Nonselective versus Selective Inhibition of Inducible Nitric Oxide Synthase in Experimental Endotoxic Shock

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The effects of two nitric oxide synthase (NOS) inhibitors with different isoform selectivity were compared in a murine model of endotoxemia. Mice challenged with 70 mg/kg intraperitoneal (ip) lipopolysaccharide (LPS) were treated 6 h after LPS with either N^G-γ-L-arginine methyl ester (L-NAME, nonselective NOS inhibitor, 10–60 mg/kg), L-canavanine (selective inhibitor of inducible NOS, 50–300 mg/kg), or saline (0.2 mL) given ip. In a subset of mice, plasma concentrations of nitrate (NO breakdown product), lipase (pancreas injury), lactate dehydrogenase, and transaminases (liver injury) were measured 16 h after LPS. Although both inhibitors reduced plasma nitrate, they produced contrasting effects on survival and organ injury. L-NAME enhanced liver damage and tended to accelerate the time of death, while L-canavanine significantly reduced mortality and had no deleterious effects in terms of organ damage. These results indicate that nonselective NOS inhibitors are detrimental in endotoxic shock and support the potential usefulness of selective inducible NOS inhibitors in this setting.

Nitric oxide (NO) is a short-lived effector molecule that is produced from L-arginine by several NO synthase (NOS) isoforms. Physiologically, small amounts of NO are produced by an endothelial constitutive NOS (ecNOS), which is involved in the regulation of vascular tone and blood flow distribution [1]. On stimulation by bacterial products such as lipopolysaccharide (LPS), and various cytokines, an inducible NOS (iNOS) is diffusely expressed, producing large amounts of NO for prolonged periods, which have been shown to play a major role in the pathophysiology of septic and endotoxic shock [1].

The recognition of NO as an important mediator of septic shock led to the proposal that the pharmacologic inhibition of NO production could represent a useful adjunct in the treatment of this condition [1]. In support of this concept, it was recently shown that mutant mice lacking the iNOS gene were conferred some protection against LPS-induced mortality [2], although this finding has not been systematically reproduced [3]. Unfortunately, such inhibition has been frequently reported to be detrimental, and recent data suggest that this deleterious potential might be a consequence of ecNOS blockade by nonselective agents [1, 4, 5]. Thus, interest is now focusing on the identification of compounds that would selectively reduce iNOS-dependent NO production [1, 4].

Indeed, beneficial effects were recently reported in experimental models of septic shock that used various putatively selective inhibitors of iNOS, such as aminoguanidine [6], L-canavanine [7], and *S*-substituted thiourea derivatives [4, 8]. However, these studies essentially focused on the hemodynamic and metabolic consequences of these inhibitors, with only a limited interest towards their influence on mortality. The present study was therefore designed to address this issue, by comparing the effects of the nonselective NOS inhibitor N^G-γ-L-arginine methyl ester (L-NAME) to those of L-canavanine, a selective iNOS inhibitor [7], on the mortality of endotoxic shock in mice.

Material and Methods

Animals

One hundred eighty-six Swiss-Webster female mice (6–8 weeks old; mean weight, 25 g) were used in this study. Mice were housed by groups of 10–15 animals with a light-night rhythm of 12 h–12 h and had free access to food and water. An adaptation period to these conditions of at least 2 weeks was observed before the animals were used for the experiments.

Experimental Setup

Effects of NOS inhibitors on endotoxin lethality. One hundred six mice were used in this experiment. At baseline, all animals were challenged with 70 mg/kg LPS intraperitoneally (ip), dissolved in 0.2 mL of normal saline. Six hours later, mice were assigned to one of the following ip treatments: L-NAME, 10 mg/kg (n = 16); L-NAME, 60 mg/kg (n = 16); L-canavanine, 50 mg/kg (n = 25);

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L-canavanine, 300 mg/kg ($n = 25$); normal saline, 0.2 mL (control group, $n = 24$). NOS inhibitors were administered 6 h after ip LPS to account for the lag period of several hours necessary for iNOS to be expressed on an induction stimulus [1]. Indeed, diffuse expression of iNOS [9] and elevation of plasma nitrogen oxides [10] have been reported in mice 6 h after ip LPS administration.

Mortality was noted at 8-h intervals until 48 h after LPS and then at 24-h intervals until day 7.

The different doses of NOS inhibitors were adapted from the results of previous experiments, in which we found that total doses of 25 mg/kg L-NAME and 100 mg/kg L-canavanine were able to efficiently blunt NO production in endotoxemic rats [7]. The dose of LPS used in the present study was chosen on the basis of pilot experiments, in which we found that this dose was responsible for a 100% lethality at 48 h.

Effects of NOS inhibitors on indicators of NO production and organ damage in endotoxic shock. Eighty mice were used in this experiment. The animals were initially challenged with 70 mg/kg LPS ip. Six hours later, they received one of the following ip treatments: L-NAME, 10 mg/kg ($n = 16$); L-NAME, 60 mg/kg ($n = 16$); L-canavanine, 50 mg/kg ($n = 16$); L-canavanine, 300 mg/kg ($n = 16$); normal saline, 0.2 mL (control group, $n = 16$). Then, 10 h later (16 h after LPS), mice were anesthetized with ip pentobarbital (200 mg/kg), the thorax was opened, and the heart was punctured with a 22-gauge needle, allowing ~0.5 mL of blood to be sampled. The sampled blood was centrifuged at 2000 *g* for 15 min. Plasma was then assayed for concentrations of alanine aminotransferase (ALT), aspartate aminotransferase (AST), lactate dehydrogenase (LDH), and pancreatic lipase, measured spectrophotometrically with a selective analyzer (Hitachi Scientific Instruments, Mountain View, CA). Plasma was also assayed for concentrations of nitrate, which were determined by a spectrophotometric method based on NADPH oxidation and adapted on an automated analyzer (Cobas; Roche, Basel, Switzerland), as previously described [11].

Materials

LPS (*Escherichia coli* O127:B8), L-NAME hydrochloride, and L-canavanine freebase were all purchased from Sigma (Buchs, Switzerland) and were freshly dissolved in isotonic saline before use.

Statistical Methods

Survival curves were compared using the log rank test, and $P < .05$ was considered significant. Comparisons between values for nitrate, AST, ALT, LDH, and lipase in the different groups were made with analysis of variance. When the F value was significant at the 5% level, further pairwise comparisons were made with saline treatment as a control. Statistical significance was assigned to $P < .05$. Results of plasma nitrate, AST, ALT, LDH, and lipase are expressed as means ± SEs.

Results

Survival experiment. Administration of LPS was followed by the rapid development (within 1 h) of signs of toxicity, as evidenced by ruffled fur, anorexia, lethargy, and tachypnea. Later in the course of endotoxic shock, mice became progressively cyanotic and deeply comatose. Death was preceded by muscular spasms and convulsions.

Figure 1 illustrates the survival curves of the different treatment groups of mice. All mice treated with saline or L-NAME (at either 10 or 60 mg/kg) died from LPS administration, death tending to occur earlier in mice receiving L-NAME (at both doses), but this difference was not statistically significant. L-canavanine afforded a significant protection against LPS-induced mortality, at either 50 mg/kg (13/25 surviving mice at day 7) or 300 mg/kg (7/25 surviving mice), without significant difference between doses.

NO production and indicators of organ damage. It was not possible to obtain plasma from mice treated with 60 mg/kg L-NAME, since all mice in this group had died at the time of blood sampling (16 h after LPS administration). Figure 2 shows plasma nitrate results in the remaining 4 groups of mice. In the control group, nitrate reached 2356 ± 91 μmol/L. Treatment with L-canavanine at either doses and L-NAME (10 mg/kg) reduced the level of plasma nitrate, this effect being most pronounced in the L-NAME group (1059 ± 105 μmol/L; $P <$ .05 vs. saline control), followed by the high dose (300 mg/kg) of L-canavanine (1536 ± 260 μmol/L; $P < .05$ vs. saline; P not significant vs. L-NAME) and the low dose (50 mg/kg) of L-canavanine (1890 ± 105 μmol/L; $P = .051$ vs. saline; P not significant vs. L-canavanine 300 mg/kg; $P < .05$ vs. L-NAME).

Figure 3 illustrates the concentrations of plasma AST, ALT, LDH, and lipase obtained in the 4 treatment groups. While L-canavanine had no significant influence on these different variables, L-NAME produced a significant increase in plasma AST, ALT, and LDH, and it tended to increase plasma lipase.

Discussion

The pharmacologic inhibition of NO production has been recently proposed as a potentially interesting adjunct to septic shock therapy [1, 12]. However, it is increasingly recognized that nonselective NOS inhibitors are more detrimental than beneficial in this setting and that the selective targeting of the inducible isoform of NOS would be preferable [1]. In a previous study [7], we reported that the survival of endotoxemic mice was markedly improved when animals were pretreated with L-canavanine, a selective inhibitor of iNOS, contrasting with no beneficial influence of pretreatment with L-NAME, a nonselective NOS inhibitor. The present study largely extends these results by showing that L-canavanine is also protective when given 6 h after LPS administration, at a time when mice already show severe signs of systemic toxicity.

Administration of the nonselective NOS inhibitor L-NAME was not protective and in fact tended to accelerate death (figure 1). In addition, L-NAME favored the development of organ injury in endotoxemic mice, as shown by a significant increase in plasma transaminases and LDH, as well as by an important

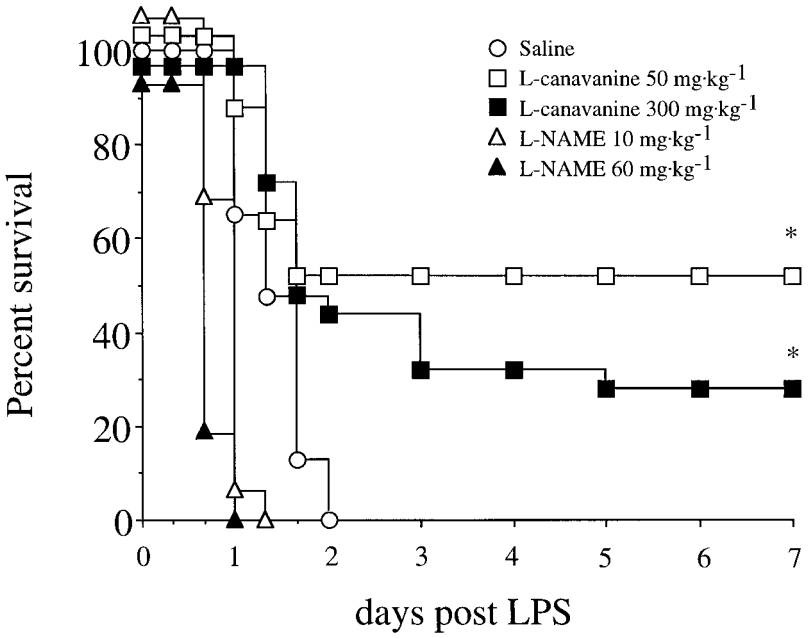

Figure 1. Survival curves obtained in mice challenged with 70 mg/kg lipopolysaccharide (LPS) intraperitoneally at baseline and treated intraperitoneally 6 h later with either normal saline, 0.2 mL (n = 24), L-canavanine, 300 mg/kg (n = 25), L-canavanine, 50 mg/kg (n = 25), N^G-γ-L-arginine methyl ester (L-NAME), 60 mg/kg (n = 16), or L-NAME, 10 mg/kg (n = 15). * $P < .05$ vs. saline treatment (log rank test).

trend toward an increase in plasma lipase (figure 3). These results fully agree with many previous studies showing that nonselective NOS inhibitors either do not influence survival [13] or enhance mortality [14–16] in septic or endotoxemic animals. The only exception is the study by Teale and Atkinson [17], who found that the nonselective NOS inhibitor monomethyl-L-arginine (L-NMMA) conferred some survival advantage when administered concomitantly with imipenem in a murine model of peritonitis. Our finding of increased signs of organ injury also confirms previous results obtained with nonselective NOS inhibitors in experimental septic shock [16, 18]. It has been proposed that these deleterious effects might reflect the loss of the regulatory functions of ecNOS on the microcirculation, platelet aggregation and endothelium-leukocytes interactions, thereby favoring tissue hypoperfusion [19, 20], microthrombi formation [21], and leukocyte infiltration [15, 16]. Taken together, these data do not support the use of nonselective NOS inhibitors in septic shock therapy.

In striking contrast with the effects of L-NAME, we found that L-canavanine, both at 50 and 300 mg/kg, afforded a significant protection against LPS-mediated mortality (figure 1) and did not reproduce the detrimental effects of L-NAME on organ damage (figure 3). However, it is noteworthy that the high dose of L-canavanine appeared somewhat less protective, since it produced a survival rate of 28% compared with 52% at the low dose. Although not statistically significant, this trend

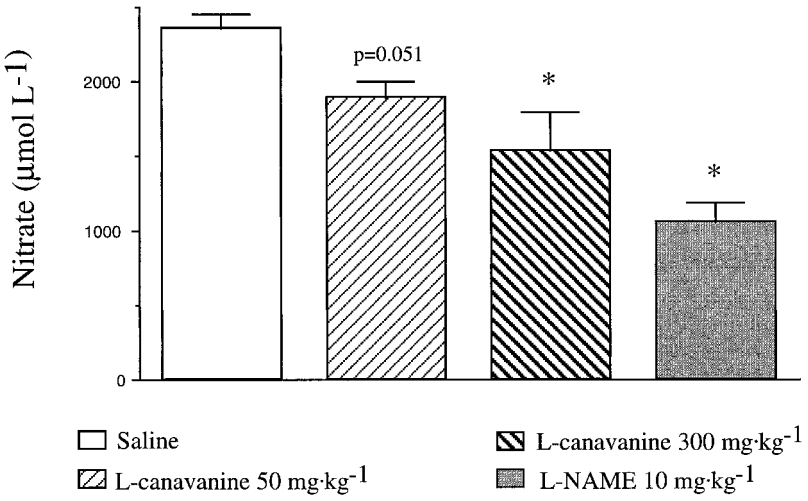

Figure 2. Plasma nitrate concentrations measured in mice challenged with 70 mg/kg lipopolysaccharide (LPS) intraperitoneally at baseline and treated intraperitoneally 6 h later with either normal saline, 0.2 mL (n = 16), L-canavanine, 300 mg/kg (n = 16), L-canavanine, 50 mg/kg (n = 16), or N^G-γ-L-arginine methyl ester (L-NAME), 10 mg/kg (n = 15). Blood samples were obtained 16 h after LPS. Data are means ± SEs. * $P < .05$ vs. saline treatment.

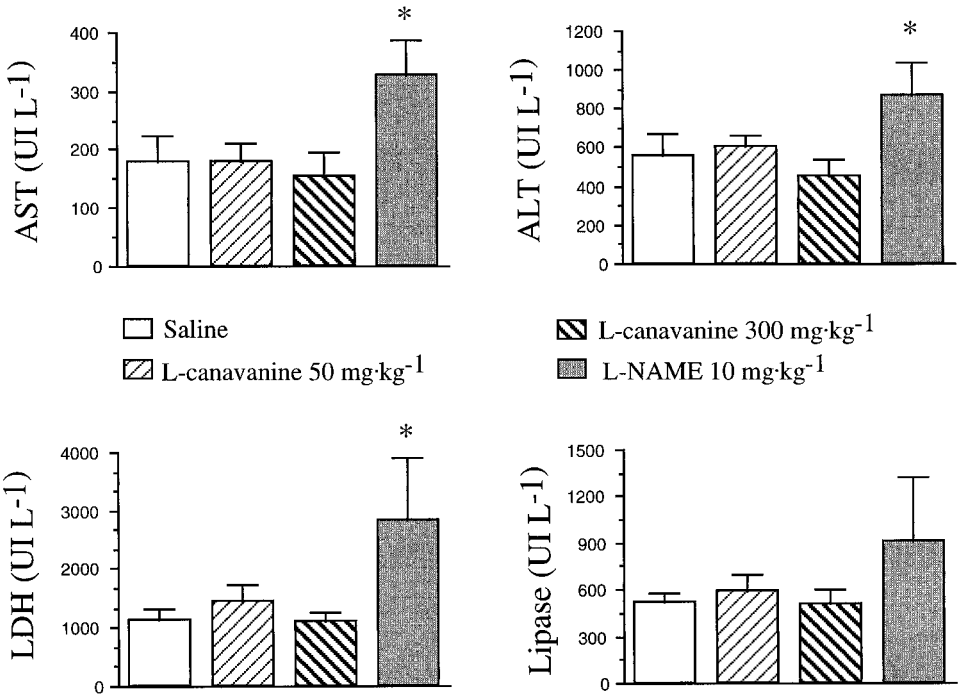

Figure 3. Plasma concentrations of aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH), and pancreatic lipase, measured in mice challenged with 70 mg/kg lipopolysaccharide (LPS) intraperitoneally at baseline and treated intraperitoneally 6 h later with either normal saline, 0.2 mL (n = 16), L-canavanine, 300 mg/kg (n = 16), L-canavanine, 50 mg/kg (n = 16), N^G-γ-L-arginine methyl ester (L-NAME), 10 mg/kg (n = 15). Blood samples were obtained 16 h after LPS. Data are means ± SEs. * $P < .05$ vs. saline treatment.

might indicate nonspecific toxicity of L-canavanine at high doses. However, this is an unlikely possibility, since the main toxicity of L-canavanine reported in rodents is pancreatic damage [22], a potential side effect that is not supported by our data of plasma lipase (figure 3). A distinct possibility might be the loss of iNOS selectivity at high doses. Indeed, very high concentrations of L-canavanine (2 m*M*) were able to inhibit the activity of ecNOS in vitro [23].

Although the mechanisms by which L-canavanine protected mice from LPS-induced mortality cannot be inferred directly from our data, several hypotheses may be advanced. First, decreasing NO production may have reduced oxidative stress by slowing the formation of peroxynitrite, a highly reactive species formed from the reaction of NO with the superoxide radical [1]. Second, the selective inhibition of iNOS may have improved tissue oxygenation and energy metabolism, either by limiting the LPS-induced fall in cardiac output, as previously reported in endotoxemic rats [7, 24], by improving microcirculatory blood flow distribution through the removal of excess vasodilator NO [1], or finally, by preventing an NO-mediated block of high-energy phosphate generation at the cellular level [25]. Indeed, we provided evidence that L-canavanine enhances ATP concentrations in various organs during rat endotoxic shock [11].

In spite of the evident protection afforded by L-canavanine, it was somewhat puzzling that it did not affect the biologic markers of tissue injury (figure 3). This may suggest that iNOS-mediated NO production was not a critical factor in the occurrence of organ damage in our conditions, at least considering the liver and the pancreas. Another possibility is that the schedule of blood sampling 16 h after LPS was too early, that is, at a time when organ injury was still insufficiently developed to detect the influence of L-canavanine. Finally, it is worth noting that plasma transaminases, LDH and lipase, while sensitive indicators of tissue damage, do not provide information regarding cell function. Therefore, the lack of effect of L-canavanine on these biologic markers does not rule out some beneficial influence of this compound on LPS-induced organ dysfunction, although this issue remains speculative.

NO production was assessed by measuring the plasma levels of nitrate, the stable oxidation product of NO in blood. In endotoxemic mice treated with saline, nitrate levels were >2000 μmol/L, indicating a massive synthesis of NO in these animals, given a normal basal concentration of plasma nitrogen oxides (nitrate and nitrite) of 60 μmol/L in mice [10]. All treatments reduced plasma nitrate (figure 2), although to different degrees, the reduction being marginally significant with the low dose of L-canavanine (−20%; $P = .051$), intermediate with the high dose of L-canavanine (−35%; $P < .05$), and most pronounced with L-NAME (−55%; $P < .05$).

These results imply that iNOS was not similarly inhibited by the different regimens. Therefore, one could argue that the contrasted effects of our treatments on survival might reflect different levels of iNOS blockade rather than selective versus nonselective NOS inhibition. Although we can not formally rule out this hypothesis, it seems unlikely, for the following reasons: while the reduction in plasma nitrate achieved with L-canavanine at a high dose (300 mg/kg) and L-NAME was not statistically different, only L-canavanine was protective; also, in studies by other investigators in endotoxemic [15] or

septic mice [13], in which treatment with the nonselective NOS inhibitors L-NAME or L-NMMA achieved reductions in plasma nitrogen oxides comparable to or even smaller than those of L-canavanine in our study, survival was either not improved [13] or depressed [15]. Taken together, these data support that selective rather than partial inhibition of iNOS was the critical factor underlying the contrasted effects of L-canavanine and L-NAME in our endotoxemic mice.

The effects of L-canavanine noted in the present study extend the results of previous works showing beneficial effects of other selective iNOS inhibitors, chemically unrelated to L-canavanine, in experimental septic shock. Aminoguanidine and one analogue, 1-amino-2-hydroxy-guanidine, reduced organ injury and metabolic acidosis in endotoxemic rats [5, 26]. Aminoguanidine was also shown to reduce bacterial translocation from the gut of endotoxemic rats [27] and to improve survival of endotoxemic mice [6]. Thiourea derivatives, such as *S*-methyl-isothiourea and aminoethylisothiourea, also produced beneficial hemodynamic and metabolic effects in endotoxic shock rats [4, 8], and *S*-methyl-isothiourea was shown to improve survival in endotoxemic mice and septic rats [4, 28]. Finally, it has been recently shown that a newly developed selective iNOS inhibitor, guanidinoethyldisulfide, caused a significant improvement in the survival rate in a lethal model of endotoxic shock in mice [29].

A number of investigations have been recently done in which various antimediator strategies have been assessed as potentially useful adjuncts to septic shock therapy. These included both inhibition of bacterial mediators, such as endotoxin, and inhibition of host inflammatory mediators, mainly cytokines such as tumor necrosis factor-α and interleukin-1 [30–32]. In substance, these studies showed that such strategies often provided favorable effects and improved survival, especially when applied before or at the time of the septic challenge in experimental animals. However, it also appeared that this protection was either largely reduced, or totally lacking, when the intervention was postponed until after the septic challenge [30]. Thus, while such therapeutic modalities might prove beneficial in the prophylaxis of septic shock in patients with sepsis or severe sepsis, their usefulness in patients with overt septic shock appears questionable. Contrastingly, we found in the present study that L-canavanine provided a significant survival advantage in endotoxemic mice when administered 6 h after the LPS challenge, at a time when animals were already seriously ill. This finding, which extends our previous observation that prophylactic L-canavanine protected mice from endotoxin lethality [7], seems therefore of potential clinical relevance, since it shows that selective iNOS inhibition remains an efficient therapy in ongoing endotoxic shock.

Several limitations to our results must be underscored. First, the hemodynamic profile of rodent endotoxemia (hypodynamic circulation) differs from that of human septic shock (hyperdynamic circulation) [33]. Thus, selective iNOS inhibition might produce different hemodynamic consequences in both types of shock, with a potential impact on outcome. Further studies should therefore be done to assess the influence of selective iNOS inhibition in hyperdynamic models of septic shock. Second, our results were obtained in an endotoxic and not bacteremic model of septic shock. This distinction is of paramount importance, in view of the properties of NO as a microbicidal agent [2]. Therefore, future studies should be designed to assess the effects of selective iNOS inhibition in experimental models of septic rather than endotoxic shock.

In conclusion, the data presented herein confirm that nonselective NOS inhibition does not give any survival advantage to endotoxemic mice and rather appears detrimental in this setting. By contrast, the significant protection afforded by L-canavanine is in agreement with convergent information obtained with other selective iNOS inhibitors in similar experimental conditions. This supports the potential usefulness of this class of agents in the adjunctive therapy of septic shock.

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