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Attenuation of endotoxin-induced multiple organ dysfunction by 1-amino-2-hydroxy-guanidine, a potent inhibitor of inducible nitric oxide synthase

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1 We have investigated the effects of (i) several guanidines on the activity of the inducible isoform of nitric oxide (NO) synthase (iNOS) in murine cultured macrophages and rat aortic vascular smooth muscle cells (RASM); and (ii) 1-amino-2-hydroxy-guanidine, the most potent inhibitor of iNOS activity discovered, on haemodynamics, multiple organ (liver, renal, and pancreas) dysfunction and iNOS activity in rats with endotoxic shock.

2 The synthesized guanidine analogues caused concentration-dependent inhibitions of the increase in nitrite formation caused by lipopolysaccaride (LPS, 1 $\mu g\ ml^{-1}$) in J774.2 macrophages and RASM cells with the following rank order of potency: 1-amino-2-hydroxy-guanidine > 1-amino-2-methyl-guanidine > 1-amino-1-methyl-guanidine > 1-amino-1,2-dimethyl-guanidine. Interestingly, 1-amino-2-hydroxy-guanidine (IC_{50}: J774.2, 68 μM; RASM, 114 μM) was more potent in inhibiting nitrite formation caused by LPS than N^G-methyl-L-arginine, but less potent than aminoethyl-isothiourea.

3 In the anaesthetized rat, LPS caused a fall in mean arterial blood pressure (MAP) from 115 ± 4 mmHg (time 0) to 98 ± 5 mmHg at 2 h ($P < 0.05$, $n = 10$) and 69 ± 5 mmHg at 6 h ($P < 0.05$, $n = 10$). The pressor effect of noradrenaline (NA, 1 mg kg^{-1}, i.v.) was also significantly reduced at 1 to 6 h after LPS (vascular hyporeactivity). Treatment of LPS-rats with 1-amino-2-hydroxy-guanidine (10 mg kg^{-1}, i.v. plus 10 mg $kg^{-1}\ h^{-1}$ starting at 2 h after LPS) prevented the delayed hypotension and vascular hyporeactivity seen in LPS-rats. However, 1-amino-2-hydroxy-guanidine had no effect on either MAP or the pressor effect elicited by NA in rats infused with saline rather than LPS.

4 Endotoxaemia for 6 h caused a significant rise in the serum levels of aspartate or alanine aminotransferase (i.e. GOT or GPT) and bilirubin, and hence, liver dysfunction. Treatment of LPS-rats with 1-amino-2-hydroxy-guanidine significantly attenuated the liver dysfunction caused by LPS ($P < 0.05$, $n = 10$). Injection of LPS also caused a rapid (almost maximal at 2 h) increase in the serum levels of urea and creatinine, and hence, renal dysfunction. This renal dysfunction was not affected by 1-amino-2-hydroxy-guanidine ($P > 0.05$; $n = 10$). Endotoxaemia also caused a dysfunction of pancreas (rise in serum levels of lipase) as well as a metabolic acidosis (falls in P_{CO_2}, HCO_3 and base excess). Both pancreatic dysfunction and metabolic acidosis were largely attenuated by treatment of LPS-rats with 1-amino-2-hydroxy-guanidine. In rats infused with saline rather than LPS, 1-amino-2-hydroxy-guanidine had no effect on liver, renal or pancreatic function ($n = 4$).

5 Endotoxaemia for 6 h resulted in a rise in the serum levels of nitrite (11.0 ± 0.8 μM, $P < 0.01$, $n = 10$), which was significantly reduced by 1-amino-2-hydroxy-guanidine (6.5 ± 0.7 μM, $P < 0.05$, $n = 10$). Endotoxaemia for 6 h was also associated with a significant increase in iNOS activity in lung and liver, which was significantly reduced in lung or liver homogenates obtained from LPS-rats treated with 1-amino-2-hydroxy-guanidine. In addition, endotoxaemia for 6 h resulted in a significant increase in myeloperoxidase activity (MPO), an indicator of neutrophil infiltration, in the liver. Treatment of LPS-rats with 1-amino-2-hydroxy-guanidine did not affect the rise in MPO-activity in the liver caused by endotoxin.

6 Thus, 1-amino-2-hydroxy-guanidine is a potent inhibitor of iNOS activity in macrophages or RASM in culture as well as in rats with endotoxic shock. Inhibition of iNOS activity with 1-amino-2-hydroxy-guanidine prevents the delayed circulatory failure and attenuates the dysfunction of liver, and pancreas, as well as the metabolic acidosis caused by endotoxaemia.

Keywords: Aminoguanidine; guanidines; circulatory shock; endothelial nitric oxide synthase; inducible nitric oxide synthase; lipopolysaccharide; endotoxic shock

Introduction

Nitric oxide (NO) is a potent endogenous vasodilator autacoid produced by NO synthase (NOS) in many mammalian cells. Three different isoforms of NOS have been isolated, sequenced, cloned and expressed. The continuous release of NO by the constitutive NOS in the vascular endothelium (eNOS) dilates blood vessels and, in concert with vasoconstrictors such as catecholamines, regulates blood vessel diameter, organ blood flow and blood pressure. In addition, endothelial NO serves to inhibit the adhesion of platelets and polymorphonuclear cells to the endothelium and, hence, contributes to the antithrombotic properties of the endothelium (Moncada & Higgs, 1993). Activation of macrophages and other cells with endotoxin or proinflammatory cytokines leads to the expression of an 'inducible' isoform of NOS (iNOS), which is not regulated by changes in intracellular calcium ('calcium-independent NOS') and produces large amounts of NO. When

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produced in high local concentrations by iNOS, NO kills bacteria and tumour cells and thus plays an important role in host defence (see Moncada & Higgs, 1993; Thiemermann, 1994).

An enhanced formation of NO following the induction of iNOS has been implicated in the pathogenesis of a number of diseases including circulatory shock of various aetiologies. For instance, an overproduction of NO contributes importantly to the severe, therapy-refractory hypotension and vascular hyporeactivity ('vasoplegia') to vasoconstrictor agents in animal models of endotoxic shock, haemorrhagic shock and the circulatory failure associated with immunotherapy (Szabo & Thiemermann, 1995). There is, however, little information regarding the effects of NOS inhibitors on organ function or the development of multiple organ dysfunction syndrome (MODS) in experimental endotoxaemia. The definition of shock does not expressly include or exclude a MODS, which is defined as the presence of altered organ function in acutely ill patients, such that homeostasis cannot be maintained without intervention. Primary MODs is a direct result of a well-defined insult in which organ dysfunction occurs early and is due directly to the specific insult. In contrast, secondary MODS develops as a consequence of the host response and is identified within the context of the systemic inflammatory response syndrome (SIRS). The progression of shock or SIRS to MODS is associated with an increase in mortality from 25–30% (in the absence of MODS) to 90–100% (see Deitsch, 1992; Bone, 1994 for review).

It has been suggested that with non-selective inhibitors of all isoforms of NOS activity (e.g. N^G-monomethyl-L-arginine, L-NMMA) the concomitant inhibition of eNOS activity in the endothelium causes excessive vasoconstriction and, thus, increases the incidence of organ ischaemia, microvascular thrombosis and mortality. Indeed, high doses of non-selective NOS inhibitors augment (i) the degree of liver injury (Harbrecht *et al.*, 1992), (ii) the hypoperfusion of the splanchnic vascular bed (Hutcheson *et al.*, 1990), (iii) microvascular thrombosis and ischaemia of the kidney (Shultz & Raij, 1992) and (iv) mortality (Wright *et al.*, 1992) in rodent models of endotoxic shock. Thus, the well-documented beneficial haemodynamic effects of non-selective NOS inhibitors may well be due to inhibition of iNOS activity, while the reported adverse effects may be due to inhibition of eNOS activity.

Aminoguanidine, a bifunctional molecule containing the guanido group of L-arginine linked to hydrazine, is a weaker inhibitor of eNOS than iNOS activity in cultured cells, isolated blood vessels and enzyme preparations *in vitro* (Corbett *et al.*, 1992; Misko *et al.*, 1993; Hasan *et al.*, 1993; Griffith *et al.*, 1993; Joly *et al.*, 1994) and attenuates the delayed circulatory failure in rats with endotoxic shock (Wu *et al.*, 1995). Other guanidines (methyl-, dimethyl-) also inhibit NOS activity, but are less potent inhibitors of iNOS activity than aminoguanidine (Hasan *et al.*, 1993).

This study compares the effects of various, newly synthesized, analogues of aminoguanidine with the ones elicited by L-NMMA (a non-selective NOS inhibitor) and aminoethylisothiourea (a relatively selective inhibitor of iNOS activity, Garvey *et al.*, 1994; Szabo *et al.*, 1994; Southan *et al.*, 1995) on the activity of iNOS expressed in macrophages and vascular smooth muscle cells activated with endotoxin and cytokines. Having identified 1-amino-2-hydroxy-guanidine as the guanidine-analogue, which is the most potent inhibitor of iNOS activity, we have elucidated the effects of 1-amino-2-hydroxy-guanidine on (i) circulatory failure (haemodynamics and vascular reactivity to vasoconstrictor agents), (ii) iNOS activity (in lung and liver), (iii) multiple organ (renal, liver and pancreatic) dysfunction, and (iv) metabolic acidosis caused by endotoxin in the anaesthetized rat. In addition, we have investigated the effects of 1-amino-2-hydroxy-guanidine on the accumulation of neutrophils, in the liver of animals with endotoxaemia.

Methods

Synthesis of analogues of aminoguanidine

1-Amino-2-hydroxyguanidine *p*-toluenesulphonate was prepared by reacting equimolar quantities of hydroxylamine with S-methylisothiosemicarbazide *p*-toluenesulphonate in methanol at room temperature for 48 h. Hydroxylamine was prepared in solution (methanol) by neutralising its hydrochloride salt with potassium hydroxide (in salt/ice bath). S-methylisothiosemicarbazide p-toluenesulphonate was prepared by refluxing equimolar amounts of thiosemicrbazide with methyl-*p*-toluenesulphonate in methanol for 18 h and precipitating the product with ether. The methylaminoguanidines were synthesized in the following manner: 1-amino-1,2-dimethyl-guanidine was prepared from 2,3-dimethyl-isothiosemicarbazide and methylamine. 2,3-dimethyl-isothiosemicarbazide was prepared by refluxing equimolar amounts from 2-methyl-thiosemicarbazide with methyl-*p*-toluenesulphonate. 1-Amino-1-methyl-guanidine was prepared by reacting equimolar amounts of 2,3-dimethyl-isothiosemicarbazide with ammonia solution in water at room temperature for 12 h. 1-Amino-2-methyl-guanidine was prepared by reacting equimolar amounts of S-methyl-isothiosemicarbazide with methylamine solution at room temperature for 12 h.

Cell culture

The mouse macrophage cell line J774.2 was cultured in Dulbecco's modified Eagle's medium (DMEM) and rat aortic smooth muscle cells (RASM) were cultured in RPMI medium, both supplemented with L-glutamine (3.5 mM) and 10% foetal calf serum (Szabo *et al.*, 1993). Cells were cultured in 96-well plates with 200 μl culture medium until they reached confluence. To induce iNOS in macrophages, fresh culture medium containing *Escherichia coli* lipopolysaccharide (LPS, 1 μg ml^{-1}, serotyp: 0127:B8) was added. In RASM, iNOS was induced by LPS (10 μg ml^{-1}) and γ-interferon (IFN-γ, 10 u ml^{-1}. Nitrite accumulation in the cell culture medium was measured after 24 h. To assess the effects of various inhibitors on nitrite production, agents were added to the cells either 15 min before or 6 h after LPS.

Measurement of nitrite production

The amount of nitrite, an indicator of NO synthesis, in the serum and in the supernatant of J774.2 or RASM were measured by the Griess reaction (Green *et al.*, 1981) by adding 100 μl of Griess reagent to 100 μl samples of unfiltered serum or supernatant. The optical density at 550 nm (OD_{550}) was measured with a Molecular Devices microplate reader (Richmond, CA, U.S.A.). Nitrite concentrations were calculated by comparison with OD_{550} of standard solution of sodium nitrite prepared in control serum or culture medium.

Cell respiration

Mitochondrial respiration, an indicator of cell viability, was assessed by the mitochondrial-dependent reduction of MTT [3-(4,5 - dimethylthiazol-2-yl) - 2,5-diphenyltetrazolium bromide] to formazan (Mossmann, 1983). Cells in 96-well plates were incubated (37°C) with MTT (0.2 mg ml^{-1} for 60 min). Culture medium was removed by aspiration and cells solubilised in dimethylsulphoxide by measurement of OD_{550} with a Molecular Devices microplate reader (Richmond, CA, U.S.A.) Formazan production was expressed as a percentage of the values obtained from untreated cells.

Measurement of haemodynamic changes

Male Wistar rats (240–320 g; Glaxo Laboratories Ltd., Greenford, Middx.) were anaesthetized with thiopentone sodium (Trapanal; 120 mg kg^{-1}, i.p.). The trachea was cannu-

lated to facilitate respiration and rectal temperature was maintained at 37°C with a homeothermic blanket (BioSciences, Sheerness, Kent, U.K.). The right carotid artery was cannulated and connected to a pressure transducer (P23XL, Spectramed, Statham, Oxnard, CA, 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 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 were given noradrenaline (NA, 1 $\mu g\ kg^{-1}$, i.v.), and 10 min later animals received vehicle for LPS (1 ml kg^{-1}, i.v., saline, $n=12$) or *E. coli* lipopolysaccharide (LPS, 10 mg kg^{-1}, i.v. in 0.3 ml of saline, $n=60$) as a slow injection over 10 min. The pressor responses to NA were reassessed at every hour after LPS injection. At 1 h ($n=5$), 2 h ($n=8$), 3 h ($n=8$), 4 h ($n=8$), 5 h ($n=8$) and 6 h ($n=15$) after LPS, blood was taken to measure the changes in the serum levels of various biochemical marker enzymes of MODS (see below). Animals in the 3 to 6 h groups received a continuous infusion of saline (saline, 0.6 ml $kg^{-1}\ h^{-1}$, $n=41$) until the end of the experiment. In a separate experiment, LPS-rats received a continuous infusion of 1-amino-2-hydroxy-guanidine (10 mg kg^{-1}, i.v. bolus loading dose, followed by a continuous infusion of 10 mg $kg^{-1}\ h^{-1}$ in 0.6 ml $kg^{-1}\ h^{-1}$ saline, $n=8$) starting at 2 h after injection of LPS. All haemodynamic parameters were recorded for a further 4 h period. In addition, two further groups of rats were anaesthetized and instrumented (as above) and treated with continuous infusions of either vehicle (0.6 ml $kg^{-1}\ h^{-1}$ saline, $n=6$) or 1-amino-2-hydroxy-guanidine (dose as above, $n=4$), but were not treated with LPS. These infusions were started at time 2 h and maintained until the end of the experiment (6 h). At 6 h after injection of LPS or vehicle, blood was taken to measure the changes in the serum levels of various biochemical marker enzymes of MODS (see below). At 1 h, 2 h, 3 h, 4 h, 5 h or 6 h after the injection of LPS, 1 ml of blood was collected from a catheter placed in the carotid artery. The blood sample was centrifuged (15,000 r.p.m. for 3 min) to prepare serum for the measurement of nitrite production by the Griess reaction (see above).

Quantification of liver, renal, lung or pancreatic injury

At 1 h, 2 h, 3 h, 4 h, 5 h or 6 h after the injection of LPS, 1.5 ml of blood was collected into a serum gel S/1.3 tube (Sarstedt, Germany) from a catheter placed in the carotid artery. The blood sample was centrifuged (6,000 r.p.m. for 3 min) to prepare serum. All serum samples were analysed within 24 h by a contract laboratory for veterinary, clinical chemistry (Vetlab Services, Sussex, U.K.). The following marker enzymes were measured in the serum as biochemical indicators of MODS:

1 *Liver dysfunction and failure* were assessed by measuring the rises in serum levels of alanine aminotransferase (GPT, a specific marker for hepatic parenchymal injury); aspartate aminotransferase (GOT, a non-specific marker for hepatic parenchymal injury) and bilirubin (a specific marker enzyme for the development of cholestasis, and, more importantly, a specific marker for the development of liver failure, see Hewett & Roth, 1995).

2 *Renal dysfunction and failure* were assessed by measuring the rises in serum 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). Moreover, rises in the serum levels of lipase served as an indicator of *pancreatic injury*. For the quantification of *lung injury*, at time 0 and 15 min, 60 min and 240 min, 100 μl of blood was collected in glass tubes (Bilbate Ltd., Daventry, U.K.) from a catheter placed in the carotid artery for subsequent blood gas analysis. Blood gases were immediately measured by using a Corning 168 pH/Blood Gas Analyser (Corning Ltd., Essex, U.K.). The blood gas analyser directly measures pH, P_{CO_2} and P_{O_2} and calculates bicarbonate (HCO_3^-), total carbon dioxide (tCO_2, which in combination with pH and P_{CO_2}, is useful in distinguishing between metabolic and respiratory disorders) and base excess (BE).

Nitric oxide synthase assay

Lungs and livers from LPS-rats treated with vehicle (control, $n=6$) or 1-amino-2-hydroxy-guanidine ($n=6$) were removed at 6 h after LPS and frozen in liquid nitrogen. Lungs and livers from rats infused with saline ($n=5$) which had not received LPS and from rats treated with LPS for 2 h ($n=5$) were also prepared for determination of iNOS activity. Lungs and livers were stored for no more than 2 weeks at −80°C before assay. Frozen organs were homogenized on ice with an Ultra-Turrax T 25 homogenizer (Janke & Kunkel, IKA Labortechnik, staufen i. Br., Germany) in a buffer composed of (mM): Tris-HCl 50, EDTA 0.1, EGTA 0.1, 2-mercaptoethanol 12 and phenylmethylsulphonyl fluoride 1 (pH 7.4). Conversion of [^{3}H]-L-arginine to [^{3}H]-L-citrulline was measured in the homogenates as described by Thiemermann *et al.* (1993). Briefly, tissue homogenates (30 μl, approx. 60 μg protein) were incubated in the presence of [^{3}H]-L-arginine (10 mM, 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 5 mM EGTA, measured the calcium-independent iNOS activity, which was taken to represent iNOS activity.

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

Assay for liver myeloperoxidase activity

Frozen liver biopsies were thawed and macerated in homogenizing buffer containing 0.5% hexadecylmethylammonium bromide (HTAB) and 10 mM 3-[N-morpholino]propanesulphonic acid (MOPS) (1 ml buffer per 50 mg tissue sample) with an Ultra-Turrax T25 blender (10 s). Homogenates were incubated (60°C for 2 h) in a water bath and then centrifuged (25,000 r.p.m. for 25 min at 4°C) in a Becker-Ultra centrifuge. Protein content of the supernatant was measured spectrophotometrically in 96-well plates with Bradford reagent (Bradford, 1976), with bovine serum albumin used as standard. Liver myeloperoxidase activity was assessed in the heat-inactivated supernatant according to Laight *et al.* (1994). The assay mixture consisted of NaH_2PO_4/Na_2HPO_4 buffer (70 μl 80 mM), tetramethylbenzidine (10 μl 16 mM), H_2O_2 (10 μl 1 mM) and heat-inactivated supernatant (10 μl). After 3 min incubation at room temperature, the reaction was stopped by addition of acetic acid (100 μl 2 M). The absorbance was determined at 650 nm with a Molecular Devices microplate reader (Richmond, CA, U.S.A.). MPO activity isolated from human leukocytes served as standard. MPO activity was expressed as u mg^{-1} protein.

Materials

Calmodulin, bacterial lipopolysaccharide (*E. coli* serotype 0.127:B8), NADPH, noradrenaline bitartrate, MOPS, H_2O_2, Na_2HPO_4, foetal calf serum, L-glutamine, tetramethylbenzidine, NaH_2HPO_4, HTAB, N^G-methyl-L-arginine,

Tris-HCl, EDTA, EGTA, 2-mercaptoethanol, acetic acid, phenylmethylsulphonyl fluoride, HEPES buffer, Bradford reagent, bovine serum albumin and Dowex 50W anion exchange resin were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). S-(2-aminoethyl)isothiourea was purchased from Aldrich (Gillingham, Dorset, U.K.). L-[2,3,4,5-^{3}H]-arginine hydrochloride was obtained from Amersham (Buckinghamshire, U.K.). Tetrahydrobiopterin (6**R**-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 samples studied. A one-way analysis of variance (ANOVA) followed by, if appropriate, a Dunnett's *post hoc* 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.

Results

Inhibition by guanidines of the increase in nitrite formation caused by endotoxin and cytokines in cultured macrophages and vascular smooth muscle cells

Activation of J774.2 macrophages with LPS resulted within 24 h in a significant increase in nitrite in the cell supernatant from 0.9 ± 0.2 μM (control) to 34.8 ± 1.0 μM (n=9). All of the NOS inhibitors tested caused a concentration-dependent inhibition of the increase in nitrite formation caused by LPS with the following rank order of potency (Figure 1a): AE-ITU (IC_{50}: 7 μM; n=9) > 1-amino-2-hydroxy-guanidine (IC_{50}: 68 μM; n=9) > L-NMMA (IC_{50}: 82 μM; n=9) > 1-amino-2-methyl-guanidine (IC_{50}: 135 μM; n=9) > aminoguanidine (IC_{50}: 150 μM; n=9) > 1-amino-1-methyl-guanidine (IC_{50}: 730 μM; n=9) > 1-amino-1,2-dimethyl-guanidine (IC_{50}: > 1000 μM; n=9). The inhibition of nitrite accumulation by these agents (up to a concentration of 1 mM) was not associated with a significant reduction in cell respiration (MTT-assay, data not shown). The inhibition by these NOS inhibitors of the increase in nitrite formation caused by LPS in J774.2 macrophages was still present when the compounds were added at 6 h after administration of LPS to these cells (Figure 1b). Activation of rat vascular smooth muscle cells with LPS plus IFN also caused a significant increase in the concentration of nitrite in the medium from 1.1 ± 0.3 μM (control) to 19.8 ± 1.1 μM (n=9). AE-ITU, L-NMMA and all of the guanidines tested caused concentration-dependent inhibitions of the formation of nitrite by vascular smooth muscle cells activated with LPS plus IFN (Figure 2a). The inhibition of these NOS inhibitors of the increase in nitrite caused by LPS and IFN still occurred when these compounds were added at 6 h after these immunostimulants to the cells (Figure 2b). As in macrophages, the inhibition of nitrite formation by these compounds was not associated with a significant inhibition of cell respiration (data not shown). Although there was some dissimilarity between the relative potencies of the agents used as inhibitors of nitrite formation in activated macrophages or smooth muscle cells, none of the NOS inhibitors tested showed a selective inhibition of nitrite formation in macrophages or smooth muscle cells. Thus, of the guanidines tested, 1-amino-2-hydroxy-guanidine was the most potent inhibitor of nitrite formation in either activated macrophages (IC_{50}: 68 μM) or rat smooth muscle cells (IC_{50}: 114 μM). The potency of 1-amino-2-hydroxy-guanidine as an inhibitor of nitrite formation was similar to that of L-NMMA, but smaller than that of AE-ITU.

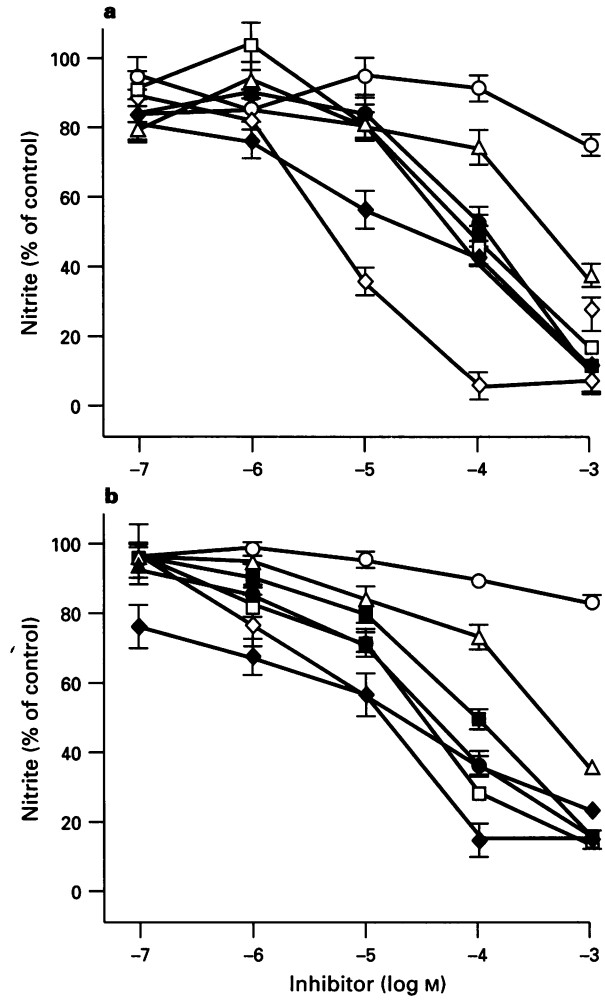

Figure 1 Effects of 1-amino-2-hydroxy-guanidine (◆, n=9), 1-amino-2-methyl-guanidine (■, n=9), 1-amino-1-methyl-guanidine (△, n=9), 1-amino-1,2-dimethyl (○, n=9), aminoguanidine (●, n=9), N^G-monomethyl-L-arginine (L-NMMA; □, n=9) and aminoethyl-isothiourea (◇, n=9) on the accumulation of nitrite in the supernatant of J774.2 macrophages activated with LPS (1 μg ml^{-1}). The above compounds were added either 15 min prior to (a) or 6 h after LPS to the cells (b). Data (nitrite as % of control) are expressed as means ± s.e.mean of n observations.

Attenuation by 1-amino-2-hydroxy-guanidine of the circulatory failure (hypotension and vascular hyporeactivity) caused by endotoxin in vivo

Baseline values for MAP and HR of the animal groups pretreated with either vehicle (control) or 1-amino-2-hydroxy-guanidine were 115 ± 5 and 112 ± 2 mmHg, and 400 ± 23 and 401 ± 12 beats min^{-1}, respectively, and were not significantly different between groups. Administration of LPS (10 mg kg^{-1}, i.v.) caused a rapid fall in MAP from 115 ± 4 mmHg to 78 ± 6 mmHg (n=10, P<0.05) at 15 min and 98 ± 5 mmHg at 120 min (prior to infusion of vehicle or the NOS inhibitor, P<0.05, n=10). After 180 min, there was a continuous further fall in MAP to 69 ± 5 mmHg at 360 min (Figure 3a). The decrease in MAP observed in rats treated with LPS was significantly greater than that observed in vehicle-treated rats, in which the MAP showed a small, gradual decline from 122 ± 4 mmHg (at time 0) to 110 ± 5 mmHg at 360 min after injection of vehicle. There was no significant effect of LPS on heart rate (data not shown).

The mean baseline values for the pressor responses to NA (1 μg kg^{-1}, i.v.) ranged from 29 ± 3 to 34 ± 3 mmHg and were not significantly different between any of the experimental groups studied. Injection of LPS resulted within 60 min in a

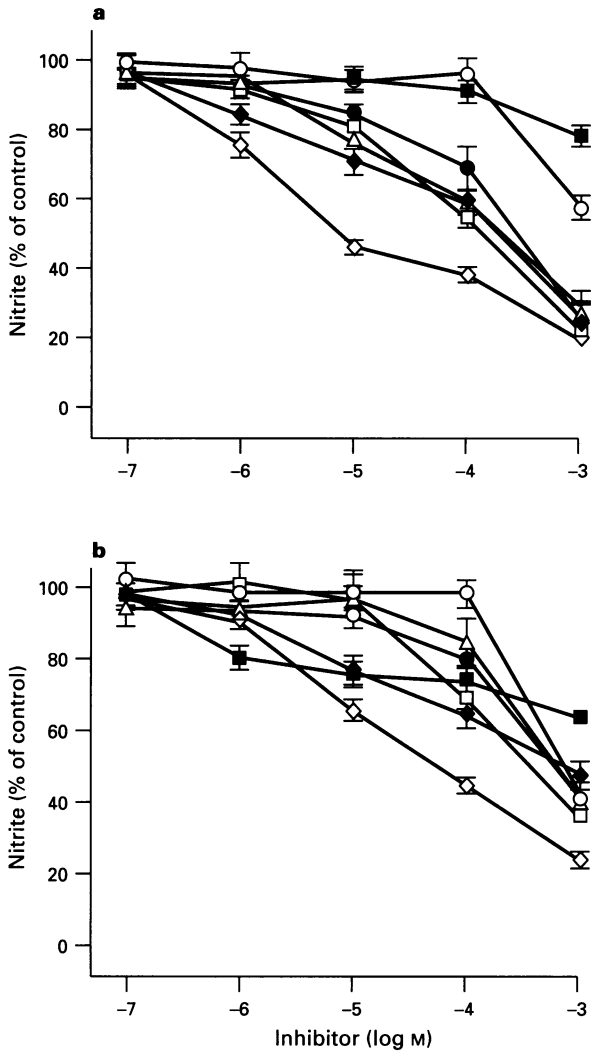

Figure 2 Effects of 1-amino-2-hydroxy-guanidine (◆, $n=9$), 1-amino-2-methyl-guanidine (■, $n=9$), 1-amino-1-methyl-guanidine (△, $n=9$), 1-amino-1,2-dimethyl (○, $n=9$), aminoguanidine (●, $n=9$), N^G-monomethyl-L-arginine (L-NMMA; □, $n=9$) and aminoethyl-isothiourea (◇, $n=9$) on the accumulation of nitrite in the supernatant of rat aortic vascular smooth muscle cells activated with LPS (1 $\mu g\,ml^{-1}$) plus interferon-γ (10 $iu\,ml^{-1}$). The above compounds were added either 15 min prior to (a) or 6 h after LPS to the cells (b). Data (nitrite as % of control) are expressed as means ± s.e.mean of n observations.

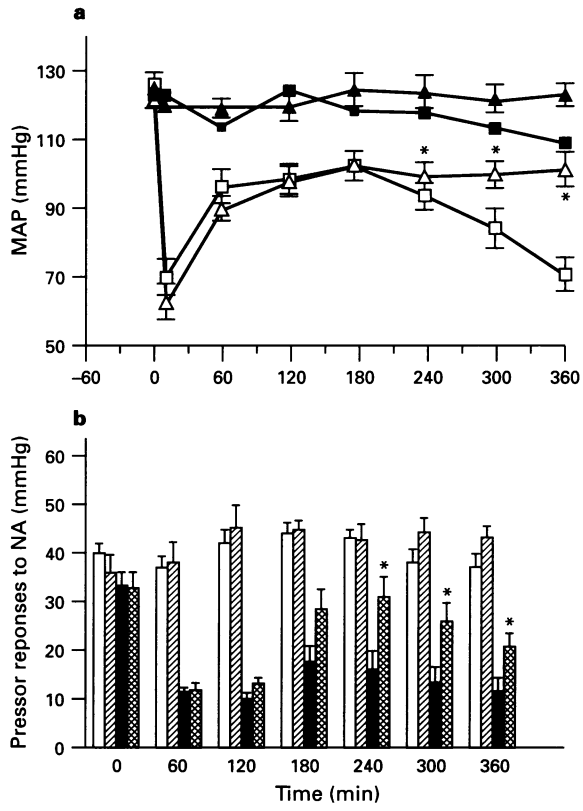

Figure 3 Effect of 1-amino-2-hydroxy-guanidine on the delayed circulatory failure caused by endotoxin in the anaesthetized rat. Depicted are the changes in (a) mean arterial blood pressure (MAP) and (b) the pressor responses to noradrenaline (NA; 1 $\mu g\,kg^{-1}$, i.v.) in rats treated with *E. coli* lipolysaccharide (LPS; 10 $mg\,kg^{-1}$, i.v. at time 0; $n=10$ per group). Different groups of LPS-rats received infusion of vehicle (saline, 0.6 $ml\,kg^{-1}\,h^{-1}$, □ or solid columns, $n=10$) or 1-amino-2-hydroxy-guanidine (10 $mg\,kg^{-1}$ plus 10 $mg\,kg^{-1}\,h^{-1}$, △ or cross-hatched columns, $n=10$) at 2 h after LPS. Separate groups of rats received vehicle rather than LPS ($n=4$ per group) and received infusions of vehicle (saline, 0.6 $ml\,kg^{-1}\,h^{-1}$, ■ or open columns, $n=10$) or 1-amino-2-hydroxy-guanidine (10 $mg\,kg^{-1}$ plus 10 $mg\,kg^{-1}$, ▲ or hatched columns, $n=10$) at 2 h after vehicle for LPS. Data are expressed as mean ± s.e.mean of n observations. *$P<0.05$ represents significant difference when compared to LPS-controls at the same time point.

significant reduction of the pressor response elicited by NA ($P<0.05$ at 60 to 360 min when compared to sham-operated rats treated with vehicle rather than LPS, $n=10$; Figure 3b).

In rats treated with vehicle rather than LPS, infusion of 1-amino-2-hydroxy-guanidine (10 mg $kg^{-1}\,h^{-1}$, starting at 120 min after injection of vehicle for LPS and continued throughout the experiment) had no effect on either MAP (Figure 3a), heart rate (data not shown) or the pressor responses elicited by NA ($n=4$; Figure 3b). In LPS-rats, however, infusion of 1-amino-2-hydroxy-aminoguanidine (10 mg $kg^{-1}\,h^{-1}$) commencing at 120 min after the onset of endotoxaemia prevented the delayed (e.g. after 180 min) fall in MAP observed in LPS-rats treated with vehicle. Thus, the MAP of LPS-rats treated with 1-amino-2-hydroxy-guanidine was significantly higher than in the respective LPS-control group at 240 to 360 min ($P<0.05$, $n=10$; Figure 3a). Injection of LPS also caused an attenuation of the pressor responses to NA from 33 ± 3 mmHg (before LPS) to 9 ± 1 mmHg at 120 min (before infusion of the NOS inhibitor). Treatment of LPS-rats with 1-amino-2-hydroxy-guanidine, however, enhanced the pressor responses afforded by NA (Figure 3b). Thus, the pressor response to NA at 240 to 360 min in LPS-rats treated with 1-amino-2-hydroxy-guanidine was significantly greater than in animals treated with LPS alone ($P<0.05$, $n=10$, Figure 3b).

Time-course of the multiple organ (liver and kidney) dysfunction and the serum nitrite formation caused by endotoxaemia

Injection of endotoxin (10 mg kg^{-1}, i.v.) resulted in a, slow, time-dependent increase in the serum levels of GOT, GPT, bilirubin and serum nitrite (Figure 4). Interestingly, the rise in the serum levels of creatinine and urea were more rapid. Thus, the plasma levels of creatinine were significantly increased at 60 min and the plasma levels of urea at 120 min after LPS (e.g. prior to commencing the infusion of the NOS inhibitor at 120 min).

Effects of 1-amino-2-hydroxy-aminoguanidine on the multiple organ (liver, kidney, pancreas, lung) dysfunction caused by endotoxaemia

Endotoxaemia for 360 min was associated with a significant rise in the plasma activites of the aminotransferases GOT and GPT as well as bilirubin (Figure 5). The rise in the plasma

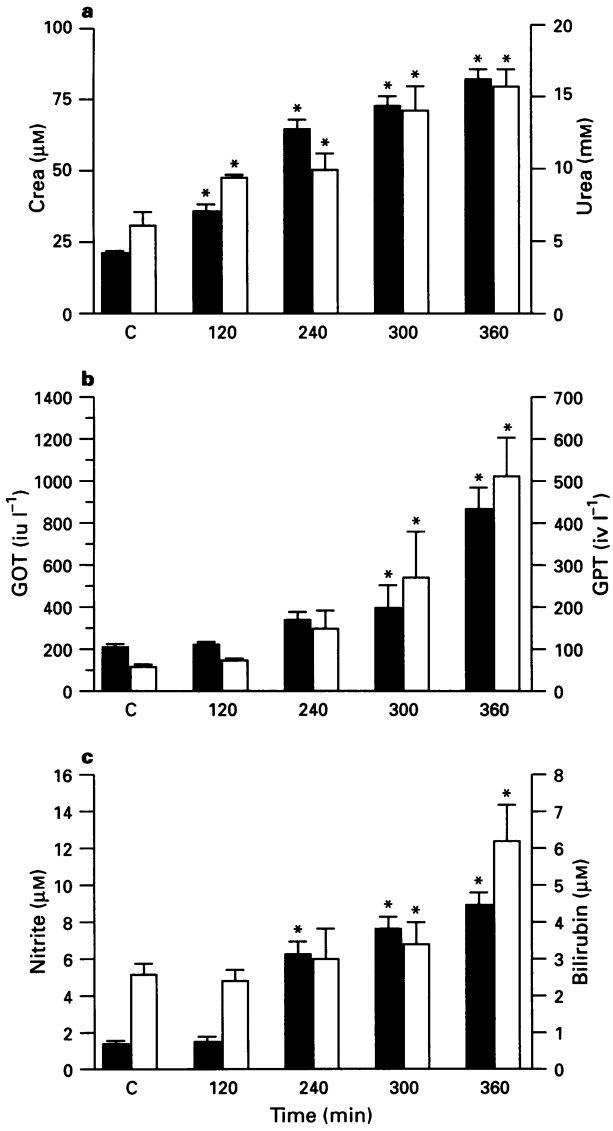

Figure 4 Time-course of the alterations in the serum levels of (a) creatinine (Crea, open columns) and urea (solid columns), (b) aspartate aminotransferase (GOT, solid columns) and alanine aminotransferase (GPT, open columns) and (c) bilirubin (open columns) and nitrite (solid columns). These enzyme activities were measured in serum obtained from rats either before ($n=4$) or at 1 h ($n=5$), 2 h ($n=8$), 4 h ($n=8$), 5 h ($n=8$) or 6 h ($n=10$) after injection of *E. coli* LPS (10 mg kg^{-1}, i.v.). Data are expressed as mean ± s.e.mean of n observations. *$P<0.05$ represents a significant increase in concentration/activity when compared to control (i.e. levels prior to injection of LPS.

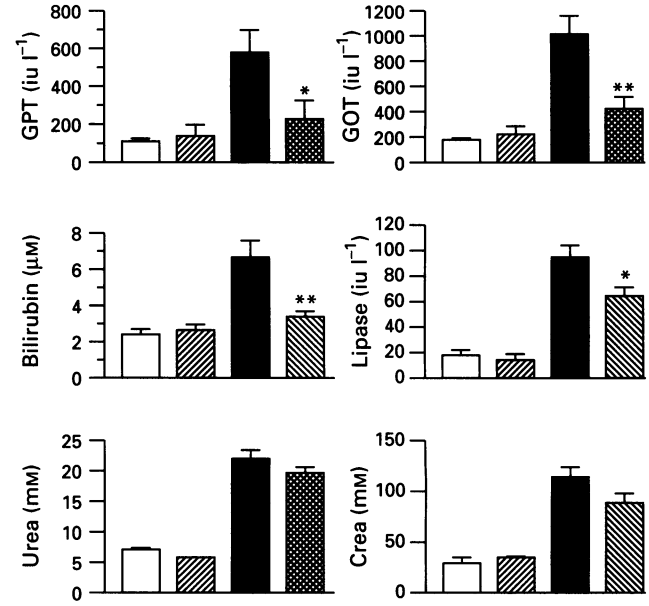

Figure 5 Effects of 1-amino-2-hydroxy-guanidine on the LPS-induced increases in the serum activities of alanine aminotransferase (GPT), aspartate aminotransferase (GPT), bilirubin, urea, creatinine (Crea), and lipase. Enzymes activities were measured in serum obtained from rats treated with vehicle rather than LPS or rats treated with *E. coli* LPS (10 mg kg^{-1}, i.v.) for 6 h. The above enzyme activities were determined in serum obtained at 6 h after injection of vehicle or LPS. Different groups of LPS-rats were infused for 4 h with vehicle (0.6 ml kg^{-1} h^{-1}, solid column, $n=10$) for 1-amino-2-hydroxy-guanidine (10 mg kg^{-1} plus 10 mg kg^{-1} h^{-1}, cross-hatched, $n=10$). Similarly, rats treated with vehicle (0.3 ml of saline) rather than LPS and received infusions of vehicle (0.6 ml kg^{-1} h^{-1}, open column, $n=4$) or 1-amino-2-hydroxy-guanidine (10 mg kg^{-1} plus 10 mg kg^{-1} h^{-1}, hatched column, $n=4$). The infusion of drug or vehicle was started at 2 h after LPS. Data are expressed as mean ± s.e.mean of n observations. *$P<0.05$ represents a significant reduction in concentration/activity when compared to LPS rats.

levels of GOT, GPT and bilirubin was abolished by treatment of LPS-rats with 1-amino-2-hydroxy-guanidine ($P<0.05$, $n=10$, Figure 5). In addition, endotoxaemia for 360 min also caused a significant increase in the plasma levels of urea and creatinine, which were not affected by treatment of LPS-rats with 1-amino-2-hydroxy-guanidine ($P>0.05$, $n=10$). Infusion of 1-amino-2-hydroxy-guanidine, however, attenuated the rise in plasma lipase activity caused by 360 min of endotoxaemia ($P<0.05$, $n=10$, Figure 5). Neither infusion of vehicle nor infusion of 1-amino-2-hydroxy-guanidine had any effect on the plasma levels of GOT, GPT, bilirubin, creatine, urea or lipase (Figure 5, $n=4$ per group).

In addition, injection of LPS caused within 15 min an acute metabolic acidosis as indicated by falls in HCO_3 and base excess (Table 1). This metabolic acidosis was compensated by 180 min by a hyperventilation (increase in respiratory rate, e.g. respiratory compensation; resulting in falls in P_{CO_2}). Treatment of LPS-rats with infusion of 1-amino-2-hydroxy-guanidine significantly attenuated the falls in HCO_3 and base excess as well as the secondary fall in P_{CO_2} ($P<0.05$ at 180 and 360 min, $n=10$, Table 1).

Attenuation by 1-amino-2-hydroxy-guanidine of the rise in plasma nitrite caused by endotoxaemia

The plasma levels of nitrite in rats infused with vehicle rather than LPS was 1.97 ± 0.36 μM ($n=4$) at 360 min. Endotoxaemia for 360 min was associated with a 5.5 fold rise in the plasma levels of nitrite ($P<0.05$, $n=10$, Figure 6). The increase in plasma nitrite caused by endotoxaemia was significantly reduced in LPS-rats treated with 1-amino-2-hydroxy-aminoguanidine ($P<0.01$, $n=10$, Figure 6). In contrast, infusion of 1-amino-2-hydroxy-aminoguanidine had no effect on the levels of plasma nitrite in rats treated with vehicle rather than LPS (Figure 6).

Inhibition by 1-amino-2-hydroxy-guanidine of iNOS activity in the lung and liver of rats with endotoxic shock

A small, calcium-independent iNOS activity was detectable in lung and liver homogenates obtained from animals treated with vehicle rather than LPS (Figure 7). Endotoxaemia for 120 min was associated with a moderate increase in iNOS activity in lung and liver homogenates, whereas LPS for 360 min caused a substantial increase in iNOS activity ($P<0.05$, $n=6$, Figure 7). However, the activity of iNOS was significantly reduced in homogenates of lung or liver obtained from LPS-rats treated with 1-amino-2-hydroxy-guanidine ($P<0.05$, $n=6$, Figure 7).

Table 1 Effects of 1-amino-2-hydroxy-guanidine on pH, Po_2, Pco_2, standard bicarbonate (HCO_3) and base excess (BE), measured in arterial blood obtained from rats treated with vehicle rather than LPS (control, $n=4$), rats treated with *E. coli* lipopolysaccharide (LPS, $n=10$) or LPS-rats with 1-amino-2-hydroxy-guanidine (AHG/LPS, 10 mg kg^{-1} bolus i.v. plus 10 mg kg^{-1}, $n=10$) commencing at 120 min after endotoxin

	pH	*Po₂ (mmHg)*	*Pco₂ (mmHg)*	*HCO₃ (mmol/l⁻¹)*	*BE (mmol/l⁻¹)*
Control					
0 min	7.34 ± 0.01	69.3 ± 1.8	47.5 ± 0.4	28.8 ± 0.6	2.2 ± 0.6
15 min	7.35 ± 0.01	70.2 ± 2.1	47.9 ± 0.3	28.6 ± 0.3	2.1 ± 0.4
60 min	7.35 ± 0.01	70.3 ± 2.6	46.1 ± 0.9	27.5 ± 0.8	1.4 ± 0.7
180 min	7.36 ± 0.01	81.7 ± 3.8	40.9 ± 1.9	26.4 ± 1.1	1.9 ± 0.8
360 min	7.38 ± 0.02	86.9 ± 5.1	41.7 ± 2.5	26.0 ± 1.1	2.1 ± 0.9
LPS					
0 min	7.34 ± 0.01	68.9 ± 1.3	48.8 ± 0.8	29.1 ± 0.7	1.5 ± 0.8
15 min	7.29 ± 0.01	78.7 ± 2.0	46.5 ± 1.4	24.2 ± 1.1	-3.0 ± 1.2
60 min	7.31 ± 0.02	79.8 ± 2.6	42.9 ± 1.9	22.4 ± 1.3	-3.9 ± 1.1
180 min	7.35 ± 0.02	79.9 ± 3.7	34.0 ± 1.3	19.6 ± 1.0	-4.8 ± 1.1
360 min	7.32 ± 0.03	78.8 ± 7.1	32.3 ± 1.8	17.5 ± 1.1	-6.8 ± 1.5
AHG/LPS					
0 min	7.34 ± 0.01	69.4 ± 0.7	51.3 ± 0.2	28.6 ± 0.5	1.5 ± 0.2
15 min	7.29 ± 0.01	76.0 ± 3.2	44.8 ± 1.5	22.6 ± 0.7	-4.0 ± 0.4
60 min	7.30 ± 0.02	73.6 ± 2.8	45.7 ± 1.6	23.0 ± 0.7	-3.7 ± 0.7
180 min	7.34 ± 0.02	76.3 ± 3.2	39.8 ± 2.0*	22.5 ± 0.8*	-2.5 ± 1.0*
360 min	7.38 ± 0.02	75.3 ± 2.3	35.2 ± 1.1*	20.8 ± 0.7*	-3.1 ± 1.0*

Data are expressed as mean ± s.e.mean of n observation. *$P<0.05$ represents significant differences when compared to LPS-controls at the same time point.

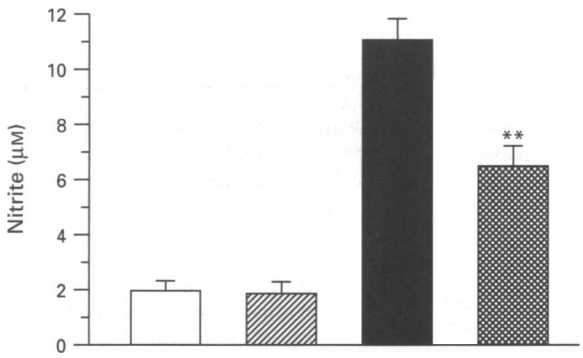

Figure 6 Effect of 1-amino-2-hydroxy-guanidine on the LPS-induced increases in the serum concentrations of nitrite. Nitrite concentrations were measured in serum obtained from rats treated with vehicle rather than LPS or rats treated with *E. coli* LPS (10 mg kg^{-1}, i.v.) for 6 h. The above nitrite concentrations were determined in serum obtained at 6 h after injection of vehicle or LPS. Different groups of LPS-rats were infused for 4 h with vehicle (0.6 ml kg^{-1} h^{-1}, solid column, $n=10$) or 1-amino-2-hydroxy-guanidine (10 mg kg^{-1} plus 10 mg kg^{-1} h^{-1}, cross-hatched column, $n=10$). Similarly, rats treated with vehicle (0.3 ml of saline) rather than LPS and received infusions of vehicle (0.6 ml kg^{-1} h^{-1}, open column, $n=4$) or 1-amino-2-hydroxy-guanidine (10 mg kg^{-1} plus 10 mg kg^{-1} h^{-1}, hatched column, $n=4$). The infusion of drug or vehicle was started at 2 h after LPS. Data are expressed as mean ± s.e.mean of n observations. *$P<0.05$ represents a significant reduction in concentration when compared to LPS-rats.

Treatment with 1-amino-2-hydroxy-guanidine does not affect the accumulation of neutrophils (myeloperoxidase activity) in rats with endotoxic shock

When compared to rats treated with vehicle rather than LPS, endotoxaemia for 360 min resulted in a pronounced increase in MPO-activity in the liver ($P<0.05$, $n=5$, Figure 8). Treatment of LPS-rats with 1-amino-2-hydroxy-guanidine did not affect the rise in MPO-activity caused by LPS in the liver ($P>0.05$, $n=5$, Figure 8).

Discussion

This study demonstrates that the guanidines 1-amino-2-hydroxy-guanidine, 1-amino-2-methyl-guanidine, 1-amino-1-methyl-guanidine and 1-amino-1,2-dimethyl-guanidine inhibit the formation of nitrite, an indicator of NO formation, by cultured macrophages and vascular smooth muscle cells activated with LPS. The inhibition by these guanidines of the nitrite formation by activated cells is due to inhibition of iNOS activity, rather than inhibition of the expression of iNOS, as these guanidines attenuated the formation of nitrite when they were given either prior to or at 6 h after LPS to the cells. Agents which inhibit the induction of iNOS, but not its activity, lose their ability to prevent the formation of nitrite when added at 6 h after LPS to the cells, as the induction of iNOS activity afforded by LPS in macrophages (Szabo *et al.*, 1993) or by LPS plus interferon-γ in vascular smooth muscle cells (Southan *et al.*, 1995) is maximal within 6 h after addition of these stimulants. We demonstrate here that 1-amino-2-hydroxy-guanidine is a more potent inhibitor of iNOS activity in activated macrophages or vascular smooth muscle cells than aminoguanidine or L-NMMA, but less potent than aminoethyl-isothiourea, the most potent and selective inhibitor of iNOS activity known (Garvey *et al.*, 1994; Southan *et al.*, 1995; Thiemermann *et al.*, 1995). In addition, this study demonstrates that 1-amino-2-hydroxy-guanidine attenuated the delayed circulatory failure (hypotension and vascular hyporeactivity to noradrenaline) caused by endotoxaemia in the anaesthetized rat. Moreover, 1-amino-2-hydroxy-guanidine abolished the rises in the serum levels of GOT and GPT (marker enzymes for a hepatic parenchymal injury) and bilirubin (a marker enzyme for cholestasis and, hence, excretory function of the liver) and, therefore, the severe liver dysfunction caused by endotoxaemia. Interestingly, 1-amino-2-hydroxy-guanidine also attenuated the increase in serum lipase activity, suggesting that this NOS inhibitor prevents the pancreatic dysfunction afforded by endotoxaemia.

It has been proposed that with non-selective inhibitors of NOS activity (e.g. L-NMMA) or with relatively selective inhibitors of eNOS activity (e.g. N$^{\omega}$-nitro-L-arginine methyl ester; L-NAME), the concomitant inhibition of eNOS activity in

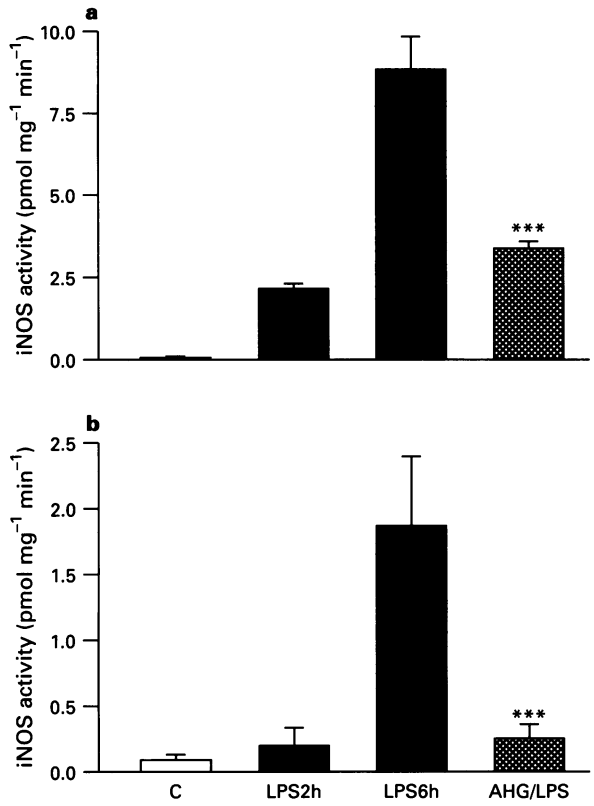

Figure 7 Treatment of rats with 1-amino-2-hydroxy-guanidine attenuates the induction of a calcium-independent iNOS activity in (a) lung and (b) liver homogenates obtained from animals with endotoxaemia. Calcium-independent iNOS activity was measured in lung homogenates obtained from rats infused with vehicle rather than LPS (control, C, open column, $n=4$) or rats treated with *E. coli* LPS ($10\,mg\,kg^{-1}$, i.v.) for 2 h (LPS2h, $n=4$) or 6 h (LPS6h, $n=10$). Different groups of LPS-rats were infused for 4 h with vehicle (saline, $0.6\,ml\,kg^{-1}\,h^{-1}$, solid column, $n=10$) or 1-amino-2-hydroxy-guanidine ($10\,mg\,kg^{-1}$ plus $10\,mg\,kg^{-1}\,h^{-1}$, cross-hatched columns, $n=10$). The infusion of vehicle or drug was started at 2 h after LPS. Data are expressed as mean ± s.e.mean of n observations. $^*P<0.05$ represents a reduction in iNOS activity by 1-amino-2-hydroxy-guanidine when compared to animals treated with LPS alone for 6 h.

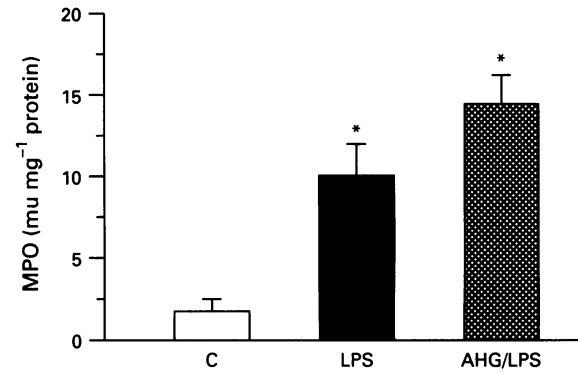

Figure 8 Effects of endotoxaemia and treatment with 1-amino-2-hydroxy-guanidine on the myeloperoxidase-activity (MPO), an indicator of neutrophil accumulation, in the liver of rats. MPO-activity was measured in liver homogenates obtained from rats infused with vehicle rather than LPS or rats treated with *E. coli* LPS ($10\,mg\,kg^{-1}$, i.v.) for 6 h. Different groups of LPS-rats were infused for 4 h with vehicle (saline, $0.6\,ml\,kg^{-1}\,h^{-1}$, solid column, $n=10$) or 1-amino-2-hydroxy-guanidine ($10\,mg\,kg^{-1}$ plus $10\,mg\,kg^{-1}\,h^{-1}$, cross-hatched column, $n=10$). Similarly rats treated with vehicle (0.3 ml of saline) rather than LPS, received infusions of vehicle ($0.6\,ml\,kg^{-1}\,h^{-1}$, open column, $n=4$) or 1-amino-2-hydroxy-guanidine ($10\,mg\,kg^{-1}$ plus $10\,mg\,kg^{-1}\,h^{-1}$, cross-hatched column, $n=4$). The infusion of vehicle or drug was started at 2 h after LPS. Data are expressed as mean ± s.e.mean of n observations. $^*P<0.05$ represents a significant increase in MPO-activity when compared to control.

the endothelium increases the incidence of organ ischaemia, microvascular thrombosis and mortality (Hutcheson *et al.*, 1990; Harbrecht *et al.*, 1992; Shultz & Raij, 1992; Wright *et al.*, 1992). In particular, it has been suggested (Harbrecht *et al.*, 1992) that inhibition of NOS activity may aggravate the hepatocellular injury caused by endotoxaemia. We demonstrate here, however, that 1-amino-2-hydroxy-guanidine attenuates the severe liver dysfunction caused by endotoxaemia in the rat. Treatment of the rats with 1-amino-2-hydroxy-guanidine did not, however, attenuate the renal dysfunction caused by endotoxaemia. This is not entirely surprising, as (i) the observed rises in creatinine and urea are already maximal within 2 h after injection of endotoxin (i.e. prior to starting the infusion of NOS inhibitors) and (ii) inhibition of iNOS activity with aminoethyl-isothiourea also had no effect on the renal dysfunction caused by endotoxin (Thiemermann *et al.*, 1995).

What, then, is the mechanism by which the NOS inhibitor, 1-amino-2-hydroxy-guanidine, reduces the dysfunction of liver and pancreas caused by endotoxaemia? Prolonged periods of endotoxaemia in the rat result in a substantial increase in iNOS activity in the liver (Salter *et al.*, 1991). In rat cultured hepatocytes, LPS, interleukin (IL)-1, tumour-necrosis factor (TNF)-α and interferon-γ synergize to cause an increase in iNOS mRNA and NO synthesis (Geller *et al.*, 1993a). Enhanced formation of NO due to induction of iNOS accounts for the reduction in the synthesis of proteins by hepatocytes (Billiar *et al.*, 1989) as well as the synthesis of prostaglandin E_2 and IL-6 by Kupffer cells (Stadler *et al.*, 1993). Large amounts of NO impair cell function by inhibiting the activity of several key enzymes, such as aconitase (tricarboxylic acid cycle), complex-1 and complex-2 (mitochondrial electron transport) and glyceraldehyde-3-phosphate dehydrogenase (glycolysis) see Morris & Billiar, 1994). The inhibition of these enzymes along with the inhibition of ribonucleotide reductase, a key enzyme in the synthesis of DNA (see Nathan, 1992), may contribute to the liver injury caused by NO following the induction of iNOS in hepatocytes. In addition to causing direct cytotoxic effects, NO may react with superoxide anions to form peroxynitrite, which in turn may contribute to the cytotoxic effects of NO (Stamler *et al.*, 1992). Indeed, the formation of peroxynitrite contributes to the inhibition of mitochondrial respiration in cultured macrophages activated with LPS to cause the induction of iNOS (Szabo & Salzman, 1995). Thus, we propose that the beneficial effects of 1-amino-2-hydroxy-guanidine on the liver dysfunction caused by endotoxaemia are due to the prevention of the above mentioned cytotoxic effects of NO. Indeed, 1-amino-2-hydroxy-guanidine attenuated the metabolic acidosis (falls in HCO_3, Po_2 and base excess) caused by endotoxaemia suggesting that this NOS inhibitor improves the oxygen extraction, possibly by preventing the inhibition of mitochondrial respiration (see above) caused by NO. As in the liver, LPS and cytokines also cause the induction of iNOS 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 iNOS in the pancreas, we speculate that the inhibition by 1-amino-2-hydroxy-guanidine of the pancreatic dysfunction caused by endotoxaemia is secondary to the inhibition of iNOS activity by this guanidine.

The formation of NO by eNOS attenuates the adhesion of polymorphonuclear cells to the endothelium (see Moncada & Higgs, 1993). As endotoxaemia results in a substantial activation of neutrophils resulting in neutropenia, it has been suggested that inhibition of NOS activity may exacerbate the adhesion of neutrophils to the endothelium resulting in microvascular plugging and organ ischaemia (see Thiemermann, 1994). This study demonstrates that endotoxaemia causes an increase in the accumulation of neutrophils within the liver.

However, inhibition of NOS activity with 1-amino-2-hydroxy-guanidine did not cause a further increase in neutrophil accumulation. Our hypothesis that the selective inhibition of iNOS activity does not enhance the degree of neutrophil activation caused by endotoxaemia is supported by the finding that the iNOS-selective NOS inhibitor S-methyl-isothiourea did not augment the degree of neutropenia caused by endotoxin in the rat (Szabo *et al.*, 1994).

In conclusion, this study demonstrates that several guanidines are inhibitors of iNOS activity in cultured macrophages and smooth muscle cells activated with LPS and cytokines. Inhibition of iNOS activity with 1-amino-2-hydroxy-guanidine attenuates the metabolic acidosis as well as the dysfunction of liver and pancreas (but not of the kidney) caused by endotoxaemia in the rat. As human hepatocytes (Geller *et al.*, 1993b; Chartrain *et al.*, 1994) and pancreatic islet cells (Kroncke *et al.*, 1993) also induce iNOS activity to produce large amounts of NO, selective inhibitors of iNOS activity may attenuate the liver and pancreatic dysfunction in patients with endotoxic shock.

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