ELSEVIER

European Journal of Pharmacology 295 (1996) 215–220

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Inhibition of nitric oxide formation with L-canavanine attenuates endotoxin-induced vascular hyporeactivity in the rat

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Received 15 June 1995; revised 13 September 1995; accepted 19 September 1995

Abstract

L-Canavanine, a selective inhibitor of inducible nitric oxide (NO) synthase, has beneficial effects on the circulatory failure of rats with endotoxin shock. To investigate the direct relationship between these beneficial effects and the inhibition of the formation of NO in response to L-canavanine in endotoxin shock in the rat, we detected changes in venous nitrosyl-hemoglobin (NO-hemoglobin) levels using an electron spin resonance (ESR) assay. Anaesthetized rats were injected with lipopolysaccharide (10 mg/kg i.v.). 1 h after the lipopolysaccharide injection, the rats were divided into four groups: a lipopolysaccharide group receiving 0.3 ml of saline hourly, an L-canavanine 10 or an L-canavanine 20 group receiving L-canavanine 10 or 20 mg/kg i.v. hourly, respectively, and an L-NAME group receiving N^G-nitro-L-arginine methyl ester (L-NAME) 15 mg/kg followed by 10 mg/kg i.v. hourly. A sham group received saline instead of lipopolysaccharide, and an L-canavanine group received L-canavanine 20 mg/kg i.v. hourly, 1 h after the saline injection. At 5 h after the lipopolysaccharide or saline injection, pressor responses to noradrenaline (1 μg/kg i.v.) were obtained. In the lipopolysaccharide group, lipopolysaccharide caused a progressive decrease in mean arterial pressure and an impairment of pressor responsiveness to noradrenaline. Administration of L-canavanine or L-NAME attenuated the endotoxin-induced hypotension and vascular hyporeactivity to noradrenaline. L-Canavanine did not alter mean arterial pressure and the pressor response to noradrenaline in the L-canavanine group. The endotoxin-induced increases in venous levels of NO-hemoglobin were significantly inhibited by L-canavanine or L-NAME. These data indicate that the beneficial hemodynamic effects of L-canavanine are associated with inhibition of the enhanced formation of NO by inducible NO synthase in a rat model of endotoxin shock. L-Canavanine is a potential agent in the treatment of endotoxin shock.

Keywords: Lipopolysaccharide; Nitric oxide (NO) synthase; Nitrosyl-hemoglobin (NO-hemoglobin); Pressor response

1. Introduction

Lipopolysaccharide and various cytokines can stimulate the activation of both constitutive nitric oxide (NO) synthase and inducible NO synthase (Szabó et al., 1993) in endothelial cells (Radomski et al., 1990), vascular smooth muscle cells (Rees et al., 1990), macrophages and neutrophils (Stuehr and Marletta, 1985; Iyengar et al., 1987). Excessive production of NO, synthesized by both isoforms of NO synthase noted above (especially by inducible NO synthase), may contribute to hypotension and vascular hyporeactivity to noradrenaline in endotoxin shock (Szabó et al., 1993). Thus, inhibition of an enhanced formation of NO by NO synthase inhibitors may improve the therapeutic outcome of endotoxin shock (Kilbourn et al., 1990). However, administration of non-selective NO synthase inhibitors, such as L-nitro-arginine (Pastor et al., 1994), N^G-methyl-L-arginine (L-NMA) (Wright et al., 1992) and N^G-monomethyl-L-arginine (L-NMMA) (Klabunde and Ritger, 1991), causes a rapid and marked increase in systemic vascular resistance and a decrease in cardiac output by inhibiting the constitutive NO synthase in endotoxin shock animals. In some cases, these side effects of non-selective NO synthase inhibitors aggravate the reduced myocardial performance and increase mortality in animals with endotoxin shock (Teale and Atkinson, 1994). Recently, it has been shown that selective inducible NO synthase

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SSDI 0014-2999(95)00593-5

inhibitors may be more desirable agents than non-selective NO synthase inhibitors to reverse the cardiovascular failure of endotoxin shock (Wu et al., 1994).

L-Canavanine is a selective inhibitor of inducible NO synthase (Iyengar et al., 1987; McCall et al., 1989; Umans and Samsel, 1992). It has been reported that L-canavanine attenuates hypotension and vascular hyporeactivity to noradrenaline in endotoxin shock in the rat, but does not affect blood pressure in normal anaesthetized rats (Burns et al., 1993; Teale and Atkinson, 1994). However, it has not been directly evidenced that these beneficial hemodynamic effects of L-canavanine are associated with inhibition of the enhanced formation of NO in vivo during endotoxin shock.

In order to determine the changes in NO levels, we used electron spin resonance (ESR) spectroscopy to detect venous nitrosyl-hemoglobin (NO-hemoglobin) levels. It has been reported that the NO-hemoglobin ESR assay is a useful method to evaluate the inhibitory effect of an NO synthase inhibitor on the enhanced formation of NO during endotoxin shock (Westenberger et al., 1990; Wang et al., 1991).

The purpose of this study was to examine the inhibitory effect of L-canavanine on the enhanced formation of venous NO-hemoglobin by using ESR spectroscopy, and to investigate the relationship between inhibition of the increases in venous NO-hemoglobin levels and the restoration of the reduced vascular reactivity to noradrenaline in response to L-canavanine in the anaesthetized rat with endotoxin shock. To test the changes in venous NO-hemoglobin levels in response to an NO synthase inhibitor by the ESR assay, N^G-nitro-L-arginine methyl ester (L-NAME), a non-selective NO synthase as L-NMMA, was also administered.

2. Materials and methods

2.1. Animal preparation

Male Sprague-Dawley rats (280–310 g) were anaesthetized with pentobarbitone sodium (65 mg/kg, i.p., followed by 26 mg/kg per h i.v.). The trachea was intubated with a 14G catheter to facilitate respiration and the rectal temperature was maintained at 36–37°C with a heat lamp. The left femoral artery was cannulated and connected to a pressure transducer (TP-300T, Nihon Kohden, Tokyo, Japan) for the measurement of mean arterial pressure on a polygraph recorder (WT-645G, Nihon Kohden, Tokyo, Japan). The right femoral vein was cannulated for taking blood samples for the ESR assay. The left femoral vein was cannulated for the intravenous infusion of pentobarbitone. Drugs were given through the tail vein.

2.2. Experimental procedure

After a 15-min stabilization period, rats were injected with lipopolysaccharide (*E. coli* 055:B5, 10 mg/kg, i.v.; dissolved in 1 ml of saline 5 min before the injection) as a slow bolus injection over 2 min. 1 h after the lipopolysaccharide injection, the rats were randomly divided into four groups: a lipopolysaccharide group ($n = 7$) receiving 0.3 ml of saline i.v. hourly; an L-canavanine 10 ($n = 6$) or an L-canavanine 20 ($n = 7$) group receiving L-canavanine 10 or 20 mg/kg i.v. hourly, at a total dose of 50 or 100 mg/kg respectively; and an L-NAME group ($n = 5$) receiving L-NAME 15 mg/kg, as an initial dose, followed by 10 mg/kg i.v. hourly, at a total dose of 55 mg/kg. A sham group ($n = 6$) was given 1 ml of saline instead of lipopolysaccharide. To investigate the cardiovascular effect of L-canavanine on the saline-treated rat, the L-canavanine group ($n = 4$) was given L-canavanine 20 mg/kg i.v. hourly, at a total dose of 100 mg/kg, 1 h after the saline injection. Because a dose of 10 mg/kg hourly of L-canavanine was not so effective as the previous report (Burns et al., 1993) to reverse the circulatory failure in rats with endotoxin shock, a dose of 20 mg/kg hourly of L-canavanine was used in this study. At 5 h after the lipopolysaccharide or saline injection, pressor responses to noradrenaline (1 μg/kg i.v.) were obtained. At the end of the study, 0.5 ml of venous blood was taken for blood counts with a blood cell auto-counter.

2.3. Determination of NO-hemoglobin by ESR

Three blood samples (0.4 ml each) were drawn from the right femoral vein at 2, 4 and 6 h after the administration of lipopolysaccharide or an equal volume (1 ml) of saline, and immediately frozen in an ESR sample tube in liquid nitrogen. ESR spectra were recorded on a JEOL JES-RE3X spectrometer (JEOL, Tokyo, Japan) at 77 K. The ESR spectrometer settings were modulation frequency, 100 kHz; modulation amplitude, 0.5 mT; scanning field, 321 ± 25 mT; receiver gain, 100; response time, 0.1 s; sweep time, 1 min; microwave power, 10 mV; and microwave frequency, 9.151 GHz. The concentrations of NO-hemoglobin in venous blood were determined by double-integration of the ESR spectrum with 0.4 ml of 10^{-4} M $CuSO_4$ as a primary standard of ESR absorption, because the ESR spectrum of NO-hemoglobin is very similar to that of $CuSO_4$ at 77 K. The calibration and calculation of the concentrations of NO-hemoglobin were performed with an ESR data system computer (JEOL, Tokyo, Japan).

2.4. Drugs

L-Canavanine, N^G-nitro-L-arginine-methyl ester (L-NAME) and lipopolysaccharide (*E. coli*, 055:B5) were

obtained from Sigma Chemical Co. (St. Louis, MO). $CuSO_4 \cdot 5\ H_2O$, obtained from WAKO Pure Chemical Industries (Osaka, Japan), was dissolved in pure water 3 h prior to the ESR assay as a standard sample. Pentobarbitone was diluted with saline to a concentration of 25 mg/ml for the intravenous infusion.

2.5. Statistical analysis

Data are expressed as means ± S.E.M. A one-way repeated-measures analysis of variance (ANOVA) followed by Scheffé's test was used to analyze time-dependent increases in venous blood NO-hemoglobin levels in each group. Comparisons among groups were performed by one-way ANOVA followed by Scheffé's test. Statistical significance was defined as $P < 0.05$. All tests were performed with the use of StatView II software.

3. Results

Lipopolysaccharide injection caused a progressive decrease in mean arterial pressure from 118 ± 2 mm Hg (at time 0) to 74 ± 3 mm Hg ($P < 0.01$). In the L-canavanine 10 and L-canavanine 20 groups, the lipopolysaccharide-induced decreases in mean arterial pressure were attenuated by the intravenous administration of L-canavanine at 6 h after lipopolysaccharide injection, and mean arterial pressure values (93 ± 3 mm Hg and 96 ± 2 mm Hg, respectively) were significantly higher than those in the lipopolysaccharide group ($P < 0.01$). There were no significant differences in mean arterial pressure between the L-canavanine 10 and L-canavanine 20 groups throughout the observation period. L-NAME caused a rapid significant increase in mean arterial pressure (occurring within 3 min) which then slowly returned to the baseline value at 6 h after lipopolysaccharide injection. In the sham and L-canavanine groups, mean arterial pressure values were unaltered during the experiment (Fig. 1, data in L-NAME and L-canavanine groups not shown).

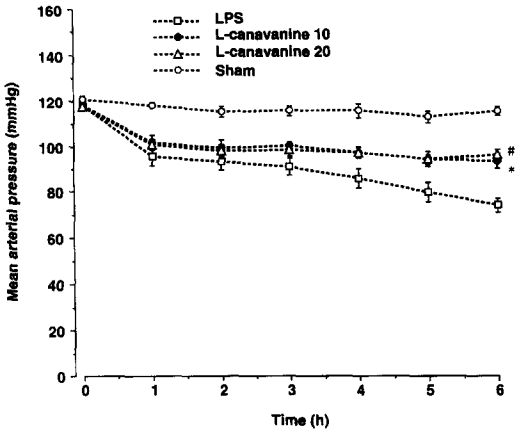

Fig. 1. L-Canavanine attenuates the progressive decrease in mean arterial pressure after lipopolysaccharide injection in the anaesthetized rat. 1 h after lipopolysaccharide injection (10 mg/kg i.v.), the lipopolysaccharide (LPS) group (□) ($n = 7$) received saline (0.3 ml i.v. hourly), the L-canavanine 10 (●) ($n = 6$) or L-canavanine 20 (△) ($n = 7$) group received L-canavanine 10 or 20 mg/kg i.v. hourly, respectively, and 1 h after saline injection (1 ml i.v.), the sham group (○) ($n = 6$) received saline (0.3 ml i.v. hourly). Saline has no effect on mean arterial pressure. Data are expressed as means ± S.E.M. of n observations. # or * $P < 0.01$ when compared to the lipopolysaccharide (LPS) group.

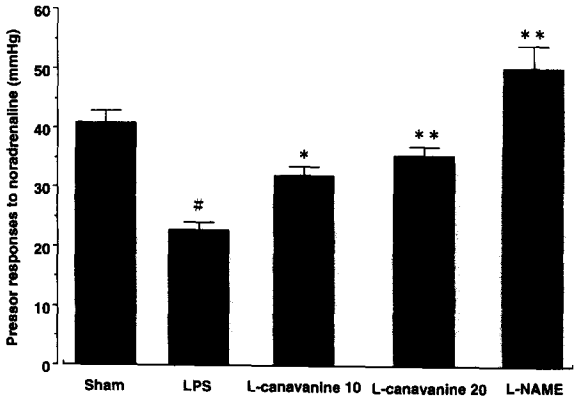

Fig. 2. 5 h after lipopolysaccharide (10 mg/kg i.v.) injection, pressor responses to noradrenaline (1 μg/kg i.v.) were significantly decreased in anaesthetized rats in the lipopolysaccharide (LPS) group ($n = 7$), # $P < 0.01$, when compared to those of rats in the sham group ($n = 6$). The rats in the L-canavanine 10 ($n = 6$) or L-canavanine 20 ($n = 7$) group received L-canavanine 10 or 20 mg/kg i.v. hourly, respectively. L-Canavanine effectively attenuated the lipopolysaccharide-induced impairment of pressor responsiveness to noradrenaline, * $P < 0.05$ or * * $P < 0.01$, respectively, when compared to the lipopolysaccharide (LPS) group. N^G-Nitro-L-arginine methyl ester (L-NAME, 15 mg/kg followed by 10 mg/kg i.v. hourly) ($n = 5$) effectively reversed the lipopolysaccharide-induced impairment of pressor responsiveness to noradrenaline in anaesthetized rats, * * $P < 0.01$, when compared to that of rats of the lipopolysaccharide (LPS) group. Data are expressed as means ± S.E.M. of n observations.

At 5 h after lipopolysaccharide injection, pressor responses to noradrenaline were significantly reduced (23 ± 2 mm Hg) in the lipopolysaccharide group, as compared with the sham group ($P < 0.01$) (Fig. 2). Administration of L-canavanine (10 mg/kg and 20 mg/kg, respectively) significantly attenuated the lipopolysaccharide-induced impairment of pressor responsiveness to noradrenaline compared with that of the lipopolysaccharide group ($P < 0.05$ and $P < 0.01$, respectively) (Fig. 2). Pressor responses to noradrenaline in the L-NAME group were significantly higher than those in the lipopolysaccharide, L-canavanine 10 and L-canavanine 20 groups ($P < 0,01$) (Fig. 2). In the L-canavanine group, L-canavanine (20 mg/kg, hourly) did not affect the pressor responses to noradrenaline compared with those of the sham group (not shown).

There were no ESR signals of NO-hemoglobin in the sham and L-canavanine groups during the observation period. In all of the lipopolysaccharide-treated groups, the NO-hemoglobin levels at 6 h after lipopolysaccharide were significantly higher than those at 2 h and 4 h (only in lipopolysaccharide and L-NAME groups; Fig. 3). The NO-hemoglobin levels in the lipopolysaccharide group at 6 h after lipopolysaccharide were about fourfold higher than those at 4 h. At 6 h after lipopolysaccharide injection, there were significant differences in the NO-hemoglobin levels between the lipopolysaccharide group and the L-canavanine 20 or L-NAME group ($P < 0.05$, Fig. 3).

In the lipopolysaccharide group, the increases in the NO-hemoglobin levels during 4–6 h after lipopolysaccharide were markedly more than those during 2–4 h after lipopolysaccharide. L-Canavanine (20 mg/kg, hourly) or L-NAME effectively inhibited the increases in the venous NO-hemoglobin levels during the 4–6 h after lipopolysaccharide (Fig. 3).

There was a significant negative correlation between pressor responses to noradrenaline and increases in venous NO-hemoglobin levels during the 4–6 h after lipopolysaccharide injection in the lipopolysaccharide, L-canavanine 10 and L-canavanine 20 groups ($r = -0.71$, $P < 0.001$) (Fig. 4).

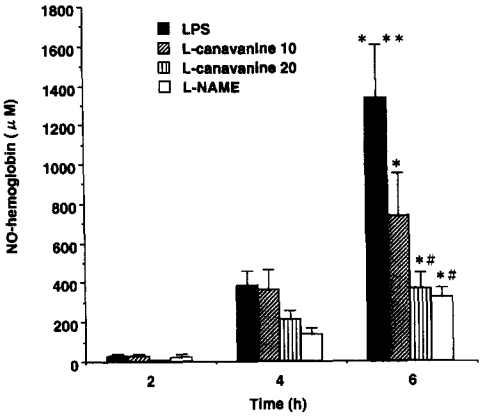

Fig. 3. Effects of L-canavanine and N^G-nitro-L-arginine methyl ester (L-NAME) on time-dependent increases in venous levels of nitrosyl-hemoglobin (NO-hemoglobin) after lipopolysaccharide injection in the anaesthetized rat. 1 h after lipopolysaccharide injection (10 mg/kg i.v.), the lipopolysaccharide (LPS) group ($n = 7$) (solid columns) received saline (0.3 ml i.v. hourly), the L-canavanine 10 ($n = 6$) (hatched columns) or L-canavanine 20 ($n = 7$) (vertically lined columns) group received L-canavanine 10 or 20 mg/kg i.v. hourly, respectively, and the L-NAME group ($n = 5$) (open columns) received L-NAME 15 mg/kg followed by 10 mg/kg i.v. hourly. Lipopolysaccharide caused a marked increase in venous levels of NO-hemoglobin at 6 h (** $P < 0.01$, when compared to 4 h). L-Canavanine (20 mg/kg i.v. hourly) or L-NAME significantly inhibited the increases in venous levels of NO-hemoglobin at 6 h after lipopolysaccharide injection. # $P < 0.05$, when compared to the lipopolysaccharide (LPS) group. * $P < 0.01$, when compared to 2 h. Data are expressed as means ± S.E.M. of n observations.

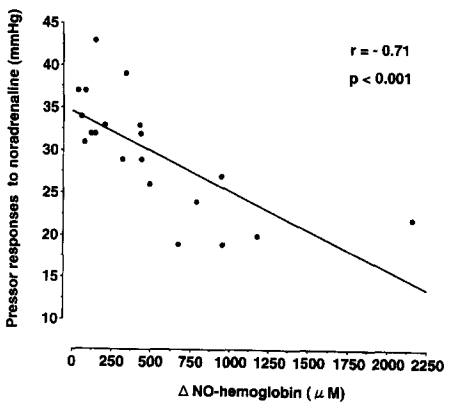

Fig. 4. Pressor responses to noradrenaline (1 μg/kg i.v.) at 5 h after lipopolysaccharide injection compared with the increases in venous nitrosyl-hemoglobin (NO-hemoglobin) levels over 4–6 h in the lipopolysaccharide (LPS), L-canavanine 10 and L-canavanine 20 groups.

Although the hemoglobin and hematocrit values (14.6 ± 0.1 g/dl and 41.3 ± 1.0%, respectively) in the L-NAME group were slightly higher than those in the other four groups (e.g. 13.8 ± 0.3 g/dl and 38.2 ± 1.5%, respectively in the sham group), there were no significant differences in hemoglobin and hematocrit between each study group.

4. Discussion

It has been demonstrated that an enhanced formation of NO contributes, at least in part, to the circulatory failure in animals (Thiemermann and Vane, 1990; Szabó et al., 1993) and in patients (Ochoa et al., 1991; Evans et al., 1993) with endotoxin shock. According to the results of many experimental investigations, an excessive NO production induced by endotoxin and/or cytokines may also be associated with tissue damage (e.g. acute lung injury) (Mulligan et al., 1991), which in turn enhances the circulatory failure of endotoxin shock. Many investigations have been made of the agents which effectively inhibit the excessive NO production by the inducible NO synthase. Dexamethasone has been reported to inhibit the induction of NO synthase, but dexamethasone has no effect on the activity of inducible NO synthase (Radomski et al., 1990; Rees et al., 1990). It has been considered that an agent which is of therapeutic value in the treatment of the circulatory failure of endotoxin shock should be a potent and selective inducible NO synthase inhibitor or an effective NO scavenger.

NO has a strong affinity for deoxyhemoglobin. NO-hemoglobin and methemoglobin are produced immediately when NO interacts with hemoglobin. NO-hemoglobin is easily detected by ESR spectroscopy, because it has a characteristic three-line, hyperfine signal at 77 K. During the early 2–8 h after lipopolysaccharide

injection, time-dependent increases in venous levels of NO-hemoglobin were observed in murine endotoxin shock by ESR, which were strongly inhibited by L-NMMA (Westenberger et al., 1990; Wang et al., 1991). Similar results were obtained in the present study. The marked changes in the increased NO formation detected by ESR were consistent with a detectable induction of NO synthase, which was only detected at 3 h after lipopolysaccharide application in vivo (Salter et al., 1991). Thus, excessive NO production by inducible NO synthase in macrophages (Stuehr and Marletta, 1985; Iyengar et al., 1987) may mainly contribute to the increased formation of NO-hemoglobin during endotoxin shock. For these reasons, determination of the amounts of NO-hemoglobin by using ESR spectroscopy is a valuable means of examining the inhibitory effect of a selective inducible NO synthase inhibitor on the enhanced formation of NO by inducible NO synthase in endotoxin shock.

Although lipopolysaccharide injection resulted in a progressive decrease in mean arterial pressure, there was no significant correlation between changes in venous levels of NO-hemoglobin and changes in mean arterial pressure in the present study (data not shown). NO-hemoglobin has a longer half-life (about 60 min) (Westenberger et al., 1990) than NO itself (a few seconds) in vivo, which may be a possible reason for the difficulty of using an absolute concentration of NO-hemoglobin at one time point to elucidate vascular tone exactly. However, we demonstrated a significant correlation between the inhibition of the increases in venous levels of NO-hemoglobin 4–6 h after lipopolysaccharide injection and the restoration of vascular hyporeactivity to noradrenaline 5 h after lipopolysaccharide injection in response to L-canavanine administration. These findings indirectly show that the rates of the increases in venous NO-hemoglobin levels may be more useful than the real venous NO-hemoglobin levels at one time point to estimate the activity of inducible NO synthase in various cells, especially in endothelial cells and vascular smooth muscle cells during endotoxin shock (Radomski et al., 1990; Rees et al., 1990; Fleming et al., 1991a, b).

This study confirmed that there were some differences in the effectiveness of L-NAME and L-canavanine to reverse hypotension and vascular hyporeactivity during endotoxin shock. L-NAME was more effective than L-canavanine in reversing hypotension and vascular hyporeactivity, but L-NAME markedly reduced pulse pressure and heart rate (data not shown), which may be due to inhibition of both constitutive NO synthase and inducible NO synthase by L-NAME. We also noted that the effect of L-canavanine on lipopolysaccharide-induced hypotension was not so potent as that reported by others (Burns et al., 1993).

This study demonstrates that the marked increase in the formation of NO, synthesized by inducible NO synthase 4–6 h after lipopolysaccharide injection, contributes to the delayed hypotension and the vascular hyporeactivity to noradrenaline during endotoxin shock, and that L-canavanine effectively attenuates hypotension and the vascular hyporeactivity to noradrenaline by inhibition of excessive NO production by inducible NO synthase in endotoxin shock in the anaesthetized rat. In conclusion, our results indicate that L-canavanine is a potential agent for the treatment of endotoxin shock and that monitoring the changes in venous levels of NO-hemoglobin by using the ESR assay may be useful to evaluate the effect of an inducible NO synthase inhibitor on the activity of inducible NO synthase during murine endotoxin shock.

Acknowledgements

The authors wish to thank Professor Masayasu Minami of the Department of Public Health, Nippon Medical School, and Mr. Masahiro Kohno of the ESR Application Laboratory, Analytical Instruments Division, JEOL, Tokyo, Japan, for their helpful advice.

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