Time course of nitric oxide production after endotoxin challenge in mice

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Braulio, Valeria B., Gabrie A. M. Ten Have, Yvonne L. J. Vissers, and Nicolaas E. P. Deutz. Time course of nitric oxide production after endotoxin challenge in mice. Am J Physiol Endocrinol Metab 287: E912–E918, 2004.—Nitric oxide (NO) regulates numerous processes during endotoxemia and inflammation. However, the sequential changes in whole body (Wb) nitric oxide (NO) production during endotoxemia in vivo remain to be clarified. Male Swiss mice were injected intraperitoneally with saline (control group) or lipopolysaccharide (LPS group). After 0, 2, 4, 6, 9, 12, and 24 h, animals received a primed constant infusion of L-[guanidino-15N2-H2]arginine, L-[ureido-15N]citrulline, L-[5-15N]glutamine, and L-[guanidino-15N 2 -2H5]phenylalanine in the jugular vein. Arterial blood was collected for plasma arginine (Arg), citrulline (Cit), glutamine (Gln), and phenylalanine (Phe) concentrations and tracer-to-tracer ratios. NO production was calculated as plasma Arg-to-Cit flux, Wb de novo Arg synthesis as plasma Cit-to-Arg flux, and Wb protein breakdown as plasma Phe flux. LPS reduced plasma Arg and Cit and increased Gln and Phe concentrations. Two peaks of NO production were observed at 4 and 12 h after LPS. Although LPS did not affect total Arg production, de novo Arg production decreased after 12 h. The second peak of NO production coincided with increased Wb Cit, Gln, and Phe production. In conclusion, the curve of NO production in both early and late phases of endotoxemia is not related to plasma Phe flux. However, because Wb Cit, Gln, and Phe fluxes increased concomitantly with the second peak of NO production, NO production is probably related to the catabolic phase of endotoxemia.

lipopolysaccharide; arginine; citrulline; glutamine; phenylalanine

THE INFLAMMATORY REACTIONS induced by inflammation are exerted via a number of mediators, including microbial products and cytokines. Lipopolysaccharide (LPS), a constituent of Gram-negative bacteria, stimulates the acute early release of cytokines such as tumor necrosis factor (TNF) and interleukin-1 (IL-1) from macrophages. These mediators, often acting synergistically, stimulate the expression and activation of NOS2 (inducible nitric oxide synthetase, iNOS), an enzyme member of the nitric oxide (NO) synthetase (NOS) family. The two other isoforms of NOS, NOS1 (neuronal NOS) and NOS3 (endothelial NOS), are also known collectively as constitutive NOS (28). NO is generated by oxidation of the terminal guanidine nitrogen of the amino acid l-arginine (Arg), with production of l-citrulline (Cit) (18, 22). NO is further oxidized to nitrite and nitrate and excreted as urinary nitrate (33).

Because direct measurements of NO are difficult, plasma and urinary nitrite/nitrate have been frequently used as indicators of NO production. However, it has become clear that these methods give only qualitative data. More recently, stable isotope methods have been introduced for quantitative measurement of NO production. By use of stable isotopes, NO production can be measured indirectly via the conversion of l-[guanidino-15N2]arginine to l-[ureido-15N]citrulline, which yields NO in stoichiometric amounts (2, 3, 9, 10, 15, 16, 27, 42).

Because of its property of provoking a generalized proinflammatory response in the infected host, endotoxin is often used to reproduce a sepsis-like condition in experimental models. Although the effects of LPS on NO synthesis have been studied extensively in vitro, the sequential changes on plasma whole body (Wb) NO production are not well characterized in vivo due to the limitations of the nitrite/nitrate method in giving kinetics information and to the complexity of other techniques, different from stable isotopes, that measure NO production in real time (27).

The aim of this study was to measure the kinetics of the systemic NO production and its precursors during 24 h after endotoxin challenge in mice by means of stable isotope techniques. In addition to Wb Arg and Cit appearance rate (Ra), we also measured the Wb glutamine (Gln) and phenylalanine (Phe) appearance rate and its plasma levels, because a considerable amount of Gln released from protein catabolism in the muscle is converted to Cit in the intestine. Arg appearance is from protein breakdown as well as from de novo Arg production from Cit in the kidney (32).

METHODS

Animals. Male Swiss mice (25–35 g) were obtained from IFFA Credo Broekman (Someren, The Netherlands). The mice were fed standard lab chow and were subjected to standard 12:12-h light-dark cycle periods (7:30 AM to 7:30 PM). Room temperature was maintained at 25°C. Water was provided ad libitum throughout the experiment. Experiments were performed in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals (39) and were approved by the Ethics Committee of Animal Research of Maastricht University.

LPS model. Mice were acclimatized overnight in an incubator (29°C, 60% humidity) before the start of the experiment. At time (t) −2 h, animals were weighed, and food was withheld. At 0 h, LPS (Escherichia coli O55:B5; 10 mg/kg freshly diluted in 0.5 ml 0.9% NaCl; Sigma, St. Louis, MO) was given by intraperitoneal injection to mice (LPS group, n = 52). Control animals (CON group, n = 55) received a corresponding volume of 150 mM NaCl (saline). At 2 h (LPS, n = 10; CON, n = 9), 4 h (LPS, n = 9; CON, n = 10), 6 h
(LPS, n = 7; CON, n = 8), 9 h (LPS, n = 10; CON, n = 10), 12 h (LPS, n = 7; CON, n = 8), and 24 h (LPS, n = 10; CON, n = 10) after LPS treatment or saline, the animals were weighed again to determine weight loss compared with t = 0 h, and the tracer infusion protocol was conducted. A third group underwent the tracer protocol for baseline measurements at 0 h without receiving LPS or saline (T0, n = 8). After injection with saline or endotoxin, the mice were transferred to a clean cage and returned to the incubator.

Tracer infusion protocol. Tracer experiments were conducted under ketamine-medetomidine anesthesia and fluid management as described in detail before (15). A primed constant infusion of stable isotopes l-[guanidino-15N-2H2]arginine ([15N2-2H2]Arg), l-[ureido-15N]citrulline ([15N]Citrull), l-[5-15N]glutamine ([15N]Gln), and l-[ring-3H3]phenylalanine ([3H3]Phe) (MassTrace, Woburn, MA) was given in the jugular vein immediately after its catheterization (Table 1). In pilot experiments, this protocol yielded steady state in 30 min (Fig. 1 and Ref. 36). Blood was collected from the carotid artery 30 min after the start of the primed continuous infusion of stable isotopes. Amino acid concentrations and tracer-to-tracee ratios (TTRs) were determined in deproteinized plasma, as previously described (36, 37). Briefly, 80 μl of plasma were added to 7 mg of dry sulfoacetylsalicylic acid and frozen in liquid nitrogen. Plasma amino acid concentrations and enrichments, calculated as TTR, were measured using a fully automated liquid chromatography-mass spectrometry system using precolumn derivatization with o-phthalaldehyde (34, 35). Detailed information on the accuracy and precision of this method was given before (35).

Calculations. Plasma Arg, Cit, Gln, and Phe Wb Rn were calculated from the arterial isotope TTR values of, respectively, [15N2-2H2]Arg, [15N]Cit, [15N]Gln, and [3H3]Phe, using the steady-state isotope dilution equation

\[ \text{Wb R}_n = I/TTR, \]

where TTR is the arterial TTR and I is the rate of infusion of the tracer (16).

The rate of NO production was measured using [15N2-2H2]Arg-to-[15N2-2H2]Cit conversion. Calculation of the plasma Arg-to-Cit flux (NO production) was made using the equation

\[ Q_{\text{Arg to Cit}} = Q_{\text{Cit}} \times TTR_{\text{Cit (M+1)}/TTR_{\text{Arg (M+1)}}}, \]

where \( Q_{\text{Cit}} \) is the plasma Cit flux (nmol/10 g body wt.\(^{-1}\).min\(^{-1}\)), estimated from the primed constant infusion of the [15N]Cit tracer, and TTRCit and TTRArg are the respective TTRs of [15N-2H2]Cit and [15N2-2H2]Arg (14).

Calculation of the plasma Cit-to-Arg flux (de novo arginine production) was made in a similar fashion (14):

\[ Q_{\text{Cit to Arg}} = Q_{\text{Arg}} \times TTR_{\text{Arg (M+1)}/TTR_{\text{Cit (M+1)}}}, \]

where \( Q_{\text{Arg}} \) is the plasma Arg flux (nmol/10 g body wt.\(^{-1}\).min\(^{-1}\)), estimated from the primed constant infusions of the [15N2-2H2]Arg tracer, and TTRArg and TTRCit are the respective TTRs of [15N]Arg and [15N]Cit.

Wb protein breakdown was calculated as plasma Phe flux (nmol/10 g body wt.\(^{-1}\).min\(^{-1}\)), estimated from the primed constant infusion of the [3H3]Phe tracer.

Wb Arg synthesis from protein breakdown was calculated by subtracting de novo Arg synthesis from the Wb Rn of Arg.

Statistics. Results are presented as means ± SE. Levels of significance were set at \( P < 0.05 \). To test changes over time within the LPS or CON groups, the data were analyzed by one-way analysis of variance followed by Tukey’s test if a significant F value was obtained. For comparison between LPS and CON groups, the data were subjected to a two-way analysis of variance, where the group measured on \( t = 0 \) was not considered. Student’s t-test was further used to assess differences between experimental conditions, and in this case differences were considered significant if \( P < 0.01 \).

RESULTS

Physical conditions. LPS-treated mice displayed visible signs of acute illness ~4 h after LPS challenge and recovered at 24 h. Characteristic responses included lethargy, exudates around the eyes and nostrils, mild diarrhea, and raised fur. There was no mortality as a consequence of LPS or saline administration. Mice from both groups lost weight during the experimental period, without significant differences between LPS and CON groups (Fig. 2).

Arterial concentrations. Arterial Arg concentrations decreased in a similar pattern in LPS and CON groups, reaching the minimal level at 12 h after LPS administration (\( P < 0.0001 \) vs. baseline values; Table 2). At 24 h, CON, but not LPS, plasma levels were restored to baseline levels (\( P < 0.01 \) vs. 12-h CON, \( P < 0.0001 \) between groups at 24 h).

Arterial Cit concentrations decreased in the LPS group after 12 h, reaching nadir at 24 h, when levels were 50% of the baseline values (\( P < 0.0001 \) vs. baseline values, \( P < 0.001 \) between groups at 24 h). No changes in time were detected in CON group.

Arterial Gln concentrations were increased by LPS treatment (\( P < 0.001 \)). Values were higher at 9 h after endotoxin induction. Control values were unchanged.
Arterial Phe concentrations increased at 4 h and at 6 h of endotoxemia ($P < 0.01$), further decreasing to baseline levels.

**Wb productions.** Mean TTRs per group are reported in Table 3.

LPS administration resulted in high elevations of NO production ($P < 0.0001$). Two peaks were observed, at 4 h and at 9–12 h. No changes in NO production were observed in the CON group (Fig. 3).

Total Wb Arg production decreased in the LPS group between 2 and 9 h ($P < 0.05$) and increased in the CON group between 12 and 24 h ($P < 0.01$). However, Wb Arg production did not differ significantly between the groups (Fig. 4). The de novo Arg production also decreased from 4 h after LPS administration, more pronouncedly at 24 h ($P < 0.05$; Fig. 4), whereas Wb Arg production coming from protein breakdown increased proportionally (24-h LPS 68.1 ± 2.3 vs. T0 50.6 ± 1.9, $P < 0.01$, vs. 24-h CON 51.9 ± 1.9, $P < 0.001$). At that time point, 82% of total Arg production was derived from protein breakdown in LPS-treated mice.

In contrast, Wb Cit production was increased as a result of LPS administration. This increase was significant at 12 h ($P < 0.01$; Fig. 5).

LPS treatment also affected Wb Gln production ($P < 0.0001$). Increases were observed at 9, 12, and 24 h (Fig. 5). No changes in time were observed in the CON group (Fig. 5).

Wb protein breakdown was significantly increased in the LPS group ($P < 0.001$). As shown in Fig. 6, two distinct elevations could be observed, the first at the early endotoxemia phase and the second at 24 h. No changes in time were observed in the CON group (Fig. 6).

**DISCUSSION**

Here, we describe the time course of systemic NO production in vivo using stable isotope techniques. The results show that endotoxemia in mice caused elevation of Wb NO production. The LPS-induced NO production occurred in a biphasic pattern, with an early peak (4 h) followed by another peak after 9–12 h after LPS administration, suggesting the presence of different responses during the first 24 h of endotoxemia in mice.

**Comparison between NO production measured by stable isotopes and plasma nitrite/nitrate.** The assumption when tracers are being used is that dilution of tracer is equal in all body pools. The true precursor pool enrichment is the amino-acyl tRNA of the amino acid studied. However, direct measurements of this pool are limited due to its small size and short half-life. Therefore, surrogate precursor pools such as arterial plasma are used. Because the enrichment of the arterial pool exceeds that of the tRNA pool, calculations always underestimate true appearance rates. In line with this, NO production measured by tracer dilution will also underestimate true production rates. Still, because it can be assumed that this underestimation is comparable in all experimental groups, comparison between groups is probably valid.

Our results are in line with studies that measured end-product nitrite/nitrate in blood as an indicator for systemic NO production. Frederick et al. (13) found increased blood nitrite/nitrate at 4 h (4-fold) and at 10 h (14-fold) after LPS administration to rats (2 mg/kg). Similar LPS doses resulted in elevations of circulating nitrite/nitrate in rats, peaking at 4 h (6-fold) and declining to baseline levels after 16 h (34). Higher LPS doses (20 mg/kg) administrated to mice resulted in greater plasma elevations of nitrite/nitrate, peaking at 12 h (44-fold increase) (23). On the other hand, NO production measured by nitrite/nitrate seems to be of a much greater magnitude than that measured by stable isotope techniques. This discrepancy between the increase in plasma nitrate and systemic NO production measured using stable isotopes was reported previously to occur in mice treated with LPS (16). Present results show a threefold increase at the two peaks of Wb NO production, thus further contributing to the hypothesis that nitrite/nitrate does not quantitatively represent acute changes in NO production. In fact, nitrite/nitrate concentrations are

![Graph](image-url)
affected by renal function (because the kidney clears NO₃ from the plasma) and by nitrate production by bacteria in the gut. NO production via Arg→Cit conversion is not influenced by these factors and is thus a more accurate measure of NO production (27).

**NOS2 in early and late endotoxemia.** The amount of NO generated by cells that express NOS2 is limited by the amount of enzyme and the availability of substrate (Arg) and cofactors such as tetrahydrobiopterin and NADPH (5). In the early steps of the inflammatory process, NOS1 and NOS3 expressed in different cell types involved in immune reactions may account for the majority of NOS activity. At that moment, only a few resident macrophages express NOS2 (19). On the other hand, a rapid induction of mRNA synthesis and NOS2 activation in response to the inflammatory stimuli triggered by LPS has also been described (6). Indeed, the expression of NOS2 mRNA in cells extracted from different organs of rats was detected as early as 20 min after in vivo LPS administration. The amount of NOS2 mRNA increased progressively thereafter, reaching a plateau at 4 h and decreasing by 24 h (26). Also, the renal glomerular NOS2 mRNA expression was shown to be increased at 60 min, reaching its peak between 2 and 4 h after endotoxin administration to mice (34). These findings are consistent with the first peak of NO production observed in the present study.

![Fig. 3. Time course of whole body (Wb) nitric oxide (NO) production in LPS (•) and CON (○) groups. Data represent means ± SE. *P < 0.01 between groups.](image)

![Fig. 4. Time course of Wb (top lines) and de novo (bottom lines) Arg production in LPS (•) and CON (○) groups. Data represent means ± SE. No significant differences between groups.](image)

The second peak of NO production occurred at the late endotoxemia phase. This effect is probably the result of a maximal NOS2-related NO production, when the enzyme is already maximally expressed, since in response to endotoxin NOS2-mediated NO production increases, whereas NOS1- and NOS3-mediated NO production was found to be reduced (15). Indeed, at the peak of chronic inflammation more than 90% of the total NOS activity could be attributed to NOS2 in activated macrophages (19). Apparently, enhanced NOS2-NO production is necessary to compensate for the reduced NOS1- and NOS3-related NO production.

Macrophages and monocytes occupy a central role in coordinating the immune response to injury and infection. LPS stimulates macrophages to release large quantities of TNF and IL-1 within the first few hours of endotoxemia. These cytokines are early mediators in the pathogenesis of the endotoxin-induced tissue injury (18). On the other hand, high mobility group-1 (HMG-1) protein was recently identified as an early mediator of inflammatory damage in a mouse model. Mice showed increased serum levels of HMG-1 from 8 to 32 h after endotoxin exposure. HMG-1 was also found to be released by cultured murine macrophages more than 8 h after stimulation with LPS, TNF, or IL-1. Moreover, a reciprocal functional relationship between the activities of the early (TNF and IL-1) and the late (HGM-1) cytokines also occurs. HMG-1
was shown to stimulate TNF synthesis in a human monocyte culture in a delayed and biphasic pattern (1, 40). Therefore, the second peak of NO production in our study could be explained by late-phase cytokine activation, such as HMG-1.

**Plasma Arg availability and NO production.** The plasma Arg flux reflects the entry of amino acid into the plasma pool from Wb protein breakdown plus de novo Arg synthesis from Cit, and, in the fed state, from that supplied from the dietary intake (41). Under catabolic conditions, protein degradation becomes a major source of Arg, whereas de novo Arg synthesis is not altered (3). Besides, it has been proposed that the maintenance of plasma Arg homeostasis is rather more dependent on a decrease in the rate of Arg degradation than on an increase in de novo Arg production (9). Indeed, in burned patients, an elevation on Arg-to-ornithine conversion as well as an elevated ornithine and Arg oxidation, with limited net de novo Arg synthesis, was described (42).

During experimental sepsis, plasma Arg levels were shown to be reduced (11, 25). Our results confirm these findings. After 12 h, plasma Arg stabilized at low levels. Concurrently, a significant decrease in plasma Cit levels was observed. The de novo Arg synthesis also decreased at that time point, suggesting that plasma Cit levels could be a limiting factor for de novo Arg production. Therefore, the increased rates of NO production seem to have occurred independently of plasma Arg levels or de novo Arg synthesis. Similarly, several human studies indicated that NO synthesis is not influenced by low plasma Arg levels (10, 35).

**Cellular Arg availability and NO production.** The apparent contradiction of low plasma Arg levels and high NO output in this study could result from a higher Arg metabolism, which is known to occur during sepsis (2). However, current knowledge of Arg metabolism in vivo is limited, due in large part to the complex interorgan and intracellular compartmentation in the liver (high arginase because of urea synthesis). Besides, increased NO production can result from LPS-induced changes on tissues where inducible NO is synthesized, namely the liver, spleen, lung, and circulating monocytes and macrophages (30). In effect, it has been postulated that the intracellular availability of Arg is the rate-limiting factor in NO production (29). At the cellular level, Arg can be obtained from exogenous sources via cationic amino acid transporters (7, 21), and endogenously from either intracellular protein breakdown or from Cit by the Cit-NO cycle, which consists of NOS, argininosuccinate synthetase, and argininosuccinate lyase (17, 41). Both Arg transport and Cit-Arg cycling have been recognized to be important for high-output production of NO in LPS-stimulated cells (24).

**Arg and Cit fluxes during endotoxemia.** The total Wb Arg production was not significantly changed by LPS treatment. This result is in agreement with another study that reported unaltered Arg fluxes in septic patients compared with normal subjects (2). Moreover, in mice treated with LPS, the renal de novo Arg production increased without repercussion on Wb Arg flux (16). The fact that Wb Cit flux was increased at 12 h after LPS indicates increased Cit production by the intestine, since the increase in Cit flux was synchronous with the increase in Gln flux. Gln can serve as a precursor for endogenous Arg synthesis, with Cit as an intermediate (32). This finding could be related to the second peak of NO production. Also, higher Cit fluxes were described to occur in pediatric septic patients (2). However, increased Cit and Gln fluxes were not in parallel with increased Wb Arg de novo synthesis. As previously suggested, Cit may not be recycled via direct flow through the plasma Arg pool. Thus part of the labeled Cit appears to be taken up and converted to Arg at a metabolic site(s) that is separated from the plasma Arg pool and does not equilibrate with it (8).

**Plasma Gln levels and Gln flux during endotoxemia.** In the present study, the arterial Gln levels were preserved after LPS treatment, consistent with other animal studies that investigated the effect of sepsis on plasma Gln levels (25, 31). In addiction, the Wb Gln flux was increased concomitantly with the second peak of NO production. During sepsis, a peripheral- to-splanchnic Gln flux was described to occur (31). After LPS treatment, Gln is known to be released by the lungs and skeletal muscle in response to the increasing demand for Gln in the liver and kidney and also by a variety of cells, including endothelial cells and macrophages (25). Moreover, Gln was found to leave the lungs in septic patients, partially as a result of protein breakdown (20). The role of Gln in NO production during sepsis is still not completely understood. Besides its conversion to Cit in the intestine, Gln may also act as an Arg precursor and a regulator of NO synthesis in LPS-activated cells (4). Increased levels of plasma Gln and Wb Gln production simultaneous with the late endotoxemia NO peak, in the current study, corroborate the notion that Gln might be required to sustain NO production.
Plasma Phe levels and Phe flux during endotoxemia. In contrast to Arg, Cit, and Gln, arterial Phe levels were increased in the early phase of sepsis, which is in agreement with previous studies in mice that showed increased plasma Phe values at 6 h after LPS administration (16) but no increments at 11 or 19 h (14, 38). In septic patients, Phe was shown to be the only amino acid whose plasma concentrations were increased (12). In the present study, the Wb Phe flux, as well as Wb Arg flux coming from protein breakdown, was markedly increased at the late phase of sepsis (24 h). The potential cause for this increment could be increased skeletal muscle catabolism. Additionally, the synchronous increases on Wb Phe, Gln, and Cit fluxes at the 12-h peak suggest interactions between these amino acids in the control of NO synthesis, independent of de novo Arg synthesis from Cit.

In conclusion, peak values of NO production were verified in both early and late phases of LPS-induced endotoxemia in mice. This effect seems not to be associated with plasma Arg kinetics. High NO output observed at the early phase of endotoxemia may be explained by increased NOS2 activity induced by early inflammatory cytokines such as TNF and IL-1, whereas late increments of NO production might be associated with the release of later inflammatory mediators (HMG-1). However, increased Wb Cit, Gln, and Phe fluxes during the catabolic phase of sepsis may also be related to the second peak of NO synthesis.

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