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Short communication

Comparison of the effects of aminoguanidine and N^{ω}-nitro-L-arginine methyl ester on the multiple organ dysfunction caused by endotoxaemia in the rat

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Abstract

This study compares the effects of aminoguanidine, a relatively selective inhibitor of inducible nitric oxide (NO) synthase, and N^{ω}-nitro-L-arginine methyl ester (L-NAME), a selective inhibitor of endothelial NO synthase, on hypotension and multiple organ dysfunction caused by endotoxaemia in the anaesthetised rat. In the sham-operated rats, L-NAME, but not aminoguanidine, caused a dose-dependent increase in blood pressure. Endotoxin caused hypotension, increases in plasma nitrite (an indicator of inducible NO synthase activity), and dysfunction of kidney, liver and pancreas. Treatment of endotoxic rats with aminoguanidine or L-NAME caused significant and sustained rises in blood pressure. The increase in plasma nitrite caused by endotoxin was inhibited by aminoguanidine, but not by L-NAME. Aminoguanidine, but not L-NAME, attenuated the renal, liver and pancreatic dysfunction caused by endotoxaemia. Thus, selective inhibition of inducible (aminoguanidine), but not endothelial NO synthase (L-NAME) attenuates the circulatory failure and the multiple organ failure caused by endotoxaemia.

Keywords: Nitric oxide (NO) synthase, inducible; Lipopolysaccharide; Multiple organ dysfunction syndrome; Liver failure; Endotoxin

1. Introduction

Nitric oxide (NO) is a vasodilator autacoid which is produced by NO synthase from L-arginine in many mammalian cells (see Moncada and Higgs, 1993). NO has many diverse biological functions in the cardiovascular, nervous and immune systems. Once formed, NO diffuses to adjacent cells where it activates soluble guanylate cyclase, resulting in the formation of cyclic guanosine monophosphate, which in turn mediates many, but not all, of the biological effects of NO. At least three isoforms of NO synthase have been cloned. The NO synthase in endothelial cells and neuronal cells are expressed constitutively, while activation of macrophages and other cells with proinflammatory cytokines or endotoxin results in the expression of an inducible isoform of NO synthase which is functionally independent of changes in intracellular calcium (see Moncada and Higgs, 1993; Thiemermann, 1994 for review). The basal release of NO from the endothelium, under physiological conditions, plays an important role in the regulation of organ blood flow and inhibits the adhesion of platelets and polymorphonuclear granulocytes to the endothelium (see Moncada and Higgs, 1993).

In endotoxic and hemorrhagic shock, an enhanced formation of NO by the inducible NO synthase, and to a lesser extent by the endothelial NO synthase (early phase of shock), contributes to hypotension and vascular hyporeactivity to vasoconstrictor agents (see Thiemermann, 1994). As endotoxaemia is also associated with tissue hypoperfusion as well as activation of platelets and neutrophils, it is conceivable that the inhibition of the release of NO by endothelial NO synthase aggravates the endotoxin-induced tissue ischaemia resulting in an increased incidence of multiple organ dysfunction syndrome (e.g. ischemic injury of kidney, liver, lung and intestine).

Currently, there is only limited information regarding the effects of NO synthase inhibitors on organ function in experimental endotoxaemia. For instance, in a model of severe liver injury, high doses of N^{G}-methyl-L-arginine (L-NMMA; 2.5 to 5 mg per mouse) augment the release of

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hepatocellular enzymes as well as the histological signs of hepatic injury (Harbrecht et al., 1992). Endotoxaemia is also associated with a substantial reduction in splanchnic blood flow. In spite of a significant induction of inducible NO synthase activity, perfused mesenteric arteries and veins obtained from endotoxin-treated rats show no hyporeactivity to vasoconstrictor agents. In addition, the vasoconstrictor effects of the thromboxane A_2 analog U46619 in mesenteric arteries and veins are markedly potentiated by L-NMMA (Mitchell et al., 1993). Moreover, higher doses of L-NMMA (10 to 50 mg/kg i.v.) enhance the intestinal injury and vascular permeability elicited by endotoxin in the rat (Hutcheson et al., 1990). N^{ω}-Nitro-L-arginine methyl ester (L-NAME) is a more potent inhibitor of endothelial NO synthase than inducible NO synthase activity (Gross et al., 1991). Thus, it is possible that with non-selective inhibitors of inducible NO synthase (e.g. L-NMMA) or with relatively selective inhibitors of endothelial NO synthase activity (e.g. L-NAME), the concomitant inhibition of endothelial NO synthase activity in the endothelium increases the incidence of organ ischaemia, microvascular thrombosis and mortality (Hutcheson et al., 1990; Harbrecht et al., 1992; Shultz and Raij, 1992; Wright et al., 1992). The well-documented beneficial haemodynamic effects of non-selective NO synthase inhibitors may well be due to inducible NO synthase inhibition, while the reported adverse effects may be due to inhibition of endothelial NO synthase (see Thiemermann, 1994).

Aminoguanidine, a bifunctional molecule containing the guanido group of L-arginine linked to hydrazine, is an inhibitor of inducible NO synthase activity with a potency similar to those reported for N^{ω}-substituted arginine analogues (Hasan et al., 1993). However, aminoguanidine is substantially less potent as an inhibitor of endothelial NO synthase activity in cultured cells, isolated blood vessels and enzyme preparations in vitro (Corbett et al., 1992; Griffiths et al., 1993; Misko et al., 1993; Tilton et al., 1993), and has beneficial effects on the delayed hypotension induced by endotoxin in the anaesthetised rat (Wu et al., 1995). This study compares the effects of aminoguanidine and L-NAME on the delayed circulatory failure (e.g. vasodilatation), the induction of the activity of inducible NO synthase (e.g. plasma nitrite levels) and the multiple organ (e.g. kidney, liver and pancreas) dysfunction syndrome caused by endotoxaemia.

2. Materials and methods

2.1. Materials

Calmodulin, bacterial endotoxin (*Escherichia coli* lipopolysaccharide serotype 0.127:B8), NADPH, aminoguanidine bicarbonate, N^{ω}-nitro-L-arginine methyl ester, and Dowex 50W anion exchange resin were obtained from Sigma Chemical Co. (Poole, Dorset, UK).

2.2. In vivo experiments

Male Wistar rats (260–340 g; A Tuck, Battlebridge, Essex, UK) were anaesthetised with thiopentone sodium (Trapanal; 120 mg/kg i.p.). The trachea was cannulated to facilitate respiration and rectal temperature was maintained at 37°C with a homeothermic blanket (BioSciences, Sheerness, Kent, UK). The right carotid artery was cannulated and connected to a pressure transducer (P23XL, Spectramed, Statham, Oxnard, CA, USA) for the measurement of phasic and mean arterial blood pressure and heart rate which were displayed on a Grass model 7D polygraph recorder (Grass Instruments, Quincy, MA, USA). The femoral vein and jugular vein were cannulated for the administration of drugs. Upon completion of the surgical procedure, cardiovascular parameters were allowed to stabilize for 15 min. After recording baseline haemodynamic parameters, animals received endotoxin (10 mg/kg i.v.) as a slow injection over 10 min. At 2 h after injection of endotoxin, animals received a bolus injection plus a continuous infusion of vehicle (saline, 0.6 ml/kg/h, $n = 7$), aminoguanidine (5 mg/kg i.v. bolus loading dose, followed by a continuous infusion of 5 mg/kg/h in 0.6 ml/kg/h saline, $n = 9$) or L-NAME (0.03 mg/kg or 0.3 mg/kg i.v. bolus loading dose, followed by a continuous infusion of 0.03 mg/kg/h or 0.3 mg/kg/h in 0.6 ml/kg/h saline, $n = 6$, respectively). All haemodynamic parameters were recorded for a further 4 h period.

In separate experiments, 4 groups of rats were anaesthetised and instrumented (as above) and treated with vehicle (1 ml/kg i.v., saline, $n = 5$). At 2 h after injection of vehicle, animals received a bolus injection plus a continuous infusion of either saline (dose as above, sham-operated control rats, $n = 5$), aminoguanidine (dose as above, $n = 3$), or L-NAME (doses as above, $n = 3$, respectively) until the end of the experiment (6 h).

At 6 h after injection of either vehicle or endotoxin, blood samples were taken to evaluate the changes in the plasma levels of biochemical markers of the multiple organ dysfunction syndrome (see below) caused by 6 h of endotoxaemia.

2.3. Quantification of kidney, liver or pancreatic injury

At 6 h after the injection of either saline or endotoxin, 1.5 ml of blood was collected from a catheter placed in the carotic artery. The blood sample was centrifuged (6000 rpm for 3 min) to prepare plasma. All plasma samples were analyzed within 24 h by a contract laboratory for veterinary, clinical chemistry (Vetlab Services, Sussex, UK). The following marker enzymes were measured in the plasma as biochemical indicators of multiple organ dysfunction. (1) *Renal dysfunction and failure* were assessed by measuring the rises in plasma levels of creatinine (an indicator of reduced glomerular filtration rate, and hence, renal failure) and urea (an indicator of impaired excretory

function of the kidney and/or increased catabolism). (2) *Liver dysfunction and failure* were assessed by measuring the rises in plasma levels of glutamate-pyruvate-transaminase (a marker for hepatic parenchymal injury); glutamate-oxalacetate-transaminase (a non-specific marker for hepatic parenchymal injury); bilirubin (a marker enzyme for the development of cholestasis, and for the development of liver failure) Hewett and Roth, 1995; and γ-glutamyl-transferase (a marker for cholestasis). (3) *Pancreatic dysfunction and failure* were assessed by measuring the rises in the plasma levels of lipase (a marker of reduced function of pancreas).

2.4. Measurement of plasma nitrite

The formation of NO (by inducible NO synthase) can easily be assessed by measuring the accumulation of nitrite in the plasma of animals treated with endotoxin. At 6 h after injection of either saline or endotoxin, 1 ml of blood was collected from a catheter placed in the carotic artery. The blood sample was centrifuged (15000 rpm for 3 min) to prepare plasma. The amounts of nitrite in the plasma were measured by the Griess reaction by adding 100 μl of Griess reagent to 100 μl samples of plasma. The optical density at 550 nm (OD_{550}) was measured using a Molecular Devices microplate reader (Richmond, CA, USA). Nitrite concentrations were calculated by comparison with OD_{550} of standard solution of sodium nitrite prepared in normal control plasma.

2.5. Statistical analysis

All values in the figures and text are expressed as mean ± S.E.M. of n observations, where n represents the number of animals or blood samples studied. A two-way analysis of variance followed by, if appropriate, a Bonferoni's test was used to compare means between groups (in vivo study). Student's unpaired t test was used to compare means between groups (in vitro study). A P-value less than 0.05 or 0.01 was considered to be statistically significant.

3. Results

Baseline values of mean arterial blood pressure ranged from 120 ± 2 to 125 ± 4 mm Hg at time 0 and were not significantly different between any of the experimental groups studied. In sham-operated control rats (without endotoxaemia), injection of aminoguanidine had no significant effect on mean arterial blood pressure, while L-NAME caused a dose-dependent increase in mean arterial blood pressure (Fig. 1a).

Injection of endotoxin resulted in a rapid fall of mean arterial blood pressure from 125 ± 4 mm Hg (time 0, prior to the injection of endotoxin) to 71 ± 6 mm Hg at 10 min

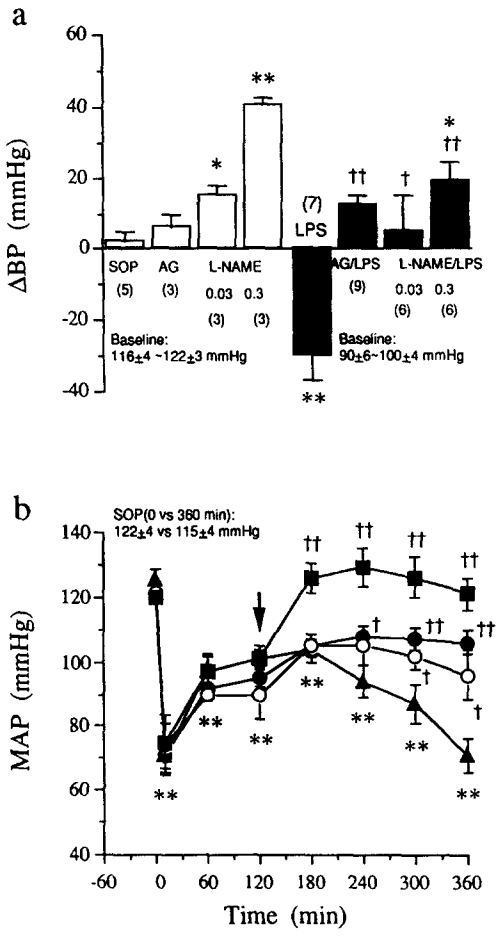

Fig. 1. Effects of NO synthase inhibitors on (a) blood pressure in rats treated with either saline or endotoxin and (b) the delayed hypotension caused by endotoxin (LPS). Depicted are (a) the changes in blood pressure (ΔBP) at the end the experimental period in different groups of animals which received infusion of saline (SOP, 0.6 ml/kg/h), aminoguanidine (AG, 5 mg/kg plus 5 mg/kg/h) or L-NAME (0.03 mg/kg plus 0.03 mg/kg/h or 0.3 mg/kg plus 0.3 mg/kg/h, respectively) at 2 h after saline (open columns) or endotoxin (solid columns) and (b) different groups of animals received infusion of vehicle (saline, 0.6 ml/kg/h, closed triangles), aminoguanidine (AG, 5 mg/kg plus 5 mg/kg/h, closed circles) or L-NAME (0.03 mg/kg plus 0.03 mg/kg/h or 0.3 mg/kg plus 0.3 mg/kg/h, open circles and closed squares, respectively) at 2 h after endotoxin. The arrow indicates the time of administration of vehicle or drugs. Data are expressed as mean ± S.E.M. of n observations which are indicated in brackets. * $P < 0.05$ and ** $P < 0.01$ represent significant differences when compared to SOP control. † $P < 0.05$ and †† $P < 0.01$ represent significant differences between endotoxin rats in the absence and presence of AG or L-NAME.

($P < 0.01$, $n = 7$) and 97 ± 6 mm Hg at 120 min (prior to administration of vehicle or NO synthase inhibitors, $P < 0.01$, $n = 7$, Fig. 1b). Due to the infusion of saline, the mean arterial blood pressure values of rats treated with endotoxin remained above 95 mm Hg from 60 to 240 min, and fell towards the end of the experimental period (360 min) to 71 ± 5 mm Hg (Fig. 1b). Thus, despite infusion of saline, the mean arterial blood pressure values of endotoxin rats were significantly lower than the ones measured

in sham-operated control rats at 10–360 min (i.e. 121 ± 4 mm Hg at 10 min and 115 ± 4 mm Hg at 360 min in sham-operated control rats). Administration of aminoguanidine at 120 min after the onset of endotoxaemia resulted in a *slow*, but sustained increase in mean arterial blood pressure (Fig. 1b). Thus, the mean arterial blood pressure of endotoxin rats treated with aminoguanidine was significantly higher than in the endotoxin control group at 240–360 min. In contrast, adminstration of L-NAME (0.03 mg/kg i.v. plus 0.03 mg/kg/h or 0.3 mg/kg i.v. plus 0.3 mg/kg/h) at 120 min after the onset of endotoxaemia caused a dose-dependent, *rapid* and sustained increase in mean arterial blood pressure (Fig. 1b).

Injection of endotoxin also caused a significant increase in the plasma levels of creatinine and urea suggesting the development of acute renal failure. This increase in plasma urea caused by endotoxin was not affected by infusion of vehicle or infusion of either aminoguanidine ($P > 0.05$, $n = 6$) or L-NAME ($P > 0.05$, $n = 5$, Fig. 2). Interestingly, the rise in the plasma levels of creatinine caused by endotoxin in vivo was attenuated by treatment of endotoxin rats with aminoguanidine ($P < 0.01$, $n = 6$), but not with L-NAME ($P > 0.05$, $n = 5$).

Endotoxaemia for 360 min was also associated with a significant rise in the plasma levels of glutamate-oxalacetate-transaminase and glutamate-pyruvate-transaminase activities as well as bilirubin and γ-glutamyl-transferase ($P < 0.01$, $n = 6$, Fig. 2) indicating the development of liver dysfunction. The rise in the plasma levels of glutamate-oxalacetate-transaminase and glutamate-pyruvate-transaminase caused by endotoxin in vivo was reduced by treatment of endotoxin rats with aminoguanidine ($P < 0.05$, $n = 6$), but not with L-NAME ($P > 0.05$, $n = 5$). Interestingly, the rise in plasma bilirubin or γ-glutamyl-transferase caused by endotoxin was not affected by aminoguanidine ($P > 0.05$, $n = 6$) or L-NAME ($P > 0.05$, $n = 5$, respectively, Fig. 2).

Administration of endotoxin also caused a significant increase in the plasma levels of lipase, an indicator of pancreatic injury ($P < 0.01$, $n = 6$, Fig. 2). This rise in the plasma levels of lipase caused by endotoxin in vivo was attenuated by treatment of endotoxin rats with aminoguanidine ($P < 0.01$, $n = 6$), but not by L-NAME ($P > 0.05$, $n = 5$, Fig. 2).

The baseline plasma nitrite level in sham-operated control rats was 1.9 ± 0.3 μM ($n = 5$). Endotoxaemia for 360 min was associated with a 5-fold rise in plasma nitrite levels (9.9 ± 1.0 μM, $P < 0.01$, $n = 6$). The increase in plasma nitrite caused by endotoxaemia was significantly reduced in endotoxin rats treated with aminoguanidine (4.8 ± 0.8 μM, $P < 0.01$, $n = 6$), but not with either dose of L-NAME (9.5 ± 1.2 μM; 10.2 ± 1.0 μM, $P > 0.05$, $n = 5$, respectively). Treatment of sham-operated control rats with vehicle or NO synthase inhibitors (aminoguanidine or L-NAME) did not affect the plasma levels of urea, creatinine, glutamate-oxalacetate-transaminase, glutamate-pyruvate-transaminase, bilirubin, γ-glutamyl-transferase, lipase or nitrite (data not shown).

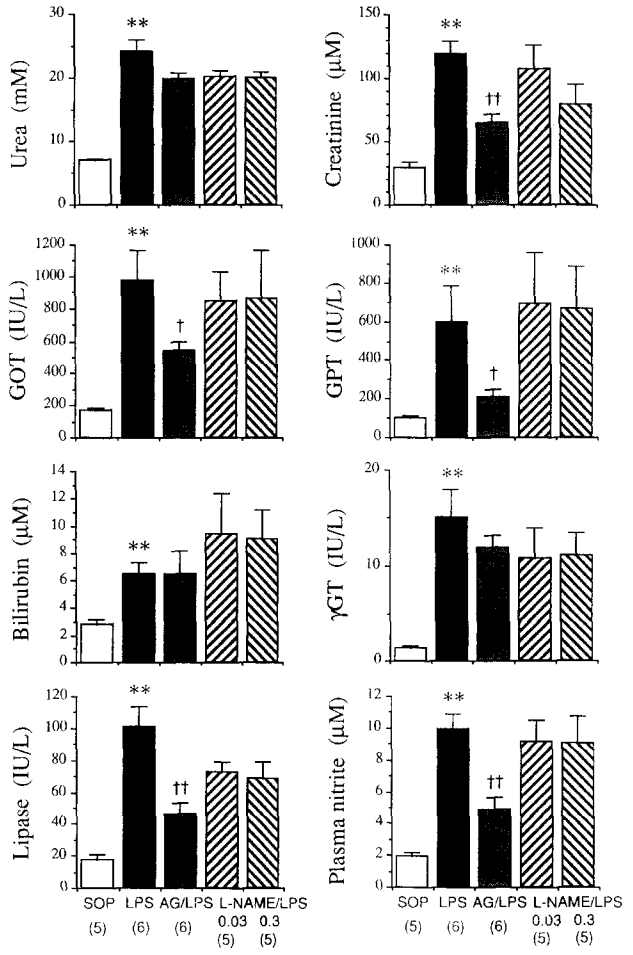

Fig. 2. Effects of NO synthase inhibitors on endotoxin-induced increases in plasma concentrations of urea, creatinine, glutamate-oxalacetate-transaminase (GOT), glutamate-pyruvate-transaminase (GPT), bilirubin, γ-glutamyl-transferase (γGT), lipase, and nitrite. These enzymes activities were measured in plasma obtained from sham-operated control rats (SOP, open columns) or rats treated with endotoxin (LPS, 10 mg/kg i.v.) for 6 h. Different groups of endotoxin rats received saline (LPS, 0.6 ml/kg/h, solid columns), aminoguanidine (AG/LPS, 5 mg/kg plus 5 mg/kg/h, stippled columns) or L-NAME (L-NAME/LPS, 0.03 mg/kg plus 0.03 mg/kg/h or 0.3 mg/kg plus 0.3 mg/kg/h, hatched columns) at 2 h after endotoxin. Data are expressed as mean ± S.E.M. of n observations which are indicated in brackets. ** $P < 0.01$ represents a significant increase in enzyme activity caused by endotoxin. † $P < 0.05$ and †† $P < 0.01$ represent significant reductions in enzyme activity in endotoxin rats treated with aminoguanidine or L-NAME when compared to endotoxin controls.

4. Discussion

Here we demonstrate that the dose of aminoguanidine used in this study does not affect the resting blood pressure (and hence, endothelial NO synthase activity) in sham-operated control rats, but inhibits the increase of plasma nitrite (an indicator of the formation of NO by inducible

NO synthase) caused by endotoxin. In contrast, L-NAME dose-dependently increases blood pressure in sham-operated control rats, but does not attenuate the rise in plasma nitrite (e.g. inducible NO synthase activity) caused by endotoxin. Thus, the doses of aminoguanidine and L-NAME used here result in a selective inhibition of inducible and endothelial NO synthase activity, respectively. Inhibition of endothelial NO synthase activity with L-NAME attenuates the hypotension without affecting the multiple organ (liver, renal or pancreatic) dysfunction caused by endotoxin. Our finding that L-NAME does not attenuate the multiple organ dysfunction associated with endotoxaemia may help to explain why (i) L-NAME did not improve survival in a murine model of endotoxic shock (Wu et al., 1995) and (ii) non-selective NO synthase inhibitors improve blood pressure without improving survival in patients with circulatory shock (Petros et al., 1991). In contrast, we show here that aminoguanidine not only reduces the hypotension, but also attenuates the multiple organ dysfunction elicited by endotoxin in the anaesthetised rat. This observation is consistent with our previous study demonstrating that aminoguanidine improves survival in a murine model of severe endotoxaemia (Wu et al., 1995).

Inhibition of NO formation with high doses of L-NMMA enhances the degree of microvascular thrombosis and the histological signs of renal cell injury in the kidney of rats with endotoxic shock (Shultz and Raij, 1992). Thus, it has been proposed that the formation of NO by either endothelial NO synthase or inducible NO synthase prevents thrombosis and excessive vasoconstriction in the kidney and, hence, is important in maintaining renal function in endotoxaemia (Shultz and Raij, 1992). We demonstrate here, however, that the selective inhibition of inducible NO synthase activity with aminoguanidine partially attenuates the rise in creatinine, but not in urea, caused by endotoxaemia. The finding that aminoguanidine did not attenuate the rise in urea caused by endotoxaemia is not entirely surprising, as the rise in urea caused by endotoxaemia in the rat is already maximal within 2 h of endotoxaemia (unpublished data), the time point at which the infusion of aminoguanidine was started. Nevertheless, we clearly demonstrate that inhibition of endothelial NO synthase activity with L-NAME neither attenuates nor aggravates the renal dysfunction caused by endotoxaemia in the rat.

Inhibition of endothelial NO synthase and inducible NO synthase activity with high doses of L-NMMA in rodents with endotoxaemia also results in (i) an augmentation of the release of the hepatocellular marker enzymes, (ii) an increase in hepatic cell injury (histology), and (iii) a reduction in liver protein synthesis (Harbrecht et al., 1992). Thus, it is possible that inhibition of NO synthase activity may aggravate the hepatocellular injury caused by endotoxaemia. We demonstrate here, however, that the selective inhibition of inducible NO synthase activity with aminoguanidine attenuates the rises in glutamate-oxalacetate-transaminase and glutamate-pyruvate-transaminase (marker enzymes for a hepatic parenchymal injury), but not of plasma bilirubin and γ-glutamyl-transferase, caused by endotoxaemia. Thus, aminoguanidine (at least partially) attenuates the degree of liver injury caused by endotoxaemia. Although the inhibition of endothelial NO synthase activity with L-NAME did not attenuate the rises in glutamate-oxalacetate-transaminase, glutamate-pyruvate-transaminase, bilirubin and γ-glutamyl-transferase, inhibition of endothelial NO synthase activity with L-NAME did not aggravate the release into the plasma of any of these marker enzymes of liver dysfunction. This finding, however, dose not preclude that an excessive inhibition of endothelial NO synthase activity (with higher doses of L-NMMA or L-NAME) may result in excessive vasoconstriction, ischemia of hepatic cells, and ultimately in an aggravation of liver injury.

There is good evidence that some disorders (e.g. diabetes) are associated with the expression of inducible NO synthase in pancreatic islet cells, which are extremely susceptible to the cytotoxic effects of NO (Kroncke et al., 1993). Although there is no evidence that endotoxaemia in the rat is associated with the induction of inducible NO synthase in the pancreas, we demonstrate here that inhibition of inducible NO synthase activity with aminoguanidine attenuates the rise in plasma lipase activity and, hence, the pancreatic dysfunction associated with endotoxic shock. Again, inhibition of endothelial NO synthase activity with L-NAME neither attenuated nor aggravated the pancreatic injury caused by endotoxaemia.

In conclusion, these results demonstrate that aminoguanidine, a selective inhibitor of inducible NO synthase activity in vivo, attenuates the circulatory failure as well as the multiple organ dysfunction syndrome associated with endotoxic shock in the rat. Inhibition of endothelial NO synthase activity with L-NAME attenuated the circulatory failure, but not the multiple organ dysfunction syndrome, caused by endotoxaemia. This strongly indicates that the beneficial haemodynamic effects of NO synthase inhibitors which have been observed, do not necessarily predict that such agents improve multiple organ dysfunction syndrome and utimately survival in animal models of circulatory shock.

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