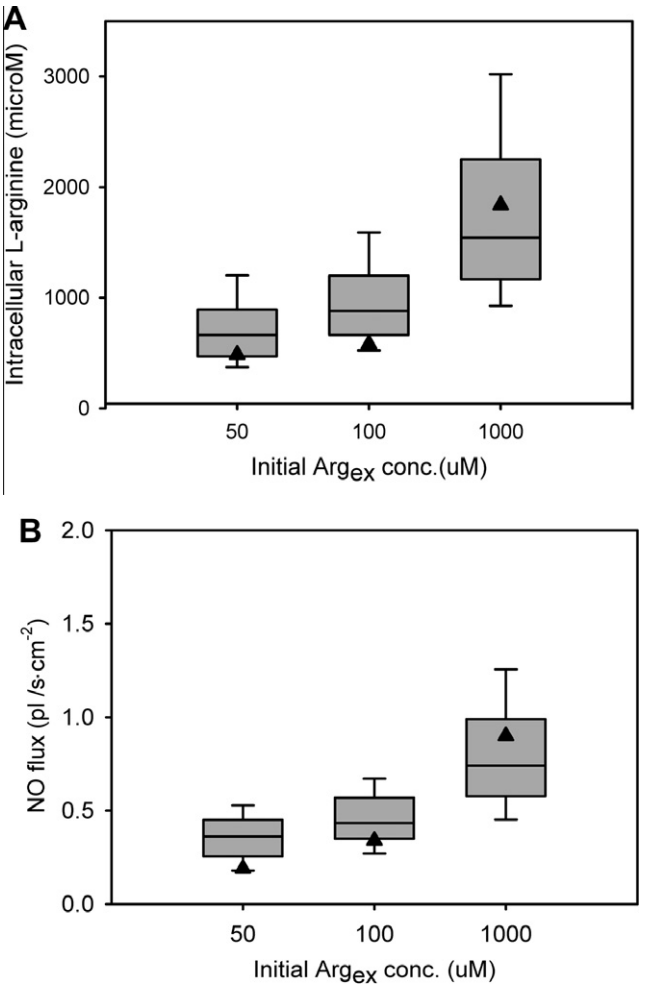

Fig. 2. Experimental and model-predicted Arg_{in} and $J_{NO,L}$ from lung epithelial cells. The boundary of the box closest to zero indicates the 25th percentile and the boundary of the box farthest from zero indicates the 75th percentile. Lines within the boxes represent median value. Vertical bars represent 10–90th percentiles. Closed black triangles represent cytomix-induced experimental results. (A) Intracellular L-arginine (Arg_{in}); (B) gas phase NO release from lung epithelial cells ($J_{NO,L}$).

Discussion

Previous studies have suggested that both L-arginine transport and arginase activity regulate NOS activity by limiting the availability of L-arginine. The purpose of this study was to establish and validate a steady state model of gas phase NO release from lung epithelial cells, and determine the relative importance of key parameters. We developed a simple model of L-arginine transport, NOS and arginase competition, NO production, diffusion and gas phase release in airway epithelial cells. Our model accurately predicts experimentally obtained values of intracellular L-arginine and gas phase NO concentration. Sensitivity analysis demonstrates that intracellular L-arginine depends strongly on transmembrane transport and arginase activity, but does not significantly impact

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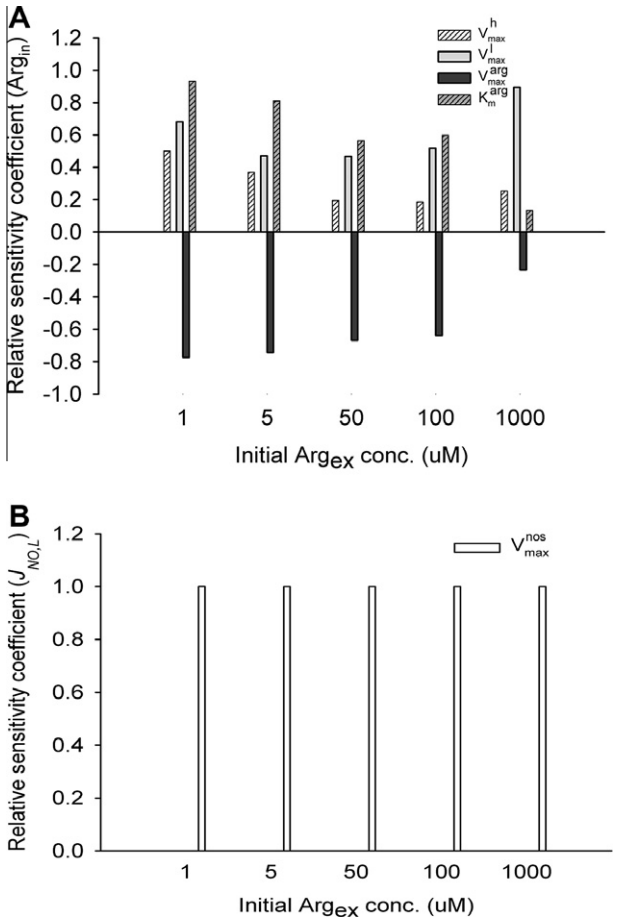

Fig. 3. Relative sensitivity coefficients. Following Latin Hypercube Sampling, all model outputs and inputs are normalized by the central or mean value, and then multiple-linear regression is used to determine the values of the relative sensitivity coefficients. Coefficients that are significantly from zero, are shown. (A) Relative sensitivity coefficients of the parameters when Arg_{in} is the output. (B) Relative sensitivity coefficients of the parameters when $J_{NO,L}$ is the output. A negative value indicates an inverse relationship between an input parameter and the output.

gas phase NO release. NOS activity is the dominant parameter impacting NO release.

Asthmatic and normal plasma arginine levels are 45 μM and 94 μM, respectively [21], which are similar to the lower levels of Arg_{ex} used in our experiments and model simulations. Interestingly, unlike iron and other molecules, the transmembrane transport of L-arginine is facilitated, resulting in higher intracellular concentrations than extracellular, as previously reported [22]. Our observation that Arg_{in} is sensitive to CAT activity (V^l_{max} and V^h_{max}) is consistent with an Arg_{in} (1–2 mM) that is significantly lower than K^{arg}_m (10 mM); that is, enhanced overall transport of L-arginine results in an increase in Arg_{in} as there is little enhanced activity from arginase and the small activity from NOS is saturated.

Furthermore, our observation that enhanced arginase activity decreases Arg_{in} suggests that enhanced arginase activity might limit the bioavailability of Arg_{in} for NOS and thus impact C^g_{no}. Indeed, increased expression of arginase in airway epithelial cells has been suggested to lead to low exhaled NO in smoking asthmatic subjects, indicating that increased arginase expression and activity may contribute to limited substrate availability for iNOS resulting in less NO production [23]. However, our model predicts that arginase activity has a negligible impact on C^g_{no}, even when Arg_{ex} is very low (<5 μM). This prediction can be explained by noting that Arg_{in} is well above the V_{max} of iNOS, and should be sufficient to saturate iNOS.

Interestingly, we did observe, both theoretically and experimentally, an increase in C^g_{no} as Arg_{ex} and Arg_{in} increase. This phenomenon has been previously reported and termed the "arginine paradox". For example, both inhaled or ingested arginine increases exhaled NO in human airways [24,25] despite Arg_{in} being much larger than K^{NOS}_m. The mechanism underlying the arginine paradox remains unclear, although several explanations have been proposed. One theory is that L-arginine concentration inside the cell is not spatially uniform, and the L-arginine concentration that NOS effectively accesses is distinct from the bulk cytosolic L-arginine (e.g., multiple intracellular arginine pools exist) [19,26]. It has been proposed that NO signaling occurs through caveolae, a subcompartment of the plasma membrane [27]. Because of this membrane-bound receptor-mediated process, NOS activation in certain cells (e.g., endothelial cells) might require extracellular L-arginine [27]. Another explanation for the arginine paradox could be the presence of the endogenous NOS inhibitor asymmetric dimethylarginine(ADMA), which could modulate NO production by antagonizing intracellular L-arginine [28,29].

An alternative theory for the arginine paradox is that NOS activity (i.e., V^{NOS}_{max}) increases as L-arginine concentration increases. This is consistent with the previous report that transmembrane L-arginine transport, which would increase intracellular L-arginine, enhances NOS activity [21] and consistent with the development of our model (Eq. (7)). Alternatively, a previous study has shown that L-arginine deficiency specifically impaired the stability of iNOS mRNA [30] or protein [31]. In another words, if the accessible L-arginine pool for NOS is depleted by arginase, NOS activity could decrease leading to a reduced C^g_{no}. However, our western blot of iNOS protein expression in A549 cells does not demonstrate a significant dependence on Arg_{ex} (data not shown). Thus, based on our results, we propose that the most probable explanation for the arginine paradox in airway epithelial cells is either sequestered intracellular L-arginine and/or NOS activity linked in a positive manner with transmembrane transport of L-arginine.

Finally, our model results suggest that V^{NOS}_{max} is the most important factor in determining NO release from lung epithelial cells. This prediction is consistent with our previous *in vitro* observations that gas phase NO release and NOS protein change in similar patterns both temporally and in magnitude [2,3]. In addition, this prediction is consistent with *in vitro* and *in vivo* observations that iNOS is expressed in the airway epithelium of asthmatic subjects, and corticosteroids decrease both iNOS expression, and exhaled NO [32–35].

For simplicity, our model neglects the NO reactions with GSH and either oxygen or superoxide, does not consider the compartmentalization of intracellular L-arginine, and the simultaneous production of superoxide by NOS when L-arginine is limited [36]. Future modeling might need to consider the above reactions as well as how the impact of inflammation as natural extensions of the model.

In summary, we constructed a steady state model of NO release from lung epithelial cells, and identified that the most important factor is NOS expression and activity. While arginase activity can impact intracellular L-arginine, it has a negligible effect on available stores of L-arginine for NOS. As such, we conclude that factors which directly impact NOS protein activity (e.g., inflammatory cytokines) will have the greatest impact on NO release from lung epithelial cells, and thus exhaled NO.

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Appendix A

A.1. Steady state differential mass balance for NO in the epithelium

A steady state differential mass balance over a thickness Δx (Fig. 1) of NO within the epithelial cell can be used to derive the differential expression for $C^{e}_{NO}(x)$. NO can diffuse into or out of the control volume obeying Fick's 1st Law at a rate of $J_{NO}A_e$ (mol NO/s, where $J_{NO} = -D_{NO,e}A_e dC^{e}_{NO}/dx$) evaluated at x and $x + \Delta x$, respectively. Within the control volume, NO is produced at a rate per unit volume $\dot{V}_{NO}$, or.

$$0 = -D_{NO,e}A_e\left(\frac{dC^{e}_{NO}}{dx}\right)^{x}_{x+\Delta x} + \dot{V}_{NO}A_e\Delta x \tag{A.1}$$

By dividing both sides of the mass balance by the volume ($A_e\Delta x$) and taking the limit as Δx approaches 0, one arrives at,

$$0 = D_{NO,e}\frac{\partial^2 C^{e}_{NO}}{\partial x^2} + \dot{V}_{NO} \tag{A.2}$$

where the first term represents diffusion of NO, and the second the production from NOS. One can then substitute Eq. (2) into Eq. (A.2), and non-dimensionalize the mass balance to arrive at,

$$0 = \frac{\partial^2 \bar{C}^{e}_{NO}}{\partial \bar{x}^2} + \alpha \tag{A.3}$$

where the overbar represents dimensionless concentration (concentration normalized by a maximum concentration) and dimensionless distance (position normalized by the thickness of the epithelial cell, L_e). α is a dimensionless group representing the ratio of the rate of NO production divided by the rate of NO diffusion,

$$\alpha = \frac{V^{NOS}_{max}/K^{NOS}_{m}}{D_{NO,e}/L^2} = \frac{\text{rate of NO production}}{\text{rate of NO diffusion}} \tag{A.4}$$

Using reasonable estimates for the parameters that comprise α ($V^{NOS}_{max} \sim 1.5\ \mu M\ min^{-1}$; $K^{NOS}_{m} \sim 10\ \mu M$ [11]; $D_{NO,e} \sim 3.3 \times 10^{-5}$ cm^2/s [37]; $L \sim 10\ \mu m$ [38]), one can readily determine that $\alpha \sim 0.000075$ or $\alpha \ll 1$. Thus, the rate of intracellular NO diffusion is much greater than the rate of NO production.

A.2. Rate of NO diffusion toward the medium compared to rate of NO production

The diffusion flux from the epithelium to medium ($J_{NO,0}$) can be expressed using a mass transfer coefficient (k_m) multiplied by the concentration difference between epithelium (C^{e}_{NO}) and medium (C^{m}_{NO}) [17],

$$J_{NO,0} = k_m(C^{e}_{NO} - C^{m}_{NO})A_m p \tag{A.5}$$

where p is the porosity of the Transwell membrane (0.005). The central value for k_m between the epithelium and the medium is described by considering the thickness and diffusion coefficients of NO in both the epithelium and medium [17], and assuming the solubility of NO in the cell and medium are equal [39],

$$k_m = \left(\frac{L/2}{D_{NO,e}} + \frac{L_m/2}{D_{NO,m}}\right)^{-1} \tag{A.6}$$

Using reasonable estimates for the parameters that comprise k_m ($L_e \sim 10\ \mu m$, $L_m \sim 1\ mm$, $D_{NO,e} \sim 3.2 \times 10^{-5}\ cm^2/s$, $D_{NO,m} \sim 9.6 \times 10^{-5}\ cm^2/s$ (the diffusion coefficient of NO in medium is approximated as that in water, which is threefold of its value in the epithelium [17])), $C^{e}_{NO} \sim 0.2$ nM [17], $C^{m}_{NO} \sim 0$ [17], and $Arg_{in} \sim 1$ mM, one can demonstrate that $\frac{h\dot{V}_{trans}Arg_{in}}{K^{NOS}_{m}+Arg_{in}}V_e \gg k_m C^{e}_{NO}A_m p$ (more than four orders of magnitude). In other words, the rate of NO production is much larger than the rate of NO diffusion into the medium.

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