

Original Article

Isotopic study of L-Arginine kinetics in the lung during *pseudomonas* sepsis in an ovine model

Hongzhi Xu¹, Davin Watson², Yong-Ming Yu¹, Daniel L Traber³, Stefani Fischer³, Joan Nichols³, Donald Deyo³, Lillian L Traber³, Joaquin Cortiella³

¹Shriners Hospital for Children and Massachusetts General Hospital, Boston 02114, USA; ²University of Massachusetts Medical School, Worcester, MA 01655; ³Department of Anesthesiology, The University of Texas Medical Branch and Shriners Hospital for Children, Galveston, TX 77555, USA

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Abstract: The objective of the study is to investigate how L-Arginine pulmonary metabolism is altered in response *Pseudomonas aeruginosa* (*P. aeruginosa*) induced septic conditions using an ovine model. Methods: Seven female sheep were infused with a primed-constant infusion of L-[¹⁵N₂-guanidino, 5, 5, ²H₂] L-Arginine for 28 hs. After the initial 4 hs of the L-Arginine infusion, a continuous infusion of live *Pseudomonas aeruginosa* bacteria started for 24 hs. A NO synthase (NOS) inhibitor, N⁶-Methyl-L-arginine (L-NMA), infusion was added during the last 4 hs of the bacterial infusion. Blood samples were taken at specific time points for isotopic enrichment during control, septic and NOS blocking phases of the study. Results: We observed that the level of total delivery of L-Arginine to the lungs was significantly decreased in septic phase after 24 hours of *pseudomonas* infusion. In contrast, the fractional uptake and metabolism of L-Arginine by the lungs was doubled during septic phase relative to the control phase ($M_{\text{ARG-basal}} = 100\%$ vs. $M_{\text{ARG-septic}} = 220 \pm 56\%$, $P < 0.05$). NO production in the lungs was also significantly increased. Infusion of L-NMA markedly blunted this elevated NO production and attenuated the total arginine metabolized in the septic lungs ($M_{\text{ARG-septic}} = 220 \pm 56\%$ vs. $M_{\text{ARG-NO blocking}} = -25 \pm 20\%$; $P < 0.05$). We demonstrated sepsis induced by *P. aeruginosa* infusion caused an increase in the fractional uptake and metabolic rate of arginine in the lungs. Furthermore, our data suggests that arginine was mainly consumed via arginine – NO pathway, which might be responsible for this enhanced arginine metabolic activity in the septic lungs.

Keywords: Arginine, sepsis, nitric oxide, isotopic study, *Pseudomonas aeruginosa*, nitric oxide synthase, fractional uptake

Introduction

Sepsis is a systemic inflammatory response to infection resulting in high morbidity and mortality rates [1, 2]. It has been well established that nitric oxide (NO) plays a pivotal role in the pathophysiology of sepsis [3]. In previous studies, it was shown that reactive nitrogen species (RNS), which is formed from NO and superoxide, play a detrimental role in acute lung injury (ALI) in the form of lipid peroxidation, protein oxidation, enzyme inactivation and eventually cell death [4]. In addition, it has demonstrated excessive NO formation plays an important role in the pathogenesis of shock in sepsis and ALI [5].

As a critical substrate of NO synthesis, L-Arginine, a semi-essential amino acid, has received

growing attention in relate to sepsis during last decade [6].

Interventions which target L-Arginine metabolism in order to manipulate NO production have been proposed as therapeutic strategies to sepsis [7-11]. However, conflicting data from human and animal studies reveal that our understanding of L-Arginine metabolism is far from clear and more detailed information about L-Arginine metabolism is needed. Therefore the aim of this study is to elucidate the altered pulmonary metabolism of L-Arginine by obtaining quantitative information in order to have a better understanding of the effects of sepsis on local arginine metabolism in the lungs.

Under physiological conditions, L-Arginine is involved in several metabolic pathways includ-

ing synthesis of not only NO but also proteins such as creatine, urea, polyamines, proline, glutamate, and agmatine etc. In sepsis, the kinetics of these metabolic pathways and the metabolic fate of L-Arginine may be significantly altered because of inflammation-induced changes in the corresponding enzymatic activities of these pathways [12-15]. Studies have shown that many of these changes are organ-specific due to the fact that metabolism of L-Arginine is highly compartmentalized [16-18]. Previously, L-Arginine metabolism in liver, kidney, intestine and whole body [19-21] during sepsis has been investigated using isotopic tracer techniques. This technique is considered the most accurate method to study the kinetics of L-Arginine pathway and NO production *in vivo* [22]. However, there are few isotopic studies of local L-Arginine metabolisms in lungs, especially under disease conditions.

In addition to the cardiovascular changes (e.g. hyperdynamic state) that occur during sepsis, the plasma level of L-Arginine has been shown to significantly decline in sepsis [23, 24]. Therefore we asked the following questions: 1) what is the total L-Arginine delivery to the lung during sepsis? 2) How does the L-Arginine metabolic rate change in septic lungs? And, 3) what is the main metabolic fate of L-Arginine? To answer these questions, we used an ovine model of pseudomonas sepsis mimicking the hyperdynamic circulation in septic patients and L-[¹⁵N₂-guanidino, 5, 5, ²H₂] L-Arginine as a tracer to examine the L-Arginine kinetics in the lung. Therefore we set out to quantitatively investigate the altered pulmonary metabolism of L-Arginine in order to have a better understanding of the effects of sepsis on pulmonary arginine metabolism.

Materials and methods

The procedures and experimental design described in this study were approved by the Animal Care and Use Committee (IACUC) of The University of Texas Medical Branch and were conducted in compliance with the guidelines for the care and use of animals established by the American Physiological Society as well as those of the National Institutes of Health.

Tetra-labeled L-[¹⁵N₂-guanidino, 5, 5-²H₂] arginine. HCl ([T4] Arg; 99% abundance), were purchased from MassTrace (Woburn, MA). N^G-Me-

thyl-L-arginine acetate salt (M7033) was purchased from Sigma-Aldrich (St. Louis, MO).

Surgical preparation

Seven female sheep (body weight = 32.4 ± 2.0 kg) were used in this study. After a 12-h fasting period, the sheep were anesthetized with 1.5-2.5 vol% halothane in oxygen, and anesthesia was maintained with halothane in oxygen (1.0-1.5 vol%). Right femoral arterial and venous catheters were placed through a femoral incision and advance to the abdominal aorta and inferior vena cava, respectively. A Swan-Ganz thermal dilution catheter (model 93A-131-7F, American Edwards Laboratories, Irvine, CA) was positioned through the jugular vein into the pulmonary artery. After a left lateral thoracotomy at the fifth intercostal space, a silastic catheter (0.062 in. ID, 0.125 in. OD; Dow Corning, Midland, MI) was inserted into the left atrium. After wound closure, the animals were weaned from ventilation and allowed to recover for at least 5 days. During this time, the sheep were monitored three times a day for appearance, adequacy of pain control, temperature, oral intake, and fecal and urinary output. If their body temperature exceeded 39.6°C, intravenous antibiotic treatment was begun and maintained until the body temperature was normal for > 24 h. All antibiotics were stopped the day before the experiment. During the recovery and study periods, the animals were held in metabolic cages with free access to food and water. The day before the experiment the animals were anesthetized with ketamine, and a urethral Foley urinary retention catheter was placed. Thereafter, all sheep were connected to continuously flushing pressure transducers (Baxter, Irvine, CA), which were attached to hemodynamic monitors (model 78304A, Hewlett-Packard, Santa Clara, CA). The animals received a continuous infusion of Ringer lactate (2 ml/kg/h), and urine was collected until the experiment was started.

Bacterial preparation

In this study, sepsis was induced by injecting the animals with live *Pseudomonas aeruginosa* (strain ISR 12-4-4) isolated from a burn patient at Brooke Army Hospital, San Antonio, TX). The bacteria were prepared from a frozen stock culture. After thawing, 0.8 ml of a stock culture solution of *P. aeruginosa* was inoculated into a

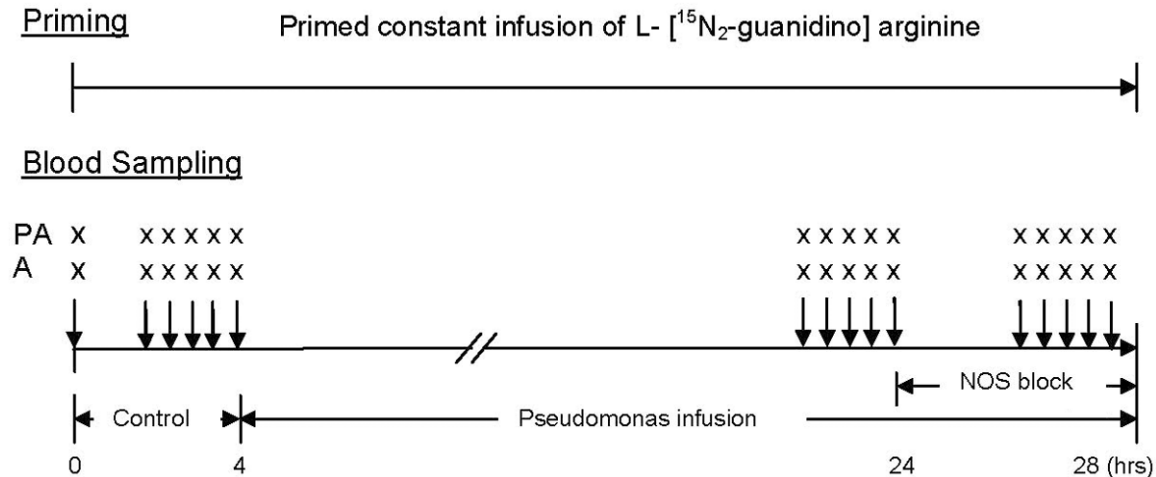


Figure 1. Experimental protocol for isotopic tracer L-[¹⁵N₂-guanidino] arginine infusion studies. Isotopic arginine tracer L-[¹⁵N₂-guanidino] was infused with a priming dose of 18 $\mu\text{mol/kg}$ and a targeted infusion rate of 0.3 $\mu\text{mol/kg/min}$ for 28 hours. After the initial 4 hours (control phase) of the L-Arginine isotope infusion, a continuous infusion of live *Pseudomonas aeruginosa* bacteria at a dose of 6×10^6 colony-forming units (CFU)/kg/hr for 24 hours (septic phase). At 24th hour, an infusion of 6.6 mg/kg/hr L-NMA started for 4 hours (NOS blocking phase). Blood samples were taken from PA and A at specific time points indicated as "x". The interval between the serial samplings (except time 0) is 10 minutes. (PA: pulmonary artery; A: aortic artery).

trypticase soy broth (Difco Laboratories, Detroit, MI) and incubated for 24 h at 37°C. After centrifugation, bacteria were harvested and the bacterial concentration was counted in a Petroff-Hausser count chamber (Hausser Scientific, Blue Bell, PA). The bacteria were then resuspended in 250 ml of 0.9% saline to a final concentration so that with an infusion rate of 2 ml/kg/h the sheep received 6×10^6 CFU/kg/hr.

Experimental protocol

Sheep received a primed-constant infusion of L-[¹⁵N₂-guanidino, 5, 5, ²H₂] L-Arginine with a priming dose of 18 $\mu\text{mol/kg}$ and a targeted infusion rate of 0.3 $\mu\text{mol/kg/min}$ for 28 hours. After the initial 4 hours (control phase) of the L-Arginine isotope infusion, a continuous infusion of live *Pseudomonas aeruginosa* bacteria at a dose of 6×10^6 colony-forming units (CFU)/kg/hr for 24 hours (septic phase). At the 20th hour, an infusion of 6.6 mg/kg/hr L-NMA started for 4 hours (NOS blocking phase). At the beginning of L-Arginine isotope infusion (0 min, one sample only) and 50 minutes before the end of each phase, a serial of blood samples from pulmonary artery (PA) and Aorta (A) were taken for determination of plateau stage isotopic enrichment. The time interval between each serial samples was 10 min (see Figure 1).

Analysis of samples

The isotopic enrichments of the labeled arginine tracer in whole blood were measured using the method described previously [25, 26]. Briefly, whole blood samples were deproteinized with 10% sulfosalicylic acid (Fisher Scientific, Fair Lawn, NJ) and stored at -70°C in a deep freezer until used for analysis. Stable isotope enrichments of arginine were measured on 500 μl of protein-free supernatant. Each sample was passed through an ion-exchange column, and then the methyl ester trifluoroacetyl derivatives of the amino acids were prepared using a procedure comparable to that of Nissim et al. [27]. The isotopic abundances were determined using on-column injection with a gas chromatograph (model 5980 series II, Hewlett-Packard, Palo Alto, CA) coupled to a mass spectrometer (model 5988A, Hewlett-Packard). Selective ion monitoring of arginine was conducted on the $[M - 20]^+$ ion with use of negative chemical ionization, with methane as the reagent gas. This ion corresponds to a loss of HF from the molecular ion. Selective ion monitoring was carried out at mass-to-charge ratio (m/z) 456 and m/z 457 for natural and [¹³C]-guanidinolarginine ($M + 1$) and m/z 458 and m/z 460 for [5, 5-²H₂] arginine ($M + 2$) and [T4] Arg ($M + 4$). A multiple linear regres-

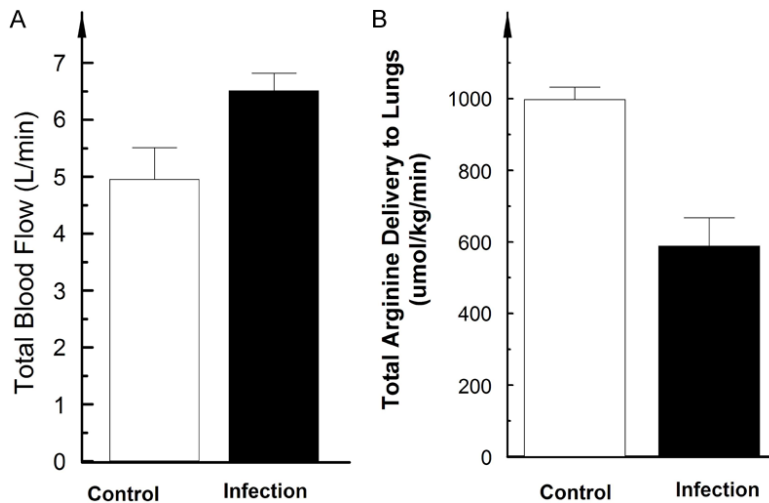


Figure 2. Blood flow and total arginine delivery to the lung during septic phase. A: Total Blood flow in the lungs during septic phase. Pulmonary blood flow was significantly elevated ($TBF_{\text{basal}} = 4.95 \pm 0.56$ vs. $TBF_{\text{septic}} = 6.51 \pm 0.31$, $*P < 0.05$) after 24 hours of pseudomonas infusion. B: Total delivery of L-arginine to the lungs. The delivery of arginine to the lungs was lower during in septic phase of the study in comparison to the non septic basal delivery ($TAD_{\text{basal}} = 997.4 \pm 34.6$ vs $TAD_{\text{septic}} = 587.7 \pm 79.2$, $*P < 0.05$).

sion approach was used to calculate the isotopic abundances of the amino acids from the mass spectrometry data.

Calculations

Details of the calculations and quantitative measurements for *in vivo* amino acid metabolism within an organ have been shown in our previous publication [28]. Briefly, the fraction of the total delivered L-Arginine being metabolized within the lungs, f_{arg} , is: $f_{\text{arg}} = (PA_{\text{arg}} \times E_{PA,\text{arg}} - A_{\text{arg}} \times E_{A,\text{arg}}) / PA_{\text{arg}} \times E_{PA,\text{arg}}$.

Where $[PA_{\text{arg}}]$ and $[A_{\text{arg}}]$ are, respectively, L-Arginine concentrations in the pulmonary artery (the input to the lungs) and aorta (output from lungs, assuming no L-Arginine metabolism in the left ventricle); $[E_{PA,\text{arg}}]$ and $[E_{A,\text{arg}}]$ are the enrichments of L-[$^{15}\text{N}_2$ -guanidino, 5, 5, $^2\text{H}_2$] L-Arginine in the pulmonary artery (PA) and aorta (A) at isotopic steady state.

Total pulmonary arterial delivery of L-Arginine metabolized in the lungs $M_{\text{arg,lung}}$, is:

$$M_{\text{arg,lung}} = [PA_{\text{arg}}] \times F_{\text{lung}} \times f_{\text{arg}}$$

Where F_{lung} is the blood flow rate of the lungs in ml/min, which is represented by the total cardiac output, measured by thermo-dilution

method of the implanted Swan-Ganz tube in the pulmonary artery.

The total NO production from the lungs TP_{nitr} , is:

$$TP_{\text{nitr}} = ([A_{\text{nitr}}] - [PA_{\text{nitr}}]) \times F_{\text{lung}}$$

The total rate of nitrate production from pulmonary arterial delivery of L-Arginine in the lungs (P_{A-N}) will provide information if the alteration of L-Arginine concentration in PA blood will affect NO production in the lungs.

Statistics

Statistical analysis was performed with analysis of variance (ANOVA) for repeated measures to detect differences within a group, and with factorial ANOVA for repeated measures to detect differences between groups, with *post hoc* analysis done with Fisher's least significant difference procedure. Bonferroni's correction for multiple comparisons was used when appropriate. Statistical significance was set at $p < 0.05$. Data are presented as means \pm SEM.

Results

Total arginine delivery to the lungs was decreased during the septic phase

After 24 hours of Pseudomonas infusion (septic phase), we observed a statistically significant elevation of total blood flow ($TBF_{\text{basal}} = 4.95 \pm 0.56$ vs. $TBF_{\text{septic}} = 6.51 \pm 0.31$, $P < 0.05$, **Figure 2A**), measured by Swan-Ganz thermal dilution catheter. However, because of lower plasma arginine concentration, total delivery to the lungs was decreased in septic phase in comparison to that of the basal phase ($TAD_{\text{basal}} = 997.4 \pm 34.6$ vs $TAD_{\text{septic}} = 587.7 \pm 79.2$, $P < 0.05$, **Figure 2B**).

Pseudomonas infusion enhanced arginine metabolism in the lungs

Using isotopic tracer techniques, we could determine the fractional uptake by the lungs at different phases. Our results showed that the

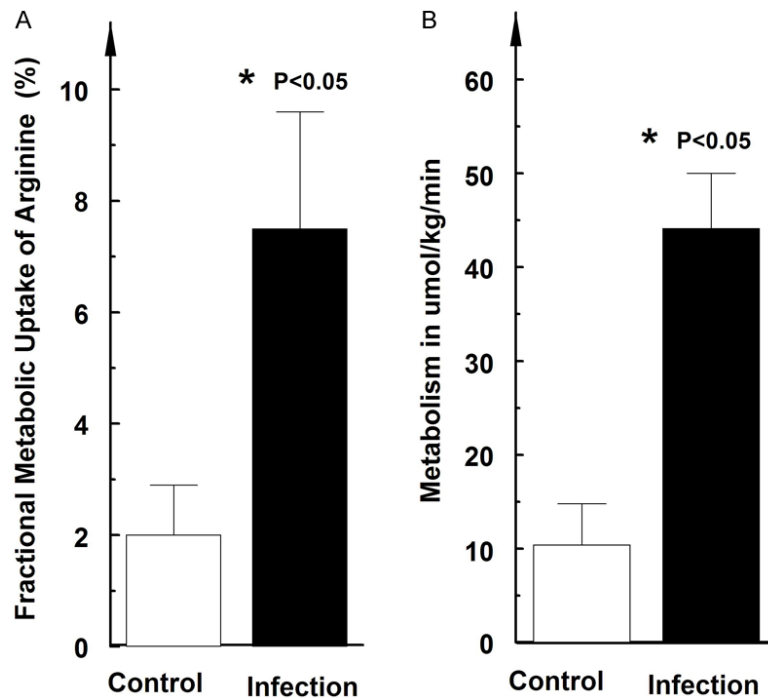


Figure 3. Arginine metabolized by the lungs during septic phase. A: The fractional uptake of L-Arginine by the lungs. L-arginine uptake in the lungs was markedly enhanced after pseudomonas infusion ($f_{\text{ARG-basal}} = 2.0 \pm 1.1$ vs. $f_{\text{ARG-septic}} = 7.5 \pm 2.1$, *P < 0.05). B: The amount of arginine metabolized by the lungs was about doubled during septic phase comparing with basal phase.

vs. $f_{\text{ARG-septic}} = 7.5 \pm 2.1\%$, P < 0.05, **Figure 3A**). As a result, despite of the decreased delivery of total arginine to the lungs, the amount of arginine metabolized by the lungs increased almost 2.2 times during septic phase comparing with basal phase ($M_{\text{ARG-basal}} = 100\%$ vs. $M_{\text{ARG-septic}} = 220 \pm 56\%$, P < 0.05; **Figure 3B**).

NO production increased in the septic lungs

NO production was assessed by measuring the total rate of ^{15}N -nitrate production. After pseudomonas infusion, we found that the rate of nitrate production was significantly elevated ($P_{\text{nitrate-basal}}^* = 10.3 \pm 3.2$ vs. $P_{\text{nitrate-septic}}^* = 43.6 \pm 5.7$, P < 0.05, **Figure 4**), indicating a significantly increased NO production from the lungs during septic phase.

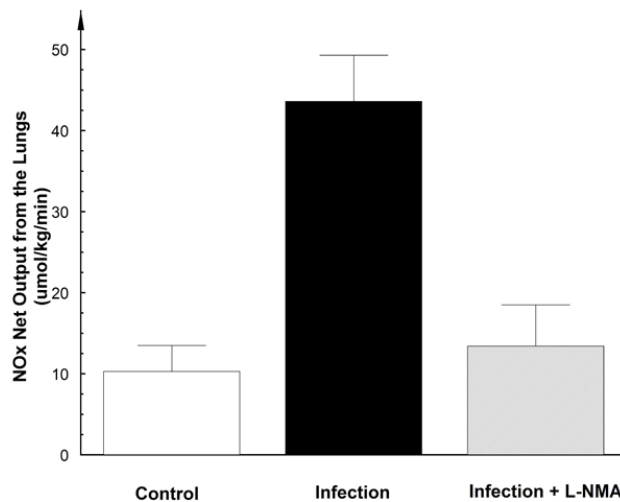


Figure 4. Nitrate Output in the lungs. After 24 hours of pseudomonas infusion, the rate of nitrate production was elevated ($P_{\text{nitrate-basal}}^* = 10.3 \pm 3.2$ $\mu\text{mol/kg/min}$ vs. $P_{\text{nitrate-septic}}^* = 43.6 \pm 5.7$ $\mu\text{mol/kg/min}$, P < 0.05), and injection of L-NAM, the production was reduced to 13.4 ± 5.1 , no significant from basal level.

The augmented arginine metabolism in the septic lungs is mostly utilized for NO production

At the last stage of experiment, we used NOS inhibitor L-NMA to test whether the hypermetabolism of arginine in septic lungs can be attributed to the increased NO production. Our data showed that L-NMA, a selective inhibitor of NO synthesis from L-arginine, markedly blunted NO production, indicated by a significant drop in nitrate output ($P_{\text{nitrate-basal}}^* = 43.6 \pm 5.7$ vs. $P_{\text{nitrate-septic}}^* = 13.4 \pm 5.1$ P < 0.05, **Figure 4**). Concomitantly, the total arginine metabolized in the septic lungs markedly attenuated to 16.4 ± 4.0 $\mu\text{mol/kg/min}$, not significantly different from the basal level). These observations suggest the enhanced arginine metabolism in the septic lungs is mostly utilized for NO production.

Discussion

Sepsis is characterized by a physiological derangement of the cardiovascular system following the systematic inflammatory response

fraction of the total delivered arginine metabolized in the lungs was markedly enhanced after pseudomonas infusion ($f_{\text{ARG-basal}} = 2.0 \pm 1.1\%$

to microbial infections. Perturbations in hemodynamics and significant changes in enzymatic activities induced by inflammatory cytokines may disrupt the homeostasis of L-Arginine and significantly alter the fate of L-Arginine locally and systemically. Despite the biochemical importance of L-Arginine, the impact of sepsis on L-Arginine metabolism, particularly in the lungs, is not fully understood. In the present study, using isotopic tracer techniques, we characterized the kinetics of L-Arginine delivery and uptake in the lungs during sepsis. We demonstrated that L-Arginine metabolism was significantly enhanced despite of reduced total delivery to the septic lungs. In addition, this increased L-Arginine metabolism is largely utilized for NO production in the septic lungs.

Infusion of *P aeruginosa* in ovine consistently induced a hyperdynamic circulatory state in our sepsis model, evidenced by elevated blood flow rate. However, the total delivery of L-Arginine to the lungs actually declined, mainly due to the markedly low plasma L-Arginine concentration during sepsis. The observed low plasma L-Arginine has been attributed to an increased of whole body consumption of L-Arginine, administration of resuscitation fluids, decreased endogenous L-Arginine production and food intake during sepsis [18, 29, 30]. Furthermore, we found that there is an increase in the fraction of L-Arginine that was metabolized within the septic lungs despite of diminished total delivery of the amino acid to the lungs. Taken together, the end result is that the metabolic rate of L-Arginine is augmented in lungs after continuous infusion of *P aeruginosa* for 24 hours. In other words, among these altered physiological variables during sepsis, increased blood flow and fractional uptake of L-Arginine by the lungs of the infected animals overrode the decrease in total delivery of L-Arginine to the lung and resulted in an increased rate of L-Arginine metabolized in the lungs. This finding agrees with previously reported evidence regarding sepsis-induced local changes in arginine metabolism in the lungs. For example, activities or gene expressions of several key enzymes in L-Arginine catabolic pathways have been shown to be significantly upregulated in septic lungs. In addition, cellular uptake of L-Arginine has been identified as a crucial regulatory mechanism that modulates L-Arginine metabolism. Huang et al. [31] reported that activities of Type-2 cationic amino acid transporter (CAT-2), a major L-Arginine

transporter in the lungs, increased in rat lung tissues under condition of sepsis. Based on our observation of increased uptake of arginine by the lung during sepsis, it is plausible that the increase expression of transporters might be an adaptive response to the low L-Arginine availability during sepsis. Interestingly, there is some evidence suggesting that cells can indirectly “sense” the changes in L-Arginine availability via changes in activity of various metabolic pathways [32].

Considering the multiple pathways for L-Arginine metabolism in the lungs, we further investigated the fate of this enhanced L-Arginine metabolism in the lungs under sepsis condition. We found that the enhanced L-Arginine metabolism was associated with increased NO production in the lung. The finding that a non-specific NOS inhibitor, L-NMA, can almost completely abolish the enhanced L-Arginine metabolism induced by sepsis, confirmed the increased metabolism of L-Arginine in lungs might be largely used for NO production. In this study, we did not examine the alterations in other arginine metabolic pathways. Previously, there are studies based on LPS-induced animal model suggesting that arginase, the enzyme in arginine-ornithine pathway, might also be activated in the lungs during sepsis in a time-specific and arginase-isoform specific patterns [33, 34]. In addition, Lortie MJ et al showed that endotoxin also caused increased ADC and ODC activities, and blockade of iNOS resulted in increased agmatine and polyamine levels [35]. Thus, together with our results, it implied that while sepsis condition may activate multiple arginine catalytic pathways in the lungs, the arginine-NO pathway is the predominant pathway that responsible for the enhanced arginine metabolism in the lungs during sepsis. NOS does not form NO directly from arginine but first forms an intermediate N (omega)-hydroxy-nor-L-arginine (NOHA). NOHA then forms citrulline and NO and is also is a potent inhibitor of arginase [36].

It is noteworthy that there were numerous studies reporting L-Arginine metabolism and NO synthesis at systemic level. However, because of its high degree of tissue-specific responses to pathological conditions, detailed information on L-Arginine metabolism at individual organ levels is still much needed in order to gain better understanding of the role of this versatile amino acid. Previously, using isotopic tracer technique, Deutz et al [21, 37] have assessed

effects of endotoxemia on local NO synthesis in different organs such as liver, kidneys, gut, and muscle in mice and porcine model. With the same technique, the present study provided the first quantitative information on how local L-Arginine metabolism in the lungs is modulated in large animal model.

Because of the complex role of NO and arginine in the pathophysiology of sepsis, it is unclear whether the local predominant activation of arginine-NO pathway in the septic lungs observed in this study is beneficial or not. Previously, administration of NO has been shown to improve pulmonary circulation and gas exchange in endotoxin shock [4]. Arginine supplement has also been reported to have beneficial effect by various mechanisms including enhancement of NOS pathway and NO production. Thus, increased NO via NOS might be regarded as a compensatory mechanism in sepsis; on the other hand, other studies showed favorable results by inhibition of NO production with nonselective and selective NOS-2 inhibitors, as increase NO production by NOS-2 is linked to systemic hypotension and oxidative stress. These seemingly contradictory results indicate that more in-depth study on arginine metabolism is needed.

In present study, we demonstrated in an animal model that sepsis induced by *P aeruginosa* infusion caused significant changes in arginine metabolism in the lungs. While the total delivery of arginine to the lungs was reduced, the fractional uptake and metabolic rate of arginine in the lungs was enhanced. Furthermore, we showed that arginine was mainly consumed via arginine – NO pathway, which might be responsible for this enhanced metabolic activity in the septic lungs. The quantitative information provided in this study will help us better understand the effect of sepsis on local arginine metabolism in the lungs. Because arginine catabolism involves multiple organs and is highly variable among these organs, more studies on arginine metabolism within each individual organ is needed to gain full insight of arginine metabolism and further design a more reliable arginine-based therapeutic intervention for sepsis.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Yong-Ming Yu or Hongzhi Xu, Shriners Burn Hospital for Children, Massachusetts General Hospital, 51 Blossom Street, Boston, MA, 02114, USA. E-mail: yyu@partners.org (YMY); hongzhi2008@gmail.com (HZX)

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