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## Bioactive products of arginine in sepsis: tissue and plasma composition after LPS and iNOS blockade

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**Lortie, Mark J., Shunji Ishizuka, Doron Schwartz, and Roland C. Blantz.** Bioactive products of arginine in sepsis: tissue and plasma composition after LPS and iNOS blockade. *Am J Physiol Cell Physiol* 278: C1191–C1199, 2000.—Blockade or gene deletion of inducible nitric oxide synthase (iNOS) fails to fully abrogate all the sequelae leading to the high morbidity of septicemia. An increase in substrate uptake may be necessary for the increased production of nitric oxide (NO), but arginine is also a precursor for other bioactive products. Herein, we demonstrate an increase in alternate arginine products via arginine and ornithine decarboxylase in rats given lipopolysaccharide (LPS). The expression of iNOS mRNA in renal tissue was evident 60 but not 30 min post-LPS, yet a rapid decrease in blood pressure was obtained within 30 min that was completely inhibited by selective iNOS blockade. Plasma levels of arginine and ornithine decreased by at least 30% within 60 min of LPS administration, an effect not inhibited by the iNOS blocker L-N<sup>6</sup>(1-iminoethyl)lysine (L-NIL). Significant increases in plasma nitrates and citrulline occurred only 3–4 h post-LPS, an effect blocked by L-NIL pretreatment. The intracellular composition of organs harvested 6 h post-LPS reflected tissue-specific profiles of arginine and related metabolites. Tissue arginine concentration, normally an order of magnitude higher than in plasma, did not decrease after LPS. Pretreatment with L-NIL had a significant impact on the disposition of tissue arginine that was organ specific. These data demonstrate changes in arginine metabolism before and after de novo iNOS activity. Selective blockade of iNOS did not prevent uptake and can deregulate the production of other bioactive arginine metabolites.

L-N<sup>6</sup>(1-iminoethyl)lysine; agmatine; polyamines; arginine decarboxylase; ornithine decarboxylase; nitric oxide; putrescine; ornithine; spermine; spermidine; lipopolysaccharide; inducible nitric oxide synthase

THE CONCENTRATION OF ARGININE in extracellular fluid is maintained within 100–200  $\mu$ M mainly by 1) absorption from the diet, 2) conversion to ornithine by the liver, and 3) synthesis from citrulline in the kidney (37). The importance of an exogenous source of this “semi-essential” amino acid is well known with respect to normal growth and wound healing. Thus, although most cells can synthesize some arginine, the cellular uptake route is thought to be of primary importance. A

number of products derived from arginine are known to be important bioactive compounds affecting a broad range of physiological and pathophysiological functions; however, regulation of the metabolic fate of this amino acid is less well defined. In the last decade, an enormous amount of interest has focused on the role of nitric oxide (NO) generated from arginine by NO synthase (NOS). NO is a highly reactive vasodilatory substance with other effects ranging from neuromodulation (47) to bacteriostasis (50, 52). Arginine is also converted to ornithine, the precursor to polyamines, via the action of arginase. The essential and ubiquitous nature of polyamines (putrescine, spermine, and spermidine) derived from ornithine decarboxylase (ODC) activity has been characterized for decades (21, 22, 28), yet the mechanisms whereby these cationic compounds regulate gene transcription and Ca<sup>2+</sup> signaling remain elusive. A regulatory role for polyamines in inducible (i) NOS induction has been reported whereby ODC activity is elevated before iNOS expression (33, 36), and the aldehyde metabolites of these compounds suppress iNOS induction (51). Evidence of agmatine production by arginine decarboxylase (ADC) in mammals is more recent, but there is increasing physiological and pharmacological evidence of central, vascular, and renal actions of this compound (18, 24, 35). Because arginine is the common substrate for these molecules, it is not surprising that feedback mechanisms for the different metabolic pathways interact. For example, N-omega-hydroxy-L-arginine, an intermediary in the production of NO from arginine, is a potent inhibitor of arginase activity (7), as is citrulline (44). Also, a report describes increased polyamine degradation mediated by exogenous agmatine (53). Work from our laboratory has shown that agmatine exerts inhibitory effects on both NOS and polyamine pathways (40, 43) and most recently that NO directly inhibits ODC activity (39).

Sepsis is characterized by renal failure and a reduction in systemic vascular resistance that is resistant to vasopressor therapy. Bacterial products such as lipopolysaccharide (LPS) trigger a number of cellular events via the immune response of cytokine release (6), including the induction of a Ca<sup>2+</sup>-independent isoform of NOS (iNOS; see Ref. 57) and massive NO production. Hypotension and impaired renal function in the (LPS) rat model of sepsis is ameliorated by administration of highly selective iNOS inhibitors (42). However, iNOS blockade (1, 23, 49) or gene deletion (26, 54, 58) cannot fully abrogate all the effects of LPS. This might be

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anticipated considering the multiple adaptive mechanisms coincided with iNOS. LPS is known to upregulate synthesis of the Na<sup>+</sup>-independent Y<sup>+</sup> transporters (9, 20), which facilitates the uptake of arginine and ornithine. Increases in arginine transport may be necessary for the generation of high NO levels (4, 48) and appears to be regulated independently from de novo iNOS expression (11, 45, 48) and polyamine synthesis (11). However, the net effect of upregulating bidirectional transporters on intracellular amino acid concentration is not clearly defined. In addition to iNOS expression, a multitude of cofactors and urea cycle enzymes that may promote and/or regulate NO production are upregulated by LPS (10, 17, 29, 32, 34, 46).

Considering the alternate pathways of arginine metabolism, the question arises as to whether selective blockade of the iNOS enzyme, at a time when arginine transport and/or synthesis is elevated, might promote the production of other bioactive compounds derived from arginine. We present herein a series of experiments in rats in which we assessed LPS-induced changes in arginine disposition with respect to iNOS induction in the presence or absence of L-N<sup>6</sup>(1-*iminoethyl*)lysine (L-NIL), a selective iNOS inhibitor. The data demonstrate that multiple arginine metabolic pathways are upregulated in the LPS model of sepsis. Furthermore, we show that early changes in plasma amino acid composition precede de novo iNOS activity. We also establish the impact of LPS with and without selective iNOS blockade on the characteristic intracellular amino acid composition of the kidney, lung, liver, and heart. Finally, we report a discordance in the temporal expression of physiological responses to LPS with respect to de novo iNOS expression, suggesting the occurrence of continuously expressed iNOS.

## METHODS

**Animal preparation.** To determine temporal changes in plasma amino acid composition, male Wistar rats (300 g) were prepared for chronic studies in awake animals by sterile surgical implantation of catheters (Silastic) in the left femoral artery and vein under short-acting anesthesia (Brevital). The catheters were exteriorized in the dorsal neck area to prevent self-induced damage and were flushed regularly with a sterile hypertonic solution of mannitol and heparin to prevent blockage. Animals were individually caged and trained to permit sampling and flushing of the catheters while in a restraining cage for at least 1 wk before studies. The arterial line was used for blood sampling and blood pressure monitoring, whereas the venous line was used for the bolus infusion of LPS (*Escherichia coli* 0111:B4, 1 mg/kg iv, freshly diluted in 1 mg/ml 0.9% NaCl; List Biological Laboratories). Some animals were pretreated with the selective iNOS inhibitor L-NIL (3 mg/kg ip; BID, Alexis, CA; see Ref. 56) for 3 days before LPS. This low-dose pretreatment regimen of L-NIL administration was previously validated by us to avoid change in systemic blood pressure, renal function, or plasma nitrates. Furthermore, because L-NIL is an analog of lysine and a substrate for the Y<sup>+</sup> transport system, a high bolus dose could potentially affect arginine uptake.

Blood samples (80  $\mu$ l) were collected from the arterial line at timed intervals in heparinized capillary tubes (Sigma) before and after LPS infusion. In all experiments, animals

were killed by rapid exsanguination while under anesthesia in accordance with good animal practice guidelines. Tissue samples were rapidly blotted on absorbent pads, dissected, and then placed on ice for enzyme studies described below or snap-frozen in liquid nitrogen and stored at  $-70^{\circ}\text{C}$  to await HPLC analysis as described below. For renal tissue, both kidneys were decapsulated and further dissected to isolate the cortex. For liver and cardiac tissue, sections of  $\sim 1$  g were dissected. Pulmonary tissue in these studies consisted of one upper and one lower lobe pooled after removal of large vessels and airways.

**Plasma and tissue sampling for HPLC studies.** For each blood sample, the plasma fraction was rapidly separated by centrifugation, and a 30- $\mu$ l aliquot was mixed with an equal volume of 10% TCA in 20 mM HCl solution containing  $10^{-4}$  M homocysteic acid as an internal standard. Proteins were denatured, and amino acids were extracted in this mixture for 24 h at  $4^{\circ}\text{C}$  before high-speed centrifugation to separate the protein pellet from the supernatant. Samples were stored at  $-70^{\circ}\text{C}$  until further processing for HPLC analysis. Processing of snap-frozen tissue for amino acid extraction involved pulverization of  $\sim 100$  mg of frozen tissue using a spring-loaded impact hammer kept frozen with liquid nitrogen. The frozen pulverized tissue was transferred to Eppendorf tubes containing 1 ml of 10% TCA in 20 mM HCl to denature the proteins before lyophilization (thereby eliminating enzyme activity and tissue water content). Samples were then resuspended in 250  $\mu$ l double distilled H<sub>2</sub>O, and the amino acids were extracted for 24 h at  $4^{\circ}\text{C}$ . After centrifugation at high speed to precipitate denatured proteins, the protein pellet was set aside for quantification, and the extract was stored at  $-70^{\circ}\text{C}$ . The tissue water content of each organ was established by determining a wet-to-dry ratio and protein content from separate samples of each organ.

**Fluorescence detection of amino acids.** For HPLC separation of amino acids, the same basic steps were used to prepare all samples for elution. The sample supernatants were transferred to a 10,000 molecular weight spin filter (Millipore) for further purification and then were extracted three times with hydrated ethyl ether to remove all traces of TCA and lipids. Plasma, tissue extracts, and appropriate known standards were then derivatized for fluorescence detection of primary and secondary amine groups with *N*-hydroxysuccinimidyl-6-aminoquinoyl carbamate as per kit instructions (AccQ tag; Waters). Elution was performed using a Hewlett-Packard 1100 series binary HPLC pump system with a 250-mm 3- $\mu$ m ODS Hypersil C<sub>18</sub> RP column (Hewlett-Packard) maintained at  $45^{\circ}\text{C}$ . Fluorescence was detected in line using a Waters 470 detector linked to the data acquisition system. Elution gradients were loosely based on the AccQ tag kit instructions.

**Spectrophotometric detection of nitrites and nitrates.** Systemic NO production was assessed from the plasma concentration of nitrites and nitrates using the Griess reaction in an automated HPLC system. Aliquots of known standards and of deproteinated plasma (10  $\mu$ l) were injected on a column of fine mesh cadmium plated with magnesium to reduce nitrate to nitrite. Postcolumn mixing with Griess reagents [1% sulfanilic acid in 5% H<sub>3</sub>PO<sub>4</sub> and 0.1% *N*-(1-naphthyl)ethylenediamide dihydrochloride] in a heated coil leading to a variable-wave ultraviolet detector enabled on-line recording of absorbance at 650 nm. The column can be bypassed to determine the ratio of nitrate and nitrite in a sample. Griess products in plasma were virtually all nitrates.

**ADC and ODC activity.** Tissue for enzymatic studies was obtained from a separate series of animals acutely anesthetized to permit intravenous LPS infusion. Animals were subsequently killed at predetermined time points after LPS

or vehicle bolus infusion (1 mg/kg), as described below. The preparation of viable renal proximal tubules by rapid mechanical and enzymatic digestion has been previously reported by us (24), and minor adaptation was suitable to isolate hepatic cells. ADC and ODC activity was assessed by the generation of radiolabeled  $\text{CO}_2$  from [ $^{14}\text{C}$ ]arginine or ornithine [C-1 labeled,  $1.5 \times 10^6$  counts  $\cdot$  min $^{-1}$  (cpm)  $\cdot$  tube $^{-1}$ , 50–60 mCi/mmol; American Radiolabeled Chemicals] using an aliquot of hepatic cells or renal proximal tubules [ $\sim 10$  mg in 1 ml DMEM (95%  $\text{O}_2$ , pH 7.4, 100  $\mu\text{M}$  arginine and ornithine, 0.05 mM pyridoxal phosphate, and 0.5 mM  $\text{MgSO}_4$ )]. Large-bore test tubes, capped with rubber stoppers and fitted with a metabolic well (Kontes) containing 300  $\mu\text{l}$  of Solvable (DuPont) as a  $^{14}\text{CO}_2$  trapping agent, were used to incubate cells at 37°C for 60 min. Enzyme activity was terminated by injecting 100% TCA through the cap after 60 min. Metabolic wells containing trapped  $^{14}\text{CO}_2$  were carefully transferred to vials containing scintillation fluid for counting. A standard Lowry test was used to determine the protein content of an aliquot. ADC and ODC activity is expressed as cpm of  $\text{CO}_2$  generated in 60 min per milligram protein per cpm added.

**iNOS PCR.** In a separate series of rats, two to three rats were killed at 0, 0.5, 1, 2, 4, 6, and 16 h post-LPS to recover the kidneys. RT-PCR analysis was used to determine iNOS mRNA in total extracts of renal cortex tissue. Ten micrograms total RNA extract were reverse transcribed with 0.1  $\mu\text{l}$  of 5 $\times$  buffer, 5  $\mu\text{l}$  dithiothreitol (0.1 M), 10  $\mu\text{l}$  dNTP (2.5 mM), 0.7  $\mu\text{g}$  oligo(dT), and 1  $\mu\text{g}$  RT in a total volume of 50  $\mu\text{l}$ . The reaction was stopped by heating the sample at 65°C for 10 min. PCR amplification was performed in a reaction volume of 100  $\mu\text{l}$  using a thermal cycler. cDNA was added to the reaction mixture containing 10  $\mu\text{l}$  of 10 $\times$  buffer, 2.5 ml dNTP (0.5 mM), 1  $\mu\text{l}$  of each primer, and 0.5  $\mu\text{l}$  *Taq* DNA polymerase (1.25 units). The iNOS primers used are based on the following rat sequence: sense 5'-GCCTCCCTCTGGAAAGA-3' and antisense 5'-TCCATGCAGACAACCTT-3'. Thirty-five cycles were performed under the following conditions: 95°C for 1 min (denaturation), 54°C for 1 min (annealing), 72°C for 2 min (extension), and 72°C for 7 min (final extension). Aliquots of the PCR products (15  $\mu\text{l}$ ) were then separated by gel electrophoresis on a 1% agarose gel stained with ethidium bromide. Rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to assess total mRNA and normalize iNOS content of each well. Densitometry evaluation (Scion) of a digitized image enabled the calculation of iNOS to GAPDH ratios.

**Statistics.** ANOVA was used to determine significant differences between paired values. Most assays were performed in duplicate or were completely reproduced in the case of HPLC detection, and mean values obtained for the same test sample were treated as a single value. Unless stated otherwise, the changes described in RESULTS were statistically significant ( $P < 0.05$ ).

## RESULTS

**Early and late phase responses.** Systemic blood pressure decreased significantly from  $103 \pm 4$  to  $90 \pm 6$  mmHg within 30 min after intravenous LPS. Pretreatment with L-NIL completely prevented the effects of LPS on blood pressure (Fig. 1). A significant increase in plasma Griess products, an index of NO production, from a baseline value of  $47 \pm 12$  to  $123 \pm 32$   $\mu\text{M}$  ( $n = 5$ ) occurred 3 h post-LPS, whereas no change was observed in either control or L-NIL-pretreated animals. In three rats, we monitored the profile of plasma Griess products over 24 h, as represented in Fig. 2. The sublethal dose of LPS used in these experiments caused

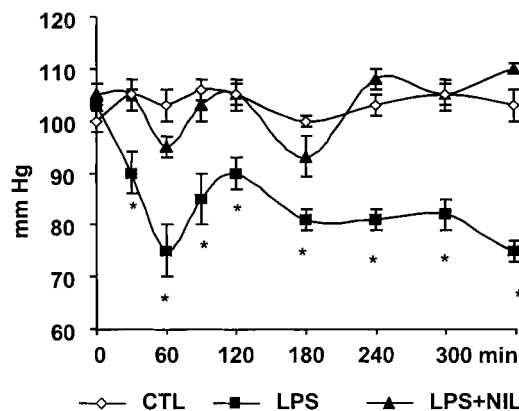


Fig. 1. Changes in arterial blood pressure after iv lipopolysaccharide (LPS) with or without L-N<sup>6</sup>(1-*iminoethyl*)lysine (L-NIL;  $n = 6$  rats; \*  $P < 0.05$ ) vs. control (CTL).

an increasing accumulation of plasma nitrate for at least 8 h that returned to normal by 24 h. Accordingly, de novo iNOS expression as determined by RT-PCR became evident in rat kidney within 60 but not 30 min after LPS, was maximal between 2 and 4 h, and was normal by 16 h (Fig. 3). Unexpectedly, a significant amount of iNOS mRNA was observed in control rat kidneys (i.e., *time 0*), resulting in a mean GAPDH-to-iNOS ratio of 2.9, a value that subsequently increased 30-fold between 1 and 6 h post-LPS.

The range in plasma concentration of arginine and arginine-derived products spans three orders of magnitude, as indicated on the scale of each graph in Fig. 4. Arginine in plasma decreased in both LPS and L-NIL + LPS-treated rats within 30 min from  $134.1 \pm 2.9$  and  $147.5 \pm 2.7$   $\mu\text{M}$  to  $91.1 \pm 4.4$  and  $100.6 \pm 3.9$   $\mu\text{M}$ , respectively. Plasma arginine levels further decreased to  $60.9 \pm 6.5$   $\mu\text{M}$  at 180 min and remained low for 6 h in the LPS group, supporting the concept that arginine uptake is upregulated by LPS. Plasma ornithine level followed a similar pattern of change, likely reflecting uptake by the same transporter, but tended to return to normal after 3 h. Another very rapid response to LPS was the transient increase in plasma spermidine concentration from  $1.74 \pm 0.11$  to  $3.28 \pm 0.15$   $\mu\text{M}$  in 60 min. Curiously, L-NIL treatment alone significantly elevated baseline spermidine levels to  $3.78 \pm 0.13$ , which subsequently decreased to control values by 120 min after LPS, as if polyamine synthesis and/or transport was not only initiated but also rapidly switched off. Spermine concentration in plasma was the lowest of the substances measured ( $0.25 \pm 0.09$   $\mu\text{M}$ ), and no significant changes were observed in either experimental condition.

A number of events appear to occur in a later phase (3–6 h) after LPS infusion. Plasma citrulline levels increased in the LPS-treated group from a baseline value of  $68.1 \pm 2.2$  to  $86.5 \pm 2.8$   $\mu\text{M}$  only after 4 h, an effect completely blocked by L-NIL pretreatment (Fig. 4). This is synchronous with increased nitrate levels in plasma and likely reflects massive NO production in tissue by iNOS. Plasma putrescine increased significantly in both LPS-treated groups but only after 3–4 h,

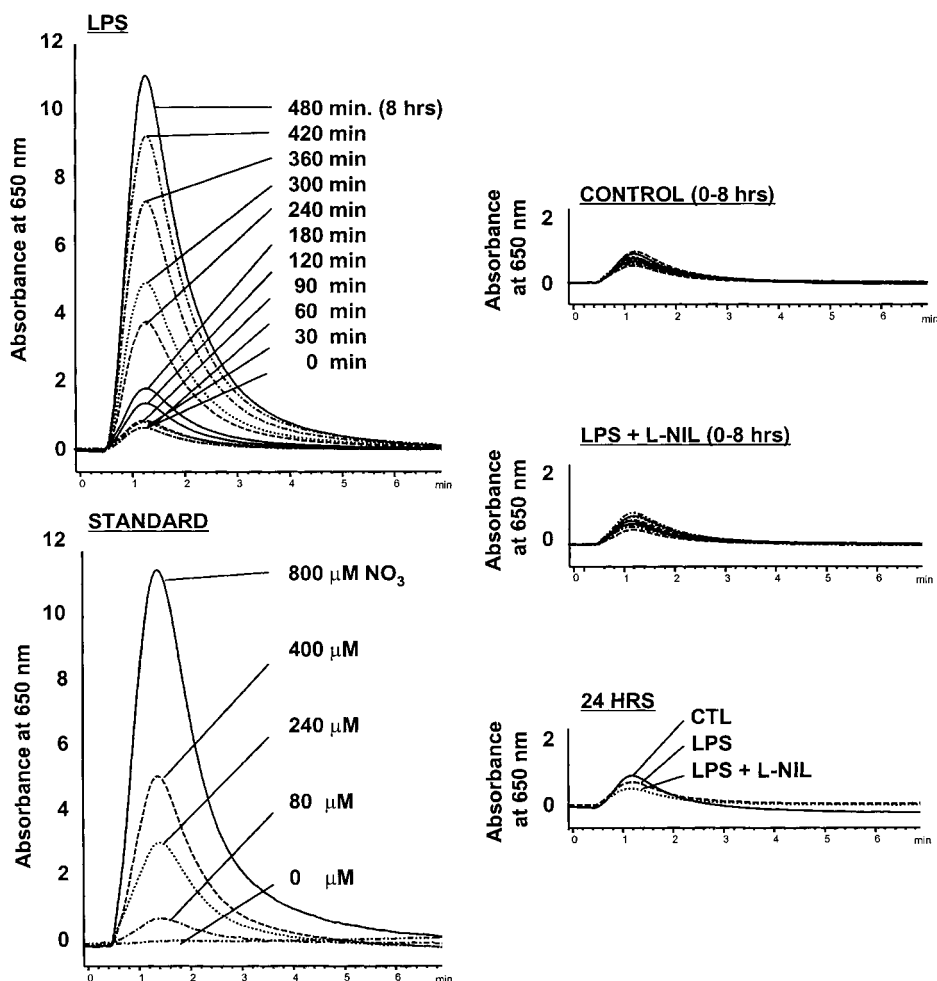


Fig. 2. Plasma nitrites increase after iv bolus of LPS, are blocked by L-NIL, and are normal within 24 h.

suggesting a delayed increase in ODC activity. Circulating agmatine levels were unchanged in animals treated with LPS alone but increased significantly after 4 h in the L-NIL-pretreated group from  $2.83 \pm 0.35$  to a maximum of  $4.69 \pm 0.21 \mu\text{M}$  after 6 h. As seen in Fig. 5, a significant increase in ODC and ADC activity was evident in proximal tubules and liver cells harvested 6 h post-LPS. ODC activity increased 48.79% in the kidney and 64.48% in the liver, whereas ADC activity increased 27.17 and 31.74%, respectively.

*Arginine and related products in tissue.* We determined the ratio of wet to dry tissue weight for each organ sampled 6 h post-LPS to evaluate potential changes in extracellular volume. No change occurred in either renal, hepatic, or cardiac tissue (wet-to-dry ratio:  $2.45 \pm 0.03$ ,  $1.79 \pm 0.01$ , and  $2.19 \pm 0.01$ , respectively). However, the appearance of edema was evident in pulmonary tissue, with the wet-to-dry tissue ratio increasing significantly from  $2.25 \pm 0.02$  to  $2.42 \pm 0.04$ , an effect that was blocked by L-NIL pretreatment. Calculations based on tissue water and protein content reveal that amino acid concentration can exceed plasma values by an order of magnitude and differs markedly among tissue types. For example, in control animals, we estimate that intracellular arginine concentration in the kidney is  $\sim 5$  vs.  $0.5$  mM in the liver, whereas values for ornithine are  $0.8$  and  $2.0$  mM, respectively. In the absence of a marker for extracellular volume in these experiments, calculations of intracellular concentration from these data underestimate actual values, particularly in tissue where there is potential for edema. Therefore, to enable comparison between tissue types, values for tissue content are expressed in relation to tissue protein (nmol/mg). The results depicted in Fig. 6 demonstrate substantial differences in amino acid composition of the kidney, heart, liver, and lung. In

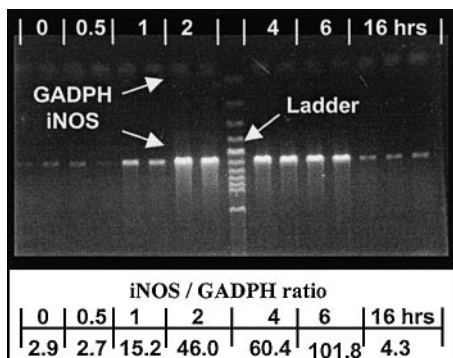


Fig. 3. Expression of inducible (i) nitric oxide synthase (NOS) mRNA in rat kidney cortex after LPS. Each lane represents kidney mRNA from an individual rat killed at the time indicated.

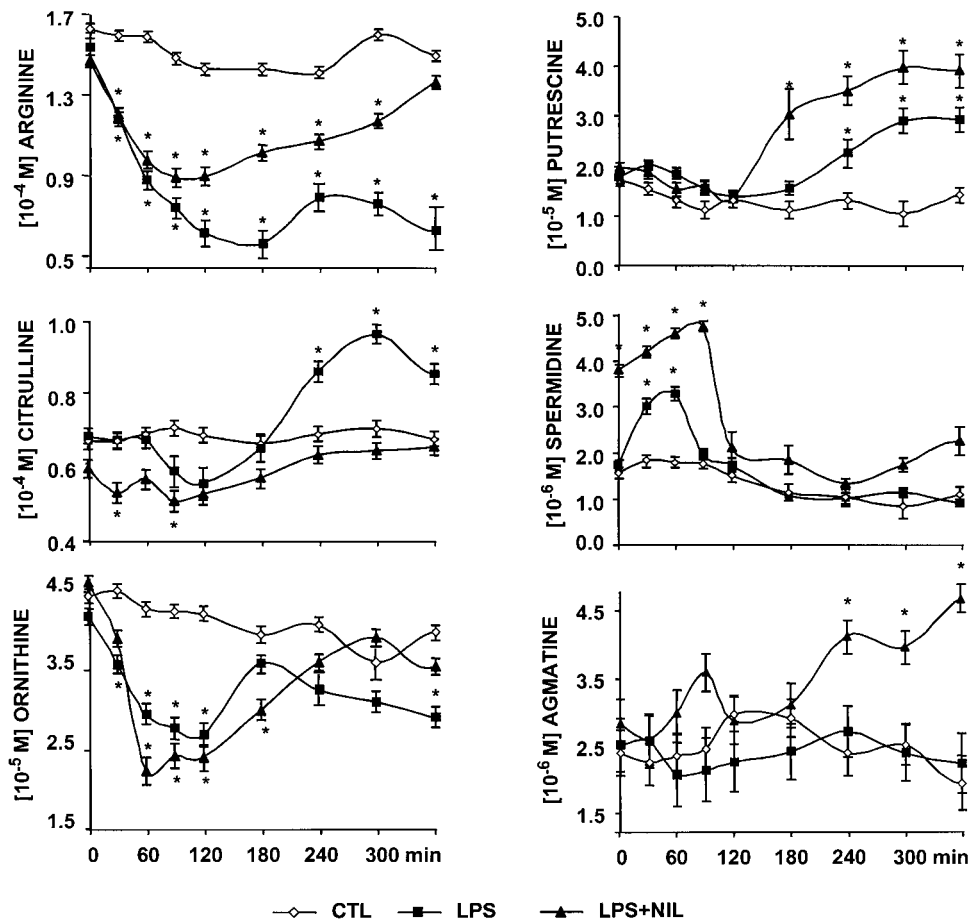


Fig. 4. Changes in plasma amino acids after iv LPS with or without L-NIL (*n* = 4; \**P* < 0.05) vs. CTL.

untreated animals, it is immediately apparent that the kidney contains substantially more arginine than the other organs tested. Also, a far greater proportion of arginine with respect to citrulline and ornithine ( $17.59 \pm$

$0.50$ ,  $4.51 \pm 0.21$ , and  $3.00 \pm 0.48$  nmol/mg, respectively) is maintained in the kidney, whereas in liver ornithine predominates ( $0.79 \pm 0.10$ ,  $1.54 \pm 0.16$ , and  $3.84 \pm 0.20$  nmol/mg, for arginine, citrulline, and ornithine, respectively). It is also evident that the organs tested contain more agmatine and polyamines in relation to arginine than was seen in plasma. Furthermore, unlike plasma, tissue putrescine content was found to be consistently lowest of the arginine-related metabolites measured.

No change in tissue arginine content could be demonstrated after LPS treatment in kidney liver or lung despite the evidence of high iNOS activity at 6 h, but a significant decrease from  $4.18 \pm 0.13$  to  $2.88 \pm 0.22$  nmol/mg did occur in the heart. Also, only a small but significant elevation in citrulline content was observed in the lung ( $2.12 \pm 0.13$  to  $2.86 \pm 0.05$  nmol/mg) and liver ( $1.54 \pm 0.16$  to  $2.11 \pm 0.09$  nmol/mg) of septic rats. Tissue ornithine content of LPS-treated rats was significantly increased from control values in kidney ( $3.00 \pm 0.48$  to  $3.98 \pm 0.30$  nmol/mg), liver ( $3.84 \pm 0.20$  to  $5.76 \pm 0.39$  nmol/mg), and lung ( $1.16 \pm 0.06$  to  $2.03 \pm 0.12$  nmol/mg). Baseline agmatine content was similar in all four tissues tested and only increased significantly in renal tissue from  $1.61 \pm 0.13$  to  $2.48 \pm 0.08$  nmol/mg. In addition, LPS treatment caused changes in tissue polyamine content that were tissue specific. In the kidney, spermidine levels increased significantly

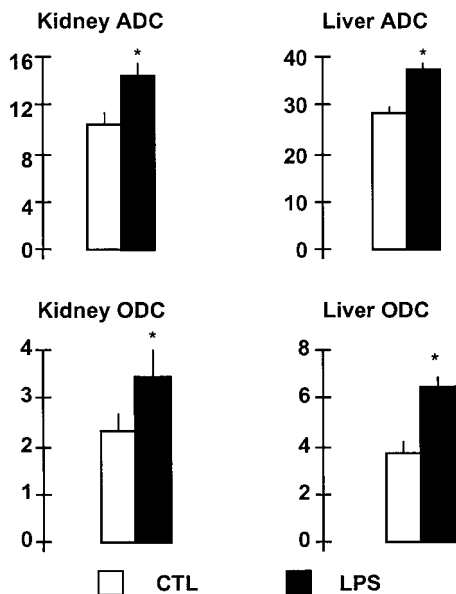


Fig. 5. Arginine (ADC) and ornithine (ODC) decarboxylase activity in kidney and liver cells from rats 6 h post-iv LPS or vehicle; *n* = 4, \**P* < 0.05 vs. CTL, y-axis =  $10^3 \text{ cpm } ^{14}\text{CO}_2 \cdot \text{mg protein}^{-1} \cdot \text{h}^{-1}$  at 37°C from  $10^6 \text{ cpm } ^{14}\text{C}$  arginine or  $^{14}\text{C}$  ornithine.

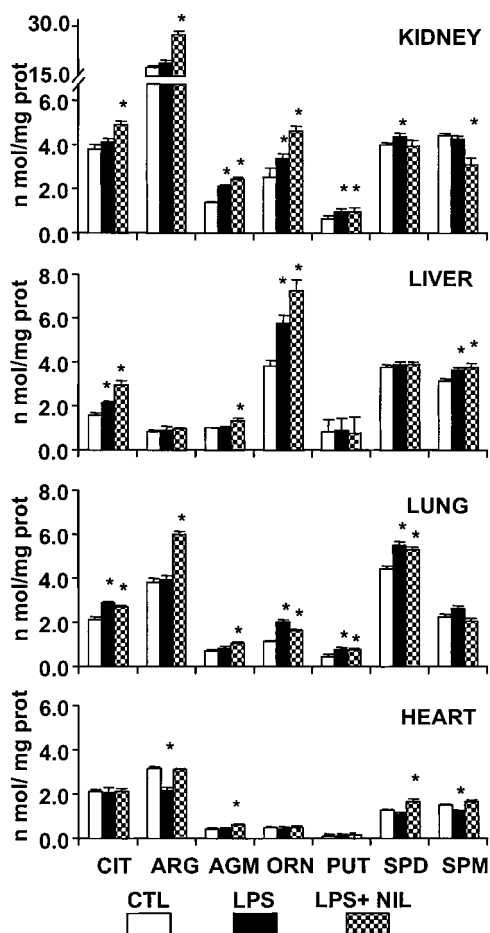


Fig. 6. Changes in tissue arginine and related metabolites after iv LPS with or without L-NIL;  $n = 4$ , \* $P < 0.05$  vs. CTL. CIT, citrulline; ARG, arginine; AGM, agmatine; ORN, ornithine; PUT, putrescine; SPD, spermidine; SPM, spermine; prot, protein.

from  $4.74 \pm 0.14$  to  $5.21 \pm 0.18$  nmol/mg. In the liver, spermine levels increased from  $3.13 \pm 0.13$  to  $3.59 \pm 0.17$  nmol/mg. In the lung, both putrescine and spermidine levels increased from  $0.46 \pm 0.10$  to  $0.74 \pm 0.15$  and  $4.45 \pm 0.13$  to  $5.47 \pm 0.19$  nmol/mg, respectively. As with arginine in cardiac tissue, spermidine and spermine levels in the heart tended to be lower after LPS treatment (significant for spermine only, from  $1.97 \pm 0.12$  to  $1.56 \pm 0.12$  nmol/mg). Clearly, LPS effected major changes in arginine metabolism beyond simply converting arginine to NO and citrulline.

Pretreatment with the iNOS inhibitor L-NIL in rats subjected to LPS had significant and unexpected effects on tissue amino acid composition. A consistent trend in tissue from L-NIL-treated animals was observed whereby agmatine content was greater than control or LPS treatment alone, as was observed in plasma. In the kidney, the levels of arginine, citrulline, ornithine, and agmatine ( $27.41 \pm 1.21$ ,  $5.80 \pm 0.20$ ,  $5.49 \pm 0.26$ , and  $2.88 \pm 0.15$  nmol/mg, respectively) were all significantly greater than corresponding values for control or LPS treatment alone. The increase in renal spermidine resulting from LPS alone was abrogated by L-NIL, and spermine content decreased significantly ( $3.71 \pm 0.33$  nmol/mg). In the liver, more citrulline, agmatine, and

ornithine ( $2.93 \pm 0.18$ ,  $1.33 \pm 0.08$ , and  $7.23 \pm 0.52$  nmol/mg, respectively) were detected than in the other experimental groups, but arginine content was unaffected and remained the lowest of the tissues tested. Pulmonary content of all the arginine products measured was greater in L-NIL-treated rats than control with the exception of spermine ( $6.00 \pm 0.12$ ,  $2.66 \pm 0.09$ ,  $1.61 \pm 0.09$ ,  $1.05 \pm 0.10$ ,  $0.73 \pm 0.11$ , and  $5.31 \pm 0.14$  nmol/mg, for arginine, citrulline, ornithine, agmatine, putrescine, and spermidine, respectively). In cardiac tissue, L-NIL prevented the decrease in arginine, spermidine, and spermine caused by LPS ( $4.11 \pm 0.11$ ,  $2.18 \pm 0.17$ , and  $2.21 \pm 0.10$  nmol/mg, respectively). As predicted, the blockade of iNOS activity caused an increase in the production of other arginine-derived bioactive products.

## DISCUSSION

*Early phase effects of LPS.* In characterizing the LPS-induced events with respect to arginine metabolism, it became clear that functional and physical changes occurred before evidence of de novo iNOS activity. Arginine and ornithine concentrations in plasma decreased rapidly and significantly after LPS, whereas that of other substances remained stable, indicating specific uptake. This finding is in accordance with in vitro studies demonstrating increased affinity and transport rates (Michaelis constant and maximal velocity) of the Y<sup>+</sup> system after LPS (3, 20, 55) and other stimuli (11). The data reported herein raise the question as to why arginine uptake precedes de novo iNOS synthesis and activity. At least two obvious explanations come to mind. 1) Arginine delivery for a quiescent substrate limited fast-response NOS. 2) Rapid arginine influx may be part of the cascade leading to de novo expression of iNOS and related enzymes. As discussed below, there is supporting evidence in the literature for both hypotheses.

We and others (2, 5, 8, and unpublished observation) have observed that arginine infusion elicits a number of responses, including NO production, and that plasma arginine is rapidly restored to normal levels, supporting the concept that uptake may be rate limiting in certain tissues in vivo. Recent studies using LPS-treated rats (13), pigs (14), dogs (19), and rabbits (41) have also demonstrated an early phase decrease in systemic arterial pressure. Although it is known that LPS may elicit the release of vasoactive substances other than NO, we unexpectedly observed that iNOS blockade with L-NIL completely abrogated this early phase hemodynamic response. How could L-NIL prevent the early phase hypotension after LPS? Possibly, L-NIL effects result from nonselective NOS blockade, yet we did not observe an increase in systemic blood pressure typical of constitutive endothelial NOS inhibitors. In fact, the selectivity of L-NIL and the dosage regimen aimed to avoid such effects. An alternate explanation derives from evidence of "constitutive" iNOS expression documented in the human airway (16) and rat kidney (31). Indeed, there are functional results reported that document an LPS-induced increase in

expired NO before increased expression of any NOS isoform (13, 14, 19, 41). It is worth noting that no further decrease in blood pressure occurs after 60 min, indicating that maximal vasodilation is achieved very rapidly and is maintained, although admittedly de novo iNOS may play a role in the later phase. Why plasma citrulline and Griess products are not elevated in the early phase remains to be explained. The amount of citrulline and Griess products generated might be relatively small with respect to plasma content or may be converted efficiently within the cell. With respect to plasma nitrite and nitrates (Griess products), alternate products of NO (i.e., peroxynitrite) may possibly play a role in early phase vasoactivity. Regardless of the NOS isoform blocked by L-NIL, these data demonstrate how the early phase hypertension after LPS is dissociated from de novo iNOS activity.

Because polyamines are known mediators of gene transcription, it is tempting to speculate that iNOS induction might require a signal from another arginine metabolite. It is interesting to observe that spermidine concentration in plasma rapidly and transiently doubled after LPS, suggesting a potential link between de novo iNOS induction and polyamine metabolism. There is supporting evidence for this concept from reports of rapid changes in polyamine content in vivo (27) and the regulation of a protein-synthesis initiation factor by spermidine (15). Furthermore, a counterregulatory mechanism has been characterized in which polyamine metabolites had an inhibitory effect on iNOS induction (51).

*Late phase effects of LPS.* A time lag of 2–3 h post-LPS occurred before the awake rats displayed visible signs of septicemia (lethargy, shivering, piloerection). This corresponds temporally to ~1 h after the appearance of de novo iNOS mRNA in tissue, most likely due to translation and processing. Accordingly, substantial increases in plasma citrulline and Griess products occur 3–4 h post-LPS, indicating large-scale NO production. It is worth noting that no further decrease in plasma arginine was detected at this critical time; in fact there appeared to be a trend toward an increase, suggesting a highly coordinated adaptation to maintain plasma arginine concentration. Alternatively, stable plasma arginine levels may indicate a shift away from dependence on uptake from plasma. Reports in the literature have addressed this issue. For example, tissue-specific expression of mRNA for argininosuccinate synthase and lyase was cosynchronous with respect to iNOS (32), and a decrease in arginase was demonstrated that coincided with increased iNOS (10). The impact of L-NIL on the events at this time period is evident from the absolute abrogation of citrulline and Griess product accumulation in plasma and a gradual return to normal of plasma arginine and ornithine within 6 h. Further experiments will be required to determine if the maintenance of low plasma arginine in the later phase results from high iNOS activity or as a mechanism of regulating uptake-dependent NO production.

Further evidence of a late phase change in arginine metabolism may be gleaned from plasma measurements of agmatine and putrescine, the products of arginine and ornithine decarboxylation. Plasma putrescine increased rapidly between 2 and 3 h after LPS, an effect that was amplified by iNOS blockade, whereas agmatine increased in the L-NIL group only. Although the source of these circulating substances cannot be determined from these experiments, we were able to demonstrate an increase in both ADC and ODC activity in freshly harvested liver and kidney 6 h after LPS. Together these data suggest that ADC and ODC activity may depend on substrate availability and/or regulation by NO itself. With respect to the latter, in vitro studies have shown that NO inactivates soluble ODC (39) and that membrane-bound ADC (38) decreases 18–24 h after iNOS induction.

*Arginine disposition in tissue.* The differences in the amino acid composition of the kidney, liver, heart, and lung reflect the specialized functional role of arginine products in these organs. Accordingly, we observed arginine levels in the kidney far in excess of other substances and a predominance of ornithine over citrulline and arginine in the liver. Such differences should be taken into account when comparing whole cell enzymatic activity from different tissue types using competitive inhibitors. Estimations based on tissue amino acid and water content reveal a large cell to plasma gradient for arginine and virtually all related products. In light of this, it is particularly difficult to imagine that arginine availability would be rate limiting for any enzyme in these organs unless there is intracellular sequestration. After LPS alone, little or no difference in arginine content of kidney, liver, and lung was observed, yet, in the presence of L-NIL, increases in intracellular arginine above control values indicate that compensatory mechanisms help sustain iNOS. Clearly, a high degree of tissue-specific adaptation involving varying degrees of transport and synthesis serves to maintain intracellular and plasma arginine levels. Depletion of arginine in the heart may have resulted from a compensatory increase in cardiac output and energy expenditure (link between the urea cycle and tricarboxylic acid cycle in the mitochondria) or a greater dependency on arginine uptake. Studies in ex vivo cardiac tissue responses to LPS have demonstrated some unique characteristics such as a lack of complete urea cycle (45, 30) and mRNA translation but no iNOS (25) activity.

In sharp contrast to observations in plasma, citrulline in the kidney, lung, and liver increased after LPS in L-NIL-treated rats. This suggests that an increase in urea cycle enzymes, concomitant with iNOS activation, promotes the generation of citrulline from ornithine. Normally, during iNOS activity the arginase pathway of ornithine synthesis would be inhibited by NO (7, 44) but not during iNOS blockade. The substantial tissue citrulline concentration and upregulation of urea cycle enzymes should therefore be taken into consideration in studies purporting to measure iNOS activity by the generation of citrulline. The induction and blockade of

iNOS resulted in some significant, tissue-specific effects on polyamine levels that could potentially exert functional effects. As noted previously, the exact role of polyamines has not been fully elucidated but includes mediation of critical cell functions such as replication and senescence. In light of this, it should be noted that the impact of sepsis and iNOS blockade could have substantially different effects in the elderly, since polyamine metabolism is known to change with age (12). In tissue, putrescine is maintained at substantially lower levels than ornithine or the polyamines in plasma, indicating that transport out of the cell may serve to regulate polyamine synthesis. Consistent with changes in plasma, agmatine levels increased in all four organs tested when iNOS activity was blocked. This again raises the possibility that L-NIL treatment increased agmatine production by abrogating inhibitory effects of NO, shunting arginine or as a feedback response to elevated ODC activity.

We conclude 1) hypotension and uptake of plasma arginine is evident within 30 min of LPS administration; 2) evidence of de novo iNOS activity first appears 3–4 h after LPS; 3) the early phase hypotension after LPS is inhibitable by L-NIL; 4) changes in plasma content of ornithine, agmatine, and polyamines occur before and after de novo iNOS activity; 5) LPS causes increased ADC and ODC activity, and blockade of iNOS results in increased agmatine and polyamine levels; 6) tissue arginine contents and the composition of arginine-derived products differ markedly among organs; and 7) substantial changes occur in a tissue-specific fashion to the amino acid composition of the kidney, lung, liver, and heart as a result of iNOS induction by LPS and blockade with L-NIL. Further studies will be needed to elucidate the time course and functional impact of redirected arginine metabolism with particular attention to specific organs and tissues.

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