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Aminoguanidine attenuates the delayed circulatory failure and improves survival in rodent models of endotoxic shock

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1 We have investigated the effects of aminoguanidine, a relatively selective inhibitor of the cytokine-inducible isoform of nitric oxide synthase (iNOS), on the delayed circulatory failure, vascular hyporeactivity to vasoconstrictor agents, and iNOS activity in a rat model of circulatory shock induced by bacterial endotoxin (*E. coli* lipopolysaccharide; LPS). In addition, we have evaluated the effect of aminoguanidine on the 24 h survival rate in a murine model of endotoxaemia.

2 Male Wistar rats were anaesthetized and instrumented for the measurement of mean arterial blood pressure (MAP) and heart rate (HR). Injection of LPS (10 mg kg^{-1}, i.v.) resulted in a fall in MAP from 115 ± 4 mmHg (time 0, control) to 79 ± 9 mmHg at 180 min ($P<0.05$, $n = 10$). The pressor effect of noradrenaline (NA, 1 μg kg^{-1}, i.v.) was also significantly reduced at 60, 120 and 180 min after LPS injection. In contrast, animals pretreated with aminoguanidine (15 mg kg^{-1}, i.v., 20 min prior to LPS injection) maintained a significantly higher MAP (at 180 min, 102 ± 3 mmHg, $n = 10$, $P<0.05$) when compared to rats given only LPS (LPS-rats). Cumulative administration of aminoguanidine (15 mg kg^{-1} and 45 mg kg^{-1}) given 180 min after LPS caused a dose-related increase in MAP and reversed the hypotension. Aminoguanidine also significantly alleviated the reduction of the pressor response to NA: indeed, at 180 min, the pressor response returned to normal in aminoguanidine pretreated LPS-rats.

3 Thoracic aortae obtained from rats at 180 min after LPS showed a significant reduction in the contractile responses elicited by NA (10^{-9}–10^{-6} M). Pretreatment with aminoguanidine (15 mg kg^{-1}, i.v., at 20 min prior to LPS) significantly prevented this LPS-induced hyporeactivity to NA *ex vivo*.

4 Endotoxaemia for 180 min resulted in a significant increase in iNOS activity in the lung from 0.6 ± 0.2 pmol mg^{-1} min^{-1} (control, $n = 4$) to 4.8 ± 0.3 pmol mg^{-1} min^{-1} ($P<0.05$, $n = 6$). In LPS-rats treated with aminoguanidine, iNOS activity in the lung was attenuated by 44 ± 5% ($n = 6$, $P<0.05$). Moreover, when added *in vitro* to lung homogenates obtained from LPS-rats, aminoguanidine and N^{ω}-nitro-L-arginine methyl ester (L-NAME; 10^{-8} to 10^{-3} M) caused a concentration-dependent inhibition of iNOS activity ($n = 3-6$, IC_{50}: 30 ± 12 and 11 ± 6 μM, respectively $P>0.05$). In contrast, aminoguanidine was a less potent inhibitor than L-NAME of the constitutive nitric oxide synthase in rat brain homogenates ($n = 3-6$, IC_{50} is 140 ± 10 and 0.6 ± 0.1 μM, respectively, $P<0.05$). In addition, the inhibitory effect of aminoguanidine on iNOS activity showed a slower onset than that of L-NAME (maximal inhibition at 90 min and 30 min, respectively).

5 Treatment of conscious Swiss albino (T/O) mice with a high dose of endotoxin (60 mg kg^{-1}, i.p.) resulted in a survival rate of only 8% at 24 h ($n = 12$). However, therapeutic application of aminoguanidine (15 mg kg^{-1}, i.p. at 2 h and 6 h after LPS) increased the 24 h survival rate to 75% ($n = 8$), whereas L-NAME (3 mg kg^{-1}, i.p. at 2 h and 6 h after LPS) did not affect the survival rate (11%, $n = 9$).

6 Thus, aminoguanidine inhibits iNOS activity and attenuates the delayed circulatory failure caused by endotoxic shock in the rat and improves survival in a murine model of endotoxaemia. Aminoguanidine, or novel, more potent selective inhibitors of iNOS may be useful in the therapy of septic shock.

Keywords: Aminoguanidine; nitric oxide; endotoxic shock; nitric oxide synthase; vascular hyporeactivity; survival rate

Introduction

The formation of nitric oxide (NO) from one of the terminal guanidino nitrogen atoms of L-arginine is catalysed by NO synthase (NOS). Three distinct isoforms of NOS have recently been cloned, sequenced and expressed (see Dinerman *et al.*, 1993; Moncada & Higgs, 1993). The NOS in endothelial cells (eNOS; M_r 130 kD) and neuronal cells (nNOS; M_r 150 kD) are expressed constitutively and their activity is regulated by changes in intracellular calcium. Under physiological conditions, the release of NO by the eNOS in endothelial cells causes vasodilatation and in concert with the sympathetic nerves, regulates blood vessel diameter, organ blood flow and blood pressure (see Moncada *et al.*, 1991). Activation of macrophages with endotoxin and/or cytokines results in the *de novo* biosynthesis of a calcium-independent (inducible) isoform of NOS (iNOS; M_r 130 kD), which produces large amounts of NO (see Moncada & Higgs, 1993). The induction of iNOS by endotoxin or cytokines greatly contributes to the bactericidal and tumouricidal effects of these cells (Stuehr & Nathan, 1989; see Nathan, 1992). However, endotoxin also induces iNOS in vascular smooth muscle cells, fibroblasts, hepatocytes, Kupffer cells, keratinocytes and megakaryocytes *in vitro*, which is reflected by induction in tissues *in vivo* such as lung, spleen, liver, heart, kidney and blood vessels (see Nathan, 1992; Moncada & Higgs, 1993; Thiemermann, 1994).

The circulatory failure in shock of various aetiologies is characterized by systemic vasodilatation, hyporeactivity to vasoconstrictor agents and organ ischaemia leading to multiple organ failure and death. An enhanced formation of NO importantly contributes to the acute (eNOS) and delayed (iNOS) hypotension and vascular hyporeactivity to

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noradrenaline in endotoxic shock (Thiemermann & Vane, 1990; Julou-Schaeffer *et al.*, 1990; Kilbourn *et al.*, 1990; Meyer *et al.*, 1992; Suba *et al.*, 1992; Wright *et al.*, 1992; Szabo *et al.*, 1993a). An enhanced formation of NO by iNOS also accounts for the hypotension associated with cytokine-chemotherapy (Kilbourn & Griffith, 1992). Thus, there is strong experimental evidence that the overproduction of NO, particularly by iNOS, is a key factor in the development of circulatory failure in shock of various aetiologies (see Thiemermann, 1994).

With non-selective inhibitors of iNOS, the concomitant inhibition of eNOS in the endothelium may increase the incidence of organ ischaemia, microvascular thrombosis and mortality (Hutcheson *et al.*, 1990; Harbrecht *et al.*, 1992; Shultz & Raij, 1992; Wright *et al.*, 1992). Thus, the beneficial haemodynamic effects of non-selective NOS inhibitors may well be due to iNOS inhibition, while the reported adverse effects may be due to inhibition of eNOS.

Aminoguanidine is a more potent inhibitor of iNOS than N^{ω}-substituted arginine analogues. In addition, aminoguanidine is less potent as an inhibitor of the constitutive NOS activity in cultured cells, isolated blood vessels and enzyme preparations *in vitro* (Corbett *et al.*, 1992; Griffiths *et al.*, 1992; Misko *et al.*, 1993; Tilton *et al.*, 1993). Here, we investigate the effects of aminoguanidine on the delayed circulatory failure (vasodilatation, hyporeactivity to vasoconstrictor agents) and induction of iNOS in endotoxic shock in the anaesthetized rat. In addition, we have evaluated the effects of aminoguanidine and N^{ω}-nitro-L-arginine methyl ester (L-NAME), a non-isoenzyme selective NOS inhibitor, on survival rate in a murine model of endotoxaemia.

Methods

Endotoxic shock

Male Wistar rats (240–300 g; Glaxo Laboratories Ltd., Greenford, Middx.) were anaesthetized with thiopentone sodium (Trapanal; 120 mg kg^{-1}, i.p.). The trachea was cannulated to facilitate respiration and rectal temperature was maintained at 37°C with a homeothermic blanket (Bio-Sciences, Sheerness, Kent). The right carotid artery was cannulated and connected to a pressure transducer (P23XL, Spectramed, Statham, U.S.A.) for the measurement of phasic and mean arterial blood pressure (MAP) and heart rate (HR) which were displayed on a Grass model 7D polygraph recorder (Grass Instruments, Quincy, MA, U.S.A.). The left femoral vein was cannulated for the administration of drugs. Upon completion of the surgical procedure, cardiovascular parameters were allowed to stabilize for 20 min. After recording baseline haemodynamic parameters, animals were treated with vehicle (0.1 N HCl neutralized to pH 7.4 with 0.1 N NaOH, $n = 10$) or aminoguanidine (15 mg kg^{-1}, i.v., $n = 10$), and 10 min later the pressor response to noradrenaline (NA, 1 $\mu g\ kg^{-1}$, i.v.) was recorded. At 20 min after injection of vehicle or aminoguanidine, animals received *E. coli* lipopolysaccharide (LPS, 10 mg kg^{-1}, i.v.) as a slow injection over 10 min and pressor responses to NA were reassessed at 30, 60, 120, and 180 min after LPS injection.

In other experiments, the pressor effects elicited by aminoguanidine in sham-operated rats (control) were compared with those in rats treated with LPS for 180 min. The effects of aminoguanidine on the delayed hypotension caused by LPS were also studied. In these experiments, rats were anaesthetized and instrumented (as above) and treated with either vehicle (control, $n = 5$) or LPS (10 mg kg^{-1}, i.v., $n = 8$). At 180 min after injection of vehicle or LPS, two doses of aminoguanidine (15 and 45 mg kg^{-1}, i.v. bolus) were administered in a cumulative manner and the haemodynamic parameters were monitored for a period of 30 min.

Organ bath experiments

At 180 min after the injection of LPS, thoracic aortae were obtained from sham-operated controls as well as from rats pretreated either with vehicle or with aminoguanidine. The vessels were cleared of adhering periadventitial fat and the thoracic aortae were cut into rings of 3–4 mm width. The endothelium was removed by gently rubbing the intimal surface. The lack of a relaxation to acetylcholine (1 μM) following pre-contraction of rings with NA (1 μM) was considered as evidence that the endothelium had been removed. The rings were mounted in 10 ml organ baths filled with warmed (37°C), oxygenated (95% O_2/5% CO_2) Krebs solution (pH 7.4) consisting of (mM): NaCl 118, KCl 4.7, KH_2PO_4 1.2, $MgSO_4$ 1.17, $CaCl_2$ 2.5, $NaHCO_3$ 25 and glucose 5.6. Indomethacin (5.6 μM) was added to prevent the production of prostanoids. Isometric force was measured with Grass FT03 type transducers (Grass Instruments, Quincy, MA, U.S.A.) and recorded on a Grass model 7D polygraph recorder (Grass Instruments, Quincy, MA, U.S.A.). A tension of 2 g was applied and the rings were equilibrated for 60 min, changing the Krebs solution every 15 min (Thiemermann *et al.*, 1993). Dose-response curves to NA (10^{-9}–10^{-6} M) were obtained in all experimental groups.

Nitric oxide synthase assay

Lungs from LPS-rats pretreated with either vehicle (saline, control) or aminoguanidine were removed at 180 min and frozen in liquid nitrogen. Lungs from sham-operated rats were also prepared for determination of baseline NOS activity. Lungs were stored for no more than 2 weeks at −80°C before assay. Frozen lungs were homogenized on ice with an Ultra-Turrax T 25 homogenizer (Janke & Kunkel, IKA Labortechnik, Staufen i. Br., Germany) in a buffer composed of: Tris-HCl 50 mM, EDTA 0.1 mM EGTA 0.1 mM, 2-mercaptoethanol 12 mM and phenylmethylsulphonyl fluoride 1 mM (pH 7.4). Conversion of [^{3}H]-L-arginine to [^{3}H]-L-citrulline was measured in the homogenates as described by Szabo *et al.* (1993). Briefly, tissue homogenates (30 μl, approx. 60 μg protein) were incubated in the presence of [^{3}H]-L-arginine (10 μM, 5 kBq/tube), NADPH (1 mM), calmodulin (30 nM), tetrahydrobiopterin (5 μM) and calcium (2 mM) for 25 min at 25°C in HEPES buffer (pH 7.5). Reactions were stopped by dilution with 1 ml of ice cold HEPES buffer (pH 5.5) containing EGTA (2 mM) and EDTA (2 mM). Reaction mixtures were applied to Dowex 50W (Na^+ form) columns and the eluted [^{3}H]-L-citrulline activity was measured by scintillation counting (Beckman, LS3801; Fullerton, CA, U.S.A.). Experiments performed in the absence of NADPH determined the extent of [^{3}H]-L-citrulline formation independent of a specific NOS activity. Experiments in the presence of NADPH, without calcium and with EGTA (5 mM), determined the calcium-independent iNOS activity.

The effects of aminoguanidine and L-NAME on iNOS activity (lungs from LPS-rats) were compared with those on nNOS activity (brains from control rats). Homogenates of lungs obtained from rats treated with LPS for 180 min and brains obtained from control rats were prepared by the above method. Lung or brain homogenates (20 μl, approximately 40 μg protein) were incubated with either vehicle (control), aminoguanidine or L-NAME (10^{-8} to 10^{-3} M) and the conversion of [^{3}H]-L-arginine to [^{3}H]-L-citrulline was measured as described above.

Protein concentration was measured spectrophotometrically in 96-well plates with Bradford reagent (Bradford, 1976), with bovine serum albumin used as standard.

Survival studies

Survival studies were performed in Swiss albino mice (T/O mice; 28–30 g; Tuck Laboratories, U.K.). LPS (60 mg kg^{-1},

i.p.) was injected in the presence of vehicle or drugs and survival was monitored every 3 h for 24 h. Different groups of animals received vehicle (saline) together with LPS ($n = 12$), LPS plus L-NAME (3 mg kg^{-1} at 2 h and 6 h after LPS, $n = 9$), or LPS plus aminoguanidine (15 mg kg^{-1} at 2 h and 6 h after LPS, $n = 8$). Thus, we chose a dose of aminoguanidine for these survival studies, which did not cause a significant rise in blood pressure in normal rats in order to evaluate whether a dose which has only minor effects on eNOS is sufficient to improve survival. Similarly, a relatively low dose of L-NAME, which causes a moderate, but not dramatic increase in blood pressure in normal rats (Hecker *et al.* 1990), was chosen for comparison.

Materials

Calmodulin, bacterial lipopolysaccharide (*E. coli* serotype 0.127:B8), NADPH, aminoguanidine bicarbonate, acetylcholine chloride, noradrenaline bitartrate, N$^{\omega}$-nitro-L-arginine methyl ester, and Dowex 50W anion exchange resin were obtained from Sigma Chemical Co. (Poole, Dorset). Aminoguanidine, like other guanidine derivatives (see Hasan *et al.* 1993 for chemical structures), contains the guanidino group that is found in the side chain of L-arginine and L-arginine based derivatives. Aminoguanidine was dissolved in 0.1 N HCl and subsequently neutralized (pH 7.4) in 0.1 N NaOH. All other solutions were made in saline or distilled water. L-[2,3,4,5-^{3}H]-arginine hydrochloride was obtained from Amersham (Buckinghamshire). Tetrahydrobiopterin (6R-L-erythro-5,6,7,8-tetrahydrobiopterin) was obtained from Dr B. Schircks Laboratories (Jona, Switzerland).

Statistical evaluation

All values in the figures and text are expressed as mean ± s.e. mean of n observations, where n represents the number of animals or blood vessels studied. A two way analysis of variance (ANOVA) followed by, if appropriate, a Bonferoni's test was used to compare means between groups. A P-value less than 0.05 was considered to be statistically significant.

Results

Aminoguanidine attenuates the cardiovascular changes caused by LPS in the anaesthetized rat

Baseline values for MAP and HR of the vehicle- and aminoguanidine-pretreated animal groups were 115 ± 4 and 112 ± 2 mmHg, and 400 ± 16 and 401 ± 17 beats min^{-1} respectively, and were not significantly different between these groups. Administration of LPS (10 mg kg^{-1}, i.v.) caused a fall in MAP from 115 ± 4 mmHg to 78 ± 6 mmHg ($n = 10$, $P < 0.05$) at 60 min. Thereafter, MAP remained significantly reduced (e.g. at 180 min, MAP was 79 ± 9 mmHg; $n = 10$; Figure 1a). Endotoxaemia for 180 min was not associated with a significant change in HR (Figure 1b). However, LPS-treatment significantly attenuated the pressor responses to NA (1 μg kg^{-1}, i.v.) at 30, 60, 120 and 180 min ($P < 0.05$, $n = 10$; Figure 1c). Administration of aminoguanidine (15 mg kg^{-1}, i.v., 20 min prior to LPS) had no significant effect on MAP or HR (Figure 1). However, LPS-rats that had been pretreated with aminoguanidine maintained significantly higher MAP values when compared with rats treated with LPS alone (Figure 1a). Pretreatment of rats with aminoguanidine did not affect the hyporeactivity to NA seen at 30 min after LPS (Figure 1c). However, at 60, 120 and 180 min after LPS, the pressor responses to NA in LPS-rats treated with aminoguanidine were significantly greater than in rats treated with LPS alone. Most notably, the pressor responses afforded by NA at 180 min in LPS-rats treated with aminoguanidine were not different from the pressor responses caused by NA prior to LPS administration, while they were markedly reduced (at 180 min) in LPS-treated rats (Figure 1c).

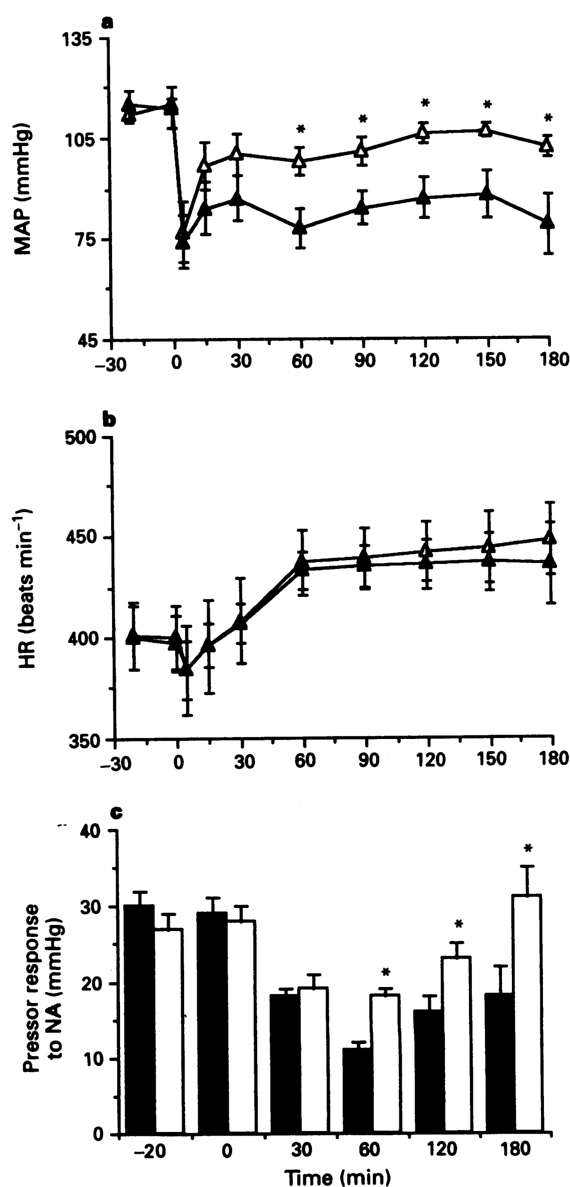

Figure 1 Aminoguanidine ameliorates the delayed hypotension and vascular hyporeactivity to noradrenaline (NA) in endotoxic shock in the anaesthetized rat. Depicted are the changes in (a) mean arterial blood pressure (MAP), (b) heart rate (HR) and (c) pressor responses to NA (1 μg kg^{-1}, i.v.) in rats treated with *E. coli* lipopolysaccharide (LPS, 10 mg kg^{-1}, i.v. at time 0). Different groups of animals were pretreated either with vehicle (saline, ▲ and solid column; $n = 10$) or aminoguanidine (15 mg kg^{-1}, i.v. for 20 min prior to LPS; Δ and open column; $n = 10$). Data are expressed as means ± s.e.mean of n observations. *$P < 0.05$ represents significant differences when compared to control at the same time point.

When injected at 180 min after LPS, aminoguanidine (15 and 45 mg kg^{-1}, i.v.) caused a dose-related rise in blood pressure and, hence, reversed the hypotension elicited by LPS in a dose-dependent manner (Figure 2). Interestingly, the rise in blood pressure caused by aminoguanidine in LPS-treated rats was significantly greater than the one produced in sham-operated controls (Figure 2).

Aminoguanidine attenuates the LPS-induced vascular hyporeactivity to noradrenaline ex vivo

In rat aortic rings obtained from sham-operated rats (control), NA (10^{-9} to 10^{-6} M) caused a dose-dependent increase

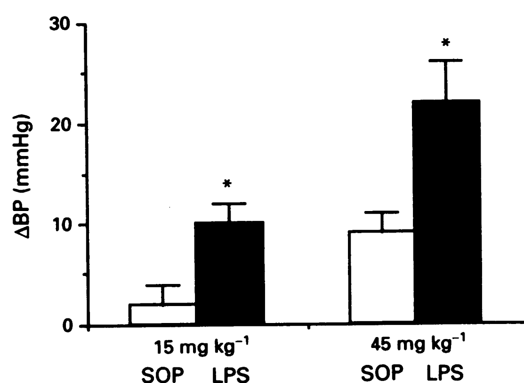

Figure 2 Comparison of the pressor responses elicited by aminoguanidine in sham-operated control rats (SOP) or rats subjected to endotoxic shock. Different groups of animals were subjected to vehicle (SOP, open column; $n = 5$) or *E. coli* lipopolysaccharide (LPS, $10\,mg\,kg^{-1}$; solid column; $n = 8$) for 180 min. Thereafter, two doses of aminoguanidine (15 and $45\,mg\,kg^{-1}$, i.v.) were administered in a cumulative manner. Aminoguanidine caused a slow rise in blood pressure (maximum approx. 30 min). Thus, the data are expressed as change in mean arterial blood pressure (MAP) observed within 30 min. Note that the higher ($45\,mg\,kg^{-1}$), but not the lower dose of aminoguanidine ($15\,mg\,kg^{-1}$) caused a significant rise in MAP in SOP rats. $^*P<0.05$ represents significant differences when compared to SOP-control.

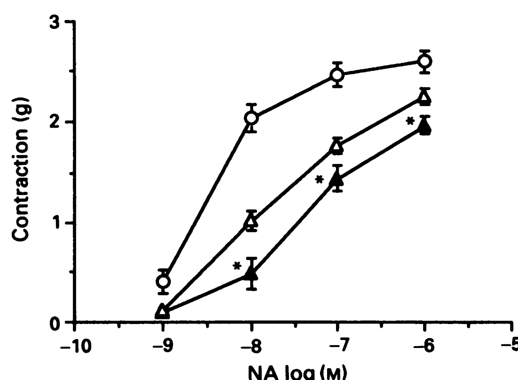

Figure 3 Aminoguanidine attenuates the endotoxin-induced vascular hyporeactivity to noradrenaline (NA) in rat aortic rings *ex vivo*. Depicted are concentration-response curves to NA ($10^{-9}-10^{-6}$ M) in aortic rings without endothelium obtained from sham-operated control rats (○; $n = 6$), from rats treated with lipopolysaccharide (LPS, $10\,mg\,kg^{-1}$, i.v.) for 180 min (▲; $n = 10$) and from LPS-rats pretreated with aminoguanidine ($15\,mg\,kg^{-1}$, i.v. at 20 min prior to LPS; Δ; $n = 10$). Data are expressed as means ± s.e.mean of n observations. Note that the contractions caused by NA after 180 min of LPS in rats pretreated with either vehicle (▲) or aminoguanidine (Δ) were significantly reduced when compared to control rats (○). $^*P<0.05$ represents significant differences between LPS-rats pretreated with vehicle and those pretreated with aminoguanidine.

in vascular tone. The contractions caused by NA were significantly reduced in aortic rings obtained from rats after 180 min of endotoxaemia ($P<0.05$ at $10^{-9}-10^{-6}$ M; Figure 3). In contrast, pretreatment with aminoguanidine of LPS-rats significantly ameliorated the vascular hyporeactivity to NA of rat aortic rings *ex vivo* ($P<0.05$ at 10^{-8} to 10^{-6} M; Figure 3). However, the contractions produced by NA in aortic rings obtained from LPS-rats pretreated with aminoguanidine were still significantly reduced when compared to control responses ($P<0.05$ at $10^{-9}-10^{-6}$ M; Figure 3).

Aminoguanidine inhibits the activity of inducible nitric oxide synthase (iNOS) in endotoxic shock

A small, calcium-independent NOS activity was detectable in lung samples from sham-operated control animals. After 180 min of endotoxaemia, there was a substantial increase in iNOS activity in lung homogenates (Figure 4). However, iNOS activity was significantly reduced (by 44 ± 5%, $P<0.05$) in lung homogenates obtained from animals pretreated with aminoguanidine at 20 min prior to LPS administration.

To elucidate whether aminoguanidine directly inhibits iNOS activity *in vitro*, iNOS activity (in lung homogenates of rats treated with LPS for 180 min) and nNOS activity (in brain homogenates of control animals) were measured after incubation for 25 min with vehicle and increasing concentrations of aminoguanidine (10^{-8} to 10^{-3} M) or L-NAME (10^{-8} to 10^{-3} M). Clearly, aminoguanidine and L-NAME were equipotent and caused a dose-related inhibition of iNOS activity (IC_{50}: 30 ± 12 and 11 ± 6 μM, respectively; $n = 3-6$, $P>0.05$; Figure 5). Interestingly, the inhibition of iNOS activity seen with aminoguanidine was slow in onset and only reached a maximal effect after a lag period of more than 90 min. In contrast, the inhibition of iNOS activity by L-NAME was immediate and reached a maximum within 30 min (Figure 6). Aminoguanidine, however, was a less potent inhibitor than L-NAME of the activity of nNOS (IC_{50}: 140 ± 10 and 0.6 ± 0.1 μM, respectively; $n = 3-6$, $P<0.05$; Figure 5).

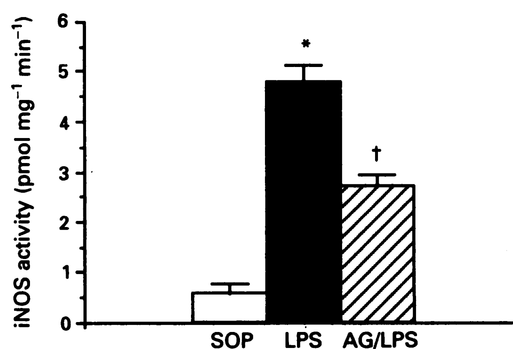

Figure 4 Pretreatment of rats with aminoguanidine inhibits the activity of a calcium-independent (inducible) isoform of nitric oxide synthase (iNOS) in lung homogenates obtained from animals subjected to 180 min of endotoxic shock. Calcium-independent iNOS activity was measured in lung homogenates obtained from sham-operated control rats (SOP, open column, $n = 4$) or rats treated with *E. coli* lipopolysaccharide ($10\,mg\,kg^{-1}$, i.v.) for 180 min. Different groups of LPS-rats were pretreated with vehicle (LPS, solid column, $n = 6$) or aminoguanidine (AG/LPS, hatched column; $15\,mg\,kg^{-1}$, i.v., $n = 6$). Data are expressed as means ± s.e.mean of n observations. $^*P<0.05$ represents a significant increase in iNOS activity when compared to control. $^{\dagger}P<0.05$ represents a significant reduction in iNOS activity when compared to LPS.

Aminoguanidine increases the survival rate of mice treated with LPS

The administration of a high dose ($60\,mg\,kg^{-1}$, i.p.) of LPS to T/O mice was associated with a 24 h survival rate of only 8% of the animals. In contrast, LPS-mice treated with aminoguanidine ($15\,mg\,kg^{-1}$, i.p. at 2 h and 6 h after LPS, $n = 8$) had a survival rate of 75% at 24 h. In contrast to aminoguanidine, treatment of LPS-mice with L-NAME ($3\,mg\,kg^{-1}$, i.p. at 2 h and 6 h after LPS, $n = 9$) did not improve the survival rate (Figure 7).

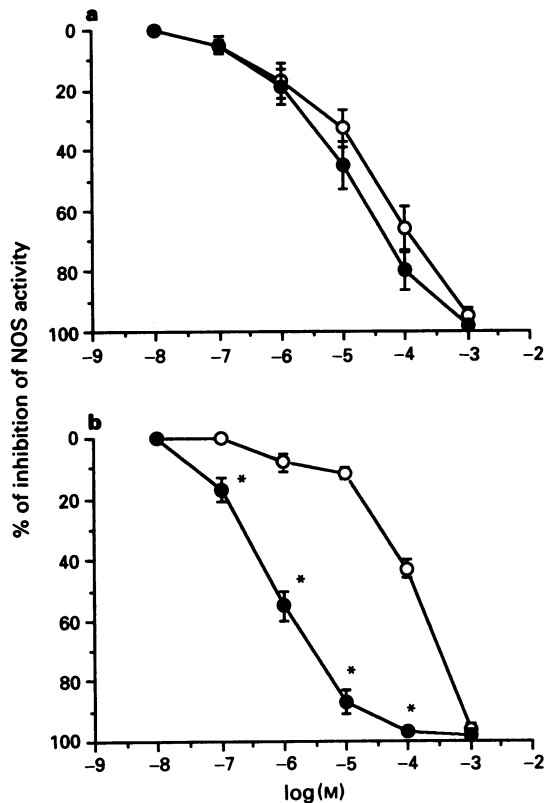

Figure 5 Aminoguanidine and N^{ω}-nitro-L-arginine methyl ester (L-NAME) dose-dependently inhibit (a) the activity of the inducible nitric oxide synthase (iNOS) in lung homogenates from LPS-rats and (b) the activity of constitutive nitric oxide synthase (nNOS) in brain homogenates from normal control rats *in vitro*. Lung homogenates were prepared from rats at 180 min after injection of *E. coli* lipopolysaccharide (10 mg kg^{-1}, i.v.) and the inhibition of iNOS activity was measured in the absence and with increasing concentrations (10^{-7}–10^{-3} M) of aminoguanidine (○) or L-NAME (●). Brain homogenates were prepared from control animals and the inhibition of nNOS activity was measured in the absence and with the same increasing concentrations of aminoguanidine or L-NAME. Data are expressed as means ± s.e.mean of n observations and as percentage inhibition of control iNOS or nNOS activity. $*P<0.05$ represents significant differences between aminoguanidine and L-NAME.

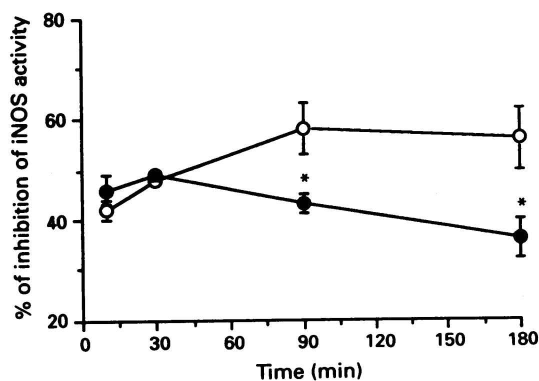

Figure 6 Time course study of inhibition of the activity of inducible nitric oxide synthase (iNOS) by aminoguanidine or N^{ω}-nitro-L-arginine methyl ester (L-NAME) in lungs obtained from rats treated with LPS for 180 min. The IC_{50} of aminoguanidine (○, $n=5$) and L-NAME (●, $n=5$) on inhibiting iNOS activity were added to the lung homogenates then incubated for 10, 30, 90, and 180 min to measure the activity of iNOS. Data are expressed as percentage inhibition of iNOS activity by comparing iNOS activity measured in the different time points to those in the absence of these inhibitors (as control). Values are means ± s.e.mean of n observations. $*P<0.05$ represents significant differences between aminoguanidine and L-NAME at the same time point.

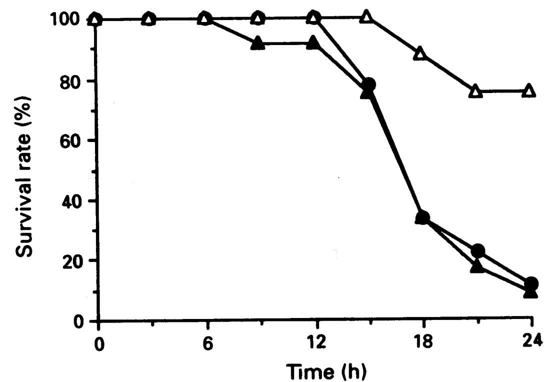

Figure 7 Aminoguanidine improves the survival rate of mice treated with *E. coli* lipopolysaccharide (LPS). T/O mice were injected with LPS (60 mg kg^{-1}, i.p.) then vehicle (saline, $n=12$, ▲), aminoguanidine (15 mg kg^{-1}, i.p., $n=8$, △), or N^{ω}-nitro-L-arginine methyl ester (L-NAME, 3 mg kg^{-1}, i.p., $n=9$, ●) were administered at 2 h and 6 h after LPS, and the survival rate was monitored every 3 h.

Discussion

This study demonstrates that aminoguanidine, a relatively selective inhibitor of the cytokine inducible isoform of nitric oxide synthase attenuates the delayed circulatory failure associated with endotoxic shock in the rat and improves survival in a murine model of endotoxaemia. In particular, we show that aminoguanidine attenuates the delayed fall in blood pressure and the vascular hyporeactivity to NA elicited by prolonged periods of endotoxaemia in the rat. In addition to causing beneficial haemodynamic effects in a rat model of endotoxic shock, aminoguanidine, but not the non-selective NOS inhibitor L-NAME, improves survival in a murine model of severe endotoxaemia.

What, then, is the mechanism by which aminoguanidine affords the beneficial effects on haemodynamics and survival in the animal models of endotoxic shock in this study? Clearly, aminoguanidine attenuates the activity of iNOS in the lung of rats subjected to prolonged periods of endotoxaemia. The observed reduction in iNOS activity is due to an inhibition by aminoguanidine of iNOS activity, since (i) aminoguanidine dose-dependently inhibited iNOS activity in lung homogenates obtained from rats treated with LPS for 180 min *ex vivo* (this study), (ii) aminoguanidine attenuated the reduction of the vasoconstrictor responses to NA (vascular hyporeactivity) in rat aortic rings from rats with endotoxic shock *ex vivo*, and (iii) aminoguanidine also attenuated the vascular hyporeactivity to phenylephrine in rat aortic rings *in vitro* (Joly *et al.*, 1994). When given at 180 min after endotoxin, aminoguanidine also reversed the severe delayed hypotension elicited by endotoxin, which is due to an enhanced formation of NO by iNOS (Wright *et al.*, 1992; Szabo *et al.*, 1993a). Several recent studies suggest that aminoguanidine is a less potent inhibitor of eNOS activity in cultured cells, isolated blood vessels and enzymes preparations *in vitro* (Corbett *et al.*, 1992; Griffiths *et al.*, 1993; Misko *et al.*, 1993; Tilton *et al.*, 1993). The hypothesis that aminoguanidine is a relatively selective inhibitor of iNOS is supported by this study. First, aminoguanidine is equipotent to L-NAME in inhibiting the iNOS activity in lung homogenates obtained from rats treated with endotoxin for 180 min, but is significantly weaker than L-NAME in inhibiting the nNOS activity in brain homogenates obtained from control rats. Second, aminoguanidine is a weaker inhibitor of eNOS than iNOS *in vivo*, for aminoguanidine did not cause an increase in blood pressure in control rats (at 15 mg kg^{-1}), at a dose which caused a significant inhibition of iNOS activity. Third, aminoguanidine did not affect the acute vas-

cular hyporeactivity to NA associated with endotoxic shock, which is due to an enhanced formation of NO by eNOS (Szabo *et al.*, 1993a). These results clearly demonstrate that low doses of aminoguanidine inhibit iNOS activity *in vivo* without causing a significant inhibition of eNOS activity. Interestingly, the inhibition of iNOS activity seen with aminoguanidine was slow in onset and reached a maximum effect only after a prolonged incubation period, while the inhibition of iNOS activity by L-NAME was immediate and reached a maximum within 30 min. Although we demonstrate that aminoguanidine is a less potent inhibitor than L-NAME of the activity of nNOS (brain homogenates; incubation period: 25 min), the time-dependency of this effect was not investigated. One could, therefore, argue that longer incubation periods of aminoguanidine with constitutive NOS preparations (nNOS or eNOS) would reveal a more potent inhibition of eNOS by aminoguanidine. This is, however, unlikely, since incubations of rat aortic rings with aminoguanidine (0.1 mM) for 1 h did not result in any inhibition of endothelium-dependent vasodilatations (to acetylcholine, calcium ionophore, or adenosine triphosphate) and hence, eNOS activity. In contrast, L-NAME (0.1 mM) caused a complete inhibition of endothelium-dependent vasodilatation under these experimental conditions (Joly *et al.*, 1994).

It has been suggested that some of the adverse effects of non-selective inhibitors of NOS observed in animal models of endotoxic shock (see introduction) are due to the inhibition of eNOS activity in the endothelium. Thus, it has been proposed that relatively selective inhibitors of iNOS activity should be superior to non-selective NOS inhibitors in improving survival in animal models of circulatory shock. We demonstrate here that aminoguanidine causes a substantial improvement in 24 h survival rate in mice treated with a high dose of endotoxin. In contrast, L-NAME, at a dose that is lower than that used for aminoguanidine but high enough to produce a rise in MAP in anaesthetized rats (Hecker *et al.*, 1990), did not improve survival in this murine model of severe endotoxaemia. It is tempting to speculate that the improvement in survival seen with aminoguanidine but not L-NAME, is due to the fact that aminoguanidine is a relatively selective inhibitor of iNOS. However, aminoguanidine has many pharmacological properties including inhibition of advanced glycosylation end products formation (Brownlee *et al.*, 1988; Edelstein & Brownlee, 1992); inhibition of histamine metabolism (Bieganski *et al.*, 1983), inhibition of polyamine catabolism (Seiler *et al.*, 1985) and inhibition of catalase (Ou & Wolff, 1993). Thus, it is difficult to conclude that the beneficial effect of aminoguanidine on survival in our murine model of endotoxaemia is solely due to inhibition of iNOS activity. Moreover, we have not investigated the dose-related effects of L-NAME in survival in our murine model of endotoxaemia and hence, cannot exclude that an improvement in survival could be achieved with either higher (more potent inhibition of iNOS activity) or lower doses (reduced side effects) of L-NAME.

Interestingly, aminoguanidine did not completely reverse the vascular hyporeactivity caused by LPS *ex vivo*. In addition, treatment of rat aortic rings with aminoguanidine *in vitro* does not completely restore the vascular hyporeactivity to phenylephrine (Joly *et al.*, 1994). We have recently demonstrated that another mediator, which is not blocked by inhibitors of NOS, may also be involved in the activation of soluble guanylyl cyclase occurring in vascular smooth muscle after LPS exposure and, hence, may also contribute to the vascular hyporeactivity (Wu *et al.*, 1994). It should be pointed out, however, that a partial inhibition of iNOS activity is sufficient to cause a substantial improvement of the haemodynamic alterations caused by endotoxin *in vivo* (Thiemermann *et al.*, 1993; Szabo *et al.*, 1993b,c).

In conclusion, this study demonstrates that aminoguanidine is a relatively selective inhibitor of iNOS *in vitro* and attenuates the delayed circulatory failure associated with endotixic shock in the rat. In addition, we demonstrate that aminoguanidine, when given therapeutically after the onset of endotoxaemia, improves survival in a murine model of severe septic shock. These results suggest that aminoguanidine may be useful in the therapy of patients with circulatory shock, and lends further support to the hypothesis that relatively selective inhibitors of iNOS activity may be superior to non-selective inhibitors of NOS activity, in the therapy of circulatory shock of various aetiologies.

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