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Effect of L-lysine on nitric oxide overproduction in endotoxic shock

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1 An enhanced production of nitric oxide (NO) from L-arginine, related to the diffuse expression of an inducible NO synthase (iNOS), contributes to the pathogenesis of endotoxic shock. Since iNOS activity depends on extracellular L-arginine, we hypothesized that limiting cellular L-arginine uptake would reduce NO production in endotoxic shock. We investigated the effects of L-lysine, an inhibitor of L-arginine uptake through system y^+, on NO production, multiple organ dysfunction and lactate levels, in normal and endotoxaemic rats.

2 Anaesthetized rats challenged with intravenous lipopolysaccharide (LPS, 10 mg kg^{-1}) received a 5 h infusion of either L-lysine (500 μmol kg^{-1} h^{-1}, $n=12$) or isotonic saline (2 ml kg^{-1} h^{-1}, $n=11$). In rats treated with saline, LPS produced a large increase in plasma nitrate and L-citrulline concentrations at 5 h, both markers of enhanced NO production. LPS also caused severe hypotension, low cardiac output and marked hyperlactataemia. All these changes were significantly reduced by L-lysine administration.

3 Endotoxaemia also caused a significant rise in the plasma levels of alanine aminotransferase (ALAT), lipase, urea and creatinine, and hence, liver, pancreatic and renal dysfunction. These changes tended to be less pronounced in rats treated with L-lysine, although the differences did not reach statistical significance.

4 Similar experiments were conducted in 10 rats challenged with LPS vehicle in place of LPS and then treated with L-lysine (500 μmol kg^{-1} h^{-1}, $n=5$) or saline (2 ml kg^{-1} h^{-1}, $n=5$) for 5 h. In these animals, all the haemodynamic and metabolic variables remained stable and not statistically different between both treatment groups, except for a slight rise in ALAT, which was comparable in L-lysine and saline-treated rats.

5 In conclusion, L-lysine, an inhibitor of cellular L-arginine uptake, reduces NO production and exerts beneficial haemodynamic effects in endotoxaemic rats. L-lysine also reduces hyperlactataemia and tends to blunt the development of organ injury in these animals. Contrastingly, L-lysine has no effects in the absence of endotoxin and thus appears to act as a selective modulator of iNOS activity.

Keywords: Nitric oxide; inducible nitric oxide synthase; endothelial constitutive nitric oxide synthase; L-lysine; L-arginine; system y^+; endotoxic shock; lipopolysaccharide

Introduction

Nitric oxide (NO) is a short lived effector molecule which 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. Upon stimulation by lipopolysacharride (LPS) and various cytokines, an inducible NOS (iNOS) is diffusely expressed, producing large amounts of NO which contribute to the cardiovascular failure characterizing septic and endotoxic shock (Szabo, 1995). The pharmacological inhibition of NO production has been therefore proposed as a new therapy in septic shock (Geroulanos *et al*., 1992; Petros *et al*., 1991; 1994). Unfortunately, such inhibition has been frequently shown to be detrimental, and recent evidence reveals that this deleterious potential is a consequence of ecNOS blockade by nonselective agents (Wright *et al*., 1992; Ruetten *et al*., 1996; Wu *et al*., 1996). Thus, interest is now focusing on the identification of compounds which would selectively reduce iNOS-dependent NO production (Szabo *et al*., 1994).

It has been shown that the sustained iNOS activity increases the cellular needs of L-arginine (L-Arg) and is thus strictly dependent on the availability of extracellular L-Arg (Schott *et al*., 1993a; Bune *et al*., 1995). In contrast, the intracellular pool of L-Arg appears to be sufficient to meet with the low output NO synthesis dependent on the ecNOS isoform (Thomas *et al*., 1989; Mitchell *et al*., 1990). Therefore, reducing cellular L-Arg uptake from extracellular sources might represent an effective way to decrease selectively NO release in conditions of iNOS activation (Southan & Szabo, 1996). Indeed, Bianchi *et al*. (1995) recently showed that the overproduction of NO occuring during rodent endotoxaemia could be largely reduced by CNI-1493, a synthetic inhibitor of cellular L-Arg uptake, while this compound was devoid of any influence on physiologically released NO.

The amino acid L-lysine (L-Lys) is a natural inhibitor of L-Arg transport, which competes with L-Arg for uptake through system y^+, the major transport system for cationic amino acids in mammalian cells (White, 1985). Therefore, we postulated that increasing extracellular L-Lys concentrations by exogenous administration would reduce L-Arg uptake and thus selectively limit iNOS-dependent NO synthesis in rats challenged with LPS.

Methods

Surgical preparation and measurements

All procedures were in accordance with the Swiss laws on animal experimentation and approved by our local ethics committee. Thirty nine male Wistar rats were anaesthetized

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with intraperitoneal sodium pentobarbitone, 50 mg kg^{-1} for induction and 10 mg kg^{-1} for maintenance, given as needed (interdigital reflex). The trachea was canulated to facilitate respiration. Polyethylene catheters (PE 50) were inserted into the right femoral vein for intravenous infusions (see experimental protocol) and into the right femoral artery for the measurement of arterial blood pressure (BP) and heart rate (HR) (Hewlett-Packard; 78342A BP monitor) which were continuously displayed on paper (WR 3310 polygraph recorder; Graphtec, Japan). A PE 50 catheter was inserted into the right internal jugular vein and a thermistor catheter was placed into the ascending thoracic aorta through the right carotid artery for the determination of cardiac output (CO) by thermodilution (Hewlett-Packard; 78231C CO monitor). Thermodilution curves were obtained following injection of small volumes (0.15 ml) of a 2.5% glucose solution at room temperature into the right atrium (Isoyama *et al.*, 1982; Liaudet *et al.*, 1996). CO was averaged from 3 subsequent determinations. Systemic vascular resistance (SVR) and stroke volume (SV) were calculated with standard formulae. CO, SVR and SV were indexed to the weight of the animals (CI, SVRI and SVI).

Arterial blood samples (1 ml) were obtained at baseline and at the end of experiments to measure the plasma concentrations of the following: nitrate, which was determined by a spectrophotometric assay based on NADPH oxidation, adapted on an automated analyser (Cobas Fara, Roche, Basel, Switzerland), as previously described (Liaudet *et al.*, 1996); lactate (YSI 23 L lactate analyser; Yellow-Springs, U.S.A.); alanine-aminotransferase (ALAT), creatinine, urea and pancreatic lipase (all measured with a Hitachi 717 selective analyser). Additional blood samples (1 ml) were taken to determine the plasma concentration of L-Lys and L-citrulline, which were measured with an amino acid analyser (Beckman 6300), and for the determination of haematocrit. The blood removed was replaced by equivalent volumes of isotonic saline.

Experimental protocol

After baseline measurements (time 0 h), endotoxaemia was induced by an i.v. infusion (given in 15 min) of 10 mg kg^{-1} LPS, dissolved in 1 ml isotonic saline. Immediately after the LPS infusion, rats randomly received a continuous infusion of either L-Lys (500 μmol kg^{-1} h^{-1}; $n=12$), or vehicle only (isotonic saline, 2 ml kg^{-1} h^{-1}, $n=17$), which was given until the end of the 5th hour following LPS administration (time 5 h). If an animal died before the end of the study, it was excluded from analysis and the experiment was repeated. Thus, the final number of rats included in the analysis was $n=12$ in the L-Lys group (no death during the protocol) and $n=11$ in the saline group (6/17 deaths during the protocol). The same experiments were conducted in 10 other rats, which were given LPS vehicle at baseline (sham rats) and then a continuous infusion of either L-Lys (500 μmol kg^{-1} h^{-1}; $n=5$) or vehicle (isotonic saline, 2 ml kg^{-1} h^{-1}; $n=5$).

Chemicals

LPS (*E. Coli* O127:B8) and L-lysine free base were purchased from Sigma (Buchs, Switzerland).

Data analysis

All values in the figures and text are expressed as mean $\pm$ s.e.mean of n observations, where n represents the number of animals studied. Experiments conducted in sham and endotoxaemic animals were analysed separately. Only the animals surviving until T5 were included in the statistical analysis. Effects of time and treatment were statistically evaluated by a two way analysis of variance, followed by Bonferroni adjustments. A P value <0.05 was considered significant.

Results

All rats from the sham experiments survived until the end of the study. In endotoxaemic animals, 12/12 rats treated with L-Lys survived for the whole study period, while 6/17 rats treated with saline died before the end of experiments and were excluded from further analysis (see above, statistical analysis). Except from 1 animal which died during the first hour, the 5 other animals died after the third hour of the experiment (2 deaths between 3 and 4 h, and 3 deaths between 4 and 5 h). In each case, death was consecutive to rapid haemodynamic deterioration. It is noteworthy that the exclusion from analysis of animals that died prematurely has, if anything, biased our results against a beneficial effect of L-Lys, since more deaths occurred in the absence than in the presence of this treatment.

Haemodynamic data

Figure 1 illustrates the time-course of mean BP, CI, HR, SVRI and SVI in rats challenged with LPS. The decline in BP and CI noted in rats treated with saline was significantly reduced by L-Lys, which also prevented the decrease in HR observed towards the end of study. Except for a small but significant decrease during the 1st hour in the saline group, SVRI did not vary significantly and was not statistically different between both groups. The decrease in SVI produced by endotoxin was blunted by L-Lys treatment, but the difference was not statistically significant ($P=0.15$).

Figure 2 shows the time-course of haemodynamic variables obtained in sham rats. All these variables remained stable and were not influenced by L-Lys administration.

Plasma nitrate and L-citrulline

In the absence of endotoxin, a slight rise in plasma nitrate was observed from baseline to the end of study in both saline and L-Lys groups, from 12 ± 1 μM to 42 ± 15 μM and from 9 ± 2 μM to 39 ± 7 μM, respectively (NS between groups). L-Citrulline remained stable, from 63 ± 5 μM to 70 ± 6 μM in the saline group and from 62 ± 4 μM to 71 ± 5 μM in the L-Lys group (NS). In endotoxaemic rats, plasma nitrate and L-citrulline significantly increased in both groups, the changes being significantly less pronounced in rats treated with L-Lys. At T5, the respective values of nitrate and L-citrulline were 271 ± 18 μM and 207 ± 13 μM in the saline group, as compared to 220 ± 13 μM and 135 ± 13 μM in the L-Lys group ($P<0.05$ between groups for both variables).

Plasma lactate, biochemical markers of organ injury and haematocrit

Figure 3 illustrates the time-course of plasma lactate, lipase, creatinine and ALAT concentrations in endotoxaemic rats. All these variables significantly increased in both groups at 5 h and these changes tended to be blunted by L-Lys, the difference being statistically significant only for lactate. In addition, LPS administration produced a significant increase in plasma urea concentration, from 4.7 ± 0.3 mM to 12.3 ± 0.9 mM and from 5.0 ± 0.2 mM to 13.4 ± 0.5 mM in saline and L-Lys groups, respectively (NS between groups). In sham rats, these variables remained stable, except for a small, but significant increase in ALAT, which was comparable in both treatment groups, and a slight increase in plasma lipase which was significant in the saline group, as depicted in Figure 4. In these animals, plasma urea did not vary significantly with time (saline: from 5.0 ± 0.3 mM to 7.0 ± 1.7 mM; L-Lys: from 5.3 ± 0.2 mM to 7.4 ± 1.2 mM, NS).

A significant decrease in haematocrit (hct) was noted in all experimental groups, which was not influenced by L-Lys. In sham rats, hct decreased from 0.46 ± 0.01 to 0.38 ± 0.01 in the saline group, and from 0.44 ± 0.02 to 0.36 ± 0.02 in the L-Lys group (NS between groups). In rats exposed to LPS, hct decreased from 0.44 ± 0.01 to 0.39 ± 0.01 (saline group) and from 0.44 ± 0.01 to 0.38 ± 0.01 (L-Lys group) (NS between groups).

Plasma L-lysine

Comparable values, ranging from 260 to 282 μM, were obtained at baseline in all treatment groups of rats. After 5 h, in sham animals, plasma L-Lys was 286 ± 20 μM in the saline group (NS vs baseline) and 1387 ± 181 μM in the L-Lys group ($P < 0.05$ vs baseline and saline), while in endotoxaemic rats, it increased both in saline and L-Lys-treated rats, to 751 ± 65 μM and 2390 ± 206 μM, respectively ($P < 0.05$ vs baseline and between groups).

Discussion

The various isoforms of NOS are characterized by different requirements in L-Arg to sustain their catalytic activity. While endothelial intracellular stores of L-Arg provide sufficient substrate for the low-output ecNOS isoform (Thomas *et al*., 1989; Mitchell *et al*., 1990), NO production by cells expressing the high-output iNOS isoform appears strictly dependent on extracellular L-Arg, and L-Arg uptake may become the rate-limiting step in NO synthesis by these cells (Schott *et al*., 1993a; Durante *et al*., 1996). L-Arg uptake is principally mediated by the Na^+-independent carrier system y^+ (White, 1985), which was recently shown to be co-induced with iNOS in different cells following exposition to LPS and cytokines, thus providing a mechanism to increase the intracellular levels of substrate during iNOS activation (Bogle *et al*., 1992; Durante *et al*., 1996).

L-Lys is an alternate substrate for system y^+, which competitively inhibits L-Arg transport (White, 1985; Bogle *et al*., 1992) and may thus limit iNOS-dependent NO production. *In vitro*, L-Lys blocks interleukin-1β (IL-1β) stimulated NO production by vascular cells (Durante *et al*., 1995) and partially reverses the NO-mediated hyporeactivity of rat aortic rings exposed to endotoxin (Schott *et al*., 1993b). Our results provide evidence that such effects of L-Lys also occur *in vivo*. L-Lys administration to endotoxaemic rats efficiently reduced NO production, as evidenced both by a significant reduction in plasma nitrate, the stable oxidation product of NO, and L-citrulline, the co-product of the NOS reaction. Since the en-

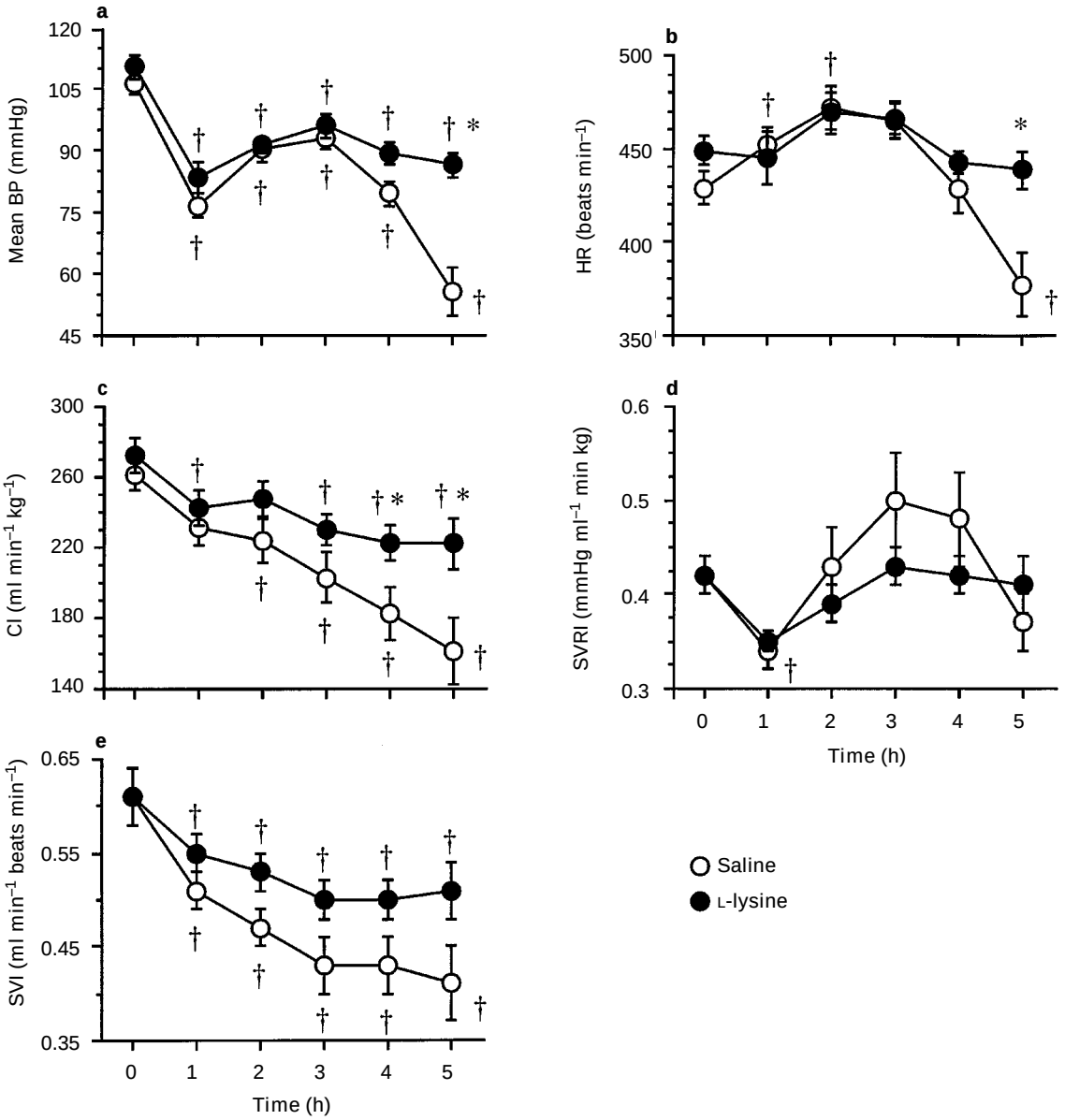

Figure 1 Time course of (a) mean blood pressure (BP), (b) heart rate (HR), (c) cardiac index (CI), (d) systemic vascular resistance index (SVRI) and (e) stroke volume index (SVI) in endotoxaemic rats. Rats received at baseline an intravenous bolus of 10 mg kg^{-1} LPS and were then treated by a continuous infusion of either isotonic saline, 2 ml kg^{-1} h^{-1} ($n = 11$) or L-lysine, 500 μmol kg^{-1} h^{-1} ($n = 12$) until the end of study. Data are expressed as means of n observations; vertical lines show s.e. mean. $^{\dagger}P < 0.05$ vs baseline. * $P < 0.05$, L-lysine vs saline treatment.

hanced NO release in endotoxic shock is related to diffuse iNOS induction (Moncada & Higgs, 1993), these data support the hypothesis that inhibition of cellular L-Arg uptake by L-Lys leads to decreased iNOS activity in these conditions. Contrastingly, in absence of endotoxin exposure, L-Lys did not affect plasma nitrate and L-citrulline, nor did it influence arterial BP, suggesting a lack of effect on ecNOS activity. Taken together, these findings are consistent with the concept that L-Lys selectively reduces iNOS-dependent NO synthesis.

The effects of L-Lys on NO production profoundly altered the course of endotoxin shock. This treatment largely prevented the delayed fall in BP induced by LPS, which is known to be mediated in large part by iNOS expression in the cardiovascular system (Szabo, 1995).

Concomitantly, the fall in CI was attenuated (Figure 1), an effect unlikely to be due to a difference in volume status between L-Lys and saline-treated rats, since (i) blood losses were similar, (ii) the infusion rate of administered fluids was identical, and (iii) a comparable decrease in hct, reflecting progressive haemodilution, was noted in both treatment groups.

The mechanisms by which L-Lys improved CI were two fold. Firstly, L-Lys prevented the late fall in HR observed in

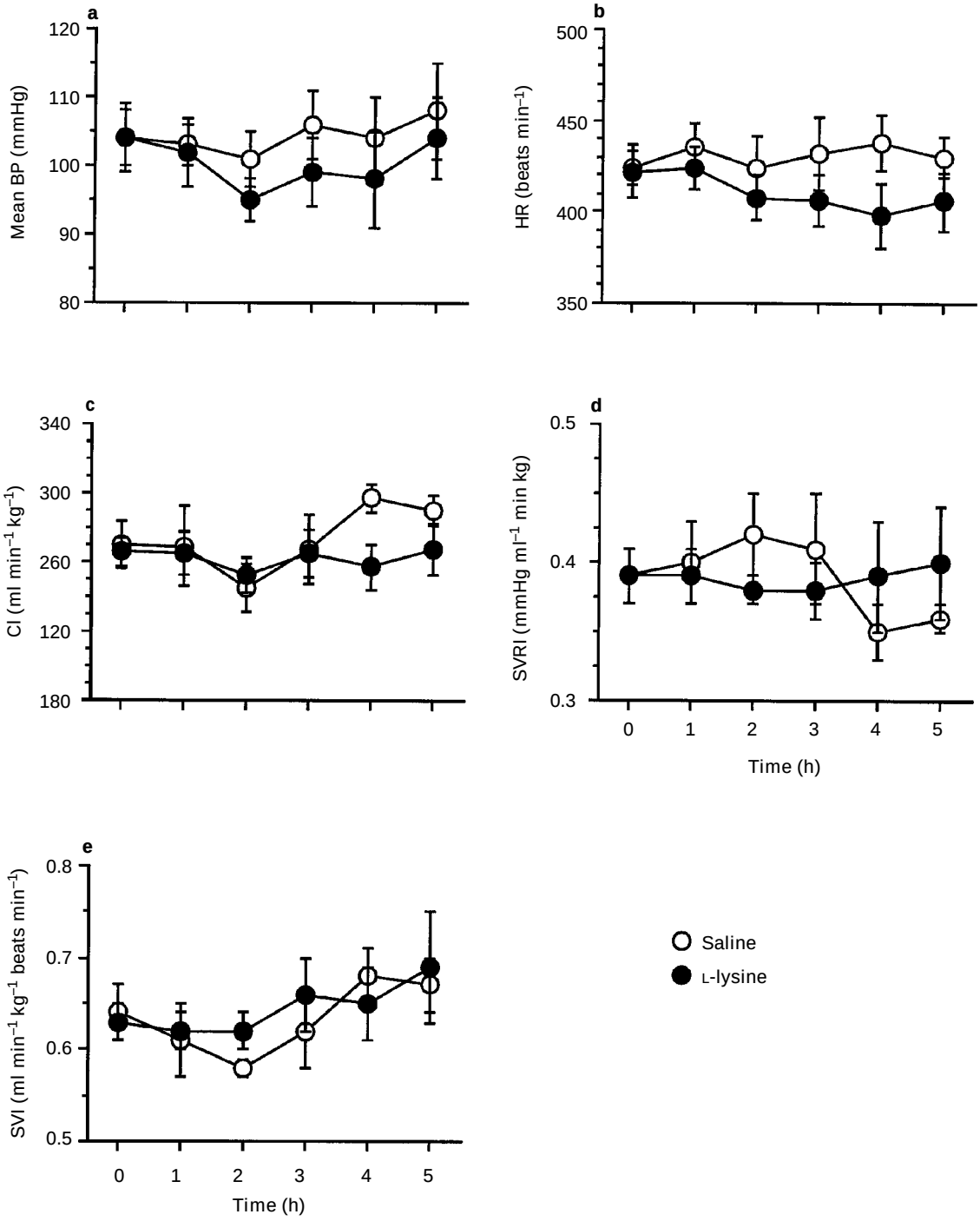

Figure 2 Time course of (a) mean blood pressure (BP), (b) heart rate (HR), (c) cardiac index (CI), (d) systemic vascular resistance index (SVRI) and (e) stroke volume index (SVI) in sham rats. Rats received at baseline an intravenous bolus of 1 ml isotonic saline and were then treated by a continuous infusion of either isotonic saline, 2 ml kg^{-1} h^{-1} ($n=5$) or L-lysine, 500 μmol kg^{-1} h^{-1} ($n=5$) until the end of study. Data are expressed as means of n observations; vertical lines show s.e.mean $^{\dagger}P<0.05$ vs baseline. $^{*}P<0.05$ L-lysine vs saline treatment.

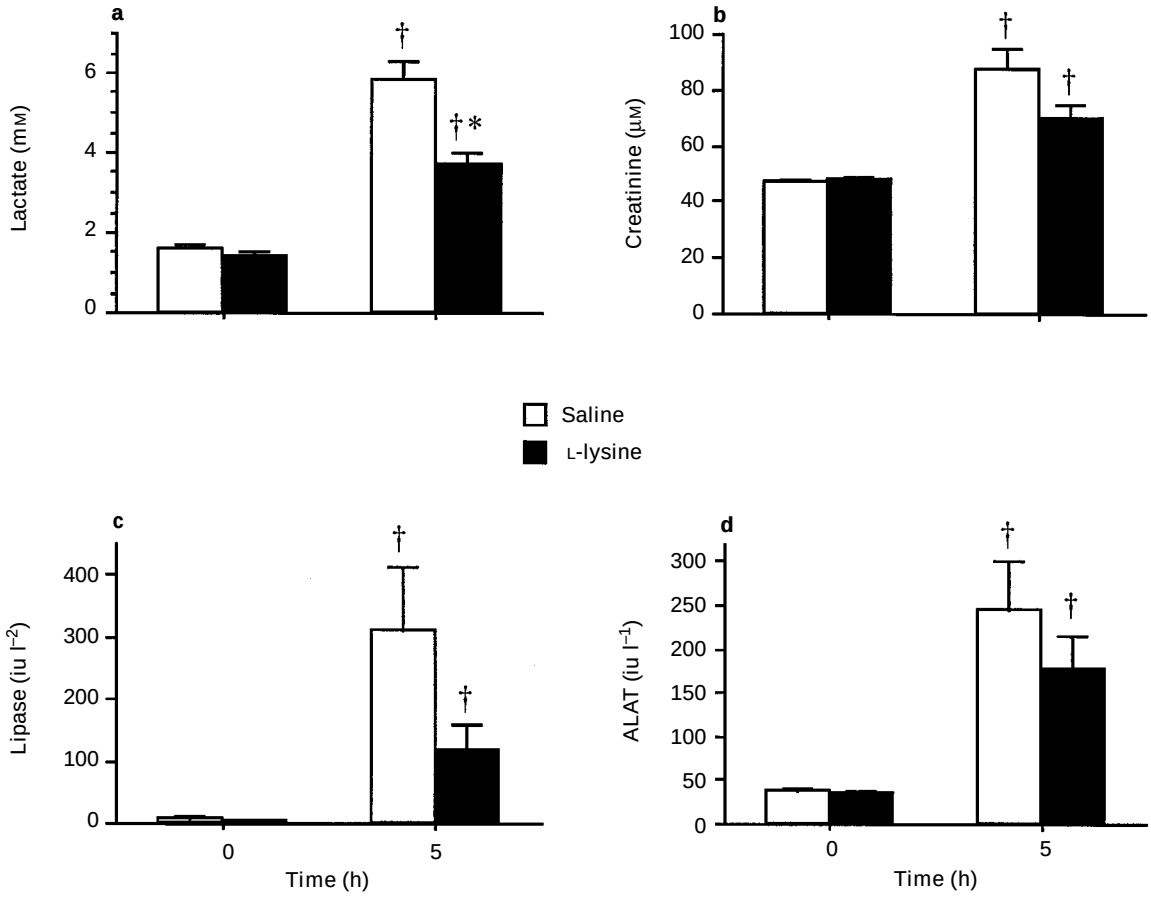

Figure 3 Arterial plasma concentrations in (a) lactate, (b) creatinine, (c) lipase and (d) alanine aminotransferase (ALAT) in rats administered 10 mg kg^{-1} LPS at baseline, followed by a continuous infusion of either isotonic saline, 2 ml kg^{-1} h^{-1} ($n=11$) or L-lysine, 500 μmol kg^{-1} h^{-1} ($n=12$) until the end of study. Data are expressed as means$\pm$s.e. mean of n observations. †$P<0.05$ vs baseline; *$P<0.05$ L-lysine vs saline treatment.

endotoxaemic rats treated with saline (Figure 1). The delayed bradycardia produced by endotoxin may reflect altered baroreceptor responses (Karzai *et al*., 1995), increased vagal activity (Levy & Blattberg, 1967; Blattberg & Levy, 1969; Halinen, 1976), and a reduced chronotropic response to β-adrenoceptor agonists (Parratt, 1973). We speculate that L-Lys prevented the fall in HR through its inhibitory effect on NO production in endotoxaemic rats. Indeed, NO decreases the spontaneous beating rate of cardiac myocytes (Balligand *et al*., 1993) and isolated cells from cardiac pacemaker tissue (Han *et al*., 1994), by reducing the L-type calcium current in these cells (Han *et al*., 1994). In addition, an enhanced NO production following iNOS expression has been shown to reduce the beating rate of cardiac myocytes exposed to interleukin 1β (Shindo *et al*., 1994). Finally, excess NO may indirectly reduce HR in endotoxaemic conditions by inhibiting both the release (Schwarz *et al*., 1995) and the biological activity (Macarthur *et al*., 1995) of catecholamines.

Secondly, treatment with L-Lys did not depress SVI (Figure 1), which in fact was 25% higher than in untreated animals (a non statistically significant difference), while increasing left ventricular afterload (higher mean BP) and improving CI. This pattern of action necessarily implies a favourable effect of L-Lys on either ventricular contractility or venous return, which have been shown indeed to be both affected by the excessive NO release following LPS exposure (Tao & McKenna, 1994; Fishman *et al*., 1997).

A further effect of L-Lys was a marked reduction in the LPS-induced hyperlactataemia (Figure 3). Since hyperlactataemia may reflect the development of tissue anaerobiosis, one may hypothesize that L-Lys partially corrected the endotoxin-induced tissue dysoxia, but this issue remains speculative. Indeed, hyperlactataemia in endotoxic shock may develop in the absence of tissue dysoxia, following increased aerobic glycolysis, inhibition of pyruvate deshydrogenase or reduced hepatic clearance of lactate (Hotchkiss & Karl, 1992). An additional finding was that L-Lys tended to limit the development of multiple organ dysfunction in endotoxaemic rats. LPS produced significant pancreatic, renal and liver dysfunction, as evidenced by the large increase in plasma lipase, creatinine and ALAT concentrations (Figure 3). These abnormalities were nonsignificantly blunted by L-Lys, which might reflect a reduction in NO-mediated cytotoxicity (Szabo, 1995; Payen *et al*., 1996). A distinct possibility is that of an improvement in tissue perfusion due to the higher BP and CI afforded by L-Lys (Figure 1).

In addition to its haemodynamic and metabolic effects, L-Lys appeared to prevent death in endotoxaemic animals. Indeed, 100% of the rats treated with L-Lys survived until the end of experiments, as compared to a 35% death rate (6/17) in rats receiving saline treatment, which corresponds to the expected mortality of this model, since we previously found a comparable death rate in another study performed in identical conditions (Liaudet *et al*., 1997). It is worth noting that the present study was not designed to assess the effects of L-Lys on survival, so that this difference in mortality was not statistically evaluated. However, this result suggests that the favourable physiological effects of L-Lys may be correlated with an improved outcome during endotoxic shock, and further studies are needed to address this particular issue.

It may seem puzzling that L-Lys only modestly reduced the plasma levels of nitrate (-20%) and L-citrulline (-35%), considering the importance of its physiological effects in en-

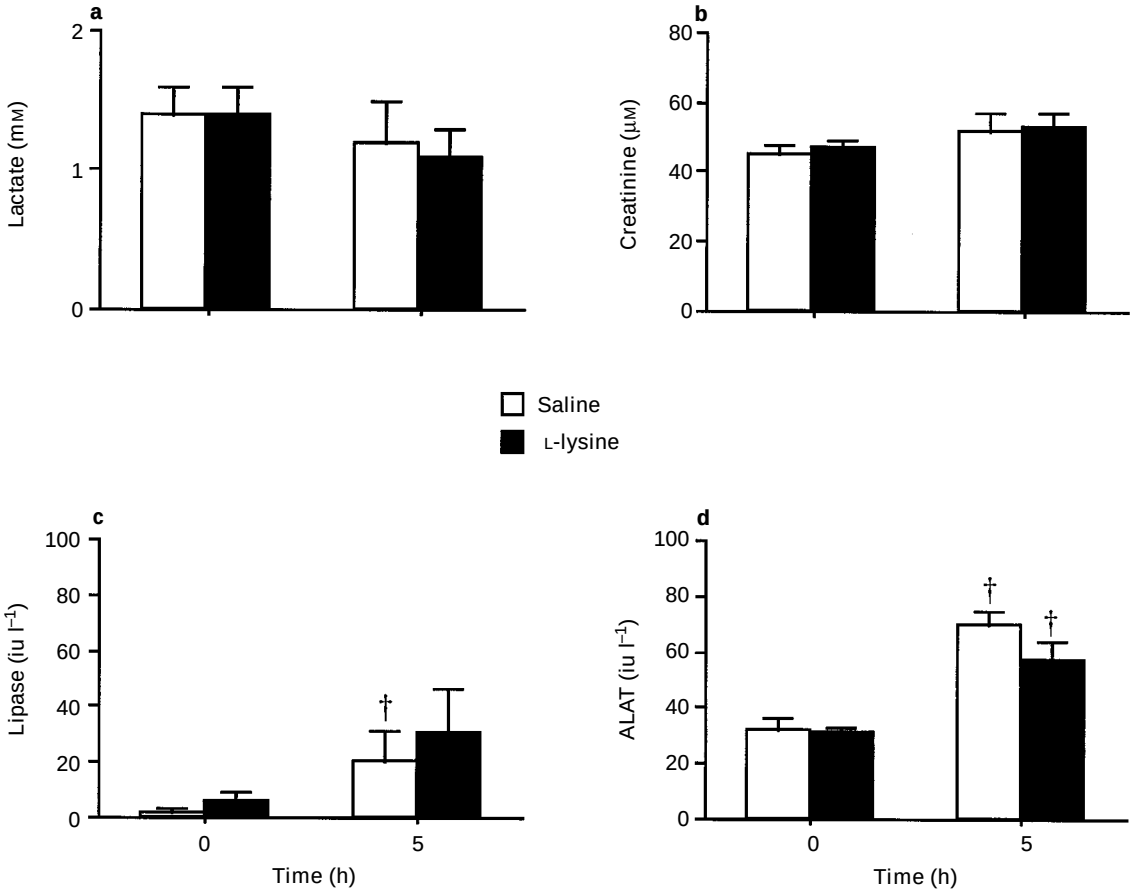

Figure 4 Arterial plasma concentrations in (a) lactate, (b) creatinine, (c) lipase and (d) alanine aminotransferase (ALAT) in rats administered 1 ml isotonic saline at baseline (sham rats), followed by a continuous infusion of either isotonic saline, 2 ml kg^{-1} h^{-1} ($n=5$) or L-lysine, 500 μmol kg^{-1} h^{-1} ($n=5$) until the end of study. Data are expressed as means $\pm$ s.e. mean. $^{\dagger}P<0.05$ vs baseline. $^{*}P<0.05$ L-lysine vs saline treatment.

dotoxaemic animals. However, the measurement of plasma nitrogen oxides (NOx, nitrate and nitrite) and L-citrulline only allows a global assessment of whole body NO production, which cannot reflect regional differences in NO biosynthesis. Thus, heterogeneous changes in NO production between different tissues may have biologically significant effects not necessarily reflected in plasma NOx and L-citrulline. As an example, it has been shown that low doses of the NO synthase inhibitor N^{G}-nitro-L-arginine-methylester (L-NAME) largely prevented hypotension in endotoxaemic rats, in the total absence of any decrease in plasma NOx (Wu *et al*., 1996).

It is noteworthy that the effects of L-Lys noted in the present study are very close to those obtained in similar conditions with various selective inhibitors of iNOS (Wu *et al*., 1995; Ruetten *et al*., 1996; Liaudet *et al*., 1996), while they markedly contrast with those of agents blocking both ecNOS and iNOS. Indeed, the latter compounds increase BP in endotoxin shock at the expense of a severe reduction in CI, while enhancing both lactic acidosis and organ damage (Harbrecht *et al*., 1992; Cobb *et al*., 1995; Liaudet *et al*., 1996). Maintenance of ecNOS appears therefore to be of critical importance in endotoxaemic conditions, while reduction of iNOS activity seems beneficial in this setting.

The downregulation of NO production by L-Lys was obtained at plasma levels 6 to 8 fold its physiological concentration, which is in agreement with the extracellular concentrations known to reduce L-Arg uptake and iNOS-dependent NO production *in vitro* (Bogle *et al*., 1992). Unexpectedly, plasma L-Lys also rose in endotoxaemic rats receiving saline only, reaching approximately 3 fold the control value. This finding probably reflects enhanced cellular efflux of L-Lys, which might be related to stimulated system y^{+} activity. Indeed, system y^{+} mediates both influx and efflux of cationic amino acids across the cellular membrane (White, 1985). Whether such spontaneous increase in plasma L-lys provides a physiological mechanism to limit L-Arg availability and NO production in endotoxic shock should be investigated.

In spite of the high plasma concentrations of L-Lys reached in our study, we did not notice any side-effect of this amino acid. Indeed, although excess L-Lys has been shown to be toxic to both kidney and pancreas (Racusen *et al*., 1985; Kitajima & Kishino, 1985), we did not find evidence of such toxicity, as attested by the time-course of plasma creatinine and lipase (Figures 3 and 4). Inhibition of the urea cycle, another potential side-effect of L-Lys administration (Kato *et al*., 1987) cannot be formally ruled out, but seems unlikely considering that L-Lys had no effect on plasma urea. Thus, administration of L-Lys at a dose of 500 μmol kg^{-1} h^{-1} appears well tolerated both by normal and endotoxaemic rats.

In conclusion, this study demonstrates for the first time that L-lysine, an inhibitor of cellular L-arginine transport through system y^{+}, reduces NO overproduction in endotoxaemia without influencing ecNOS-dependent NO synthesis, suggesting a selective modulation of iNOS activity. Treatment of endotoxaemic rats with this amino acid is associated with beneficial haemodynamic consequences, without demonstrable deleterious side effects. L-Lysine deserves further interest as a potentially effective agent for septic shock therapy.

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