Is the L-Arginine–Nitric Oxide Pathway Involved in Endotoxemia-Induced Muscular Hypercatabolism in Rats?

P. Pernet, C. Coudray-Lucas, J. Le Boucher, L. Schlegel, J. Giboudeau, L. Cynober, and C. Aussel

We investigated the role of the nitric oxide (NO) synthase (NOS) pathway in muscular metabolism during endotoxemia in four groups of male Wistar rats. Two groups were injected with the lipopolysaccharide (LPS) of *Escherichia coli* (3 mg/kg), with one group treated using N^G-nitro-L-arginine methylester ([L-NAME] 85 mg/kg/d) and the other not. The two control groups included one treated with L-NAME and the other not. After 24 hours of fasting, the rats were fed by controlled enteral nutrition and killed on day 3. The results showed that (1) NOS inhibition was detrimental during endotoxemia, increasing lethality from 20% to 80.5%, and (2) NOS inhibition did not modify the hypercatabolic state consecutive to endotoxemia, particularly at the muscular level (nitrogen balance, total-body and muscular weight loss, and muscular protein and glutamine concentrations). However, myofibrillar catabolism was delayed in the LPS-NAME group. In conclusion, NO production is of major importance for survival after an endotoxemic challenge, but contributes weakly to the metabolic response of muscle to injury.

NITRIC OXIDE (NO) is a versatile molecule produced by mammalian cells, formed from L-arginine and molecular oxygen by NO synthase (NOS) and yielding L-citrulline as a coproduct. Several types of NOS, constitutive (cNOS) or inducible (iNOS), have been described and are selectively inhibited by arginine analogs.[1]

Endotoxemic shock is mainly characterized by acute cardiovascular collapse resulting from the systemic response to bacterial infection[2] and results in hypotension, a lack of response to vasoconstrictors, intestinal damage, and multiorgan failure. During endotoxemia or septic shock, increased NOS activity leads to NO overproduction, which is responsible for some of the associated hemodynamic disorders.[1] In addition to hypotension and intestinal damage, one of the major characteristic responses to injury is muscular weight loss, net proteolysis, and a decrease in the muscle glutamine concentration due to a high rate of transfer from the muscle to the splanchnic area, kidneys, and immune cells.[3] These processes depend on hormones and are modulated by cytokines, particularly tumor necrosis factor alpha, for which NO acts as a second messenger.[4] Endotoxins and cytokines can act as signals to increase NO production.[1] In addition, several recent studies indicate that muscle tissue produces NO[5-10] and increases NOS activity in various pathological conditions, with upregulation of cNOS and expression of iNOS.[9-10] However, whether increased NO production is involved in the muscular hypercatabolic response during injury remains to be clarified.

The purpose of the present study was to evaluate the implication of the L-arginine-NO pathway in muscular metabolism during septic shock. The well-described and reproducible model of endotoxemia in young rats was used to mimic a septic shock–like syndrome. To determine the role of NO in muscular changes, NOS was selectively inhibited with N^G-nitro-L-arginine methylester (L-NAME) during the 3 days of the hypermetabolic phase in response to lipopolysaccharide (LPS) injection.

From the Service de Biochimie A, Hôpital Saint-Antoine, Paris; Groupe de Recherche et d'Etudes en Nutrition et Métabolisme Hépatique; and Institut National de la Santé et de la Recherche Médicale U402, Centre Hospitalier Universitaire Saint-Antoine, Paris, France.

Submitted February 17, 1998; accepted July 21, 1998.

Supported in part by a Clintec-European Society of Parenteral and Enteral Nutrition research fellowship.

Address reprint requests to P. Pernet, MD, Hôpital Saint-Antoine, Service de Biochimie A, 184 rue du Faubourg Saint-Antoine, 75571 Paris cedex 12 France.

0026-0495/99/4802-0009$10.00/0

MATERIALS AND METHODS

Animals

Eighty-three male Wistar rats (Centre d'Elevage René Janvier, Saint-Denis-les-Laval, France) aged 4 weeks and weighing 90 to 110 g were used for three consecutive experiments. They were housed individually in stainless steel wire-bottom metabolic cages on a reversed 12-hour light/dark cycle (8 AM to 8 PM) in a thermostatically controlled room (22°C). They were acclimatized for 5 days to standard laboratory conditions with free access to water and rat chow (Usine d'Alimentation Rationnelle [UAR] AO3; Epinay-sur-Orge, France) before beginning the experiments. Animal care complied with the rules of the Saint-Antoine Hospital. In addition, three of the investigators (J.G., L.C., and C.C.-L.) are authorized by the French Ministry of Agriculture to use this experimental model.

Study Design

After acclimatization, the rats were randomly allocated into four groups defined by the presence or absence of LPS administration and NOS inhibition with L-NAME (Table 1). Injections were performed intraperitoneally (volume, 1 mL/100 g body weight). The following groups were used: LPS-NAME, 36 rats injected first with 85 mg/kg L-NAME (Sigma-Aldrich, Saint Quentin Fallavier, France) dissolved in saline and 30 minutes later with 3 mg/kg endotoxin from *Escherichia coli* 0127:B8 (Sigma-Aldrich) dissolved in saline; LPS, 18 rats injected first with saline and 30 minutes later with 3 mg/kg endotoxin from *E. coli* 0127:B8 dissolved in saline; T-NAME, 13 rats first injected with 85 mg/kg L-NAME dissolved in saline and 30 minutes later with saline; and T, 16 rats injected twice with saline. The LPS-NAME group was made larger than the others because of the high mortality rate expected.[11,12]

After injections, the rats were fasted for 24 hours with free access to water (day 0 to day 1) to aggravate the effects of injury and induce long-lasting metabolic alterations.[13] Enteral nutrition (Osmolite; Abbott, Rungis, France) was then administered for 48 hours from day 1 to day 3 in three daily gavages (210 kcal/kg/d, 1.2 g N/kg/d).[14] In the T-NAME and LPS-NAME groups, L-NAME (85 mg/kg/d) was mixed with the enteral nutrition to maintain NOS inhibition till death. Finally, the surviving rats were killed by decapitation on day 3.

From day 0 to day 3, the rats were monitored for total body weight variation and urine was collected daily and stored at −20°C for determination of nitrogen and 3-methylhistidine (3-MH) excretion. A 50-μl vol of 1‰ sodium ethylmercurithiosalicylate solution (Prolabo,

Table 1. Definition of Four Experimental Groups According to the Presence or Absence of NOS Inhibition and Endotoxemia

Parameter	T (n = 17)	T-NAME (n = 13)	LPS (n = 20)	LPS-NAME (n = 36)
First injection, time 0				
Saline	+	–	+	–
L-NAME	–	+	–	+
Second injection, time 0 + 30 min				
Saline	+	+	–	–
LPS	–	–	+	+

Paris, France) was added to each urine collection container to prevent bacterial growth. At death, the anterior tibialis and extensor digitorum longus were promptly removed, weighed, and stored at −80°C until determination of total protein and glutamine concentrations.

Measurements

Urinary nitrogen. Total urinary nitrogen levels were measured by chemiluminescence on an Antek 8000 N (Antek Instrument, Dusseldorf, Germany). Day-to-day nitrogen balance was calculated (difference between nitrogen intake and urinary nitrogen excretion).

Urinary 3-MH. In the rat, 3-MH is chiefly excreted in the urine in acetylated form. Therefore, samples were hydrolyzed with HCl 6 mol/L (vol/vol) at 100°C for 24 hours before quantification by ion-exchange chromatography and ninhydrin detection using a model 6300 analyzer (Beckman, Palo Alto, CA).

Muscle glutamine. Muscles were homogenized in 10% trichloroacetic acid containing 0.5 mmol/l EDTA. After centrifugation, glutamine concentrations were measured in supernatants by ion-exchange chromatography on a Beckman 6300 analyzer.

Total muscle protein. After homogenization of the muscles and centrifugation, total muscle protein concentrations were measured by the method of Gornall et al[15] on centrifugation precipitates after treatment with NaOH 1 mol/L.

Statistical Analysis

Data are expressed as the mean ± SD. Comparisons were made using the χ^2 test for the mortality rate and one-way ANOVA for all other parameters. When ANOVA results indicated an overall significant difference among the four groups, the Newman-Keuls test was performed to determine which groups differed. Differences were considered significant at a P level less than .05.

RESULTS

Mortality Rate

Deaths occurred only in groups injected with LPS, within 24 hours after treatment. The results showed that NOS inhibition is deleterious in endotoxemic rats: the mortality rate in the LPS-NAME group (80.5%) was statistically higher ($\chi^2 = 19.48$, $P < .001$) than in the LPS group (20%). Moreover, deaths occurred earlier in the LPS-NAME group, with 62% of deaths (18 of 29) in the first 6 hours versus 0% (none of four) in the LPS group. Two rats were accidentally killed during gavages in the LPS group.

Consequently, 50 rats had a full 3-day follow-up period (T, n = 16; T-NAME, n = 13; LPS, n = 14; LPS-NAME, n = 7).

Weight Variation

No statistically significant differences were found among the animals for day-to-day total body weight variation (data not shown). In particular, no difference among the groups appeared during the 2 days of enteral nutrition.

Nitrogen Balance

Day-to-day nitrogen balance is presented in Fig 1. On day 1, nitrogen balance was more negative ($P < .01$) in the two injured groups (LPS and LPS-NAME) compared with the control groups (T and T-NAME). This difference persisted for the first 24 hours of renutrition ($P < .05$), but only the LPS-NAME group maintained a negative nitrogen balance on day 2 that was still not statistically different versus the LPS group ($P = .06$). On day 3, nitrogen balance was positive in all groups, with no difference between groups.

Myofibrillar Catabolism

Day-to-day total urinary excretion of 3-MH was used to evaluate myofibrillar catabolism (Fig 2). On day 1, increased excretion was found in the LPS group compared with all other groups ($P < .01$), with no difference between LPS-NAME, T, and T-NAME. On day 2, myofibrillar catabolism in the LPS group remained higher compared with the T and T-NAME groups ($P < .01$), but the highest excretion rate for 3-MH was

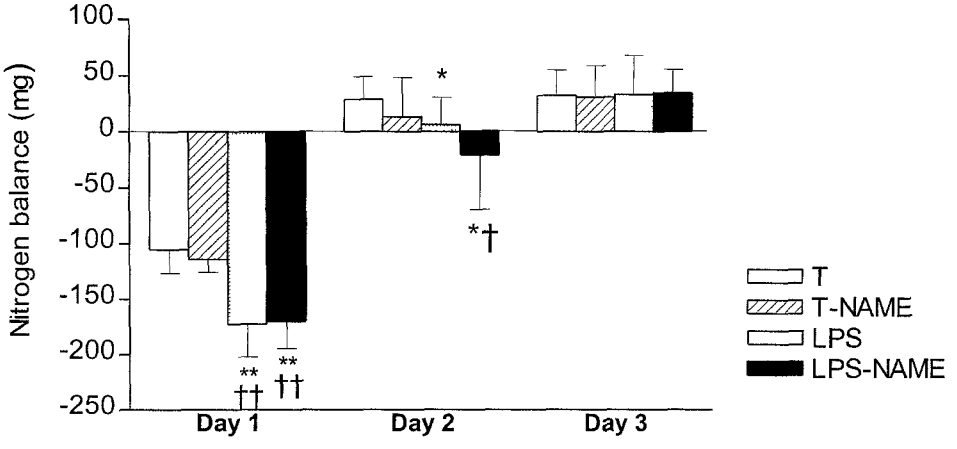

Fig 1. Effect of endotoxemia and NOS inhibition on day-to-day nitrogen balance. Results are the mean ± SD. T, n = 16; T-NAME, n = 13; LPS, n = 14; LPS-NAME, n = 7. *$P < .05$ and **$P < .01$ *v* T; †$P < .05$ and ††$P < .01$ *v* T-NAME.

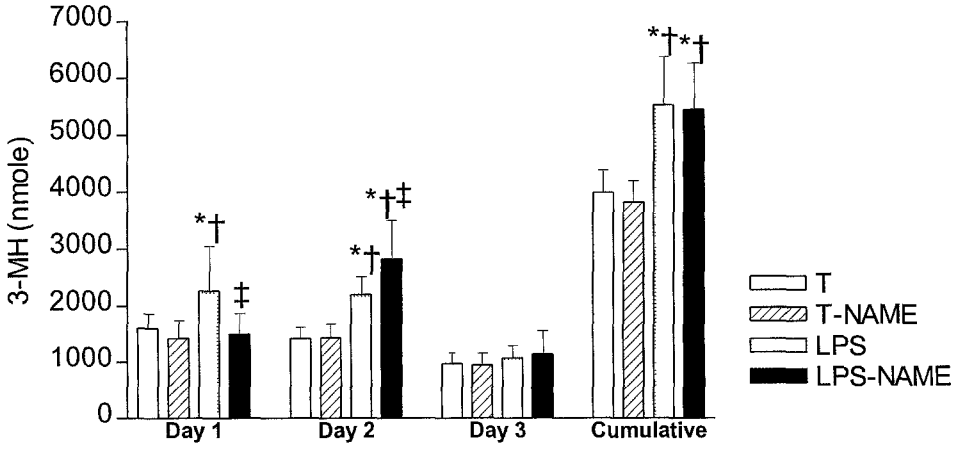

Fig 2. Day-to-day and 3-day cumulative urinary 3-MH excretion. Results are expressed as nmol/24 h or nmol/3 d. T, n = 16; T-NAME, n = 13; LPS, n = 14; LPS-NAME, n = 7. *$P < .01$ *v* T; †$P < .01$ *v* T-NAME; ‡$P < .05$ *v* LPS.

found in the LPS-NAME group ($P < .01$ *v* all others). On day 3, the groups were not different. Three-day cumulative values showed that 3-MH urinary excretion was higher in both endotoxemic groups versus controls ($P < .01$), with no difference between LPS and LPS-NAME groups. This figure was the same when comparisons were made cumulatively for day 1 and day 2, ie, during the high myofibrillar catabolism period.

Muscular Parameters

On day 3, the anterior tibialis weight was decreased in both endotoxemic groups compared with the T group. In addition, the anterior tibialis weight was also lower in the LPS-NAME group compared with the T-NAME group (Table 2). No differences were found for the protein concentration in muscle. Muscular glutamine concentrations were decreased in the LPS and LPS-NAME groups compared with the T and T-NAME groups ($P < .01$). The same statistical significance was found in extensor digitorum longus for the weight and protein and glutamine concentrations as observed in anterior tibialis (data not shown).

DISCUSSION

Despite the plethora of biological functions proposed for NO, studies relating NO to skeletal muscle metabolism are few, and a role for NO in muscular metabolism during injury has not been investigated. However, it is well documented that LPS injection is responsible for induction of the iNOS isoform[7,8,16] and upregulation of the NOS isoform[9] with increased muscular NO production. In addition, it has been shown that NO production is implicated in decreased muscular strength[9] and modulates glucose transport.[10,16] Given that during septic shock, skeletal muscle undergoes catabolism for energy production and inflammatory protein synthesis, we investigated the relationship between NO production and alteration in muscle protein metabolism. Accordingly, an endotoxemic rat model developed by our group[14] was used to evaluate the effect of NOS inhibition on the induced hypercatabolic state especially at the muscular level.

Weanling rats were chosen because young animals (4 weeks old) are in an anabolic phase and more sensitive to injury. Given that the rat is resistant to trauma and early enteral feeding induces rapid recovery,[13,17] induction of a hypercatabolic state in this model requires more than endotoxemia, ie, 24-hour fasting followed by low-calorie hyponitrogenous nutrition. Controlled hyponutrition with enteral feeding was also advantageous, enabling us to form pair-fed groups, because endotoxemia generates anorexia[18] and NO can modulate food intake.[19] Hence, this method of feeding avoids the influence of variable food intake on the metabolic changes observed.

L-NAME was chosen because it is the inhibitor most frequently used in similar studies in rats using experimental models of injury. At the muscular level, it has been shown that L-NAME can inhibit NO production in vivo[20,21] and in vitro,[16] particularly using the oral route and a dose similar to that used in the present study.[21] We also verified that the active metabolite of L-NAME, L-nitro-arginine,[22] which is the true inhibitory molecule, was found in anterior tibialis and extensor digitorum longus in muscle amino acid chromatograms.

In our experimental model, long-term administration of 85 mg/kg L-NAME in healthy rats for 3 days did not induce any significant modifications compared with untreated healthy rats. This indicates that L-NAME has no action per se on the parameters studied, and therefore, any differences observed

Table 2. Muscular Weight, Total Protein, and Free Glutamine on Day 3 in Anterior Tibialis (mean ± SD)

Parameter	T (n = 16)	T-NAME (n = 13)	LPS (n = 14)	LPS-NAME (n = 7)
Muscular weight (mg/100 g)	369 ± 17	358 ± 23	349 ± 26*	333 ± 23†‡
Protein (mg/g muscle)	178 ± 18	165 ± 27	171 ± 16	158 ± 30
Glutamine (μmol/L)	7,284 ± 1,028	7,114 ± 1,441	5,649 ± 928†§	4,876 ± 284†§

*$P < .05$, †$P < .01$ *v* T.
‡$P < .05$, §$P < .01$ *v* T-NAME.

between the two injured groups, such as lethality, can be attributed to NOS pathway–LPS interaction.

Endotoxin from *E. coli* 0127:B8 3 mg/kg has been shown to be a nonlethal dose in adult rats.[14] Hence, the mortality rate of 20% in the LPS group we observed is probably related to the use of young animals, which are usually more sensitive to injury. Concerning the early high mortality rate observed in the LPS-NAME group, data from the present experiments are consistent with previous studies showing a detrimental role for NO inhibition during endotoxemia.[11,12] In the present experiment, because deaths in the LPS-NAME group were observed within the 6 first hours after injections, these could be related to an early aggravation of cardiovascular failure consequent to increased plasma levels of tumor necrosis factor alpha and interleukin-6 that are reported to be involved in this lethality.[12,23] No attempt was made to measure plasma cytokine levels in the present study.

Although NOS inhibition severely impairs the cardiovascular system during endotoxemia, the present study provides evidence that the hypercatabolic state and its important muscular component are not clearly aggravated. The nitrogen balance showed that endotoxemia induces a catabolic state for 2 days that is not significantly modified by NOS inhibition. Related to this hypermetabolism, the glutamine depletion found in muscles of endotoxemic rats[14] also is not significantly worsened by NOS inhibition.

Muscle weight loss observed during endotoxemia is known to be related to myofibrillar catabolism, with transfer of amino acids from the muscle to splanchnic areas for energy production and inflammatory protein synthesis. In our model, this muscle weight loss, found at the same level in the LPS and LPS-NAME groups, shows no further action due to L-NAME treatment. NOS inhibition also has no effect on the muscular protein concentration.

However, daily urinary 3-MH excretion shows that myofibrillar catabolism in endotoxemic rats is modified by L-NAME treatment. NOS inhibition delays the urinary 3-MH excretion peak in endotoxemic rats from day 1 to day 2 without increasing the cumulative excretion observed during the 3-day follow-up period. This transiently delayed 3-MH excretion was not previously reported. It may also indicate that NOS inhibition induced a strong deleterious effect on renal and/or hepatic function. A profound renal insufficiency in the LPS-NAME group does not seem to be involved, since diuresis and urinary nitrogen and creatinine excretion were not different in the LPS and LPS-NAME groups during the 3-day follow-up period (data not shown). However, the precise state of renal function is unknown on day 1, ie, when 3-MH excretion is altered in the LPS-NAME group, because plasma creatinine and creatinine clearance were not evaluated at that time. A defect in hepatic acetylation of 3-MH may also be involved, because 3-MH in rats is chiefly excreted in the acetylated form. This possibility deserves further investigation.

In conclusion, NOS expression appears to be of major importance for survival after an endotoxemic challenge, but seems weakly contributive to the metabolic response of muscle to injury.

REFERENCES

1. Moncada S, Higgs A: The L-arginine–nitric oxide pathway. N Engl J Med 329:2002-2012, 1993
2. Glauser MP, Zanetti G, Baumgartner JG, et al: Septic shock: Pathogenesis. Lancet 338:732-735, 1991
3. Souba WW, Austgen TR: Interorgan glutamine flow following surgery and infection. J Parenter Enteral Nutr 14:90S-93S, 1990 (suppl)
4. Moncada S, Palmer RMJ, Higgs EA: Nitric oxide: Physiology, physiopathology and pharmacology. Pharmacol Rev 43:109-141, 1991
5. Boczkowski J, Lanone S, Ungureanu-Longrois D, et al: Induction of diaphragmatic nitric oxide synthase after endotoxin administration in rats. Role on diaphragmatic contractile dysfunction. J Clin Invest 98:1550-1559, 1996
6. Kobzik L, Reid MB, Bredt DS, et al: Nitric oxide in skeletal muscle. Nature 372:546-548, 1994
7. Okuda S, Kanda F, Kawahara Y, et al: Regulation of inducible nitric oxide synthase expression in L6 rat skeletal muscle cells. Am J Physiol 272:C35-C40, 1997
8. Thompson M, Becker L, Bryant D, et al: Expression of the inducible nitric oxide synthase gene in diaphragm and skeletal muscle. J Appl Physiol 81:2415-2420, 1996
9. El Dwairi Q, Comtois A, Guo Y, et al: Endotoxin-induced skeletal muscle contractile dysfunction: Contribution of nitric oxide synthases. Am J Physiol 274:C770-C779, 1998
10. Kapur S, Bédard S, Marcotte B, et al: Expression of nitric oxide synthase in skeletal muscle: A novel role for nitric oxide as a modulator of insulin action. Diabetes 46:1691-1700, 1997
11. Minnard EA, Shou J, Naama H, et al: Inhibition of nitric oxide synthesis is detrimental during endotoxemia. Arch Surg 129:142-148, 1994
12. Tiao G, Rafferty J, Ogle C, et al: Detrimental effect of nitric oxide synthase inhibition during endotoxemia may be caused by high levels of tumor necrosis factor and interleukin-6. Surgery 116:332-338, 1994
13. Vaubourdolle M, Coudray-Lucas C, Jardel A, et al: Action of enterally administered ornithine α-ketoglutarate on protein breakdown in skeletal muscle and liver of the burned rat. J Parenter Enteral Nutr 15 517-520, 1991
14. Lasnier E, Coudray-Lucas C, Le Boucher J, et al: Ornithine alpha-ketoglutarate counteracts thymus involution and glutamine depletion in endotoxemic rats. Clin Nutr 15:197-200, 1996
15. Gornall AG, Bardawil CJ, David MM: Determination of serum protein by means of the biuret reaction. J Biol Chem 177.751-766, 1949
16. Bédard S, Marcotte B, Marette A. Cytokines modulate glucose transport in skeletal muscle by inducing the expression of inducible nitric oxide synthase. Biochem J 325·487-493, 1997
17. Mochizuki H, Trocki O, Dominioni L, et al: Mechanism of prevention of postburn hypermetabolism and catabolism by early enteral feeding. Ann Surg 200:297-301, 1984
18. Sharma RJ, Macallan DC, Sedgwick P, et al: Kinetics of endotoxin-induced acute-phase protein gene expression and its modulation by TNF-α monoclonal antibody. Am J Physiol 262:R786-R793, 1992
19. Morley JE, Flood JF: Evidence that nitric oxide modulates food intake in mice. Life Sci 49:407-411, 1991
20. Zhang B, Knight KR, Guida E, et al: Timing of dexamethasone or the nitric oxide synthase inhibitor, nitro-L-arginine methyl ester, is critical for effective treatment of ischaemia-reperfusion injury to rat skeletal muscle. Clin Sci 93:167-174, 1997
21. Roberts CK, Barnard RJ, Scheck SH, et al: Exercise stimulated glucose transport in skeletal muscle is nitric oxide dependent. Am J Physiol 273:E220-E225, 1997
22. Pfeiffer S, Leopold E, Schmidt K, et al: Inhibition of nitric oxide synthesis by N^G-nitro-L-arginine methyl ester (L-NAME)· Requirement for bioactivation to the free acid N^G-nitro-L-arginine. Br J Pharmacol 118:1433-1440, 1996
23. Damas P, Ledoux D, Nys M: Cytokine serum level during severe sepsis in human: IL6 as a marker of severity. Ann Surg 215:356-362, 1992