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Systemic Arginine Depletion After a Murine Model of Surgery or Trauma

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Abstract

Background—Arginine metabolism and availability after surgery or trauma (ST) is an important modulator of immune responses. Arginine levels are significantly depleted in human trauma patients. Diets containing arginine administered to surgery patients have restored immune function. We hypothesized an arginase-dependent depletion of arginine in a murine model of ST. In addition, we hypothesized a systemic arginase release in human trauma patients.

Methods—Male mice were anesthetized and a laparotomy with bowel manipulation was used as a model of ST. Plasma was collected after ST for analysis of arginase activity and arginine, ornithine, and citrulline. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) in plasma were measured after ST. Also, arginase activity was determined in human plasma from 4 healthy controls and 8 trauma patients.

Results—Arginase activity increased maximally at 2–4 hours after ST, and arginine was significantly reduced after ST. Citrulline was significantly decreased at 8 and 12 hours after ST. Plasma AST and ALT did not significantly vary from control mice after ST. In addition, on day 1 after intensive care unit admission, human trauma patients exhibited a significant increase in arginase activity.

Conclusions—The biological consequences of arginine depletion remain incompletely understood. These data are consistent with data showing that patients given arginine-containing diets experience reduced morbidity. Understanding of arginine metabolism after ST may lead to therapies aimed at improving clinical outcome after ST.

Keywords

surgery; injury; trauma; arginase; infection

Clinical Relevancy Statement

Arginine metabolism after surgery or trauma plays a critical role in the regulation of immune responses as diverse as vasodilation, wound healing, and T cell function. The mechanisms for which arginine supplementation improves outcome after surgery remain largely unknown. Here, we describe novel findings that show systemic levels of arginine are depleted by arginase released into the circulation. Furthermore, we show that arginase is also increased in plasma of human trauma patients. These findings suggest a novel mechanism of arginine depletion by extracellular arginase. Understanding arginine/arginase metabolism after injury will help to develop strategies to decrease morbidity and mortality.

Introduction

Trauma is a major source of morbidity and mortality worldwide.¹ In the United States, it is the fifth largest cause of death and the number one cause of death in people younger than age 44.² A significant cause of increased morbidity, mortality, and cost associated with trauma is the development of infections. Similar to trauma, infectious complications after elective surgery are proportional to the degree of physical injury and increase morbidity, mortality, and cost. Understanding the mechanisms that lead to increased susceptibility to infections after trauma and/or surgery is a worthwhile strategy that should result in the development of successful therapies.

L-arginine is a semi-essential or conditionally essential amino acid in adults during times of catabolic stress, and replacement is advocated during illnesses, including surgery or trauma (ST) and possibly in sepsis.³ Arginine is also the substrate for both nitric oxide synthase production of nitric oxide (NO), a biological molecule involved in processes as diverse as vasodilation, and bacterial cell killing.^{4,5} In addition, arginine plays multiple other essential biological roles, including being an essential amino acid during T lymphocyte activation, and is the substrate for the production of polyamines and hydroxyproline and is thus involved in collagen deposition and wound healing.^{6,7} All of these biological functions can be negatively affected by the absence or depletion of arginine.

Arginine is catabolized by the enzyme arginase, of which there are 2 isoenzymes: arginase-1 and arginase-2. Classically, arginase-1 is observed in the liver, where its constitutive expression forms an integral part of the urea cycle. In 2001, our laboratory was the first to report the presence of arginase-1 in immune tissues (such as the spleen) and demonstrated that it could be induced as part of a response to physical injury in mice and was also observed in peripheral blood mononuclear cells in humans after surgery or trauma.^{8,9} More recently, we were able to isolate unique arginase-1-expressing cell subpopulations, characterized as CD11b⁺ and GR-1⁺ splenocytes in mice after ST and CD16⁺ cells in human peripheral blood mononuclear cells after trauma.^{10,11} Furthermore, in humans, the CD16⁺ cells also had increased expression of CD66b, characterizing these cells as arginase-1-expressing activated neutrophils.¹¹ Also, human neutrophils have been shown to constitutively express arginase-1.¹²

In 2001, we also forwarded the hypothesis that arginase-1 could be responsible for the depletion of arginine in stress states, explaining the mechanisms by which arginine deficiency developed.^{8,9} This hypothesis has gained increased acceptance, particularly with the report that blunting both the appearance of CD11b⁺/Gr1⁺ cells and arginase-1 induction after trauma was associated with improved production of NO after trauma in response to endotoxin.¹³ The increase in cells expressing arginase implies that arginine depletion occurs at a cellular level in tissues and does not address the role of arginase release into the systemic environment.

The experiments in this article take a different approach in that we have focused our work on understanding how arginase could help explain systemic arginine depletion. We report that contrary to previous belief, arginase release in plasma correlates with early depletion of systemic arginine. This systemic increase in arginase can be observed both in a mouse model of moderate ST and in humans after trauma, which we show is accompanied in mice with a significant decrease in systemic arginine.

Our work thus provides novel insights as to how arginine depletion may occur systemically after physical injury. Furthermore, our work demonstrates a possible novel mechanism of arginine deficiency, albeit active systemic enzymatic destruction. Our work raises the possibility that therapies aimed at restoring early arginine availability may play a significant role in reestablishing arginine homeostasis.

Materials and Methods

The current experimental protocol was approved by the University of Pittsburgh Institutional Animal Care and Use Committee and Division of Laboratory Animal Research. All reagents were purchased from Sigma Aldrich (St Louis, MO) unless otherwise noted.

Animal Model of ST

C57BL/6 male mice aged 6–8 weeks were purchased from Jackson Laboratories (Bar Harbor, ME) and were kept in a pathogen-free facility under 12-hour light/dark cycles in a temperature range from 20–22°C. Mice were allowed a 2-week acclimation period, and food and water were available ad libitum. A mouse model of physical injury as described previously was used in this study.^{10,13} Briefly, under aseptic conditions, a midline laparotomy incision was made after administering anesthetic (430 mg/kg ketamine and 34 mg/kg xylazine). The bowels were teased for 30 seconds. The incision was closed in 2 layers, and animals were placed on warming pad until fully recovered. Mice receiving anesthesia only served as controls. Time course experiments were collected at 1, 2, 4, 8, 12, and 24 hours after ST, and the timing after ST was started when mice were placed on the warming pad.

Collection of Plasma

Mice were anesthetized using isoflurane, and whole blood was collected by cardiac puncture. Blood was placed into tubes containing sodium heparin, centrifuged, and plasma aliquoted and frozen immediately at –80°C for down-stream analysis.

Quantification of Amino Acids

Arginine, citrulline, and ornithine were extracted and derivatized from plasma (25–100 µL) using the EZ:fast LC-MS for amino acid analysis kit from Phenomenex (Torrance, CA) according to the manufacturer's instructions, as described previously.¹⁴ These samples were processed with high-pressure liquid chromatography (HPLC; Varian ProStar Pumps, Model 210 [Varian, Inc, Walnut Creek, CA]) and mass spectrometry (MS; Varian 1200L Quadrupole MS/MS) using a Phenomenex EZ:fast 4-µm AAA-MS column (250 × 2.0 mm).

Arginase Activity Assay

Arginase activity (conversion of L-arginine to L-ornithine) was assessed in nonhemolyzed plasma samples as described previously.¹⁵ Briefly, arginase was activated by incubation of serum with MnCl₂ at 55°C for 20 minutes. Carbonate buffer (100 mM) and 100 mM L-arginine were added and samples were incubated for 40 minutes at 37°C. Background ornithine concentration was assessed by running the same samples in parallel but excluding the 40-minute incubation at 37°C. The reaction was stopped immediately by adding glacial

acetic acid, and ninhydrin solution (2.5 g ninhydrin, 40 mL 6M phosphoric and 60 mL glacial acetic acid) was added. Standards and samples were heated at 95°C for L-ornithine-dependent color development. The colorimetric reaction with L-ornithine was measured with a spectrophotometer at 515 nm (Spectramax 340; Molecular Devices, Sunnyvale, CA). Arginase assay was linear with time, and activity was normalized to total protein in each sample quantified by the Bradford method.¹⁶

Human Study of Arginase in Plasma

Trauma patients were admitted to the intensive care unit (ICU) at Presbyterian Hospital of the University of Pittsburgh Medical Center. The institutional review board (IRB) of the University of Pittsburgh approved this study protocol, and informed consent to participate was obtained from patients or next of kin as per IRB regulations.

Statistical Analysis

For all comparisons, data were normal and variances were not different. For single comparison of 2 groups, a Student *t* test was used. One-way analysis of variance with Tukey-Kramer multiple comparison tests was used to compare 3 or more groups. The differences were considered significant at a *P* value < .05. All data are presented as mean $\pm$ SEM. GraphPad Prism (GraphPad Software, La Jolla, CA) was used for the analyses.

Results

Arginase Activity in Plasma Is Significantly Increased After ST

We have extensively reported the presence of arginase activity in immune cells, including circulating myeloid cells (possibly neutrophils) in humans and in CD11b⁺/Gr-1⁺ cells in splenic tissues after ST in male mice.^{10,11} Arginase expressed in these cells can deplete arginine in the local environment, but this does not explain a decrease in circulating arginine. We hypothesized that arginase release occurred systemically after ST. We thus measured arginase activity in plasma at 1, 2, 4, 8, 12, and 24 hours after ST. In support of our hypothesis, arginase activity in plasma was increased to 6.6 ± 2.1 μ M ornithine/min/mg by 1 hour and significantly elevated to 19.9 ± 1.7 , 26.6 ± 1.9 , and 12.5 ± 2.01 μ M ornithine/min/mg at 2, 4, and 8 hours after ST, respectively, vs 0.401 ± 0.19 μ M ornithine/min/mg in control mice (Figure 1; *P* < .05). Maximal arginase activity was observed from 2–4 hours after ST (26.6 ± 1.9 μ M ornithine/min/mg). The arginase activity started to decrease at 8 hours after ST and was elevated but not significantly higher than in controls by 12 and 24 hours after ST (6.7 ± 1.4 and 2.1 ± 0.6 , respectively; Figure 1).

Plasma AST and ALT Are Not Elevated Early After ST

Arginase-1 is not only expressed in immature PMN myeloid cells but also constitutively expressed in hepatocytes. To exclude the possibility of liver damage causing increased arginase activity that we observed above, we measured systemic circulation of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), which would increase if there were a significant amount of liver damage. As shown in Figure 2, ALT and AST levels were not significantly elevated at 0.5, 1, 2, or 4 hours after ST relative to control mice (*P* > .05). These data suggest that systemic arginase release triggered by trauma is not coming from liver tissue damage.

Arginine Levels in Plasma in Mice Decrease Early After ST

We have previously reported that CD11b⁺/Gr-1⁺ cells after ST that appear in the spleen are capable of depleting arginine locally. These findings, however, do not explain systemic changes observed in circulating arginine. We thus measured plasma arginine levels at 1, 2,

4, 8, 12, and 24 hours in mice subjected to ST. Indeed, a progressive decrease in arginine plasma levels was observed for the first 8 hours after ST. Arginine in plasma was significantly decreased relative to control mice at 1, 2, 4, and 8 hours after ST (Figure 3A; $P < .05$). A nadir was observed at 4 hours (14.9 ± 6.8 vs 127 ± 9.7 μM in controls), demonstrating an 88% decrease in circulating arginine when compared to control mice.

To determine whether the decrease in plasma arginine was a result of increased arginase activity, we correlated arginase activity and arginine using a regression analysis. Figure 3B shows a tight negative correlation between plasma arginine and plasma arginase activity ($P < .01$; Pearson correlation coefficient = -0.92). These data are compatible with the hypothesis that circulating arginase is biologically active and responsible for the decrease in arginine.

Citrulline Levels in Plasma Are Decreased After ST

Citrulline is a precursor for the formation of arginine and is used by the kidney to generate and release arginine to the systemic circulation. Thus, we measured citrulline levels to determine if they were affected by ST at the same intervals in which we measured arginine. Figure 4 demonstrates that citrulline levels were lower than nontrauma controls at 1, 2, and 4 hours after injury and were significantly less than nontrauma controls at 8 and 12 hours after ST (42.8 ± 3.1 in control mice vs 24.3 ± 3.7 and 23.1 ± 2.1 μM , respectively; $P < .05$).

Ornithine Levels in Plasma Do Not Decrease After ST

Ornithine is a product of the metabolism of arginine by arginase. Thus, we hypothesized that, in contrast to citrulline and in response to the decrease in arginine, ornithine plasma levels should be either maintained or increased after ST. As seen in Figure 5, ornithine levels in plasma showed an initial nonsignificant decrease relative to controls at 1 hour (70.6 ± 7.8 vs 37.3 ± 1.9 μM , respectively; $P > .05$), followed by an increase at 4 hours after injury, which also did not reach statistical significance (99.5 ± 17 μM). Thus, in contrast to citrulline and arginine plasma levels, ornithine levels remained stable and tended to increase, data that are again compatible with a biologically active systemic arginase.

Systemic Arginase in Human Trauma Patients

We have previously demonstrated that a significant decrease in circulating arginine is characteristically observed in trauma patients and, depending on the severity of injury, may last for more than a week.⁹ In addition, we have demonstrated that trauma activates circulating neutrophils, which show signs of degranulation.¹¹ As studied in mice, we thus tested the hypothesis that there is a similar increase in circulating arginase activity in humans. Four healthy controls and 8 trauma patients were enrolled in a study to evaluate arginase activity in human plasma on day 1 after admission. As seen in Table 1, in 4 healthy male controls with an average age of 36.5 years (range, 30-54 years), arginase activity in plasma was 0.26 μM ornithine/min/mg (Figure 6). The 8 male trauma patients, with an average age of 44.8 years (range, 20-60 years), had suffered a motor vehicle accident, fall, or crush injury. Mean injury severity score was 31.2 (range, 17-40), and arginase activity in trauma patients on day 1 was significantly elevated compared to healthy controls (Figure 6; 2.3 ± 0.83 vs 0.26 ± 0.12 , respectively; $P < .05$). Thus, similar to mice, we demonstrate that in humans, trauma is associated with increased systemic release of arginase.

Discussion

Arginine supplementation in the form of diets also containing ω -3 fatty acids and nucleotides fed to patients after elective surgery results in a significant decrease in infections, complications, and cost.¹⁷⁻¹⁹ These diets clearly show beneficial effects and

should be advocated for patients undergoing high-risk elective surgery. Arginine-containing diets may also benefit trauma patients, although the degree of evidence of benefit in trauma (as opposed to elective surgery) is still fragmentary and will only be solved by adequately designed studies. The mechanisms of how these diets exert the clinical benefits observed remain unclear, a fact that limits their utilization and acceptance. To solve this, our laboratory has dedicated its efforts at systematically analyzing the metabolism of arginine after physical injury.

A decrease in circulating arginine has been described under conditions of stress, including different forms of trauma. This decrease is proportional to the severity of injury and is a well-accepted observation in the literature.⁹ Previously, other investigators have suggested that the fact that arginine levels drop after injury is a sign that arginine is “conditionally essential” and should prompt its replacement in the diet.^{20,21} The mechanisms behind the decrease in circulating arginine and the biological consequences that stem from this have been unclear until now.

This article demonstrates that there is an early decrease in circulating arginine likely as a result of the release of circulating arginase. This increase in arginase activity is observed both in mice after moderate trauma and in humans. Our data suggest that arginase release early after injury does not come from the liver, which is the organ where most constitutive arginase is concentrated, as we have been unable to demonstrate a concomitant release in AST and ALT up to 4 hours after injury when arginine levels are most severely depleted. In separate publications, we have demonstrated in humans that activation of neutrophils by trauma results in degranulation and may last for up to several weeks.¹¹ Thus, coupled with previous reports, the data presented in this article raise the possibility that circulating arginase occurs as a result of neutrophil activation. However, these data do not rule out the possibility of arginase release from other sources such as the vascular endothelium, and further work remains to elucidate the source of circulating arginase.

Arginine levels decrease only temporarily in the model of surgical trauma. We have previously demonstrated that increased arginase activity is proportional to the severity of injury with only modest increases (1.5- to 2-fold) after minor elective surgery but a 10-fold or more increase in severe trauma.⁹ The model of trauma reported in this article is modest and constitutes the performance of a laparotomy with a modest manipulation of the bowel. Most animals survive this procedure indefinitely. We have previously determined that arginase expression correlates with the severity of injury. Thus, it is not surprising that in the model of moderate surgical trauma used in this article, systemic arginine depletion is limited as arginase release is also limited to a few hours.

The biological consequences of arginine depletion are incompletely studied. Despite this, it is logical to hypothesize that the immune system could potentially be affected. This is particularly possible because NO produced by myeloid cells through the induction of nitric oxide synthase and the function of activated T cells depends on arginine availability.^{6,21} To help study the biological activity of arginase, numerous researchers have used a potent enzymatic inhibitor, nor-N-hydroxy-L-arginine (nor-NOHA), to inhibit arginase activity, including coronary microvascular function in type 2 diabetic rats and an ischemia-reperfusion model of injury.^{22,23} To better study arginine availability, we have coupled this model of trauma with an infection with *Listeria monocytogenes*. Our initial work shows an increased susceptibility to infection in mice after ST. Furthermore, pharmacological blockade of arginase with nor-NOHA may attenuate the increased susceptibility of infection to trauma (data not shown). We hypothesize that the decrease in the number of bacteria may result from restored production of NO or T cell function, due to an increased availability of arginine. Further work remains to determine whether nor-NOHA restores arginine

availability, NO production, and T cell function and whether, by doing this, we can establish a definitive role of arginase as a cause of increased susceptibility to infection after trauma.

In conclusion, our article demonstrates that arginase release likely significantly explains the decrease in early systemic arginine. Further work should lead to demonstrating that the source of systemic arginase after trauma comes from neutrophil activation. This work lends support to the utilization of arginine-containing diets as a mechanism to overcome early arginine depletion after surgery or trauma.

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Figure 1.

Arginase activity in plasma after surgery or trauma (ST). C57BL/6 male mice were subjected to ST or control anesthesia only, and plasma arginase activity (μM ornithine/min/mg) was measured in controls or at 1, 2, 4, 8, 12, and 24 post-ST. Data are shown as mean $\pm$ SEM. * $P < .05$ vs control group. $n = 3$ per treatment, representative of 2 independent experiments.

Figure 2.

Mouse plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels after surgery or trauma (ST). C57BL/6 male mice were subjected to ST or control anesthesia only, and levels of AST and ALT were measured in the plasma in control mice or 0.5, 1, 2, and 4 hours after ST. Data displayed as mean $\pm$ SEM.

Figure 3.

Mouse plasma levels of arginine after surgery or trauma (ST). C57BL/6 male mice were subjected to ST or control anesthesia only. Levels of arginine in plasma were analyzed by high-performance liquid chromatography (A) (* $P < .05$ vs control group) and correlation of arginase activity and arginine levels in mice at baseline and 1, 2, 4, 8, 12, and 24 hours after ST (B) ($P < .01$ of correlation arginine vs arginase activity; Pearson correlation coefficient = -0.92). Data displayed as mean $\pm$ SEM, $n = 3$ per group of 2 independent experiments.

Figure 4.

Systemic citrulline in mouse plasma after surgery or trauma (ST). C57BL/6 male mice were subjected to ST or control anesthesia only. Levels of citrulline in plasma in control mice or 1, 2, 4, 8, 12, and 24 hours post-ST were analyzed by high-performance liquid chromatography. * $P < .05$ vs control group; data shown as mean $\pm$ SEM.

Figure 5. Ornithine in mouse plasma after surgery or trauma (ST). C57BL/6 control mice received anesthesia only or were subjected to ST, and concentrations of ornithine in plasma were determined by high-performance liquid chromatography. Data shown as mean $\pm$ SEM.

Figure 6.

Arginase activity in healthy controls and trauma patients. Arginase activity (μM ornithine/min/mg) was measured in healthy controls and trauma patients on day 1 after admission to the intensive care unit. $*P < .05$ vs healthy controls. Bars represent 90th and 10th percentiles; box shows interquartile range, and line shows median.

Table 1

Demographics of Healthy Controls and Trauma Patients

	No.	Age (Range), y	Sex	Injury Severity Score (Range)
Healthy controls	4	36.5 (30-54)	Male	NA
Trauma patients	8	44.8 (20-60)	Male	31.2 (17-40)

NA, not applicable.