British Journal of Pharmacology (1995) 116, 2845-2851

The multiple organ dysfunction syndrome caused by endotoxin in the rat: attenuation of liver dysfunction by inhibitors of nitric oxide synthase

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1 We have investigated whether (i) endotoxaemia caused by *E. coli* lipopolysaccharide in the anaesthetized rat causes a multiple organ dysfunction syndrome (MODS; e.g. circulatory failure, renal failure, liver failure), and (ii) an enhanced formation of nitric oxide (NO) due to induction of inducible NO synthase (iNOS) contributes to the MODS. In addition, this study elucidates the beneficial and adverse effects of aminoethyl-isothiourea (AE-ITU), a relatively selective inhibitor of iNOS activity, and N^G-methyl-L-arginine (L-NMMA), a non-selective inhibitor of NOS activity on the MODS caused by endotoxaemia.

2 In the anaesthetized rat, LPS caused a fall in mean arterial blood pressure (MAP) from 117 ± 3 mmHg (time 0) to 97 ± 4 mmHg at 2 h ($P<0.05$, $n=15$) and 84 ± 4 mmHg at 6 h ($P<0.05$, $n=15$). The pressor effect of noradrenaline (NA, 1 $\mu g\ kg^{-1}$, i.v.) was also significantly reduced at 1 to 6 h after LPS (vascular hyporeactivity). Treatment of LPS-rats with AE-ITU (1 $mg\ kg^{-1}$, i.v. plus 1 $mg\ kg^{-1}\ h^{-1}$ starting at 2 h after LPS) caused only a transient rise in MAP, but significantly attenuated the delayed vascular hyporeactivity seen in LPS-rats. Infusion of L-NMMA (3 $mg\ kg^{-1}$, i.v. plus 3 $mg\ kg^{-1}\ h^{-1}$) caused a rapid and sustained rise in MAP and attenuated the delayed vascular hyporeactivity to NA. Neither AE-ITU nor L-NMMA had any effect on either MAP or the pressor effect elicited by NA in rats infused with saline rather than LPS.

3 Endotoxaemia for 6 h was associated with a significant rise in the serum levels of aspartate or alanine aminotransferase (i.e. GOT or GPT), γ-glutamyl-transferase (γGT), and bilirubin, and hence, liver dysfunction. Treatment of LPS-rats with AE-ITU significantly attenuated this liver dysfunction (rise in GOT, GPT, γGT and bilirubin) ($P<0.05$, $n=10$). In contrast, L-NMMA reduced the increase in the serum levels of γGT and bilirubin, but not in GOT and GPT ($n=5$). Injection of LPS also caused a time-dependent, but 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 either AE-ITU ($n=10$) or L-NMMA ($n=5$). In rats infused with saline rather than LPS, neither AE-ITU ($n=4$) nor L-NMMA ($n=4$) had any significant effect on the serum levels of GOT, GPT, γGT, bilirubin, creatinine or urea.

4 Endotoxaemia for 6 h resulted in a 4.5 fold rise in the serum levels of nitrite (9.13 ± 0.77 μM, $P<0.01$, $n=15$), which was significantly reduced by treatment with AE-ITU (6.32 ± 0.48 μM, $P<0.05$, $n=10$) or L-NMMA (5.10 ± 0.40 μM, $P<0.05$, $n=5$). In addition, endotoxaemia for 6 h was also associated with a significant increase in iNOS activity in lung and liver homogenates, which was significantly reduced in lung or liver homogenates obtained from LPS-rats treated with either AE-ITU or L-NMMA.

5 Thus, AE-ITU or L-NMMA (i) inhibits iNOS activity in LPS-rats without causing a significant increase in MAP in rats infused with saline and, hence inhibition of endothelial NOS activity, and (ii) attenuates the delayed circulatory failure as well as the liver dysfunction caused by endotoxaemia in the rat. Thus, an enhanced formation of NO may contribute to the development of liver failure in endotoxic shock.

Keywords: Aminoethyl-isothiourea; circulatory shock; endothelial nitric oxide synthase; inducible nitric oxide synthase; lipopolysaccharide; N^G-methyl-L-arginine; endotoxic shock

Introduction

An enhanced formation of nitric oxide (NO) due to the induction of the inducible isoform of NO synthase (iNOS) has been implicated in the pathogenesis of a number of diseases including circulatory shock of various aetiologies (see Moncada & Higgs, 1993). An overproduction of NO contributes to the severe, therapy-refractory hypotension and vascular hyporeactivity to catecholamines in endotoxic shock, haemorrhagic shock and the circulatory failure associated with immunotherapy (see Thiemermann, 1994). There is, however, little information regarding the effects of NOS inhibitors on organ function in experimental endotoxaemia. The progression of shock to a multiple organ dysfunction syndrome (MODS) is associated with a substantial increase in mortality (Deitsch, 1992).

It has been suggested (Wright *et al.*, 1992) that with non-selective inhibitors of all isoforms of NOS (e.g. N^G-methyl-L-arginine; L-NMMA) the concomitant inhibition of endothelial NOS (eNOS) activity causes excessive vasoconstriction and, hence, increases the incidence of organ ischaemia, microvascular thrombosis and mortality. Indeed, high doses of non-selective NOS inhibitors augment the degree of liver injury (Harbrecht *et al.*, 1992) and microvascular thrombosis and ischaemia of intestine (Hutcheson *et al.*, 1990) and kidney (Shultz & Raij, 1992) as well as mortality (Wright *et al.*, 1992) in rodent models of endotoxin shock. Thus, the beneficial haemodynamic effects of non-selective NOS inhibitors may

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well be due to inhibition of iNOS activity, while the reported adverse effects may be due to inhibition of eNOS activity (Thiemermann, 1994).

Here we compare the effects of aminoethyl-isothiourea (AE-ITU), a relatively selective inhibitor of iNOS activity (Garvey *et al.*, 1994; Szabo *et al.*, 1994; Southan *et al.*, 1995), and L-NMMA, a non-selective inhibitor of iNOS and eNOS activity (Hibbs *et al.*, 1987; Rees *et al.*, 1989; Gross *et al.*, 1990) on (i) iNOS activity and (ii) MODS (renal and liver dysfunction) caused by endotoxaemia *in vivo*.

Methods

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 cannulated to facilitate respiration and rectal temperature was maintained at 37°C with a homeothermic blanket (BioSciences, Sheerness, Kent). 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 challenged with 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, $n=14$) or *E. coli* lipopolysaccharide (LPS, 10 mg kg^{-1} i.v. in 0.3 ml of saline, $n=30$) as a slow injection over 10 min. The pressor responses to NA were reassessed at every hour after LPS injection. At 2 h after injection of LPS, animals received a continuous infusion of the vehicle for AE-ITU or L-NMMA (saline, 0.6 ml kg^{-1} h^{-1}, $n=15$), AE-ITU (1 ml kg^{-1} h^{-1} i.v. bolus loading dose, followed by a continuous infusion of 1 mg kg^{-1} h^{-1} in 0.6 ml kg^{-1} h^{-1} saline, $n=10$) or L-NMMA (3 mg kg^{-1} i.v. bolus loading dose, followed by a continuous infusion of 3 mg kg^{-1} h^{-1} in 0.6 ml kg^{-1} h^{-1} saline, $n=5$). All haemodynamic parameters were recorded for a further 4 h period. Another group of animals received LPS for 2 h only ($n=6$).

In separate experiments, 3 groups of rats were anaesthetized, instrumented (as above) and treated with continuous infusions of either vehicle (0.6 ml kg^{-1} h^{-1} saline, $n=6$), AE-ITU (dose as above, $n=4$), or L-NMMA (dose as above, $n=4$). These infusions were started at time 2 h and maintained until the end of the experiment (6 h).

Quantification of liver or kidney injury

At 2 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 were analysed within 24 h by a contract laboratory for veterinary, clinical chemistry (Vetlab Services, Sussex). 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); 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); and γ-glutamyltransferase (γGT, a marker for cholestasis). (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).

Measurement of serum nitrite

At 2 h or 6 h after 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. The amounts of nitrite in the serum were measured by the Griess reaction (Green *et al.*, 1981) by adding 100 μl of Griess reagent to 100 μl samples of unfiltered serum as previously described (De Kimpe *et al.*, 1995). 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 a standard solution of sodium nitrite prepared in controls serum.

Nitric oxide synthase assay

Lungs from LPS-rats treated with vehicle (control), AE-ITU or L-NMMA were removed at 6 h after LPS and frozen in liquid nitrogen. Lungs from rats infused with saline which had not received LPS and from rats treated with LPS for 2 h were also prepared for determination of iNOS activity. Lungs were stored for no more than 2 weeks at −80°C before assay. Frozen lungs were homogenized on ice with an Ultra-Turrax T 25 homogenizer (Janke & Kunkel, IKA Labortechnik, staufen i. Br., Germany) in a buffer composed of: Tris-HCl 50 mM, EDTA 0.1 mM, EGTA 0.1 mM, 2-mercaptoethanol 12 mM and phenylmethylsulphonyl fluoride 1 mM (pH 7.4). Conversion of [^{3}H]-L-arginine to [^{3}H]-L-citrulline was measured in the homogenates as described by Thiemermann *et al.* (1993). Briefly, tissue homogenates (30 μl, approx. 60 μg protein) were incubated in the presence of [^{3}H]-L-arginine (10 μM, 5 kBq/tube), NADPH (1 mM), calmodulin (30 nM), tetrahydrobiopterin (5 μM) and calcium (2 mM) for 25 min at 25°C in HEPES buffer (pH 7.5). Reactions were stopped by dilution with 1 ml of ice cold HEPES buffer (pH 5.5) containing EGTA (2 mM) and EDTA (2 mM). Reaction mixtures were applied to Dowex 50W (Na^+ form) columns and the eluted [$^+$H]-L-citrulline activity was measured by scintillation counting (Beckman, LS3801; Fullerton, CA, Fullerton 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.

Materials

Calmodulin, bacterial lipopolysaccharide (*E. coli* serotype 0.127:B8), NADPH, noradrenaline bitartrate, N^G-methyl-L-arginine, Tris-HCl, EDTA, EGTA, 2-mercaptophanol, phenylmethylsulphonyl fluoride, HEPES buffer, Bradford reagent, bovine serum albumin and Dowex 50W anion exchange resin were obtained from Sigma Chemical Co. (Poole, Dorset). 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 (6R-L-erythro-5,6,7,8-tetrahydrobiopterin) was obtained from Dr B. Schircks Laboratories (Jona, Switzerland).

Statistical evaluation

All values in the figures and text are expressed as mean ± s.e.mean of n observations, where n represents the number of animals or blood samples studied. A two-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 betwen groups (*in vitro* study). A P-value less than 0.05 or 0.01 was considered to be statistically significant.

Results

Effects of NOS inhibitors on the circulatory failure caused by endotoxaemia

Baseline values of MAP ranged from 117±3 (n=15) to 123±2 mmHg (n=5) and were not significantly different between any of the experimental groups studied. Injection of LPS (10 mg kg^{-1} i.v.) resulted in a rapid fall of MAP, which remained above 95 mmHg from 60 to 240 min, and fell towards the end of the experimental period (Figure 1a). Thus, despite infusion of saline, the MAP values of LPS-rats were significantly lower than the ones measured in rats infused with saline alone (Table 1). In addition, the mean baseline values for the pressor responses to NA (1 μg kg^{-1}, i.v.) ranged from 29±3 to 36±3 mmHg and were not significantly different between any of the experimental groups studied. Endotoxaemia resulted in a substantial, time-dependent attenuation of the pressor responses elicited by NA (n=15, Figure 1b).

Administration of AE-ITU (1 mg kg^{-1} i.v. plus 1 mg kg^{-1} h^{-1}) commencing at 120 min after the onset of endotoxaemia resulted in a rapid, but transient increase in MAP (Figure 1a). Injection of LPS also caused within 120 min an attenuation of the pressor responses elicited by NA by 52% (Figure 1b). The subsequent treatment of LPS-rats with AE-ITU, however, enhanced the pressor responses afforded by NA at 300 min (n=10, P<0.05) and 360 min (n=10, P<0.05; Figure 1b). Thus, the pressor response to NA at 300 min and 360 min in LPS-rats treated with AE-ITU was significantly greater than in animals treated with LPS alone (P<0.05, Figure 1b). In rats infused with saline rather than LPS, however, injection of AE-ITU had no significant effect on either MAP or the pressor responses to NA (Table 1).

Administration of L-NMMA (3 mg kg^{-1} i.v., plus (3 mg kg^{-1} h^{-1}) starting at 120 min after the onset of endotoxaemia resulted in a rapid and sustained increase in MAP (Figure 1a). Thus, the MAP of LPS-rats treated with L-NMMA was significantly higher than in the LPS-control group at 130 to 300 min. In this animal group, injection of LPS also reduced within 120 min the pressor responses to NA by 53%. The subsquent injection of L-NMMA enhanced the pressor responses afforded by NA at 180 to 360 min after injection of LPS (Figure 1b). Thus, the pressor effect of NA at 180 to 360 min in LPS-rats treated with L-NMMA was significantly greater than in animals treated with LPS alone (P<0.05, Figure 1b).

In rats infused with saline rather than LPS, L-NMMA caused a transient increase in MAP (within 15 min, data not shown). However, the MAP of these rats was at the end of the experiment not significantly different from the MAP values measured prior to commencing the infusion of L-NMMA (Table 1).

Effects of AE-ITU or L-NMMA on the liver dysfunction caused by endotoxaemia

Endotoxaemia for 360 min (n=15), but not for 120 min (n=6), was associated with a significant rise in the serum activities of the aminotransferases GOT and GPT (Figure 2) and bilirubin (Figure 3a). This rise in the serum levels of GOT and GPT caused by LPS *in vivo* was abolished by treatment of LPS-rats with AE-ITU (P<0.05, n=10), but not with L-NMMA (P>0.05, n=5, Figure 2). Interestingly, the rise in serum bilirubin caused by LPS was abolished by treatment of LPS-rats with either AE-ITU or L-NMMA. Endotoxaemia also caused a time-dependent increase in total serum γGT activity (Figure 3b). This increase in serum γGT caused by LPS was attenuated by AE-ITU (P<0.05, n=10) or L-NMMA (P<0.05, n=10). In rats infused with saline rather than LPS, neither AE-ITU nor L-NMMA had any effect on the serum levels of GOT, GPT, bilirubin or γGT (Table 2).

Effects of AE-ITU or L-NMMA on the renal dysfunction caused by endotoxaemia

Injection of LPS caused a time-dependent, but rapid, increase in the serum levels of creatinine and urea. Interestingly, the rise in serum urea was already maximal after 120 min of en-

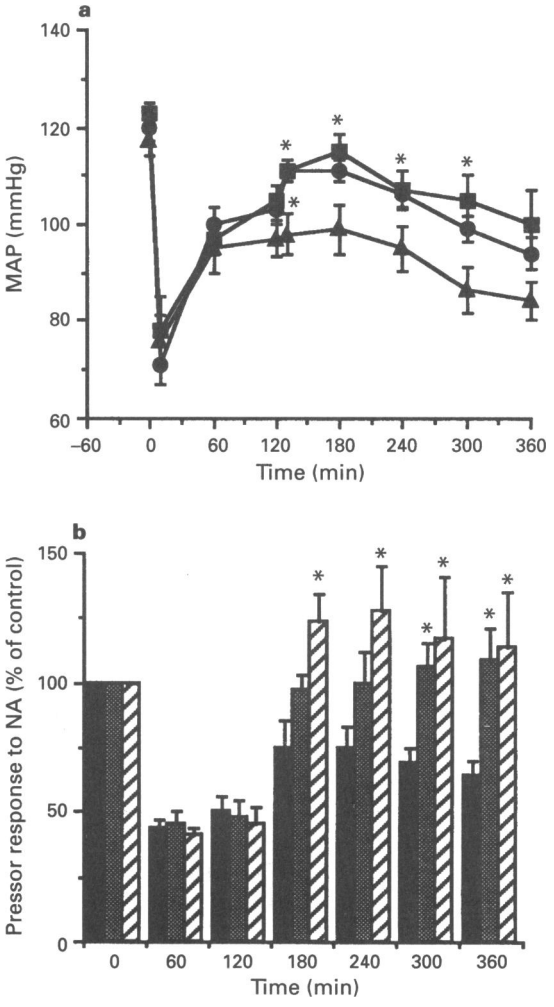

Figure 1 Effects of NOS inhibitors on the delayed hypotension caused by endotoxin in the anaesthetized rat. Depicted are the changes in (a) mean arterial blood pressure (MAP) and (b) the pressor responses to noradrenaline (NA; 1 μg kg^{-1}, i.v.) in rats treated with *E. coli* lipolysaccharide (LPS; 10 mg kg^{-1}, i.v. at time 0). Please note that the baseline responses to NA ranged from 29±3 to 36±3 mmHg and were not significantly different between any of the animal groups studied. Different groups of animals received infusion of vehicle (saline, 0.6 ml kg^{-1} h^{-1}, ▲ or solid columns, n=15), aminoethyl-isothiourea (AE-ITU; 1 mg kg^{-1} plus 1 mg kg^{-1} h^{-1}, ● or stippled columns, n=10) or N^{G}-methyl-L-arginine (L-NMMA; 3 mg kg^{-1} plus 3 mg kg^{-1} h^{-1}, ■ or hatched columns, n=5) at 2 h after LPS. Data are expressed as mean±s.e.mean of n observations. *P<0.05 represent significant difference when compared to LPS-controls at the same time point.

dotoxaemia (Figure 4a). The increase in serum urea and creatinine caused by LPS was not affected by infusion of either AE-ITU ($P>0.05$, $n=10$, Figure 4) or L-NMMA ($P>0.05$, $n=5$, Figure 4). In rats infused with vehicle rather than LPS, neither AE-ITU nor L-NMMA had any effect on serum creatinine or urea (Table 2).

Table 1 Effects of injection of vehicle ($n=6$), AE-ITU ($n=4$) or L-NMMA ($n=4$) on mean arterial blood pressure (MAP) and the pressor responses to noradrenaline (NA) in rats infused with saline rather than LPS

Groups		*Time* (min) 0	120	240	360
Vehicle	MAP (mmHg)	119±4	112±5	118±6	117±4
	NA (mmHg)	35±4	37±4	39±5	41±6
AE-ITU	MAP (mmHg)	122±6	118±4	118±6	120±7
	NA (mmHg)	34±3	36±2	42±7	45±8
L-NMMA	MAP (mmHg)	122±4	109±8	114±8	118±7
	NA (mmHg)	33±3	36±4	37±5	46±6

Note that in rats which did not receive LPS, infusion of the NOS inhibitors AE-ITU or L-NMMA did not cause a significant increase in either MAP or the pressor responses elicited by NA. For abbreviations, see text.

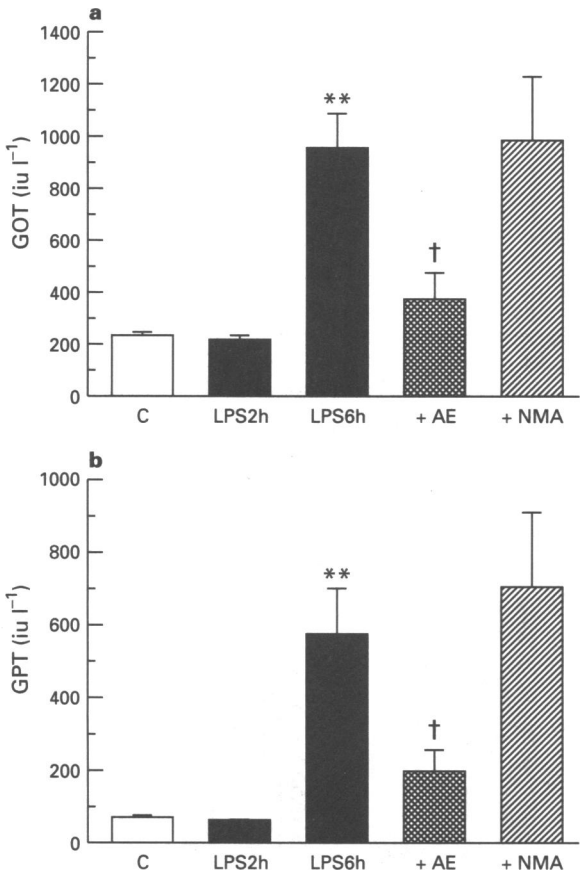

Figure 2 Effects of NOS inhibitors on LPS-induced increases in the serum activities of (a) aspartate aminotransferase (GOT) and (b) alanine aminotransferase (GPT). These enzymes activities were measured in serum obtained from rats infused with vehicle rather than LPS (control, C, open column, $n=6$) or rats treated with *E. coli* LPS (10 mg kg^{-1}, i.v.) for 2 h (LPS2h, solid column, $n=6$) or 6 h. Different groups of LPS 6h-rats were infused for 4 h with vehicle (LPS6h, 0.6 ml kg^{-1} h^{-1}, solid column, $n=15$), aminoethyl-isothiourea (+AE, 1 mg kg^{-1} plus 1 mg kg^{-1} h^{-1}, stippled column, $n=10$) or N^{G}-methyl-L-arginine (+NMA, 3 mg kg^{-1} plus 3 mg kg^{-1} h^{-1}, hatched column, $n=5$). The infusion of drugs was started at 2 h after LPS. Data are expressed as mean±s.e.mean of n observations. **$P<0.01$ represents a significant increase in enzyme activity caused by LPS. †$P<0.05$ represents a significant reduction in enzyme activity when compared to LPS 6h-rats.

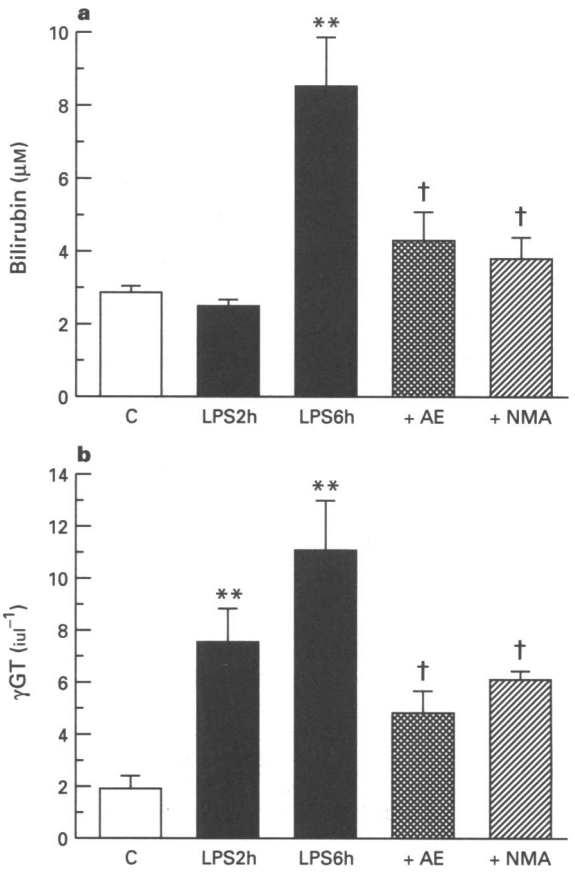

Figure 3 Effects of NOS inhibitors on LPS-induced increases in the serum activities of (a) bilirubin and (b) γ-glutamyl-transferase (γGT). Enzyme activities were measured in serum obtained from rats treated with vehicle rather than LPS (control, C, open column, $n=6$) or rats treated with *E. coli* LPS (10 mg kg^{-1}, i.v.) for 2 h (LPS2h, solid column, $n=6$) or 6 h. Different groups of LPS 6h-rats were infused for 4 h with vehicle (LPS6h, 0.6 ml kg^{-1} h^{-1}, solid column, $n=15$), aminoethyl-isothioura (+AE, 1 mg kg^{-1} plus 1 mg kg^{-1} h^{-1}, stippled column, $n=10$) or N^{G}-methyl-L-arginine (+NMA, 3 mg kg^{-1} plus 3 mg kg^{-1} h^{-1}, hatched column, $n=5$). The infusion of drugs or vehicle was started at 2 h after LPS. Data are expressed as mean±s.e.mean of n observations. **$P<0.01$ represents a significant increase in bilirubin or γGT activity caused by LPS. †$P<0.05$ represents a significant reduction in bilirubin or γGT activity when compared to LPS 6h-rats.

Table 2 Effects of vehicle ($n=6$), AE-ITU ($n=4$) or L-NMMA ($n=4$) on the serum levels of GOT, GPT, bilirubin, γGT, urea or creatinine in rats infused with vehicle (saline) for LPS (without endotoxaemia).

Groups	*GOT* ($iu\,l^{-1}$)	*GPT* ($iu\,l^{-1}$)	*Bilirubin* (μM)	*γGT* ($iu\,l^{-1}$)	*Urea* (μM)	*Creatinine* (μM)
Vehicle	221±20	63±6	2.8±0.3	1.8±0.5	3.9±0.2	29±3
AE-ITU	140±32	61±10	2.7±0.7	1.3±0.3	4.4±0.1	30±4
L-NMMA	166±38	60±3	2.3±0.3	1.7±0.7	4.0±0.6	27±2

The above parameters were measured at 360 min after injection of vehicle for LPS. For abbreviations, see text.

Effects of AE-ITU or L-NMMA on the rise in serum nitrite caused by endotoxaemia

In rats treated with vehicle for LPS, the baseline levels of nitrite in the serum was 1.98±0.22 μM ($n=6$). Endotoxaemia for 360 min was associated with a 4.5 fold rise in serum nitrite levels (9.13±0.77 μM, $P<0.01$, $n=15$). The increase in serum nitrite caused by endotoxaemia was significantly reduced (but not abolished) in LPS-rats treated with either AE-ITU (6.32±0.48 μM, $P<0.05$, $n=10$) or L-NMMA (5.10±0.40 μM, $P<0.05$, $n=5$).

Effects of AE-ITU or L-NMMA on the increase in iNOS activity in the lung and liver caused by endotoxaemia

A small calcium-independent iNOS activity was detectable in lung and liver homogenates obtained from rats infused with saline rather than LPS (Figure 5). Endotoxaemia for 120 min was associated with a moderate increase of iNOS activity in lung and liver homogenates, whereas LPS for 360 min caused a substantial increase of iNOS activity in both organs ($P<0.05$, $n=5-6$). However, the activity of iNOS was significantly re-

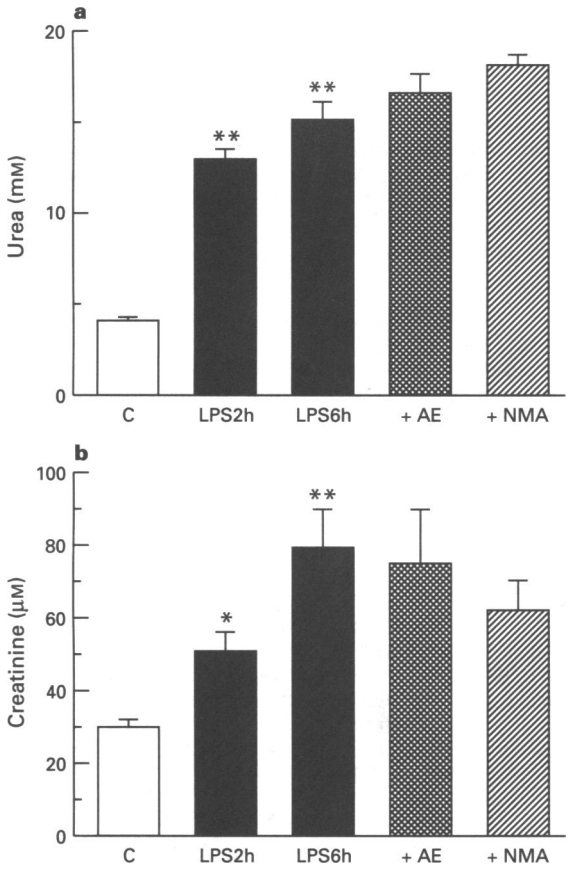

Figure 4 Effects of NOS inhibitors on LPS-induced increases in serum concentrations of (a) urea and (b) creatinine. These activities were measured in serum of rats infused with vehicle rather than LPS (control, C, open column, $n=6$) or rats treated with *E. coli* LPS ($10\,mg\,kg^{-1}$, i.v.) for 2h (LPS2h, solid column, $n=6$) or 6h. Different groups of LPS 6h-rats were infused for 4h with vehicle (LPS6h, $0.6\,ml\,kg^{-1}\,h^{-1}$, solid column, $n=15$), aminoethyl-isothiourea (+AE, $1\,mg\,kg^{-1}$ plus $1\,mg\,kg^{-1}\,h^{-1}$, stippled column, $n=10$) or N^G-methyl-L-arginine (+NMA, $3\,mg\,kg^{-1}$ plus $3\,mg\,kg^{-1}\,h^{-1}$, hatched column, $n=5$). The infusion of drug or vehicle started at 2h after LPS. Data are expressed as mean±s.e.mean of n observations. $^*P<0.05$ and $^{**}P<0.0.01$ represent a significant increase in urea or creatinine activity caused by LPS.

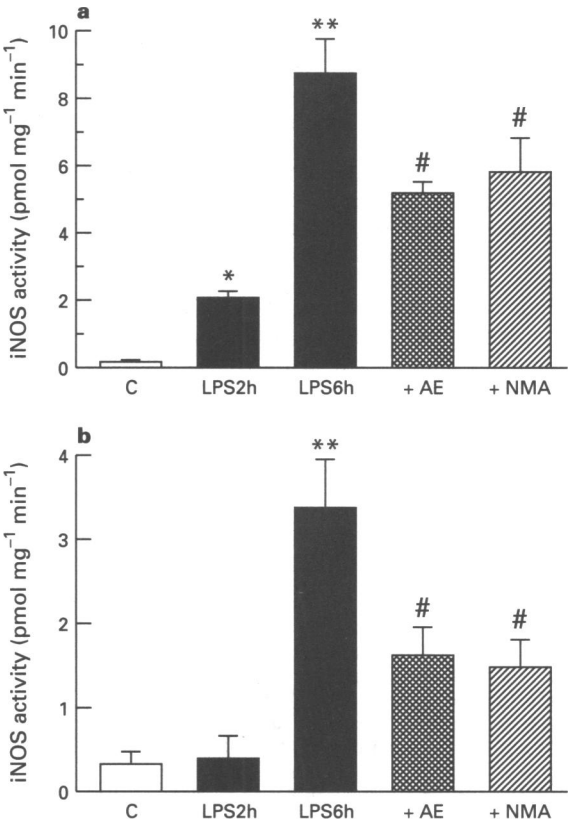

Figure 5 Treatment of rats with NOS inhibitors attenuates the induction of a calcium-independent iNOS activity in (a) lung and (b) liver homogenates obtained from animals with endotoxaemia. Calcium-independent iNOS activity was measured in lung homogenates obtained from rats infused with vehicle rather than LPS (control, C, open column, $n=6$) or rats treated with *E. coli* LPS ($10\,mg\,kg^{-1}$, i.v.) for either 2h (LPS2h, solid column, $n=6$) or 6h. Different groups of LPS 6h-rats were infused for 4h with vehicle (LPS6h, $0.6\,ml\,kg^{-1}\,h^{-1}$, solid column, $n=10$), aminoethyl-isothiourea (+AE, $1\,mg\,kg^{-1}$ plus $1\,mg\,kg^{-1}\,h^{-1}$, stippled column, $n=6$) or N^G-methyl-L-arginine (+NMA, $3\,mg\,kg^{-1}$ plus $3\,mg\,kg^{-1}\,h^{-1}$, hatched column, $n=5$). The infusion of vehicle or drug was started at 2h after LPS. Data are expressed as mean±s.e.mean of n observations. $^*P<0.05$ and $^{**}P<0.01$ represent a significant induction in iNOS activity caused by LPS. $\#P<0.05$ represents a significant reduction in iNOS activity when compared to LPS 6h-rats.

duced in lung and liver homogenates obtained from LPS-rats treated with either AE-ITU ($P<0.05$, $n=5-6$) or L-NMMA ($P<0.05$, $n=5$).

Discussion

This study demonstrates that the NOS inhibitors, AE-ITU or L-NMMA, attenuate the delayed hypotension and vascular hyporeactivity to NA caused by endotoxaemia in the anesthetized rat. In addition, the iNOS-selective NOS inhibitor AE-ITU abolished the rises in the serum levels of GOT and GPT (marker enzymes for a hepatic parenchymal injury), bilirubin and γGT (marker enzymes for cholestasis and, hence, an impairment of the excretory function of the liver) and, therefore, the severe liver dysfunction caused by endotoxaemia. Interestingly, the non-isoenzyme-selective NOS inhibitor L-NMMA also attenuated the rise in the serum levels of bilirubin and γGT, but not the rise in GPT or GOT, caused by endotoxaemia. Thus, L-NMMA attenuated, but did not abolish, the liver dysfunction caused by endotoxic shock.

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^{ω}-nitro-L-arginine methyl ester; L-NAME), the concomitant inhibition of eNOS activity in the endothelium increases the incidence of organ ischaemia, microvascular thrombosis and mortality (Hutcheson *et al.*, 1990; Cobb *et al.*, 1992; Harbrecht *et al.*, 1992; Shultz & Raij, 1992; Wright *et al.*, 1992). Indeed, in mice with chronic hepatic inflammation (due to injection of *Corynebacterium pavum*), a subsequent injection of LPS causes severe injury which is augmented by treatment of these animals with high dose (100–200 mg kg^{-1}) of L-NMMA (Harbrecht *et al.*, 1994). Thus, it has been suggested that inhibition of NOS activity may aggravate the hepatocellular injury caused by endotoxaemia. We demonstrate here, however, that both AE-ITU and to a lesser degree, L-NMMA, attenuate the severe liver dysfunction caused by endotoxaemia in the rat.

Our finding here that neither AE-ITU nor L-NMMA attenuated the renal dysfunction caused by endotoxaemia is not entirely surprising, for 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). Although it has been suggested that inhibition of NOS activity enhances renal dysfunction by causing excessive vasoconstriction and microvascular thrombosis, infusions of lower doses of L-NMMA (this study) or L-NAME (Wu *et al.*, 1995) do not increase the rises in the serum levels of creatinine and urea.

What, then, is the mechanism by which NOS inhibitors reduce the liver dysfunction caused by endotoxaemia? Expression of iNOS activity in hepatocytes and Kuppfer cells results in (i) a reduction in the synthesis of proteins (Billiar *et al.*, 1989; Curran *et al.*, 1991), prostaglandins and IL-6 (Stadler *et al.*, 1993); and (ii) inhibition of mitochondrial respiration (see Morris & Billiar, 1994), possibly due to the formation of peroxynitrite (Stamler *et al.*, 1992; Szabo & Salzman, 1995). Thus, we propose that the beneficial effects of NOS inhibitors on the liver dysfunction elicited by endotoxaemia are due to the prevention of the above-mentioned cytotoxic effects of NO.

Our finding that the degree of inhibition of iNOS activity in the liver caused by L-NMMA and AE-ITU were similar, while AE-ITU but not L-NMMA prevented the rise in the serum levels of GOT and GPT was somewhat surprising. It is likely that these beneficial effects of AE-ITU are independent of the inhibition of iNOS activity. Nevertheless, the beneficial effects of AE-ITU and L-NMMA on the rises in the serum levels of γGT and bilirubin are due to inhibition of iNOS rather than eNOS activity, for neither of these NOS inhibitors caused a signficant rise in blood pressure in rats without endotoxaemia. Our hypothesis that inhibition of eNOS activity alone does not improve liver function in endotoxic shock is supported by the finding that N^{ω}-nitro-L-arginine methyl ester (L-NAME) causes pronounced rises in blood pressure without reducing or augmenting the degree of liver injury in rats with endotoxic shock (Wu *et al.*, 1995).

In conclusion, this study demonstrates that inhibition of iNOS activity with AE-ITU or L-NMMA attenuates the liver dysfunction, but not the renal dysfunction, associated with prolonged periods of endotoxaemia in the rat. As human hepatocytes also induce iNOS activity resulting in the formation of large amounts of NO, selective inhibitors of iNOS activity may attenuate the liver dysfunction in patients with endotoxic shock.

This work was supported by a grant from Cassela AG (Germany). C.T. is supported by a grant from the Commission of the European Communities (Biomed I, BMHI, CT 92/1893). C.C.W. is supported by the National Defense Medical Centre of Taiwan, Taiwan. H.R. is a fellow of the Deutsche Forschungsgemeinschaft (Ru595/1-1).

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(Received June 7, 1995
Revised August 4, 1995
Accepted August 7, 1995)