LABORATORY INVESTIGATIONS

Effect of l-lysine on nitric oxide production in ovine endotoxaemia†

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Summary
Excess production of nitric oxide contributes to the refractory hypotension associated with sepsis and is dependent upon precursor availability, l-arginine. Endothelial uptake of l-arginine by the y⁺ transporter can be inhibited by another cationic amino acid, l-lysine. This study was undertaken to determine if excess nitric oxide production associated with ovine endotoxaemia could be reduced by the administration of l-lysine. A known nitric oxide synthase inhibitor, NG-nitro-l-arginine-methyl ester (l-NAME), which selectively inhibits constitutive ecNOS activity, was used as a positive control.

Methods
These experiments were performed in accordance with United Kingdom Home Office regulations concerning experimentation on live animals.

ANIMAL PREPARATION

After an overnight fast 18 Soay sheep (10–24 kg) were anaesthetized with thiopental (12.5 mg kg⁻¹), intubated and ventilated to normocarbia with 60% oxygen in air. Anaesthesia was then maintained with halothane (1.5%) and a fentanyl infusion (2–4 μg kg⁻¹ h⁻¹). A 16-gauge cannula was inserted into the left common carotid artery and a balloon-tipped thermodilution catheter (Abbott Critical Care, Chicago, USA) advanced into a pulmonary artery from the left internal jugular vein. The rumen was decompressed by gastrostomy and a urinary catheter inserted to measure hourly urine output.

The majority of l-arginine transport into endothelial cells occurs via a saturable, low affinity, high capacity cationic amino acid transport system (y⁺) which can be inhibited by other cationic amino acids such as l-lysine and l-ornithine. The uptake of l-arginine by vascular endothelial cells may become rate limiting for nitric oxide production when excessive sustained production of nitric oxide occurs because of iNOS activation. As intra-cellular stores of l-arginine appear to be sufficient for constitutive ecNOS production of nitric oxide, inhibition of l-arginine uptake could potentially allow selective inhibition of the iNOS enzyme whilst not affecting ecNOS activity. This appears to occur in rodent models of endotoxaemia, but has not previously been examined in a large animal model.

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Keywords: anaesthetic gases, nitric oxide; protein, amino acids, l-lysine; protein, amino acids, l-arginine; complications, endotoxaemia; complications, sepsis; model, sheep

Nitric oxide is a potent vasodilator synthesized from the semi-essential amino acid l-arginine by a family of enzyme isoforms. Endothelial constitutive nitric oxide synthase (ecNOS) has a homeostatic role on blood vessel tone via the action of nitric oxide on soluble guanylate cyclase present in vascular smooth muscle. In sepsis or endotoxaemia, however, cytokine mediators induce expression of a calcium independent isoform of nitric oxide synthase (iNOS) which produces excessive uncontrolled amounts of nitric oxide. This can cause refractory vasodilatation and hypotension leading to multi-organ dysfunction and death. Recently l-arginine analogues have been used as nitric oxide synthase inhibitors to increase vascular tone in human sepsis. These agents act as non-specific inhibitors of nitric oxide synthase and some (notably N⁶-monomethyl-l-arginine, l-NMMA) also inhibit l-arginine uptake. Studies in animal models of sepsis have recently suggested that non-specific nitric oxide synthase inhibition can have detrimental effects, possibly because of the co-inhibition of constitutive ecNOS.
Animals were then placed in a sitting-recumbent position under a heated blanket to maintain normothermia. Saline (308 mMol) (10–15 ml kg\(^{-1}\) h\(^{-1}\)) was administered i.v. to maintain normovolaemia.

**EXPERIMENTAL PROCEDURE**

After a one hour period of stabilization, baseline measurements were taken and *Escherichia coli* endotoxin (Serotype 0127:B8, Sigma Chemicals, Poole) 2 μg kg\(^{-1}\) was administered i.v. over 30 min. After a further 2 h animals were randomly assigned to one of three groups of equal size (six animals per group). These groups received:

1. L-lysine 500 mg kg\(^{-1}\) administered i.v. over 1 h followed by the same dose infused over 8 h,
2. L-NAME 25 mg kg\(^{-1}\) administered i.v. over 1 h followed by the same dose infused over 8 h, or
3. Saline (308 mMol) 50 ml administered i.v. over 1 h followed by 50 ml infused over 8 h.

L-lysine (Sigma Chemicals, Poole) and L-NAME (Alexis Corporation, Nottingham) were administered as the hydrochloride salt dissolved in 50 ml saline (308 mMol). L-NAME at a dose of 25 mg kg\(^{-1}\) has previously been shown to produce a reasonable degree of nitric oxide synthase block in ovine endotoxaemia,\(^{17}\) and L-lysine 500 mg kg\(^{-1}\) was chosen after consultation with the pharmacy department of the John Radcliffe Hospital, Oxford, as a large dose is unlikely to cause directly toxic effects based on data from drug company development of total i.v. feeding regimens.

**DATA COLLECTION**

Animals were studied for a period of 10 h after endotoxin administration, to allow sufficient time for full induction of the inducible isoform of nitric oxide synthase.\(^{1,4}\) Systemic and pulmonary arterial pressure, central venous and pulmonary arterial occlusion pressure and cardiac output (by triplicate cold saline (308 mMol) injection) were measured every 30 min for 5 h, then hourly for a further 5 h. At the same time intervals samples were taken for arterial blood–gas analysis (Radiometer ABL 330, Copenhagen, Denmark) and for the determination of serum nitrate concentrations.

**NITRATE ANALYSIS**

Blood samples for nitrate analysis were immediately centrifuged at 3000 rpm for 15 min and the plasma fraction frozen at −40°C for later analysis. Samples were analysed using a semi-automated modification of the Braman technique.\(^{18}\) In brief, 1 ml of plasma was deproteinated by addition of 1 ml 0.1 mol litre\(^{-1}\) zinc sulphate solution and centrifugation at 3000 rpm for 10 min. Aliquots (0.5 ml) were then injected into hot (85°C), acidic, 0.1 mol litre\(^{-1}\) vanadum III chloride solution which reduces inorganic nitrate and nitrite to nitric oxide. This was then eluted in a constant stream of nitrogen and the nitric oxide concentration measured by a chemiluminescence analyser (Ecophysics CLD 700AL, Düren, Switzerland). Total serum nitrite/nitrate was calculated on-line using in-house developed software and nitrate standards. This technique has an intra-assay coefficient of variation of 2% and an inter-assay variation of 10%.

The nitro group of L-NAME interferes with nitrate determination by this method so a second technique was developed for analysis of samples from the L-NAME treated group. This was based on the Sigma quality control test procedure for nitrate reductase. 300 μl of plasma was added to 300 μl of pH 7.5 KH\(_2\)PO\(_4\)/KHPO\(_4\) buffer, 50 μl of 2 mmol litre\(^{-1}\) NADPH, 50 μl of 50 μmol litre\(^{-1}\) FAD, and 50 μl of 1 unit ml\(^{-1}\) aspergillus nitrate reductase (all reagents from Sigma Chemicals, Poole). This was incubated at room temperature for 1 h, followed by the addition of 500 μl of 0.2 mol litre\(^{-1}\) zinc sulphate solution and 70 μl 2 mol litre\(^{-1}\) sodium hydroxide to deproteinize the sample. The resulting solution was analysed for nitrite content using a standard Greiss reaction.\(^{19}\) The intra-assay coefficient of variation for this method is 2.2% with an inter-assay variation of less than 5%. Buffer and serum spiked with L-NAME were tested over a range of concentrations and no interference of nitrate/nitrite determination detected. The two methods were verified for concordance using serum from control animals and equivalent results obtained. Results for serum nitrate alone were consistently ≤0.6 μmol litre\(^{-1}\) or undetectable and nitrate to nitrite conversion was ≥90%.

**DATA ANALYSIS**

Results are expressed as mean (SEM). Systemic and pulmonary vascular resistance were calculated using standard formulae and cardiac index was calculated using the following formula for ovine body surface area\(^{20}\): \[\text{body surface area (m}^2) = 0.082 \times \text{body weight}^{25} \text{(kg)}\]. Treatment groups (L-NAME or L-lysine administration) were compared with the control group (normal saline administration) using a two factor repeated measures analysis of variance from the start of treatment onwards, and performed on a Macintosh LC II microcomputer running StatView II software (Abacus Concepts, Berkeley, California). Change in serum nitrate in the control group was assessed by one factor repeated measures analysis of variance.

A P value of less than 0.025 was taken to be significant to account for multiple analysis against the control group.

**Results**

Two animals died in the L-lysine treated group. Deaths occurred after a sustained period of low output cardiac failure and occurred at 450 and 500 min after endotoxin administration. They could not be attributed to a biochemical or hypoxic/pH related cause, nor were they typical of endotoxaemia. There were no deaths in the other two groups. For comparison of the L-lysine treated and control groups, statistical significance was calculated twice, once for the four animals surviving to the end of the study period and once for all six animals up to the last measurement before the first death (420 min after endotoxin administration). These results were equivalent and the latter figure is quoted.
CARDIOVASCULAR

The administration of endotoxin caused changes typical of acute endotoxaemia with a marked increase in pulmonary vascular resistance and a reduction in cardiac index. By 2h, the start time for l-lysine or l-NAME infusions, haemodynamic variables had returned towards baseline levels and a typical sepsis-like syndrome of decreasing arterial pressure and systemic vascular resistance was developing.

The administration of l-NAME caused an increase in systemic and pulmonary vascular resistance ($P<0.0001$) and mean arterial pressure ($P<0.0001$) (figs. 1–3). Cardiac index decreased sharply at the start of l-NAME administration, but the overall reduction was not significantly different from the control group (fig. 4). l-lysine administration had no significant effect on systemic or pulmonary vascular resistance, mean arterial pressure or cardiac index (figs. 1–4).

SERUM NITRATES

In control animals mean serum nitrate concentration increased from a baseline value of 11.6 $\mu$mol litre$^{-1}$ (SEM 1.2 $\mu$mol litre$^{-1}$) to 13.8 $\mu$mol litre$^{-1}$ (SEM 1.8 $\mu$mol litre$^{-1}$) ($P<0.0001$, one factor analysis of variance). l-NAME administration caused a decrease in mean serum nitrate concentration from 9.5 $\mu$mol litre$^{-1}$ (SEM 0.7 $\mu$mol litre$^{-1}$) to 5.9 $\mu$mol litre$^{-1}$ (SEM 0.2 $\mu$mol litre$^{-1}$) ($P<0.0001$ vs controls). In the l-lysine treated group mean serum nitrate concentrations increased from a baseline value of 11.4 $\mu$mol litre$^{-1}$ (SEM 0.9 $\mu$mol litre$^{-1}$) to 13.8 $\mu$mol litre$^{-1}$ (SEM 0.6 $\mu$mol litre$^{-1}$) and was not significantly different from the control group (fig. 5).

Discussion

Severe human sepsis is characterized by a hyperdynamic circulation and reduced systemic vascular resistance which can be refractory to conventional vasoconstrictor therapy. Nitric oxide synthase block has been demonstrated to increase vascular resistance

Figure 1  Change in systemic vascular resistance (dyn s$^{-1}$ cm$^{-5}$) with time, $n=6$ per group (mean (SEM)). Arrow indicates start of l-NAME, l-lysine or normal saline infusion. (ns = no significant difference and ***$P<0.0001$ vs control group).

Figure 2  Change in pulmonary vascular resistance (dyn s$^{-1}$ cm$^{-5}$) with time, $n=6$ per group (mean (SEM)). Arrow indicates start of l-NAME, l-lysine or normal saline infusion. (ns = no significant difference and ***$P<0.0001$ vs control group).

Figure 3  Change in mean arterial pressure (mm Hg) with time, $n=6$ per group (mean (SEM)). Arrow indicates start of l-NAME, l-lysine or normal saline infusion. (ns = no significant difference and ***$P<0.0001$ vs control group).

Figure 4  Change in cardiac index (1 min$^{-1}$ m$^{-2}$) with time, $n=6$ per group (mean (SEM)). Arrow indicates start of l-NAME, l-lysine or normal saline infusion. (ns = no significant difference vs control group).
and systemic arterial pressure both in human and animal sepsis, but may have detrimental effects because of inhibition of the constitutive enzyme ecNOS. In vitro studies have demonstrated that the uptake of normal concentrations of L-arginine can be inhibited by other cationic amino acids such as L-lysine in concentrations of 1–10 mmol litre \(^{-1}\). By inhibiting L-arginine uptake rather than nitric oxide synthase production directly, it may be possible to inhibit the excessive production of nitric oxide by iNOS, whilst allowing low level constitutive production of nitric oxide by ecNOS to continue. This is possible because the \(K_{m}\) value for iNOS is some 10 times greater than that for the ecNOS enzyme. Indeed, Bianchi and colleagues have demonstrated that a new tetravalent guanylhydrazone, CNI-1493, which inhibits cytokine-inducible L-arginine transport in murine macrophages, but does not affect constitutive nitric oxide production, conferred protection against lethal endotoxin challenge in mice. Furthermore, Liaudet and colleagues have recently demonstrated that L-lysine (0.5 mmol kg \(^{-1}\) for 5 h) administered to endotoxin treated rats caused inhibition of nitric oxide production by iNOS, but not by ecNOS, and also tended to limit signs of organ dysfunction. We have previously demonstrated that nitric oxide production in this ovine model of endotoxaemia is limited by L-arginine availability and we therefore postulated that the administration of exogenous L-lysine might reduce nitric oxide production in this large animal model.

Administration of L-NAME, a recognized nitric oxide synthase inhibitor, caused a significant increase in mean arterial pressure and systemic/pulmonary vascular resistance compared with control animals. These results are similar to the effect of nitric oxide synthase inhibition in other studies of experimental endotoxaemia. The haemodynamic effects were accompanied by a significant reduction in serum nitrate concentrations, the stable end metabolite of nitric oxide. The initial small offset in serum nitrate concentrations in the L-NAME treated group is probably because of differences in the assays used and would not affect the statistical analysis. More importantly, the reduction in serum nitrates in this group cannot be ascribed to an effect of the assay itself as the increased nitrate production noted in the control group was still present when these samples were re-analysed using the same aspergillus nitrate reduction assay. These data suggest that the haemodynamic changes after L-NAME administration resulted from a marked decrease in nitric oxide production.

The administration of L-lysine, on the other hand, had no significant effect on mean arterial pressure, systemic/pulmonary vascular resistance, cardiac index or serum nitrate concentrations indicating that L-lysine administration had no significant effect on nitric oxide production in this model.

Although serum concentrations of lysine were not measured, i.v. administration of L-lysine at a dose of 500 mg kg \(^{-1}\) (equivalent to 2.7 mmol kg \(^{-1}\)) should produce an extra-cellular concentration of at least 4 mmol litre \(^{-1}\), a concentration which in vitro produces near maximal inhibition of L-arginine uptake. Intra-cellular stores of L-arginine would not be sufficient to maintain nitric oxide production for the 10 h of the study period and L-arginine production does not appear to occur from protein degradation. Intra-cellular recycling of L-citrulline to L-arginine can occur via argininosuccinate synthetase and argininosuccinate lyase but this cannot sustain the increased nitric oxide production associated with endotoxin stimulation in vitro. The disparity between Liaudet’s and our own findings is interesting and may be because of a species difference. In vivo studies in rodents demonstrate that these animals respond to endotoxin by producing extremely large amounts of nitric oxide with a many fold increase in serum nitrate concentrations (peak 250–900 \(\mu\)mol litre \(^{-1}\)). The increase in serum nitrates (and therefore of nitric oxide production) was more modest in our ovine model, which in this respect is closer to the changes found in human sepsis and is one of the reasons that many authors consider ovine endotoxaemia to be one of the best models of human sepsis. If nitric oxide production is increased only modestly then partial L-arginine uptake inhibition (coupled with some degree of intra-cellular recycling from L-citrulline) may not be sufficient to produce a clinically significant reduction in nitric oxide production. The dose of L-lysine used in the present study, was some five times greater than that used by Liaudet and colleagues and was associated with a 33% late mortality. It is possible, therefore, that whatever mechanism was responsible for these toxic effects could mask haemodynamic changes resulting from a decreased nitric oxide production. This would not, however, explain the lack of effect on nitric oxide production as measured by serum nitrate concentrations.

It appears unlikely, therefore, that L-lysine or other cationic amino acids which act to reduce L-arginine uptake, could be used clinically to reduce nitric oxide production in human sepsis.

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