British Journal of Pharmacology (1996) 119, 1411-1421

Role of nitric oxide in the circulatory failure and organ injury in a rodent model of Gram-positive shock

Ken. M. Kengatharan, Sjef J. De Kimpe & [1]Christoph Thiemermann

The William Harvey Research Institute, St Bartholomew's Hospital Medical College, Charterhouse Square, London, EC1M 6BQ

1 The pathological features of Gram-positive shock can be mimicked by the co-administration of two cell wall components of *Staphylococcus aureus*, namely lipoteichoic acid (LTA) and peptidoglycan (PepG). This is associated with the expression of the inducible isoform of nitric oxide synthase (iNOS) in various organs. We have investigated the effects of dexamethasone (which prevents the expression of iNOS protein) or aminoguanidine (an inhibitor of iNOS activity) on haemodynamics, multiple organ dysfunction syndrome (MODS) as well as iNOS activity elicited by LTA + PepG in anaesthetized rats.

2 Co-administration of LTA (3 mg kg^{-1}, i.v.) and PepG (10 mg kg^{-1}, i.v.) resulted in a significant increase in the plasma levels of tumour necrosis factor-α (TNFα, maximum at 90 min) as well as a biphasic fall in mean arterial blood pressure (MAP) from 120±3 mmHg (time 0) to 77±5 mmHg (at 6 h, $n=8$; $P<0.05$). This hypotension was associated with a significant tachycardia (4-6 h, $P<0.05$) and a reduction of the pressor response elicited by noradrenaline (NA, 1 μg kg^{-1}, i.v., at 1-6 h; $n=8$, $P<0.05$). Furthermore, LTA + PepG caused time-dependent increases in the serum levels of markers of hepatocellular injury, glutamate-pyruvate-transaminase (GPT) and glutamate-oxalacetate-transaminase (GOT). In addition, urea and creatinine (indicators of renal dysfunction) were increased. There was also a fall in arterial oxygen tension (Pa_{O_2}), indicating respiratory dysfunction, and metabolic acidosis as shown by the significant drop in pH, Pa_{CO_2} and HCO_3^-. These effects caused by LTA + PepG were associated with the induction of iNOS activity in aorta, liver, kidney and lungs as well as increases in serum levels of nitrite + nitrate (total nitrite).

3 Pretreatment of rats with dexamethasone (3 mg kg^{-1}, i.p.) at 120 min before LTA + PepG administration significantly attenuated these adverse effects as well as the increases in the plasma levels of TNFα caused by LTA + PepG. The protective effects of dexamethasone were associated with a prevention of the increase in iNOS activity (in aorta, liver, lung, kidney), the expression of iNOS protein (in lungs), as well as in the increase in the plasma levels of total nitrite.

4 Treatment of rats with aminoguanidine (5 mg kg^{-1} + 10 mg kg^{-1} h^{-1}) starting at 120 min after LTA + PepG attenuated most of the adverse effects and gave a significant inhibition of iNOS activity (in various organs) as well as an inhibition of the increase in total plasma nitrite. However, aminoguanidine did not improve renal function although this agent caused a substantial inhibition of NOS activity in the kidney.

5 Thus, an enhanced formation of NO by iNOS importantly contributes to the circulatory failure, hepatocellular injury, respiratory dysfunction and the metabolic acidosis, but not the renal failure, caused by LTA + PepG in the anaesthetized rat.

Keywords: Nitric oxide synthase; lipoteichoic acid; peptidoglycan; multiple organ injury; circulatory failure; septic shock

Introduction

Septic shock can be defined as sepsis with hypotension resulting in impaired tissue perfusion despite adequate fluid resuscitation. The multiple organ by dysfunction syndrome (MODS) which is normally evident in septic shock is characterized by an impaired organ function in acutely ill patients such that homeostasis cannot be maintained without intervention. Despite significant improvements in critical care, septic shock remains the major cause of death in non-coronary intensive care units (see Bone, 1994). The progression of shock to MODS is associated with an increase in the mortality such that with the number of organs failing (from 1-4), mortality progressively increases from 30% (in the absence of MODS) to 100% (Fry *et al.*, 1980; see Deitsch, 1992; Baue, 1993 for review). Traditionally recognised as a consequence of Gram-negative bacteraemia, septic shock is also caused by Gram-positive organisms, fungi and probably viruses and parasites. Although relatively rare in the 1970s, the incidence of Gram-positive septic shock has increased markedly over the past 15 years, and today between 1/3 and 1/2 of all cases of sepsis are caused by Gram-positive organisms (Bone, 1994).

Endotoxin, a component of the outer membrane of Gram-negative bacteria, has been identified as the prime initiator of Gram-negative bacterial septic shock. In contrast to endotoxic shock, we know relatively little about the mechanisms by which Gram-positive bacteria, which lack endotoxin, cause shock and MODS. However, lipoteichoic acid (LTA) from *Staphylococcus aureus* causes the induction of a calcium-independent isoform of nitric oxide (NO) synthase (iNOS) in vascular smooth muscle cells and macrophages *in vitro* and the signal transduction pathway leading to the expression of iNOS by LTA in cultured macrophages (J774.2) involves the activation of tyrosine kinase and the nuclear transcription factor NFκB (Auguet *et al.*, 1992; Cunha *et al.*, 1993; De Kimpe *et al.*, 1995a; Kengatharan *et al.*, 1996a). *Staphylococcus aureus* LTA causes circulatory failure as well the induction of iNOS protein and activity in the rat lung (De Kimpe *et al.*, 1995a). Unlike endotoxin, administration of LTA does not cause MODS or death in the anaesthetized rat suggesting that other components of the cell-wall of Gram-positive bacteria are involved in the pathogenesis of Gram-positive septic shock (De Kimpe *et al.*, 1995b).

Peptidoglycan (PepG), another cell wall component of *Staphylococcus aureus*, causes some of the features of septic shock such as complement-activation and release of tumour necrosis factor-α (TNFα) but does not alone cause a severe circulatory failure, MODS or death in the rat (Bone, 1994; De Kimpe *et al.*, 1995b). Although *Staphylococcus aureus* PepG

[1] Author for correspondence.

does not induce iNOS activity in cultured J774.2 macrophages, PepG synergizes with LTA in causing the expression of iNOS protein and activity in these macrophages (De Kimpe *et al.*, 1995b). Furthermore, when administered into anaesthetized rats, PepG and LTA synergize to cause iNOS expression, circulatory failure and MODS (De Kimpe *et al.*, 1995b).

In rodent models of endotoxic shock, an enhanced formation of NO, particularly by iNOS, contributes to a delayed circulatory failure and (to a lesser extent) MODS (see Thiemermann, 1995; Ruetten *et al.*, 1996). Therefore, this study was designed to evaluate whether an enhanced formation of NO by iNOS contributes importantly to the circulatory failure and MODS caused by the co-administration of LTA plus PepG in the anaesthetized rat. We have, therefore, investigated the effects of (i) dexamethasone, which prevents the induction of iNOS by endotoxin (Radomski *et al.*, 1990) or LTA (Kengatharan *et al.*, 1996a), and (ii) aminoguanidine, a selective inhibitor of iNOS activity (Corbett *et al.*, 1992; Griffiths *et al.*, 1993; Misko *et al.*, 1993) on these adverse effects caused by LTA plus PepG in the anaesthetized rat. Preliminary accounts of this study have been presented to the British Pharmacological Society (Kengatharan *et al.*, 1996b,c).

Methods

Haemodynamic measurements

Male Wistar rats (240–300 g; Glaxo Laboratories Ltd, Greenford, Middlesex) 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, U.K.). The right carotid artery was cannulated and connected to a pressure transducer (Sensnor 840, Horten, Norway) for the measurement of mean arterial blood pressure (MAP) and heart rate (HR) which were recorded throughout the experiment and displayed on a MacLab 8 recording system (AD Instruments, London, UK). The jugular vein was cannulated for the administration of drugs.

Experimental protocol

Upon completion of the surgical procedure, cardiovascular parameters were allowed to stabilize for 30 min. After recording the baseline haemodynamic parameters, animals were injected with a submaximal dose (with respect to the pressor response) of noradrenaline (NA, 1 $\mu g\ kg^{-1}$, i.v., Thiemermann *et al.*, 1993) and 10 min later animals received vehicle (1 ml kg^{-1}, i.v. saline) or LTA (from *Staphylococcus aureus*, DSM 20233; 3 mg kg^{-1} in 0.3 ml saline, i.v.) plus PepG (from *Staphylococcus aureus*, 10 mg kg^{-1} in 0.3 ml saline, i.v.) given as a slow infusion over 30–45 min in order to prevent a rapid fall in MAP. The pressor responses to NA were reassessed at every hour after injection of LTA + PepG by determining the area under the pressor response curve with the chart analysis software contained within the MacLab 8 computer recording system and expressed as mmHg min. As a maximal increase in the plasma levels of TNFα was detected 90 min after injections of LTA + PepG (De Kimpe *et al.*, 1995b), a blood sample (200 μl) was obtained at this time point to measure the levels of TNFα in the plasma. At 1 h, 2 h, 4 h or 6 h after injections of LTA + PepG, blood was taken (in separate animals for each time point) for the measurement of changes in (i) the serum levels of various biochemical markers of MODS, (ii) arterial blood gases (see below), and (iii) the plasma levels of nitrite + nitrate (see below). Subsequent to the collection of one blood sample for the determination of markers of MODS, the animals were killed by an overdose of anaesthetic and the lungs, liver, aorta and kidney were removed for the determination of iNOS activity (see below). In addition, iNOS protein expression was measured in the lungs by Western (immuno) blot analysis (see below).

In separate experiments, animals received either dexamethasone (3 mg kg^{-1} in 0.3 ml saline, i.p.) at 120 min before LTA + PepG or aminoguanidine (5 mg kg^{-1} bolus i.v. plus 10 mg kg^{-1} h^{-1} in 0.4 ml kg^{-1} h^{-1} saline i.v. infusion for the duration of the experiment) at 120 min after the injection of LTA + PepG. Aminoguanidine was given 120 min after LTA + PepG since pretreatment of rats with aminoguanidine (at the same dose) attenuated the expression of iNOS protein in lungs caused by LTA + PepG (see below). Three further groups of rats were anaesthetized and instrumented as described above and treated with saline (0.6 ml per rat), dexamethasone (3 mg kg^{-1}, i.p., 120 min before the injection of saline) or aminoguanidine (5 mg kg^{-1} bolus, i.v., plus 10 mg kg^{-1} h^{-1} in 0.4 ml kg^{-1} h^{-1} saline, i.v. infusion 30 min before saline injection), but were not treated with the bacterial cell wall components (i.e. sham controls). At 6 h after injection of saline, blood was taken for the measurement of (i) serum levels of various biochemical markers of MODS, and (ii) arterial blood gases. In addition, iNOS activity was determined in lungs, liver, kidney and aorta.

Quantification of liver, kidney and lung injury

At 1 h, 2 h, 4 h or 6 h after the injection of LTA + PepG, 1.5 ml of blood was collected into a serum gel S/1.3 tube (Sarstedt, Germany) from the catheter placed in the carotid artery. The blood sample was centrifuged (6,000 r.p.m. for 3 min) to obtain serum. All serum samples were analysed within 24 h by a contract laboratory for veterinary clinical chemistry (Vetlab Services, Sussex, U.K.). The following markers were measured in the serum as a biochemical marker of MODS. (i) Liver injury was assessed by measuring the rises in serum levels of glutamate-pyruvate-transaminase (GPT, a specific marker for hepatic parenchymal injury), glutamate-oxalacetate-transaminase (GOT, a non-specific marker for hepatic parenchymal injury) and bilirubin (a specific marker for the development of liver failure or cholestasis, see Deitsch, 1992; Baue, 1993). (2) Renal dysfunction was assessed by measuring the rises in serum levels of creatinine (an indicator of reduced glomerular filtration rate, and therefore, renal failure) and urea (an indicator of impaired excretory function of the kidney and/or increased catabolism; see Deitsch, 1992; Baue, 1993). (3) For the evaluation of acid base balance and blood gases, 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 measured pH, $PaCO_2$ and PaO_2 and calculates bicarbonate ion (HCO_3^-) concentration.

Measurement of nitric oxide synthase activity

Lung, liver, aorta and kidney were removed at 6 h after LTA + PepG and immediately frozen in liquid nitrogen and stored at −80°C until determination of iNOS activity. Frozen organs/tissues were homogenized on ice with an Ultra-Turrax T 25 homogeniser (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 homogenates as described by Szabo *et al.* (1993a). Briefly, tissue homogenates (30 μl, approx. 100 μg protein) were incubated in the presence of L-arginine/[^{3}H]-L-arginine (10 μM, 7.5 kBq per tube), β-Nicotinamide adenine dinucleotide phosphate NADPH, 1 mM), calmodulin (300 u ml^{-1}), tetrahydrobiopterin (5 μM), L-valine (50 mM) and EDTA (1 mM) for 30 min at 25°C in Tris-buffer (total reaction volume: 100 μl). Reactions were stopped by addition of 1 ml ice-cold HEPES buffer (pH 5.5) containing EGTA (2 mM) and EDTA (2 mM). Reaction mixtures were applied to Dowex 50W (sodium form) containing columns and the eluted [^{3}H]-L-citrulline 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 specific NOS activity. Experiments in the presence of NADPH, without calcium (due to the presence of EDTA), measured the calcium-independent (i.e. induced) NOS 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 as standard.

Measurement of plasma nitrate + nitrite

Nitrate and nitrite are the primary oxidation products of NO reacting with water and, therefore, the nitrate + nitrite (total nitrite) concentration in plasma was used as an indicator of changes in NO production *in vivo* (Green *et al.*, 1981). The blood sample was centrifuged (15,000 r.p.m. for 3 min) to remove cells and particles. Then, the nitrate in the sample was enzymatically converted to nitrite according to a method described by Schmidt *et al.* (1992) before measuring the (total) nitrite content. Briefly, nitrate was stochiometricaly reduced to nitrite by incubation of sample aliquots (10 μl) for 15 min at 37°C, in the presence of nitrate reductase (1 iu ml^{-1}, EC 1.6.6.2), NADPH (500 μM) and Flavine adenine dinucleotide (FAD, 50 μM) in a final volume of 80 μl. When nitrate reduction was complete, the unused NADPH which interferes with the subsequent nitrite determination, was oxidised with lactate dehydrogenase (100 iu ml^{-1}) and sodium pyruvate (100 mM), in a final reaction volume of 100 μl and incubated for 5 min at 37°C. Subsequently, the total nitrite in the plasma was assayed by adding 100 μl of Griess reagent (4% sulphanilamide and 0.2% naphtylenediamide in 10% phosphoric acid) to the 100 μl sample (Green *et al.*, 1981). The difference in optical density between 550 nm and 650 nm was measured with a spectrophotometer (Molecular Devices, Richmond, CA, U.S.A.). Total nitrite concentrations (μM) were calculated by comparison with the optical density of standard solutions of sodium nitrate (also stoichiometrically converted to nitrite) prepared in plasma.

Western (immuno) blot analysis

Lungs were homogenized on ice with an Ultra-Turrax T 25 homogeniser in an extraction buffer (pH 7.4) consisting of Tris-HCl (50 mM), EDTA (10 mM), Triton X-100 (1% v/v), and the protease inhibitors pepstatin A (50 μM), leupeptin (0.2 mM) and phenylmethylsulphonlyfluoride (1 mM). The homogenates were centrifuged (5000 × *g* for 15 min at 4°C) and the supernatant was boiled for 10 min with gel-loading buffer (Tris 20 mM, EDTA 2 mM, sodium dodecyl sulphate (SDS) 2% w/v, glycerol 20% v/v, 2-mercaptoethanol 10% v/v and bromophenol blue 2 mg ml^{-1}, pH = 6.8) in a ratio of 1 : 1 (v/v). The proteins in the samples, together with molecular weight markers (SDS-7B; Sigma), were resolved by one dimensional gel electrophoresis (7.5% SDS gel). After transfer to nitrocellulose by electrophoresis, the membranes were primed overnight at 4°C with a polyclonal antibody raised to macrophage iNOS developed in rabbits (a generous gift from Dr Claire Bryant, William Harvey Research Institute, U.K.). The blots were then incubated as appropriate with anti-rabbit IgG linked to horseradish peroxidase. All antibodies were used at a 1 : 1000 dilution. Subsequently, the Western blots were developed with diaminobenzamine or detected by enhanced chemiluminescence (BioRad, U.K.). The protein bands were later quantified by a densitometer (Model GS-700 Imaging Densitometer, BioRad, Little Chalfont, Buckinghamshire, U.K.).

Measurement of the plasma levels of TNFα

The content of TNFα in the plasma was measured with a mouse TNFα enzyme-linked immunoabsorbant assay (ELISA) kit from Genzyme (Cambridge, MA, U.S.A.) which has already successfully been used to quantitate natural rat TNFα (De Kimpe *et al.*, 1995b). The samples were measured according to the instructions provided with the ELISA kit by the supplier. Briefly, binding of TNFα in the serum samples was detected by a peroxidase-conjugated polyclonal anti-mouse TNFα antibody using tetramethylbenzidine as a substrate. Following acidification (sulphuric acid, 0.5 M final concentration) the absorbance of each well was measured at 450 nm by a 96-well microplate reader (Molecular Devices, Richmond, CA, U.S.A.).

Drugs and materials

Unless otherwise stated all compounds were obtained from Sigma Chemical Co. (Poole, Dorset). L-[2,3,4,5-^{3}H]-arginine hydrochloride was obtained from Amersham (Little Chalfont, Buckinghamshire). Tetrahydrobiopeterin (6**R**-L-erythro-5,6,7,8-tetrahydrobiopterin) was obtained from Dr B. Schircks Laboratoire (Jona, Switzerland). Thiopentone sodium (Intraval sodium) was obtained from Rhone Merieux Ltd. (Harlow, Essex). Nitrate reductase (from *Aspergillus species*) and lactate dehydrogenase (from rabbit muscle) were from Boehringer-Mannheim (Nottingham). *Staphylococcus aureus* PepG was a kind gift from Dr Damon Smith (Therapeutic Antibodies Ltd, London). Aminoguanidine was dissolved in 0.1 N HCl and subsequently neutralized to pH = 7.4 with 0.1 N NaOH. All other drug solutions were made in non-pyrogenic saline (0.9% NaCl; Baxter Healthcare Ltd., Thetford, Norfolk) and care was taken to prevent endotoxin contamination.

Data analysis

All data are presented as mean ± s.e.mean of *n* independent experiments. Statistical analysis was performed by (one- or two-way) analysis of variance (ANOVA). When appropriate, this was followed by Dunnett's test (one-way ANOVA), Bonferoni's test (one-way ANOVA) or Fisher's test (two-way ANOVA) for multiple comparisons of single means. A P value less than 0.05 was considered statistically significant.

Results

Effects of aminoguanidine and dexamethasone on the circulatory failure induced by LTA + PepG

Baseline values for MAP and HR ranged from 113 to 125 mmHg and from 380 to 450 beats min^{-1} ($n = 4-8$), respectively. The parameters were not significantly different between any of the experimental groups studied. Injection of LTA + PepG resulted in a fall in MAP from 122 ± 3 mmHg (at time 0) to 83 ± 5 mmHg at 60 min. Thereafter, MAP recovered to 106 ± 6 mmHg at 120 min. After 120 min, there was a further progressive fall in MAP to 77 ± 5 mmHg at 360 min (Figure 1a). Furthermore, LTA + PepG caused a time-dependent increase in HR (Figure 1b). The mean baseline values for the pressor response to NA (1 μg kg^{-1}, i.v.) ranged from 33 to 39 mmHg min ($n = 4-8$) and were not significantly different between any of the experimental groups studied. LTA + PepG caused a significant reduction in the pressor response to NA (at 60 min to 360 min) (Figure 1c). Measurement of iNOS activity in aortic homogenates revealed a significant ($P < 0.05$) increase in iNOS activity in aortae obtained from rats treated with LTA + PepG compared to saline-treated rats (Figure 2).

Treatment of rats with dexamethasone (3 mg kg^{-1}, i.p.) 120 min before LTA + PepG significantly prevented the acute (at 60 min, $P < 0.05$) as well as the delayed fall in MAP (at 240–360 min, $P < 0.05$) (Figure 1a). Administration of aminoguanidine (5 mg kg^{-1} + 10 mg kg^{-1} h^{-1}, i.v.) commencing at 120 min after the injection of LTA + PepG significantly attenuated the delayed fall in MAP (Figure 1a). The bolus injection of aminoguanidine (5 mg kg^{-1}) administered before the start of the continuous infusion did not cause an immediate increase in MAP (data not shown). In addition, the tachycardia induced by LTA + PepG was significantly prevented by dexamethasone or aminoguanidine ($P < 0.05$ for 180–

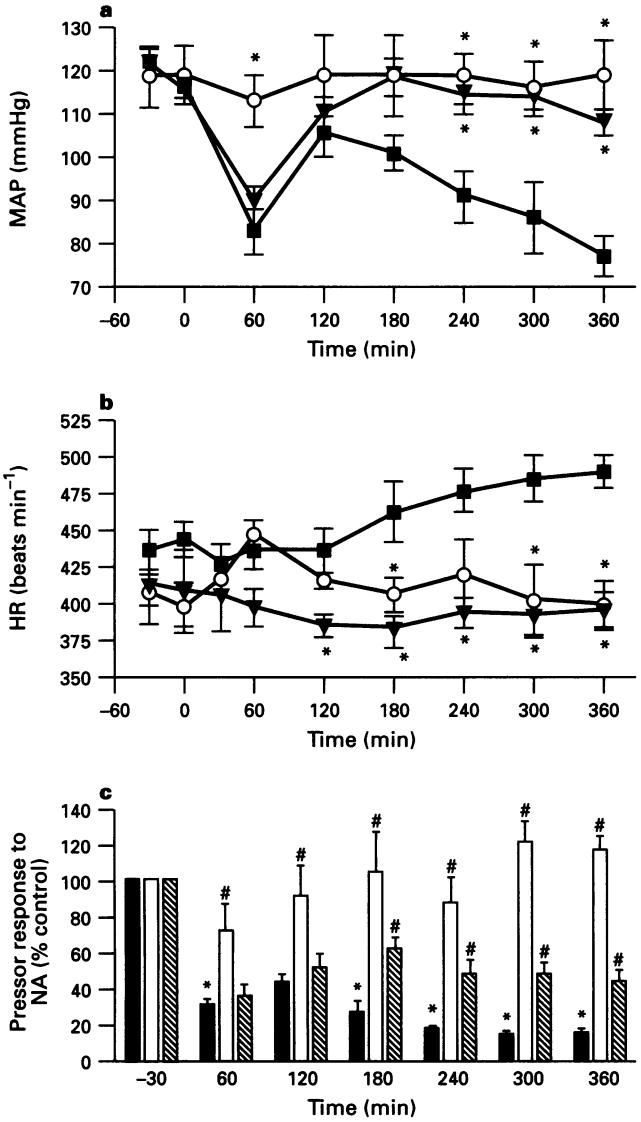

Figure 1 Effects of dexamethasone or aminoguanidine on (a) hypotension (mean arterial pressure), (b) tachycardia (heart rate) and (c) reduced pressor responses to noradrenaline (NA, 1 $\mu g kg^{-1}$, i.v.) elicited by staphylococcal lipoteichoic acid (LTA, 3 $mg kg^{-1}$, i.v., bolus) and peptidoglycan (PepG, 10 $mg kg^{-1}$, i.v. given over 30–45 min). Rats were treated with LTA + PepG (a,b: ■; c: solid columns) or LTA + PepG and dexamethasone (3 $mg kg^{-1}$, i.p., 2 h before LTA + PepG) (a,b: ○; c: open columns), or LTA + PepG and aminoguanidine (5 $mg kg^{-1}$ bolus plus 10 $mg kg^{-1} h^{-1}$ infusion, i.v., 2 h after LTA + PepG) (a,b: ▲; c: hatched columns). Data are expressed as mean ± s.e.mean ($n = 6–8$). (a), (b): $^{*}P < 0.05$ vs. LTA + PepG control. (c): $^{*}P < 0.05$ vs. time 0 and $^{\#}P < 0.05$ vs. LTA + PepG alone.

360 min) (Figure 1b). Dexamethasone and aminoguanidine also inhibited ($P < 0.05$) the development of the vascular hyporeactivity caused by injection of LTA + PepG (Figure 1c). At the end of the experiment, the pressor responses to NA were larger in rats pretreated with dexamethasone than in those treated with aminoguanidine. Also, the increase in iNOS activity at 360 min caused by LTA + PepG in the aorta was significantly ($P < 0.05$) reduced by dexamethasone and aminoguanidine (Figure 2). In saline-treated rats, neither dexamethasone nor aminoguanidine did not affect the MAP, HR, or pressor response to NA (Table 1).

Treatment of rats with LTA + PepG also caused 60% mortality by 360 min compared to 100% survival in the saline-treated animals. Most of the animals died between 4 h and 6 h after administration of LTA + PepG. Dexamethasone and aminoguanidine significantly reduced the mortality induced by

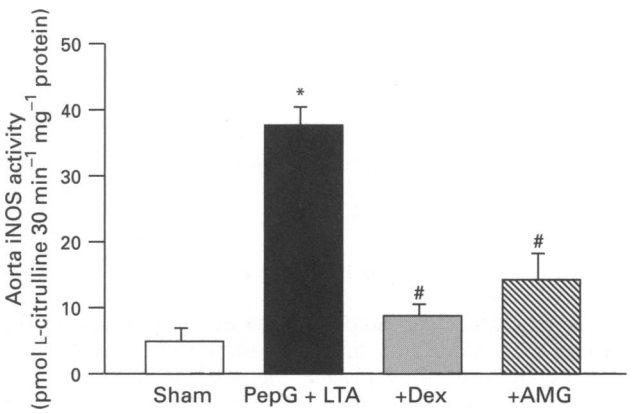

Figure 2 Effects of dexamethasone and aminoguanidine on the increase in the activity of inducible nitric oxide synthase (iNOS, solid columns) caused by staphylococcal lipoteichoic acid (LTA, 3 $mg kg^{-1}$, i.v. bolus) and peptidoglycan (PepG, 10 $mg kg^{-1}$, i.v. given over 30–45 min) in aortic homogenates. The conversion of [^{3}H]-L-arginine to [^{3}H]-L-citrulline was measured in the absence of calcium and in the presence of EGTA (1 mM) in homogenates from aortae obtained from rats 6 h after the injection of LTA + PepG (solid column) or LTA + PepG and dexamethasone (Dex, 3 $mg kg^{-1}$, i.p., 2 h before LTA + PepG, stippled column), or LTA + PepG and aminoguanidine (AMG, 5 $mg kg^{-1}$ bolus plus 10 $mg kg^{-1} h^{-1}$ infusion, i.v., 2 h after LTA + PepG, hatched column). Aortae were obtained from rats 6 h after injection of LTA + PepG or vehicle (sham, open column). Data are expressed as mean ± s.e.mean ($n = 5–8$). $^{*}P < 0.01$ vs. sham-operated animals and $^{\#}P < 0.01$ vs. LTA + PepG alone.

LTA + PepG (100% survival for dexamethasone group and 75% for the aminoguanidine-treated group). Administration of dexamethasone or aminoguanidine to saline-treated rats did not cause death.

Effects of aminoguanidine and dexamethasone on acid-base balance and blood gases and on the expression of iNOS protein and activity in the lung

The baseline values for blood gases (pH, PaO_2, $PaCO_2$, HCO_3^-) were not significantly different in any of the experimental groups compared to the vehicle-treated animals (pH = 7.38 ± 0.02, $PaO_2 = 70 \pm 2$ mmHg, $PaCO_2 = 43 \pm 4$ mmHg, $HCO_3^- = 24 \pm 1$ mmol l^{-1}, $n = 8$). LTA + PepG caused significant falls in PaO_2, pH and HCO_3^- (Figure 3). In animals receiving LTA + PepG, $PaCO_2$ progressively fell throughout the experiment from 46 ± 2 mmHg (baseline) to 39 ± 3 mmHg at 360 min ($P < 0.05$, $n = 8$). However, the observed alterations in $PaCO_2$ were not significantly different from the ones found in vehicle-treated control animals at 360 min (Table 2). Furthermore, LTA + PepG caused a progressive increase in iNOS activity in lung homogenates (Figure 3b). This was associated with a time-dependent increase in the expression of a 130 kDa protein recognised by the iNOS specific-antibody as determined by Western blot analysis (Figure 3c). When expressed as a percentage of the amount of iNOS protein induced by LTA + PepG within 6 h (measured by densitometric analysis of the respective Western blots, densitometry units measured at 6 h were taken as 100%), the expression of iNOS protein at 1 h after LTA + PepG was 0% ($n = 3$), at 2 h was 20 ± 8% ($n = 3$) and at 4 h was 85 ± 12% ($n = 3$).

Treatment of rats with dexamethasone or with aminoguanidine significantly prevented the falls in PaO_2, pH and HCO_3^- caused by LTA + PepG (Figure 3). In addition, the increase in iNOS activity (Figure 3b) induced by LTA + PepG in the lung was significantly prevented by dexamethasone or reduced by aminoguanidine. Dexamethasone, but not aminoguanidine, also prevented the expression of iNOS protein in the lung caused by LTA + PepG (Figure 3c). In contrast, when aminoguanidine was given to the rats 30 min before LTA + PepG

Table 1 Effects of treatment of rats with dexamethasone ($3\,mg\,kg^{-1}$, i.p.) or aminoguanidine ($5\,mg\,kg^{-1} + 10\,mg\,kg^{-1}\,h^{-1}$) on mean arterial blood pressure (MAP), heart rate (HR) and the pressor response to noradrenaline (NA) in rats infused with saline rather than PepG and LTA

		Time (min)			
Group		*0*	*120*	*240*	*360*
		113±3	112±4	112+6	117±3
Sham	MAP (mmHg)	407±18	390±19	401±13	409±8
(saline)	HR (beats min^{-1})	41±4	45±5	50±5	46±4
	NA (mmHg.min)				
		119±5	126±4	120±5	117±7
Dexamethasone	MAP (mmHg)	432±11	418±17	397±19	409±12
	HR (beats min^{-1})	50±5	46±4	45±3	47±7
	NA (mmHg.min)				
		125±9	125±6	122±5	118±3
Aminoguanidine	MAP (mmHg)	409±28	403±13	406±18	399±15
	HR (beats min^{-1})	38±7	33±5	39±4	44±3
	NA (mmHg.min)				

Note that rats which did not receive lipoteichoic acid (LTA) + peptidoglycan (PepG), an injection of dexamethasone of infusion of aminoguanidine did not cause a significant change in MAP, HR or the pressor responses elicited by NA over time. Data are expressed as mean±s.e.mean ($n=4-8$). None of the parameters measured in the dexamethasone or aminoguanidine treated rats were significantly different from the corresponding values in the saline (sham) treated animals ($P>0.05$).

(at the same dosage regimen), a significant reduction in the expression of iNOS protein was observed (65±5% of LTA + PepG control, $P<0.05$, $n=3$ (Figure 3c).

Treatment of animals with only dexamethasone or aminoguanidine (without the subsequent injection of LTA + PepG) did not result in any significant alterations in acid-base balance or blood gases (Table 2).

Effects of aminoguanidine and dexamethasone on liver injury and the induction of iNOS activity caused by LTA + PepG

Treatment of rats with LTA + PepG caused a time-dependent increase in the serum levels of the transaminases GPT (Figure 4a), GOT (Figure 4b) and in total bilirubin (Figure 4c). Thus, substantial increases in GPT, GOT and bilirubin were observed at 240 min after injection of LTA + PepG and at 360 min the serum levels of GPT and GOT levels were about 10 times that observed in vehicle-treated rats. This increase in the serum levels of the transaminases and bilirubin (total) was associated with a time-dependent increase in hepatic iNOS activity (Figure 4d).

Treatment of rats with dexamethasone or aminoguanidine significantly ($P<0.01$) prevented the increase in GPT and GOT (Figure 4a and b). However, dexamethasone, but not aminoguanidine, attenuated the increase in total bilirubin caused by LTA + PepG (Figure 4c). The enhanced iNOS activity in liver homogenates caused by LTA + PepG was reduced by dexamethasone or aminoguanidine (Figure 4d).

Treatment of animals with dexamethasone or aminoguanidine (without the subsequent injection of LTA + PepG) did not affect the serum levels of GPT, GOT or total bilirubin (Table 2).

Effects of aminoguanidine and dexamethasone on the induction of renal dysfunction and iNOS activity caused by LTA + PepG

Treatment of rats with LTA + PepG caused a delayed increase in the serum levels of urea (at 1 h: 7.5±0.9, 2 h: 9.4±0.5, 4 h: 15±0.6, and at 6 h: 17±10 $\mu mol\ l^{-1}$, $n=4$ at each time point) and creatinine (at 1 h: 42±3, 2 h: 45±10, 4 h: 58±5, and at 6 h: 65±2 $\mu mol\ l^{-1}$, $n=4$ at each time point) (Figure 5a). At 360 min, the serum levels of urea and creatinine were approximately 3 times that observed in vehicle-treated rats. This increase in the serum levels of urea and creatinine was associated with a significant increase in iNOS activity measured in kidney homogenates at 360 min (Figure 5b).

Although, dexamethasone and aminoguanidine both attenuated the iNOS activity in kidney homogenates to a similar degree (Figure 5b), dexamethasone, but not aminoguanidine, prevented the increase in the serum levels of urea and creatinine (Figure 5a).

Dexamethasone and aminoguanidine did not influence the serum levels of urea in rats treated with saline rather than LTA + PepG (Table 2).

Effects of aminoguanidine and dexamethasone on the maximal rise in plasma TNFα levels and on the increase in total plasma nitrite levels caused by LTA + PepG

The plasma levels of TNFα were below the detection limit ($35\ pg\ ml^{-1}$) in serum obtained from rats before the injection of LTA + PepG. At 90 min after LTA + PepG, there was a significant increase in the plasma levels of TNFα (38±4 $ng\ ml^{-1}$, $n=8$). Treatment of rats with dexamethasone significantly prevented the rise in TNFα caused by LTA + PepG (9.5±2.3 $ng\ ml^{-1}$, $n=5$, $P<0.05$ compared to LTA + PepG control). In the rats receiving aminoguanidine 120 min after LTA + PepG (i.e. delayed treatment), the TNFα levels at 90 min (32.3±2 $ng\ ml^{-1}$, $n=5$) were not significantly different from the TNFα levels in animals receiving LTA + PepG only. When aminoguanidine was given to the rats at 30 min before LTA + PepG (pretreatment), the TNFα levels at 90 min were also not significantly different from the ones measured in animals given LTA + PepG only at the same time point (33±1 $ng\ ml^{-1}$, $n=5$).

Injection of rats with LTA + PepG resulted in a time-dependent increase in the plasma concentration of nitrite + nitrate (Figure 6). The nitrite + nitrate levels increased significantly at 240 min after injections of LTA + PepG and reached 8 times the plasma nitrite levels compared to the sham-operated rats at 360 min (Figure 6). This increase was significantly inhibited by pretreatment of animals with dexamethasone or delayed treatment with aminoguanidine (Figure 6). In the group of animals receiving aminoguanidine, the plasma level of nitrite + nitrate at 120 min (i.e. before administration of this iNOS inhibitor, 64±4 μM, $n=6$) was not significantly different from the ones measured at 120 min in rats which had received LTA + PepG only. The baseline plasma levels of nitrite + nitrate were not significantly influenced by dexamethasone or aminoguanidine in rats treated with saline rather than LTA + PepG (Table 2).

Discussion

This study demonstrates that treatment of rats with either dexamethasone or aminoguanidine attenuated the circulatory failure (hypotension, tachycardia, vascular hyporeactivity to noradrenaline) caused by co-administration of *Staphylococcus aureus* LTA and PepG. In addition, dexamethasone and aminoguanidine both attenuated the LTA + PepG induced respiratory dysfunction, as indicated by a fall in Pa_{O_2}, a rises in the serum levels of GOT (10 fold) and GPT (1 which are biochemical markers of hepatocellular inj various species including the rat (see Baue, 1993).

What, then, is the mechanism by which dexamethas aminoguanidine exert these beneficial effects on hae namics, MODS and acid/base balance in this model of positive shock? Dexamethasone prevents the expressi

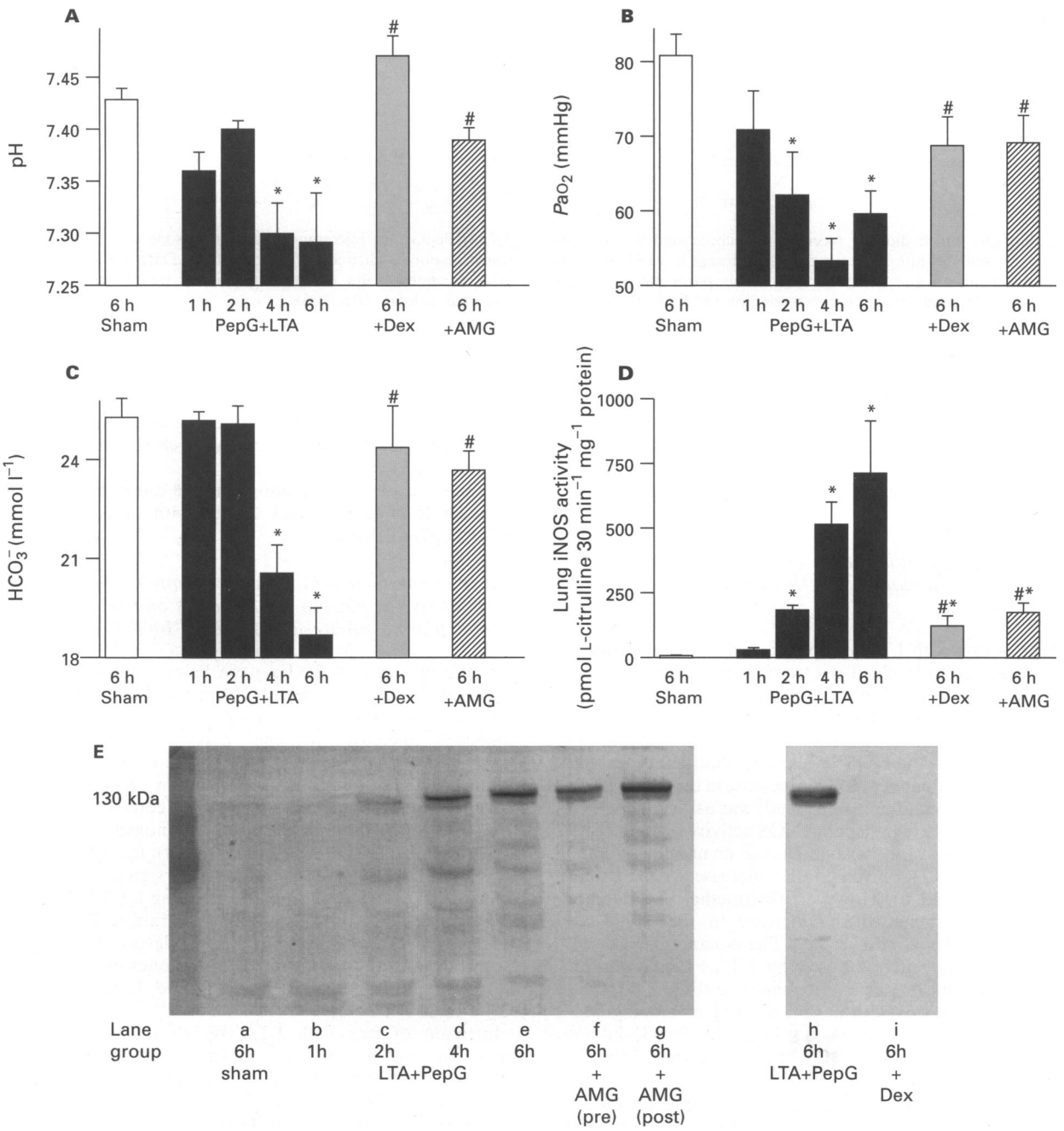

Figure 3 Staphylococcal lipoteichoic acid (LTA) and peptidoglycan (PepG) cause a time-dependent (A,B: solid columns) (A) fall ir pH, (B) fall in arterial oxygen tension (Pa_{O_2}), (C) bicarbonate ion (HCO_3^-) concentration, (D) increase in the activity of inducibl nitric oxide synthase (iNOS), and (E) expression of iNOS protein in lung homogenates (lanes b–e, h). The fall in Pa_{O_2} and th induction of iNOS activity caused by LTA (3 mg kg^{-1}, i.v., bolus) and PepG (10 mg kg^{-1}, i.v. given over 30–45 min) was inhibitec by dexamethasone (Dex, 3 mg kg^{-1}, i.p., 2 h before LTA + PepG, A,B: stippled columns), or by aminoguanidine (AMG, 5 mg kg^{-} bolus plus 10 mg kg^{-1} h^{-1} infusion, i.v., 2 h after LTA + PepG, A,B: hatched columns). Arterial blood samples and lungs were obtained from rats at respective time points after injection of LTA + PepG or vehicle (sham, open columns). Western (immuno) blo analysis of lung homogenates with an iNOS specific antibody revealed a time-dependent expression of iNOS protein induced by LTA + PepG (lanes b–e, h) which was inhibited by dexamethasone (Dex, lane i), but not by aminoguanidine (AMG-post, lane g) However, in lung homogenates obtained from rats given aminoguanidine (at the same dose) 30 min before LTA + PepG, there was a significant reduction in iNOS protein expression (AMG-pre, lane f). Data are expressed as mean ± s.e.mean ($n = 5–8$) for Pa_{O_2} and iNOS activity. This immunoblot is representative of 4 separate experiments. $^*P < 0.05$ vs. sham-operated animals (open columns and $^\#P < 0.05$ vs. LTA + PepG alone at 6 h.

iNOS protein and activity caused by LTA in cultured macrophages (Kengatharan *et al.*, 1996a) and in the rat (De Kimpe *et al.*, 1995a). Here we demonstrate that dexamethasone prevented the expression of iNOS activity in homogenates of aorta, liver, lung and kidney; and of iNOS protein in the lung of rats challenged with LTA + PepG. Moreover, dexamethasone prevented the increase in the plasma levels of nitrite + nitrate caused by co-injection of these cell-wall components of Gram-positive bacteria. We, therefore, propose than an enhanced formation of NO by iNOS also contributes to the circulatory failure, respiratory dysfunction, liver injury, and metabolic acidosis caused by LTA + PepG in the anaesthetized rat. This hypothesis is based on the findings that (i) the hypotension and vascular hyporeactivity to noradrenaline

Table 2 Effects of vehicle (0.6 ml per rat), aminoguanidine (5 mg kg^{-1} + 10 mg kg^{-1} h^{-1},i.v.) or dexamethasone (3 mg kg^{-1},i.p.) on (i) acid-base balance and blood gasses (ii) serum levels of biochemical markers of liver injury and renal failure and, (iii) on the plasma levels of nitrite + nitrate in rats treated with saline rather than LTA + PepG

		Experimental group	
Parameter	*Saline*	*Aminoguanidine*	*Dexamethasone*
GPT (iu l^{-1})	63 ± 6	53 ± 3	59 ± 11
GOT (iu l^{-1})	221 ± 20	204 ± 30	250 ± 60
Bilirubin (μmol l^{-1})	4 ± 0.5	3.1 ± 0.4	2.5 ± 0.8
Urea (mmol l^{-1})	6 ± 1	5 ± 1	6 ± 1
Creatinine (μmol l^{-1})	35 ± 4	18 ± 2*	16 ± 3*
Nitrate + nitrite (μM)	63 ± 3	50 ± 4	43 ± 3
pH	7.43 ± 0.01	7.39 ± 0.02	7.38 ± 0.02
Pa_{O_2} (mmHg)	81 ± 3	79 ± 4	84 ± 6
Pa_{CO_2} (mmHg)	38.0 ± 0.9	41.3 ± 2.6	43.3 ± 1.8
HCO_3^- (mmol L^{-1})	25.3 ± 0.6	22.6 ± 0.7	22.4 ± 0.4

The above parameters were measured 360 min after injection of vehicle or lipoteichoic acid (LTA) + peptidoglycan (PepG). For abbreviations, see text. Data are expressed as mean ± s.e.mean (n = 4–8). *P < 0.05 vs saline-treated animals.

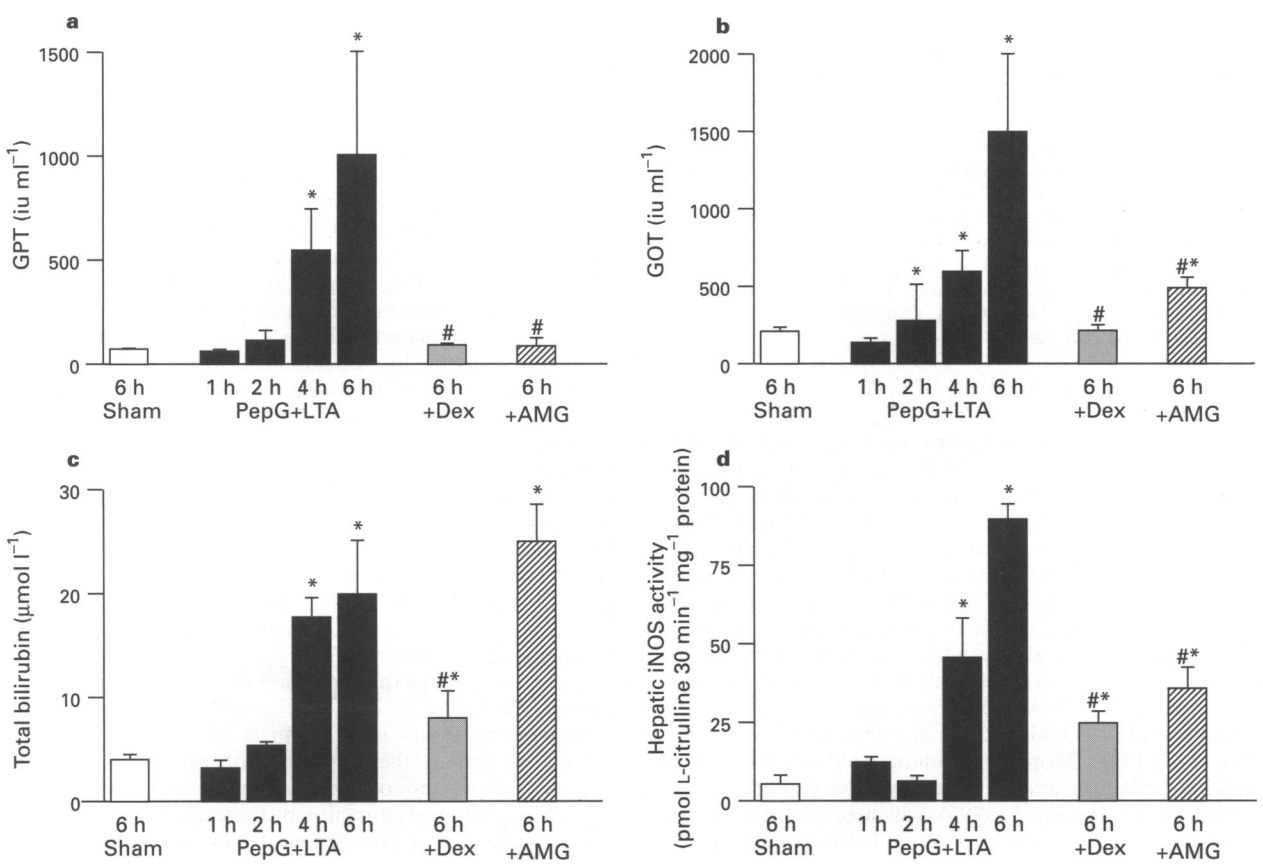

Figure 4 Staphylococcal lipoteichoic acid (LTA) and peptidoglycan (PepG) induce a time-dependent increase in the serum levels of (a) glutamate-pyruvate-transaminase (GPT), glutamate-oxalacetate-transaminase (GOT), and (c) total bilirubin as well as (d) an increase in inducible nitric oxide synthase activity in liver homogenates (iNOS) (a,b: solid columns). The increase in the serum levels of GPT, GOT and the induction of iNOS activity caused by LTA (3 mg kg^{-1}, i.v., bolus) and PepG (10 mg kg^{-1}, i.v. given over 30–45 min) was reduced by dexamethasone (Dex, 3 mg kg^{-1}, i.p., 2 h before LTA + PepG, a,b: stippled columns), or by aminoguanidine (AMG, 5 mg kg^{-1} bolus plus 10 mg kg^{-1} h^{-1} infusion, i.v., 2 h after LTA + PepG, a,b: hatched columns). In contrast, dexamethasone, but not aminoguanidine, attenuated the rise in the serum levels of bilirubin caused by LTA + PepG. GPT, GOT and bilirubin levels were measured in serum samples obtained from rats at respective time points after injection of LTA + PepG or vehicle (sham, open columns). Data are expressed as mean ± s.e.mean (n = 5–8). *P < 0.05 vs. sham-operated animals and $^{\#}P$ < 0.05 vs. LTA + PepG alone at 6 h.

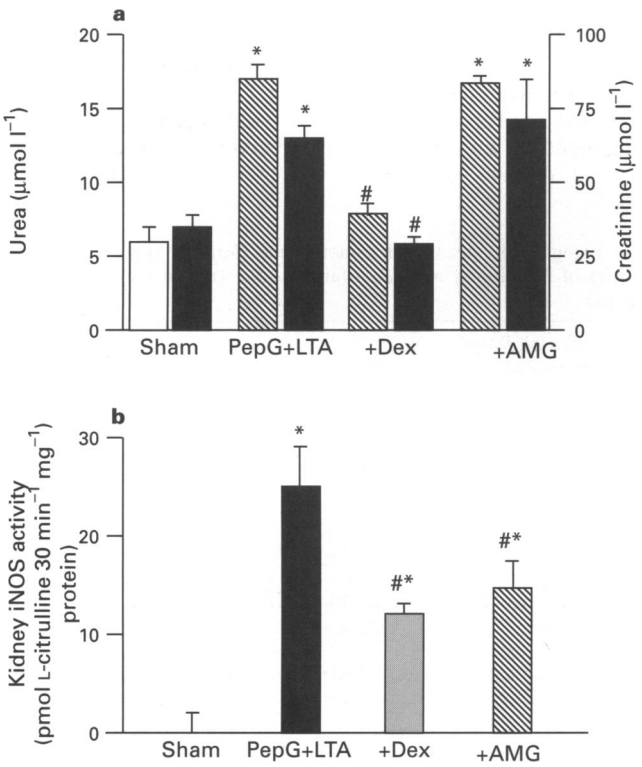

Figure 5 Effects of dexamethasone and aminoguanidine on the increase in (a) serum levels of urea (hatched columns) and creatinine (solid columns) as well as (b) inducible nitric oxide synthase activity (iNOS; solid column) in kidney homogenates caused by staphylococcal lipoteichoic acid (LTA, 3 mg kg^{-1}, i.v., bolus) and peptidoglycan (PepG, 10 mg kg^{-1}, i.v. given over 30–45 min). Dexamethasone (Dex, 3 mg kg^{-1}, i.p., 2 h before LTA + PepG; b, stippled column), but not aminoguanidine (AMG, 5 mg kg^{-1} bolus plus 10 mg kg^{-1} h^{-1} infusion, i.v., 2 h after LTA + PepG, hatched column), prevented the rise in serum levels of urea and creatinine observed at 6 h after the injection of LTA + PepG (a: solid columns). The increase in iNOS activity induced by LTA + PepG was inhibited by dexamethasone and aminoguanidine. Data are expressed as mean ± s.e.mean (n = 5–8). *P < 0.05 vs. sham-operated animals and $^{\#}P$ < 0.05 vs. LTA + PepG alone.

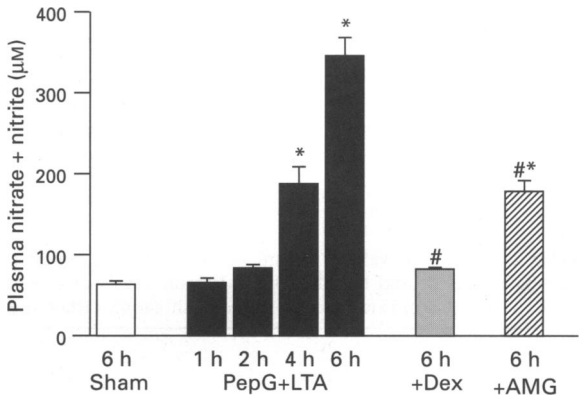

Figure 6 Staphylococcal lipoteichoic acid (LTA) and peptidoglycan (PepG) induce a time-dependent increase in plasma levels in nitrite + nitrate (solid columns). This increase nitrite + nitrate caused by LTA (3 mg kg^{-1}, i.v., bolus) and PepG (10 mg kg^{-1}, i.v. given over 30–45 min) was inhibited by dexamethasone (Dex, 3 mg kg^{-1}, i.v., 2 h before LTA + PepG, stippled column), or by aminoguanidine (AMG, 5 mg kg^{-1} bolus plus 10 mg kg^{-1} h^{-1} infusion, i.v., 2 h after LTA + PepG, hatched column). The levels of nitrite + nitrate in plasma obtained from rats at respective time points after the injection of LTA + PepG or vehicle (sham, open columns) were measured by use of the modified Griess method. Data are expressed as mean ± s.e.mean (n = 5–8). *P < 0.05 vs. sham-operated animals (open columns) and $^{\#}P$ < 0.05 vs. LTA + PepG alone at 6 h.

(circulatory failure) were associated with increases in total plasma nitrite and induction of iNOS activity in the aorta, (ii) the development of liver injury and lung dysfunction was associated with time-dependent increases in iNOS activity in these organs, and (iii) prevention of iNOS induction with dexamethasone attenuated these adverse effects. In addition, this hypothesis is also strongly supported by our finding that inhibition of iNOS activity with aminoguanidine in rats challenged with LTA + PepG also resulted in similar improvements in haemodynamics and organ function. Aminoguanidine is a selective inhibitor of iNOS activity *in vitro* (Corbett *et al.*, 1992; Misko *et al.*, 1993; Griffiths *et al.*, 1993) and in the rat *in vivo* (Wu *et al.*, 1995). Moreover, aminoguanidine also reduces the circulatory failure and liver dysfunction caused by endotoxin in the rat (Wu *et al.*, 1995), which is secondary to an enhanced formation of NO by iNOS (Thiemermann *et al.*, 1995). Collectively, this shows that increased production of NO and iNOS contributed to the circulatory shock and organ injury elicited by LTA + PepG.

This study demonstrates that the delayed vascular hyporeactivity to noradrenaline (at 3 to 6 h) caused by LTA + PepG was abolished by dexamethasone and attenuated by aminoguanidine. Thus, the formation of NO by iNOS contributes to (but is not solely responsible for) the hyporesponsiveness to pressor agents in rats treated with LTA + PepG. Interestingly, prolonged incubation of porcine pulmonary arter rings with heat-inactivated group B *streptococci* also resulted in induction of iNOS activity and vascular hyporeactivity to the contractile responses elicited by noradrenaline. This vascular hyporeactivity is prevented by pretreatment of the vessels with dexamethasone or the protein synthesis inhibitor cycloheximide, attenuated by the NOS inhibitor N^{G}-nitro-L-arginine methyl ester (L-NAME), and augmented by the addition of L-arginine to the medium (Villamor *et al.*, 1995). Thus, whole Gram-positive bacteria (Villamor *et al.*, 1995), LTA alone (De Kimpe *et al.*, 1995a) as well as LTA + PepG (this study) cause the induction of iNOS in vascular smooth muscle, which contributes to the hyporesponsiveness of the vasculature to vasopressor agents.

This study also demonstrates that dexamethasone and aminoguanidine attenuate the fall in Pa_{O_2}, hence, respiratory dysfunction, caused by co-administration of LTA + PepG suggesting that NO mediates this adverse effect. The respiratory dysfunction in septic shock is charaterized by a reduction in pulmonary oxygen uptake resulting in a fall in Pa_{O_2} (Deitsch, 1992). Endotoxaemia is associated with an impairment of hypoxic pulmonary vasoconstriction resulting in an increase in pulmonary shunt fraction and, hence, the development of a ventilation/perfusion mismatch (Theissen *et al.*, 1991). Consistent with our observations, infusions of *Staphylococcus aureus* leads to a delayed fall in Pa_{O_2} in rats (Stewart *et al.*, 1994). Furthermore, in a rat model of endotoxaemia, increased release of superoxide anions and NO by activated alveolar macrophages contribute to the formation of a potent oxidant peroxynitrite (Beckman *et al.*, 1990) which subsequently causes extensive lung injury (Wizemann *et al.*, 1994). Thus, in our model, preventing the formation of peroxynitrite by dexamethasone and aminoguanidine may have contributed to the attenuation of lung dysfunction by these agents. However, there is also evidence that production of NO may serve to detoxify superoxide anions or hydrogen peroxide, an argument which has been used to support the hypothesis that endogenous NO may help to attenuate organ injury (Kanner *et al.*, 1991; Miles *et al.*, 1996; Stamler, 1996).

Pathogenesis of organ dysfunction and metabolic acidosis caused by PepG + LTA in vivo

Circulatory shock (of various aetiologies) often results in a marked defect in tissue oxygen extraction resulting in organ

hypoxia and an increased venous oxygen concentration. This defect in tissue oxygen extraction is – in combination with an impairment of respiratory function – also the underlying cause for the development of the metabolic acidosis. Our finding that dexamethasone and aminoguanidine attenuated liver injury, lung dysfunction, metabolic acidosis and the induction of iNOS (e.g. in lung and liver) strongly implies that an enhanced formation of NO by iNOS contributes to the organ injury/dysfunction and metabolic acidosis caused by LTA + PepG in the rat. What, then, are the mechanisms by which an enhanced formation of NO by iNOS contributes to cell injury and ultimately organ failure?

The local generation of large amounts of NO e.g. by activated macrophages serves to kill bacteria or tumour cells as part of the host defence (see Nathan, 1992). Thus, it is not surprising that the generation of excessive amounts of NO (by iNOS) by cells which are under physiological conditions, not exposed to (large amounts of) NO is cytotoxic to these cells (suicide mechanism). Indeed, large amounts of NO cause an autoinhibition of mitochondrial respiration by inhibiting several key enzymes in the mitochondrial respiratory chain (e.g. NADH-ubiquinone reductase, succinate-ubiquinone oxidoreductase) or in the Kreb's cycle (e.g. cis-aconitase) resulting in a shift in glucose metabolism from aerobic to anaerobic pathways (see Morris & Billiar, 1994). In addition, NO inhibits the activities of a variety of enzymes including glyceraldehyde-3-phosphate dehydrogenase (GAPDH, Zhang & Snyder, 1992), hepatic cytochrome P_{450} reductase (Wink *et al.*, 1993), cyclo-oxygenase and lipoxygenase (Kanner *et al.*, 1992), ribonucleotide reductase (Lepoivre *et al.*, 1991), and NOS itself (Rogers & Ignarro, 1992). Inhibition of these enzymes may well contribute to the inhibition by NO (from iNOS) of the synthesis of proteins by hepatocytes (Curran *et al.*, 1991) and of prostaglandin E_2 and interleukin-1 by Kupffer cells (Stadler *et al.*, 1993).

Dexamethasone, but not aminoguanidine, reduced the rises in the serum levels of creatinine and urea and, hence, the development of acute renal failure caused by LTA + PepG in the rat (see Baue, 1993). The finding that both dexamethasone and aminoguanidine caused a similar reduction in iNOS activity in the kidney, strongly suggests that the acute renal failure caused by LTA + PepG is not mediated by NO from iNOS. The mechanism(s) by which dexamethasone reduces the renal dysfunction caused by LTA + PepG in unclear. The observed impairment in renal function may, in principle, be due to (i) either a hypoperfusion of glomeruli (e.g. due to either a reduction in renal perfusion pressure or the development of cortico-medullary shunts) or (ii) acute tubular necrosis (Deitsch, 1992). Dexamethasone prevented the acute fall in blood pressure as well as the renal dysfunction caused by LTA + PepG (this study). Thus, there is circumstantial evidence that the acute hypotension caused by LTA + PepG contributes to the acute renal failure observed in this study. However, we know little about the pathophysiology of the acute tubular necrosis caused by LTA + PepG or even by endotoxin.

Non-specific effects of dexamethasone and aminoguanidine

Although we have used dexamethasone and aminoguanidine as 'pharmacological tools' to elucidate the role of NO from iNOS in the circulatory failure and MODS caused by LTA + PepG, neither dexamethasone nor aminoguanidine are specific for prevention of iNOS induction or inhibition of iNOS activity, respectively. Indeed, dexamethasone exerts numerous effects (see Fantuzzi & Ghezzi, 1993) which may contribute to the beneficial effects of this glucocorticoid in our model of shock and MODS caused by LTA + PepG. These include (i) prevention of the expression of cyclo-oxygenase (COX II) caused by endotoxin (Masferrer *et al.*, 1992) or LTA (Abate *et al.*, 1996), (ii) prevention of the generation of platelet-activating factor (PAF; Parente & Flower, 1985), (iii) attenuation of the formation of TNFα (Beutler *et al.*, 1986; Flower, 1988; this study), (iv) prevention of the expression of adhesion molecules as well as activation of neutrophils (Cronstein *et al.*, 1992). In addition to inhibiting iNOS activity, aminoguanidine also inhibits polyamine catabolism (Seiler *et al.*, 1985), catalase activity (Ou & Wolff, 1993), histamine metabolism (Bieganski *et al.*, 1983) as well as the formation of glycosylation end products (Brownlee *et al.*, 1988; Edelstein & Brownlee, 1992). Our finding that pretreatment of rats with aminoguanidine prevents the LTA + PepG induced expression of iNOS protein and activity (lung), without affecting TNFα production, highlights the fact that aminoguanidine exerts non-specific effects which are unrelated to inhibition of iNOS activity. One could argue that this effect of aminoguanidine is due to inhibition of NO formation, as NO itself interferes with the synthesis of iNOS protein (Niemann *et al.*, 1994; Weisz *et al.*, 1996). This, however, may not be the case, as the NOS inhibitors, N^G-methyl-L-arginine, L-NAME, aminoguanidine or aminoethyl-isothiourea inhibited the formation of nitrite in murine macrophages or in the plasma from rats challenged with endotoxin, but only aminoguanidine and aminoethyl-isothiourea prevented the expression of iNOS protein (Ruetten *et al.*, 1996).

In conclusion, this study provides strong evidence that an enhanced formation of NO by iNOS importantly contributes to (i) circulatory failure (hypotension, tachycardia and to a lesser degree vascular hyporeactivity to the pressor effects elicited by noradrenaline), (ii) liver injury and dysfunction, (iii) respiratory dysfunction and (iv) metabolic acidosis caused by co-administration of LTA and PepG in the anaesthetised rat. However, an enhanced formation of NO by iNOS may not contribute to the acute renal failure caused by administration of these two constituents of the cell wall of the Gram-positive bacterium *Staphylococcus aureus*.

The authors would like to acknowledge Prof Sir John Vane for helpful comments and discussions during the preparation of this manuscript and Dr Clarie Bryant for the iNOS specific polyclonal antibody.

References

ABATE, A., KENGATHARAN, M., RUETTEN, H., HIRSCHELMANN, R., THIEMERMANN, C. & VANE, J.R. (1996). Induction of cyclooxygenase-2 by lipoteichioic acid in bovine endothelial cells involves the activation of NFκB. *Br. J. Pharmacol.*, **117**, 239P.

AUGUET, M., LONCHAMPT, M.O., DELAFLOTTE, S., GOULIN-SCHULZ, J., CHABRIER, P.E. & BRAQUET, P. (1992). Induction of nitric oxide synthase by lipoteichoic acid from staphylococcus aureus in vascular smooth muscle cells. *FEBS Lett.*, **297**, 183–185.

BAUE, A.E. (1993). The multiple organ or system failure syndrome. In *Pathophysiology of Shock, Sepsis and Organ Failure*. ed. Schlag, G. & Redl, H., pp. 1004–1018. Berlin: Springer Verlag.

BECKMAN, J., BECKMAN, T., CHEN, J., MARSHALL, P. & FREEMAN, B. (1990). Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide. *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 1620–1624.

BEUTLER, B., KROCHIN, N., MILSARK, I.W., LUEDKE, C. & CERAMI, A. (1986). Control of cachectin (tumour necrosis factor) synthesis: mechanisms of endotoxin resistance. *Science*, **232**, 977–980.

BIEGANSKI, T., KUSCHE, J., LORENZ, W., HEXTERBERG, R., STAHLKNECHT, C.D. & FEUSSNER, K.D. (1983). Distribution and properties of human intestinal diamine oxidase and its relevance for the histamine metabolism. *Biochem. Biophys. Acta*, **31**, 196–203.

BONE, R.C. (1994). Gram-positive organisms and sepsis. *Arch. Intern. Med.*, **154**, 26–34.

BRADFORD, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Anal Biochem.*, **72**, 248–254.

BROWNLEE, M., CERAMI, A. & VLASSARA, H. (1988). Advanced glycosylation end products in tissue and the biochemical basis of diabetic complications. *N. Eng. J. Med.*, **318**, 1315–1321.

CORBETT, J.A., TILTON, R.G., CHANG, K., HASAN, K.S., IDO, Y., SON, J.R. & MCDANIEL, M.L. (1992). Aminoguanidine, a novel inhibitor of nitric oxide formation, prevents diabetic vascular dysfunction. *Diabetes*, **41**, 552–556.

CRONSTEIN, B.N., KIMMEL, S.C., LEVIN, R.I., MARTINUK, F. & WEISSMAN, W. (1992). A mechanism for the anti-inflammatory effects of cortico-steroids: the glucocorticoid receptor regulates leukocyte adhesion to endothelial cells and expression of endothelial leukocyte adhesion molecule-1 and intracellular adhesion molecule-1. *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 9991–9995.

CUNHA, F.Q., MOSS, D.W., LEAL, L.M.C.C., MONCADA, S. & LIEW, F.Y. (1993). Induction of macrophage parasiticidal activity by staphylococcus aureus and exotoxins through the nitric oxide synthase pathway. *Immunology*, **78**, 563–567.

CURRAN, R.D., FERRARI, F.K., KISPERT, P.H., STADLER, J., STUEHR, D.J., SIMMONS, R.L. & BILLIAR, T.R. (1991). Nitric oxide and nitrix oxide generating compounds inhibit hepatocyte protein synthesis. *FASEB J.*, **5**, 2085–2092.

DE KIMPE, S.J., HUNTER, M.L., BRYANT, C.E., THIEMERMANN, C. & VANE, J.R. (1995a). Delayed circulation failure due to the induction of nitric oxide synthase by lipoteichoic acid from staphylococcus aureus in anaesthetised rats. *Br. J. Pharmacol.*, **114**, 1317–1323.

DE KIMPE, S.J., KENGATHARAN, M., THIEMERMANN, C. & VANE, J.R. (1995b). The cell wall components peptidoglycan and lipoteichoic acid from staphylococcus aureus act in synergy to cause shock and multiple organ failure. *Proc. Natl. Acad. Sci. U.S.A.*, **92**, 10359–10363.

DEITSCH, E.A. (1992). Multiple organ failure. *Ann. Surg.*, **216**, 117–134.

EDELSTEIN, D. & BROWNLEE, M. (1992). Mechanistic studies of advanced glycosylation end product inhibition by aminoguanidine. *Diabetes*, **41**, 26–29.

FANTUZZI, G. & GHEZZI, P. (1993). Glucocorticoids as cytokine inhibitors: role in neuroendocrine control and therapy of inflammatory diseases. *Med. Inflamm.*, **2**, 263–270.

FLOWER, R.J. (1988). Lipocortin and the mechanism of action of the glucocorticoids. *Br. J. Pharmacol.*, **94**, 987–1015.

FRY, D.E., PEARLSTEIN, L., FULTON, R.L. & POLK, H.C. (1980). Multiple system organ failure: the role of incontrolled infection. *Arch. Surg.*, **115**, 136–140.

GREEN, L.C., RUIZ DE LUZURIAGA, K. & WAGNER, D.A. (1981). Nitrate biosynthesis in man. *Proc. Natl. Acad. Sci. U.S.A.*, **78**, 7764–7768.

GRIFFITHS, M.J.D., MESSENT, M., MACALLISTER, R.J. & EVANS, T.W. (1993). Aminoguanidine selectively inhibits inducible nitric oxide synthase. *Br. J. Pharmacol.*, **110**, 963–968.

KANNER, J., HAREL, S. & GRANIT, R. (1991). Nitric oxide as an antioxidant. *Arch. Biochem. Biophys.*, **289**, 130–136.

KANNER, J., HAREL, S. & GRANIT, R. (1992). Nitric oxide, an inhibitor of lipid oxidation by lipoxygenase, cyclooxygenase and haemoglobin. *Lipids*, **27**, 46–49.

KENGATHARAN, M., DE KIMPE, S.J. & THIEMERMANN, C. (1996a). Analysis of the signal transduction in the induction of nitric oxide synthase by lipoteichoic acid in macrophages. *Br. J. Pharmacol.*, **117**, 1163–1170.

KENGATHARAN, M., DE KIMPE, S.J. & THIEMERMANN, C. (1996b). Pre-treatment with dexamethasone or delayed treatment with aminoguanidine ameliorates the circulatory failure and organ injury in a rat model of Gram-positive shock. *Br. J. Pharmacol.*, **118**, 2P.

KENGATHARAN, M., DE KIMPE, S.J., THIEMERMANN, C. & VANE, J.R. (1996c). Effect of aminoguanidine on the circulatory failure and organ injury elicited by staphylococcal lipoteichoic acid and peptidoglycan in the anaesthetised rat. *Br. J. Pharmacol.*, **117**, 53P.

LEPOIVRE, M., FIESCHI, F., COVES, J., THELANDER, L. & FONTECAVE, M. (1991). Inactivation of ribonucleotide reductase by nitric oxide. *Biochem. Biophys. Res. Commun.*, **179**, 442–448.

MASFERRER, J.L., SEIBERT, K., ZWEIFEL, B.S. & NEEDLEMAN, P. (1992). Endogenous glucocorticoids regulate an inducible cyclooxygenase enzyme. *Proc. Natl. Acad. Sci. U.S.A.*, **89**, 3917–3921.

MILES, A.M., BOHLE, D.S., GLASSBRENNER, P.A., HANSER, B., WINK, D.A. & GRISHAM, M.B. (1996). Modulation of superoxide-dependent oxidation and hydroxylation reactions by nitric oxide. *J. Biol. Chem.*, **271**, 40–47.

MISKO, T.P., MOORE, W.M., KGOTEN, T.P., NICKOLS, G.A., CORBETT, J.A., TILTON, R.G., MCDANIEL, M.L., WILLIAMSON, J.R. & CURRIE, M.G. (1993). Selective inhibition of the inducible nitric oxide synthase by aminoguanidine. *Eur. J. Pharmacol.*, **233**, 119–125.

MORRIS, S.M. JR. BILLIAR, T.R. (1994). New insights into the regulation of inducible nitric oxide syntheses. *Am. J. Physiol.*, **266**, E829–E839.

NATHAN, C. (1992). Nitric oxide as a secretary product of mammalian cells. *FASEB J.*, **6**, 3051–3064.

NIEMANN, A., BJORKLUND, A. & EIZIRIK, D.L. (1994). Studies on the molecular regulation of the inducible form of nitric-oxide synthase (iNOS) in insulin-producing cells. *Molec. Cellular. Endocrinol.*, **106**, 151–155.

OU, P. & WOLFF, S.P. (1993). Aminoguanidine: a drug proposed for prophylaxis in diabetes inhibits catalase and generates hydrogen peroxide in vitro. *Biochem. Pharmacol.*, **46**, 1139–1144.

PARENTE, L. & FLOWER, R.J. (1985). The generation of lyso-PAF in experimental inflammation. In *Inflammatory Mediators*. ed. Higgs, G.A. & Williams, T.J. pp. 65–71. Basingstoke: Macmillan Press.

RADOMSKI, M.W., PALMER, R.M.J. & MONCADA, S. (1990). Glucocorticoids inhibit the expression of an inducible, but not the constitutive nitric oxide synthase in vascular endothelial cells. *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 10043–10047.

ROGERS, N.E. & IGNARRO, L.J. (1992). Constitutive nitric oxide synthase from cerebellum is reversibly inhibited by nitric oxide formed from L-arginine. *Biochem. Biophys. Res. Commun.*, **189**, 242–249.

RUETTEN, H., SOUTHAN, G.J., ABATE, A. & THIEMERMANN, C. (1996). Attenuation of endotoxin-induced multiple organ dysfunction by 1-amino-2-hydroxy-guanidine, a potent inhibitor of inducible nitric oxide synthase. *Br. J. Pharmacol.*, (in press).

SCHMIDT, H.H.H.W., WARNER, T.D., NAKANE, M., FORSTERMANN, U. & MURAD, F. (1992). Regulation and subcellular location of nitric oxide synthases in RAW264.7 macrophages. *J. Exp. Pharmacol. Ther.*, **41**, 615–624.

SEILER, N., BOLENIUS, F.N. & KNODGEN, B. (1985). The influence of catabolic reactions on polyamine excretion. *Biochem. J.*, **225**, 219–226.

STADLER, J., HARBRECHT, B.G., DI SILVIO, M., CURRAN, R.D., JORDAN, M.L., SIMMONS, R.L. & BILLIAR, T.R. (1993). Endogenous nitric oxide inhibits the synthesis of cyclooxygenase products and interleukin-6 by rat Kupffer cells. *J. Leukoc. Biol.*, **53**, 165–172.

STAMLER, J.S. (1996). A radical vascular connection. *Nature*, **380**, 108–111.

STEWART, K.D., BRACKETT, D.J., LERNER, M.R., ARCHER, L.T. & WILSON, M.F. (1994). Comparison of *Staphylococcus aureus* and *Escherichia coli* infusion in conscious rats. *J. Surg. Res.*, **56**, 60–66.

SZABO, C., MITCHELL, J.A., THIEMERMANN, C. & VANE, J.R. (1993a). Nitric oxide mediated hyporeactivity to noradrenaline precedes nitric oxide synthase induction in endotoxin shock. *Br. J. Pharmacol.*, **108**, 786–792.

SZABO, C., THIEMERMANN, C., WU, C.C., PERRETTI, M. & VANE, J.R. (1993b). Inhibition of nitrix oxide synthase induction by endogenous glucocorticoids accounts for endotoxin tolerance in vivo. *Proc. Natl. Acad. Sci. U.S.A.*, **91**, 271–275.

THEISSEN, J.L., LOICK, H.M., CURRY, B.B., TRABER, L.D., HERNDON, D.N. & TRABER, D.L. (1991). Time course of hypoxic pulmonary vasoconstriction after endotoxin infusion in unanaesthetised sheep. *J. Appl. Physiol.*, **70**, 2120–2125.

THIEMERMANN, C. (1995). Selective inhibition of the activity of inducible nitric oxide synthase in septic shock. *Prog. Clin. Biol. Res.*, **392**, 383–392.

THIEMERMANN, C., RUETTEN, H., WU, C.C. & VANE, J.R. (1995). The multiple organ dysfunction syndrome caused by endotoxin in the rat – attenuation of liver dysfunction by inhibitors of nitric oxide synthase. *Br. J. Pharmacol.*, **116,** 2845–2851.

THIEMERMANN, C., WU, C.C., SZABO, C., PERRETTI, M. & VANE, J.R. (1993). Role of tumour necrosis factor in the induction of nitric oxide synthase in a rat model of endotoxin shock. *Br. J. Pharmacol.*, **110,** 177–182.

VILLAMOR, E., PEREZ-VIZCAINO, RUIZ, T., LEZA, J.C., MORO, M. & TAMARGO, J. (1995). Group B *Streptococcus* and *E.coli* LPS-induced NO-dependent hyporesponsiveness to noradrenaline in isolated intrapulmonary arteries of neonatal piglets. *Br. J. Pharmacol.*, **115,** 261–266.

WEISZ, A., CICATIELLO, L. & ESUMI, H. (1996). Regulation of the mouse inducible-type nitric oxide synthase gene promoter by interferon-γ, bacterial lipopolysaccharide and N^G-monomethyl-L-arginine. *Biochem. J.*, **316,** 209–215.

WINK, D.A. OSAWA, Y., DARBYSHIRE, J.F., JONES, C.R., ESHENAUR, S.C. & NIMS, R.W. (1993). Inhibition of cytochromes P450 by nitric oxide and a nitric oxide releasing agent. *Arch. Biochem. Biophys.*, **300,** 115–123.

WIZEMANN, T.M., GARDNER, C.R., LASKIN, J.D., QUINONES, S., DURHAM, S.K., GOLLER, N.L., OHNISHI, S.T. & LASKIN, D.L. (1994). Production of nitric oxide and peroxynitrite in the lung during acute endotoxemia. *J. Leukoc. Biol.*, **56,** 759–768.

WU, C.C., CHEN, S.-J., SZABO, C., THIEMERMANN, C. & VANE, J.R. (1995). Aminoguanidine attenuates the delayed circulatory failure and improves survival in rodent models of endotoxic shock. *Br. J. Pharmacol.*, **114,** 1666–1672.

ZHANG, J. & SNYDER, S.H. (1992). Nitric oxide stimulates auto-ADP-ribosylation of glyceraldehyde-3-phosphate dehydrogenase. *Proc. Natl. Acad. Sci. U.S.A.*, **89,** 9382–9385.

(Received May 22, 1996
Revised August 19, 1996
Accepted September 4, 1996)