Production of arginine by the kidney is impaired in a model of sepsis: early events following LPS


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Lortie, Mark J., Joseph Satriano, Francis B. Gabbai, Sonia Thareau, Ser Khang, Aihua Deng, Donald P. Pizzo, Scott C. Thomson, Roland C. Blantz, and Karen A. Munger. Production of arginine by the kidney is impaired in a model of sepsis: early events following LPS. Am J Physiol Regul Integr Comp Physiol 287: R1434–R1440, 2004. First published August 12, 2004; doi:10.1152/ajpregu.00373.2004.—Lipopolysaccharide (LPS) is a potent agent used experimentally to elicit the innate physiological responses observed in human sepsis. We have previously shown that LPS causes depletion of plasma arginine before inducible nitric oxide synthase (iNOS) activity, indicating that changes in arginine uptake and/or production rather than enhanced consumption are responsible. Because the kidney is the primary source of circulating arginine and renal failure is a hallmark of septicemia, we determined the time course of changes in arginine metabolism and kidney function relative to iNOS expression. LPS given intravenously to anesthetized rats caused a decrease in mean arterial blood pressure after 120 min that coincided with increased plasma nitric oxide end products (NOx) and iNOS expression in lung and liver. Interestingly, impairment of renal function preceded iNOS activity by 30–60 min and occurred in tandem with decreased renal arginine production. The baseline rate of renal arginine production was ~60 μmol·h⁻¹·kg⁻¹, corresponding to an apparent plasma half-life of ~20 min, and decreased by one-half within 60 min of LPS. Calculations based on the systemic production and clearance show that normally only 5% of kidney arginine output is destined to become nitric oxide and that ~25% of LPS-impaired renal production was converted to NOx in the first 4 h. In addition, we provide novel observations indicating that the kidney appears refractory to iNOS induction by LPS because no discernible enhancement of renal NOx production occurred within 4 h, and iNOS expression in the kidney was muted compared with that in liver or lung. These studies demonstrate that the major factor responsible for the rapid decrease in extracellular arginine content following LPS is impaired production by the kidney, a phenomenon that appears linked to reduced renal perfusion.

inducible nitric oxide synthase; sepsis; lipopolysaccharide; ornithine

DETERTIMENTAL EFFECTS OF NITRIC OXIDE (NO) derived from inducible NO synthase (NOS; iNOS) have been implicated in the cardiovascular hyporesponsiveness and loss of organ function typical of sepsis and endotoxic shock (12, 13, 15). Reduction in extracellular arginine occurs in the setting of sepsis (24), and it may be reasonable to suspect that large-scale iNOS activity results in substrate depletion. However, in a time course study of early events surrounding iNOS induction, our laboratory has previously demonstrated that rapid reduction in plasma argi-
production. To study responses within the limits of renal autoregulation and to minimize systemic vascular effects, the dose of LPS used in this study was tailored to be the minimal dose that reproducibly impaired renal function in anesthetized rats. We measured arginine and related compounds to determine differences in plasma composition between artery and vein of the kidney at 30-min intervals to assess changes in whole body and renal amino acid disposition. Last, we assessed systemic and kidney NOS activity, iNOS and endothelial NOS (eNOS) expression in three organs, and the renal disposition of nitric oxide end products (NOx). The results obtained shed new light on the role of the kidney in the dynamics of arginine production and systemic turnover before and after LPS administration.

METHODS

Animal preparation. Male Wistar rats (n = 6) were anesthetized (Inactin, 100 mg/kg ip), and a tracheal tube was inserted to allow easy free breathing. Animals were then prepared for acute studies on a thermo-controlled platform by catheterizing the jugular vein, left femoral artery, the left renal vein, and left ureter. The femoral artery line was used for blood sampling and blood pressure [mean arterial blood pressure (MAP)] monitoring, while the jugular line was used for volume replacement (PBS, 1.5 ml/h), [3H]inulin infusion (New England Nuclear, MA; 1 μCi/ml in PBS), and LPS administration. The renal vein was catheterized by using a modified 23-G hypodermic needle placed in the lumen of the vein, a method validated by us as having no impact on renal function. Animals were allowed to stabilize for 45 min before the first of two 30-min control blood sampling and urine collection periods was initiated. The animals then received a bolus infusion of LPS (E. Coli 0111:B4, List Biological Laboratories, CA), and eight 30-min sampling periods ensued. To ensure reproducibility, frozen aliquots of reconstituted LPS (1 mg/ml 0.9% NaCl, 4°C) were thawed immediately before injection. While the potency of solubilized LPS may be somewhat reduced by freezing, we determined in pilot studies that a dosage of 1 mg/kg infused intravenously in 1 min resulted in the consistent physiological responses described herein. On termination of the study, animals were killed in accordance with National Institutes of Health guidelines as approved by San Diego-Veterans Affairs San Diego Healthcare System Institutional Animal Care and Use Committee.

Plasma and urine sampling. Urine was collected in preweighed polyethylene tubes, and volume was calculated from the weight difference. Blood samples (80 μl) were collected from the arterial and renal vein lines at 30-min intervals in heparinized capillary tubes (Sigma). For each blood sample, the plasma fraction was rapidly separated by centrifugation. Plasma and urine were ultrafiltered (40 μl) by using acid washed centrifugal devices to remove albumin and any other solids (10K molecular weight cutoff; Millipore). All samples were stored at −20°C until further processing.

Sample analysis. Aliquots of plasma and urine (10 and 2 μl, respectively) were mixed with 5 ml of scintillation fluid to determine [3H]inulin content. Glomerular filtration rate (GFR) was calculated from the clearance of inulin ([Uinulin]/[Pinulin]) and renal plasma flow (RPF) derived from the renal extraction of inulin ([Uinulin]/[V]/ΔAV[Pinulin]). Plasma concentration of NOx (NO₂ + NO₃) was established by using the colorimetric Griess reaction in an automated HPLC system. Known standards, plasma or 20X diluted urine (2.5 μl), were injected on a microcolumn of fine mesh magnesium plated cadmium to reduce NO₂ to NO₃ (>98% efficiency). Postcolumn mixing with Griess reagents [1% sulfanilic acid in 5% H₃PO₄ and 0.1% N-(1-naphthyl) ethylene diamine dihydrochloride] in a heated coil leading to a variable wave absorbance detector (650 nm) enabled the determination of total plasma NOx. We have found that this technique increases the accuracy and sensitivity of NOx measurements by 10- to 100-fold over reductase enzyme-based assay kits.

For the measurement of amino acids, a 10-μl aliquot of each plasma and urine sample as well as appropriate standards were derivatized for fluorescence detection of primary and secondary amine groups with p-hydroxymercuriindolyl-6-aminoquinoyl carbamate as per kit instruction (AccQ tag, Waters). The elution gradient was loosely based on the AccQ tag kit instructions. Elution was performed by using a Hewlett Packard 1100 series binary HPLC pump system with a 250-mm, 3-μm ODS Hypersil C-18 RP column (Hewlett Packard) maintained at 45°C. Fluorescence was detected in line by using a Waters 470 detector linked to the data-acquisition system.

Western blot. In separate experiments, a series of rats were killed 4 h after either LPS (n = 3) or vehicle infusion (n = 3). A sample of renal, hepatic, and pulmonary tissue was rapidly harvested and then transferred to chilled PBS to be minced and rinsed. The tissue was then further homogenized in chilled lysis buffer (phosphatase buffered saline, 1% Triton X-100, 0.1% SDS, 1 mM EDTA, 0.5% deoxycholic acid, 4 mM NaF, and Complete protease inhibitor cocktail; Roche Diagnostics, Mannheim, Germany). Homogenates were centrifuged at high speed for 20 min at 4°C, and the supernatant was assayed for soluble protein content (DC Protein Assay, Bio-Rad, Hercules, CA). Protein extract (60 μg) was separated on a 3–8% SDS polyacrylamide gel and then transferred to a nitrocellulose membrane (InVitrogen, Carlsbad, CA). The membrane was blocked overnight with 5% nonfat dry milk, washed, and incubated for 2 h in a primary solution containing monoclonal murine antibody for iNOS (1:2,000, BD Bioscience). After washing, the membrane was incubated with horseradish peroxidase-labeled anti-mouse IgG (1:5,000, Santa Cruz Biotechnologies, Santa Cruz, CA). The membrane was then washed and developed with ECL-plus (Amersham Pharmacia Biotech, Piscataway, NJ). The gels were subsequently stripped and reprobed by using a murine eNOS monoclonal antibody (1:2,000, BD Bioscience) and again by using a monoclonal murine β-actin antibody (Sigma monoclonal, 1:5,000). The radiogram densitometry for each protein band was determined under identical conditions for each tissue type (i.e., same exposure of radiogram and digital imaging parameters) using image-J software provided by the National Institutes of Health.

Statistics. Data for each animal have been normalized to whole body (2 kidney) renal function expressed per kilogram body weight. Parameters that integrate functional and composition measurements, such as renal disposition, filtered load, and excretion, were calculated for each individual animal. Analysis of variance was used to determine significant changes in repeated measures over time. The P values of significant changes from control (unless otherwise specified) were determined from paired sample t-tests for each time point. HPLC assays were performed in duplicate (two separate derivatizations for amino acid analyses and repeated measurement for NOx), and mean values obtained for the same sample were treated as a single value.

RESULTS

Systemic hemodynamics and renal function. LPS caused a sustained reduction in MAP after 120 min (from 122 ± 3 to 108 ± 6 mmHg, P < 0.05, Fig. 1A). A reduction in RPF preceded changes in systemic blood pressure, decreasing from 31.6 ± 1.9 to 17.5 ± 3.4 ml min⁻¹kg⁻¹ (P < 0.01) within 60 min of LPS infusion and persisted over the subsequent collection periods (Fig. 1C). Similarly, GFR remained unchanged from control (9.5 ± 0.4 ml min⁻¹kg⁻¹) in the first 30 min postinfusion but decreased significantly by 60 min to 3.5 ± 0.6 ml min⁻¹kg⁻¹ (P < 0.01), a level that was sustained for 240 min (Fig. 1D). Changes in urine flow rates followed a slightly different profile from hemodynamics, whereby urine output at 60 min decreased from 36.0 ± 8.6 to 12.2 ± 3.2 ml min⁻¹kg⁻¹ (P < 0.01) and then gradually returned to 1.9 to 17.5 ml min⁻¹kg⁻¹.
arginine was 1,029 nmol/kg; pilot experiments without LPS, the kidney production rate of arginine, citrulline, or ornithine, indicating that the highly efficient reabsorption from filtrate was not compromised by arginine metabolism, was consistently greater than the corresponding renal venous value (89.5 ± 5.4 vs. 54.4 ± 3.3 μM; P < 0.05), resulting from a net uptake of 991.4 ± 255.6 nmol·min⁻¹·kg⁻¹ by the kidney. The concentration of arterial citrulline increased at 180 min following LPS (from 89.5 ± 5.1 to 120 ± 6.7 μM; P < 0.05), concurrent with increasing plasma NOx and lower MAP. Interestingly, comparison of the baseline filtered load for citrulline by the kidney (695.7 ± 153.2 nmol·min⁻¹·kg⁻¹) with the corresponding renal uptake highlights a phenomenon whereby more citrulline is taken up by the kidney than is filtered.

NOx. LPS caused an increase in the concentration of NOx in arterial plasma from baseline (33.8 ± 6 μM) that was significant after 90 min and fourfold higher by 240 min (73.6 ± 11.9 and 150.3 ± 50.6 μM, respectively, both P < 0.01; see Fig. 3A). During the control period, no difference was observed between arterial and venous plasma NOx, indicating that the absolute amount of NO produced by the kidney is normally minimal. Systemic NOx levels increased as a response to LPS, but no significant difference in renal arteriovenous plasma NOx concentration was observed. We also assessed renal
excretion of NOx to determine whether increased urinary excretion could account for the apparent lack of kidney iNOS activity. Before LPS, the average concentration of urinary NOx was 1.7 ± 0.4 mM and, when factored for individual urine flow, amounted to a renal excretion rate of 56.9 ± 11.8 nmol·min⁻¹·kg⁻¹ (see Fig. 3C). Changes in the concentration of NOx in urine closely paralleled changes in urine flow rates described above, resulting in a transient reduction of excretion rate to 10.1 ± 4.0, 8.5 ± 3.0, and 28.7 ± 12.5 nmol·min⁻¹·kg⁻¹ at 60, 90, and 120 min, respectively (P < 0.05 vs. control), thereafter returning to baseline values. Last, we observe that excretion rates of NOx are a minor fraction of the filtered load by the kidney (774 ± 146 and 2,365 ± 994 nmol·min⁻¹·kg⁻¹ for control and 240 min, respectively), indicating that most filtered arginine is reabsorbed (Fig. 3B).

**Immuochemistry.** A histogram of the densitometry analysis of iNOS and eNOS expression in liver, lung, and kidney 4 h post-LPS or vehicle is seen in Fig. 4. Multiple protein bands resolved at or near the appropriate molecular weight for iNOS, which prompted us to verify for possible cross reactivity of the antibody with other NOS isoforms. Stripping and probing the same gels with eNOS monoclonal antibody confirmed that the higher molecular weight band was due to iNOS antibody cross reacting with eNOS protein and allowed us to more positively identify the isoform of interest. Densitometry measurements...
observations pertaining to arginine metabolism in the kidney have revealed that iNOS protein increased significantly within 4 h of LPS in liver and lung but not kidney tissue. The expression of iNOS in the kidney was 4.86 ± 1.01 and 7.91 ± 1.66 normalized relative densitometry units in control vs. LPS-treated animals, values that were not statistically different. In comparison, values for liver and lung increased from 9.47 ± 2.62 to 146.75 ± 36.10 and 13.36 ± 1.70 to 123 ± 13.6 normalized relative densitometry units, respectively (both P < 0.01), or 14- and 8.5-fold increases, respectively. No significant difference in eNOS expression between control and LPS treatment was observed (from 98.91 ± 13.09 to 110.68 ± 15.72, from 87.11 ± 15.73 to 72.29 ± 19.76, and from 11.86 ± 2.87 to 10.00 ± 1.90 relative densitometry units in liver, lung, and kidney, respectively).

**DISCUSSION**

This study initially proposed to address why extracellular arginine levels quickly decreased in the LPS model of sepsis. Utilizing repeated measurements for arginine, citrulline, ornithine, and NOx in arterial and renal venous plasma, we were able to determine the renal disposition of each compound by factoring those values with corresponding renal function parameters. The results of this study have verified that arginine is avidly produced by the kidney under control conditions and that LPS caused plasma concentration to decrease before the appearance of de novo iNOS activity. The net rate of renal arginine synthesis was abruptly reduced by over 50%, concurrently with compromised renal function, and was of a magnitude that could readily account for reduced extracellular arginine concentration. This study also provides interesting new observations pertaining to arginine metabolism in the kidney such as the basolateral uptake of citrulline by the kidney, substrate availability for NO production, and changes in kidney production of ornithine. Lastly, unanticipated results were obtained with respect to NO in the kidney, whereby a comparatively minor change in iNOS expression and no discernible change in NOx production occurred within 4 h of LPS administration, although systemic iNOS induction and activity were clearly evident. Taken together, these data provide a more complete picture of the complex nature of arginine metabolism in the kidney, both in control conditions and during the initial loss of renal function resulting from LPS.

As seen in Figs. 1A and 3A, evidence of systemic iNOS activity followed a delay of 90–120 min, resulting in lower systemic blood pressure and elevated plasma NOx. However, clear evidence of compromised kidney function preceded this phenomenon by 30–60 min. The fact that the kidney constitutes the primary source of circulating arginine is not new, but what is perhaps less well known is the high rate of systemic turnover of this amino acid. As seen in Fig. 2C, the net renal production of arginine under basal conditions is almost 1,000 nmol·min⁻¹·kg⁻¹ or 60 µmol·h⁻¹·kg⁻¹, whereas the content of extracellular fluid is ~40 µmol/kg (assuming a 25% extracellular fluid volume-to-body weight ratio and an extracellular concentration of 160 µM). Thus complete turnover time for circulating arginine may be as little as 40 min under control conditions. Clearly a sustained reduction in the net renal production of arginine by ≥50%, as observed within 60 min following LPS, could account for the ~30% reduction in extracellular concentration occurring simultaneously. In fact, it is surprising that plasma arginine concentration does not decrease more; however, sepsis has been characterized as catabolic and may result in decreased amino acid utilization for protein synthesis and possibly even provide a temporary alternate source of arginine via proteolysis.

The question naturally arises as to what causes kidney arginine production to decrease in the timeframe that we observe, before expression of iNOS. Although this study was not designed to determine what caused RPF and GFR to decrease, the fact that these changes occurred simultaneously with impaired arginine production makes it likely that the phenomena are linked. Indeed, one could postulate that a reduction in the delivery of citrulline to the kidney as a result of impaired renal function could attenuate arginine synthesis. This is appealing because the molar uptake of citrulline almost exactly matched the net production of arginine. Citrulline uptake by the kidney always exceeded the filtered load by nearly 30%, signifying that some transport of citrulline occurs beyond the glomerulus (possibly the basolateral aspect of proximal tubule epithelia) and that GFR alone might not dictate citrulline availability. In support of this, Gekle and Silbernagl (5, 6) describe basolateral citrulline transport in the everted toad bladder, a longstanding model of renal tubule epithelia and that a carrier-mediated citrulline transporter has been characterized in epithelia of the rat gut (27). The fact that citrulline is taken up from postglomerular plasma may help explain how the nonfiltering kidney could persist as a net producer of arginine, something that is of interest in the clinical setting of acute and chronic renal failure.

The liver is considered a major source of circulating ornithine due to very high arginase activity (3, 7, 19). In the present study, we demonstrate that the renal contribution of this amino acid is nontrivial under control conditions, approaching 25% of...
the arginine synthetic rate. Changes in renal ornithine release appear consistent with reports of arginase induction in many tissues, including the kidney by LPS (20, 22), and are in agreement with our laboratory’s past observation that the ornithine content of rat kidney cortex is elevated 6 h post-LPS (18). Although the present study does not allow us to specifically elaborate on changes in arginase activity or induction, the data that we provide may be more relevant because we measure the net result of ornithine production by the kidney. Of particular interest is the fact that the renal contribution of ornithine to the net result of ornithine production by the kidney. Of particular interest is the fact that the renal contribution of ornithine increased threefold within 4 h of administering LPS, suggesting that a shift in the balance of urea cycle products might alter net arginine production. However, the time course of the gradual increase in ornithine production by the kidney does not reflect the rapid changes in extracellular arginine and, therefore, does not support the idea that increased arginase activity impaired arginine production.

An indication of the relative proportion of arginine destined for NO production may be gleaned from the excretion rate and change in plasma content of NOx. As verified in this study, normal plasma NOx is stably maintained at a concentration of 30–40 µM. If we assume that the majority of NO eventually becomes extracellular NOx and is excreted in urine, the kidney excretion rate (~50 nmol·min⁻¹·kg⁻¹) corresponds to only 5% of kidney arginine production (~1,000 nmol·min⁻¹·kg⁻¹) under normal conditions. Furthermore, the accumulation of extracellular NOx between 120 and 240 min (~60 µM or 15 µmol/kg, assuming an extracellular fluid volume of 25%) should reflect the increase in arginine consumed, because excretion of NOx was comparatively insignificant. Thus increased production of NO would account for only 12.5% of the normal (120 µmol/kg in 2 h) or 25% of the LPS-impaired kidney arginine production. This suggests that even a large-scale reduction in extracellular arginine might not be a limiting factor for NO production in vivo. Difficult questions regarding the production of NO by the normal and septic kidney are raised by this study, because no effect of LPS on these parameters occurred within 4 h. Although the implications were not discussed, a similar result was published in which mice given LPS intraperitoneally did not show increased NO production by the kidney (8). Alternate chemical pathways for the reaction of NO to products other than NOx might be considered as a sink over a brief period, although it is difficult to imagine how complete sequestration might occur selectively in the kidney, whereas, in other tissue, NO is fated to become plasma NOx. If the kidney is refractory to LPS-induced NO production, it may help to explain the paradoxical reduction in RPF observed in sepsis because hormonal and other vasoconstrictor mechanisms are activated in an attempt to maintain cardiovascular homeostasis.

There is little doubt that NO plays an important regulatory role in kidney function, although the molar quantities generated appear to be small. Some expression of iNOS is evident in normal kidneys, but the functional effects of iNOS activity is not clearly defined. Studies have shown that tubule transport differs between wild-type and iNOS/−/− mice (28) and that iNOS may modulate eNOS activity (1, 28). Nevertheless, two facts are clear from the immunochemistry performed in this study: 1) under control conditions, there is comparatively less eNOS and iNOS expressed in the kidney than in liver and lung; and 2) unlike liver and lung, the dose of LPS used in this study did not induce iNOS expression in the kidney. Because NO is such a potent vasodilator and arginine supply in kidney would appear to be limitless, it is not unreasonable to think that unique mechanisms could have evolved in the kidney response to bacterial LPS that moderate iNOS expression. In fact, studies have shown that renal cells in culture resist iNOS induction by bacteria (23), that systemically administered LPS did not accumulate in kidney (17), and that kidney epithelia is rich in enzymes that can detoxify LPS (4). We and others have clearly shown elevated iNOS mRNA in renal tissue following LPS (18), suggesting a further possibility that posttranscriptional regulation may prevent or delay iNOS expression in the kidney.

In rats given a relatively low-dose intravenous bolus of LPS, we assessed changes in renal arginine metabolism to gain insight into the rapid decline in circulating arginine following LPS. We succeeded in reproducing hallmark characteristics of sepsis, such as reduced systemic blood pressure and elevated plasma NOx in a time course consistent with de novo iNOS induction. Changes in kidney function and arginine production resulting from LPS preceded the increase in NO synthesis. Considering the high turnover rate of circulating arginine described herein, it is clear that rapid and sustained impairment of renal arginine production is the major factor responsible for decreased plasma content at this early time point. The question remains as to what chain of events causes net kidney arginine output to decrease, but it appears likely that a causative link exists with the reduction in renal perfusion.

GRANTS

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