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Effects of the endothelin receptor antagonist, SB 209670, on circulatory failure and organ injury in endotoxic shock in the anaesthetized rat

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1 This study investigates the effects of the non-selective ET_A/ET_B receptor antagonist, SB 209670, on systemic haemodynamics, renal function, liver function, acid-base balance and survival in a rat model of endotoxic shock.

2 Injection of *E. coli* lipopolysaccharide (LPS, 10 mg kg^{-1}, i.v.) resulted in increases in the serum levels of tumour necrosis factor-α (TNF-α, maximum 60 min after LPS), endothelin-1 (ET-1; maximum 120 min after LPS), and interferon-γ (IFN-γ, maximum 180 min after LPS).

3 Injection of LPS also resulted in a fall in blood pressure from 113 ± 3 mmHg (time = 0) to 84 ± 4 mmHg at 360 min ($n = 15$) as well as a hyporeactivity to the vasoconstrictor responses elicited by noradrenaline (NA, 1 $\mu g\ kg^{-1}$, i.v.). Pretreatment of rats with a continuous infusion of SB 209670 (3 mg kg^{-1}, i.v. bolus + 100 $\mu g\ kg^{-1}$, i.v. infusion commencing 15 min prior to LPS) significantly augmented the hypotension as well as the vascular hyporeactivity to NA caused by endotoxaemia.

4 Pretreatment of LPS-rats with SB 209670 (3 mg kg^{-1}, i.v. bolus given 15 min prior to LPS) or infusion of SB 209670 (bolus dose and infusion as above) resulted in a reduction in 6 h-survival from 71% (control) to 30% and 13%, respectively.

5 Endotoxaemia for 4 h resulted in rises in the serum levels of urea and creatinine (indicators of renal failure), but not in the serum levels of bilirubin, GPT and GOT (indicators of liver dysfunction and/or hepatocellular injury). Pretreatment of LPS-rats with SB 209670 (3 mg kg^{-1}, i.v. bolus 15 min prior to LPS) significantly augmented the serum levels of creatinine, bilirubin, GPT and GOT caused by endotoxin. In addition, endotoxaemia caused, within 15 min, an acute metabolic acidosis (falls in pH, HCO_3^- and base excess) which was compensated by hyperventilation (fall in $PaCO_2$). Pretreatment of LPS-rats with SB 209670 (3 mg kg^{-1}, i.v. bolus) significantly augmented the metabolic acidosis caused by LPS.

6 Thus, the non-selective ET_A/ET_B receptor antagonist, SB 209670, augments the degree of *(i)* hypotension, *(ii)* vascular hyporeactivity to noradrenaline, *(iii)* renal dysfunction and *(iv)* metabolic acidosis caused by endotoxin in the anaesthetized rat. In contrast to rats treated with LPS alone, LPS-rats treated with SB 209670 exhibited liver dysfunction and hepatocellular injury. We propose that the release of endogenous ET-1 serves to maintain blood pressure and subsequently organ perfusion in septic shock.

Keywords: Endotoxic shock; vascular hyporeactivity; endothelin receptors; tumour necrosis factor-α; interferon-γ; cytokines; SB 209670

Introduction

Endothelin-1 (ET-1) is a potent vasoconstrictor peptide produced by endothelial cells from its precursor big-endothelin-1 by endothelin-converting enzyme-1. Two human endothelin receptors have been cloned and expressed, namely ET_A (Arai *et al.*, 1990) and ET_B (Sakurai *et al.*, 1990). The vasoconstrictor effects of ET-1 are primarily mediated by activation of the ET_A receptor, although ET_B receptors (located on the vascular smooth muscle) also mediate vasoconstriction in certain vascular beds in animals (Bigaud & Pelton, 1992) and, to a much lesser degree, in man (Davenport 1995). Activation by ET-1 (and other members of the ET family of peptides) of the ET_B receptor located on the vascular endothelium results in a release of nitric oxide and prostacyclin, both of which attenuate the vasoconstrictor effects caused by ET-1 *in vivo* (Thiemermann *et al.*, 1989; Whittle *et al.*, 1990).

An increase in the circulating serum levels of ET-1 has been documented in many cardiovascular disorders including circulatory shock. Pronounced rises in the serum levels of ET-1 occur in experimental endotoxaemia in rats (Sugiura *et al.*, 1989), dogs (Nakamura *et al.*, 1991), pigs (Pernow 1989) and sheep (Morel *et al.*, 1989). More importantly, enhanced ET-1 serum levels have also been documented in humans subjects with sepsis and septic shock (Pittet *et al.*, 1991; Takakuwa *et al.*, 1994). In man, the serum levels of ET-1 correlate positively with the severity of endotoxaemia (Pittet *et al.*, 1991) and are lower in survivors than in non-survivors of septic shock (Takakuwa *et al.*, 1994). The question as to whether elevated serum levels of ET-1 are merely a surrogate marker of circulatory shock or whether endogenous ET-1 contributes to the underlying pathophysiology of the multiple organ dysfunction syndrome (MODS) associated with septic shock is still controversial.

The definition of circulatory shock is independent of the presence or absence of MODS, which is defined as the presence of an altered organ function in acutely ill patients, such that homeostasis cannot be maintained without intervention. Most importantly, the progression of shock to MODS is associated with an increase in mortality from 25–30% (in the absence of MODS) to 90–100% (see Baue, 1993).

This study investigated the effects of the non-selective $ET_A/$

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ET_B receptor antagonist, SB 209670 (Ohlstein *et al.*, 1994; Douglas *et al.*, 1995) on *(i)* systemic haemodynamics, *(ii)* vascular hyporeactivity, *(iii)* renal function, *(iv)* liver function and integrity, *(v)* acid-base balance, and *(vi)* survival in a rat model of endotoxic shock.

Methods

Measurement of haemodynamic changes

Male Wistar rats (240–320 g; Glaxo Laboratories Ltd., Greenford, Middlesex) were anaesthetized with thiopentone sodium (Trapanal; 120 mg kg^{-1}, i.p.) and anaesthesia was maintained by supplementary injections of thiopentone sodium (approximately 1–2 mg kg^{-1} h^{-1}, i.v., as required). The trachea was cannulated to facilitate respiration and rectal temperature was maintained at 37°C with a homeothermic blanket (BioScience, Sheerness, Kent, U.K.). The right carotid artery was cannulated and connected to a pressure transducer (P23XL, Spectramed, Stratham, 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.

In the first set of experiments, rats were pretreated with the vehicle for SB 209670 (saline) and received (15 min later) vehicle for LPS (saline) and 2 and 4 h later received bolus injection of ET-1 (0.03 nmol kg^{-1}, i.v., $n=7$). A different set of animals received SB 209670 (3 mg kg^{-1}, i.v., $n=7$) and, 15 min later, vehicle for LPS (saline) and then 2 and 4 h later, bolus injections of ET-1 (dose as above). In both series of experiments, the maximal transient falls and subsequent maximal rises in MAP caused by ET-1 were evaluated to ensure that the dose of SB 209670 used in this study was sufficient to attenuate both the vasopressor (mediated by the ET_A-receptor) and vasodilator responses (mediated by the ER_B-receptor) elicited by ET-1. This experiment was of particular importance, as lower doses of SB 209670 (e.g. 1 mg kg^{-1}, i.v.) selectively attenuate the depressor, but not the pressor response, caused by injection of exogenous ET-1 in the anaesthetized rat (Douglas *et al.*, 1995).

In the second series of experiments, animals were anaesthetized and instrumented as above. After recording baseline parameters, animals were challenged with a submaximal (with respect to the pressor response) dose (Thiemermann *et al.*, 1993) of noradrenaline (NA, 1 μg kg^{-1}, i.v.). Different group of animals received (5 min later) infusions of vehicle (saline, 0.6 ml kg^{-1} h^{-1}, $n=21$), or SB 209670 (3 mg kg^{-1} plus 100 μg kg^{-1} min^{-1} in 0.6 ml kg^{-1} h^{-1} saline, $n=8$) or bolus injections of SB 209670 (3 mg kg^{-1}, i.v., plus 0.6 ml kg^{-1} h^{-1} saline, $n=10$). Fifteen min after treatment with SB 209670, animals received either vehicle (saline, 1 mg kg^{-1}, i.v., $n=6$) or *E. coli* lipopolysaccharide (LPS, 10 mg kg^{-1}, i.v.) as a slow injection over 10 min. The pressor response to NA was reassessed at every hour after LPS. All haemodynamic parameters were recorded for a further 6 h period.

In the presence of SB 209670 (infusion or bolus), more than 70% of animals with endotoxaemia died between 4 and 6 h after LPS, whereas in LPS-control rats only 25% died. To investigate the effects of SB 209670 on the organ function and integrity in endotoxic shock, a separate set of experiments was conducted in which the period of endotoxaemia was limited to 4 h. In these experiments, rats were anaesthetized and instrumented (as above) and received either vehicle (saline, 0.6 ml kg^{-1} h^{-1}) or LPS (10 mg kg^{-1} i.v.). Rats which had received vehicle or LPS were subsequently treated either with vehicle (for SB 209670) or SB 209670 (3 mg kg^{-1}, i.v., plus 0.6 ml kg^{-1} h^{-1} saline). Four hours after injection of LPS or vehicle, blood was taken to measure the changes in the serum levels of various biochemical marker enzymes of organ function and integrity (see below).

Quantification of renal and liver function and injury

Four hours after the injection of LPS, and immediately before killing the animals with an overdose of anaesthetic, 1.5 ml of blood was collected from a catheter placed in carotid artery. The blood sample was centrifuged (6,000 r.p.m. for 3 min; Biofuge 15, Heraeus, Cologne, Germany) to prepare serum. All serum samples were analysed within 24 h by a contract laboratory for veterinary clinical chemistry (Vetlab Services, Horsham, Sussex, U.K.).

Liver function and integrity were assessed by measuring the rise in the 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); bilirubin (a specific marker for the development of cholestasis, and, more importantly, a specific marker for the development of liver failure, see Baue, 1993); and γ-glutamyl transferase (γGT, a specific maker for cholestasis).

Renal function was assessed by measuring the rise in the 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). In addition, the serum levels of creatine phosphokinase (CK) were measured as a non-specific indicator of muscle, brain or myocardial injury.

Evaluation of acid-base balance and blood gases

At time 0, 15 min, 60 min and 240 min after injection of LPS, 100 μl of blood was collected in glass tubes (Bilbate Ltd., Daventry, Northants, U.K.) from a catheter placed in the carotid artery for subsequent blood gas analysis. Blood gases were immediately measured with a Corning 168 pH/Blood Gas Analyser (Corning Ltd, Halstead, Essex, U.K.). The blood gas analyser directly measures pH, $PaCO_2$ and PaO_2 and calculates bicarbonate (HCO_3^-), total carbon dioxide (tCO_2, which in combination with pH and $PaCO_2$, is useful in distinguishing between metabolic and respiratory disorders) and base excess (BE).

Measurement of serum nitrite

Four hours after injection of LPS, 1 ml of blood was collected from the arterial catheter. The blood samples was centrifuged (15,000 r.p.m. for 3 min; Biofuge 15, Heraeus, Cologne, Germany) to prepare serum. The amount of nitrite, an indicator of NO formation, in the serum was measured by the Griess reaction (Green *et al.*, 1981; Gross *et al.*, 1990) by adding 100 μl of Griess reagent (1% sulphanilamide and 0.1% naphthylethylenediamide in 5% phosphoric acid) to 100 μl samples of serum. The optical density at 550 nm (OF_{550}) was measured with a Molecular Devices microplate reader (Anthos Labtec Instruments, Richmond, CA, U.S.A.). Nitrite concentration were calculated by comparison with OF_{550} of standard solution of sodium nitrite prepared in normal control serum.

Measurement of the serum levels of tumour necrosis factor-α, interferon-γ and ET-1

The time-course of the changes in the serum levels of ET-1, TNF-α or IFN-γ were measured in rats treated with LPS (10 mg kg^{-1}, i.v.). The content of TNF-α in serum samples (50 μl) was determined by ELISA (Mouse TNF-α ELISA kit, Genzyme, Cambridge, MA, U.S.A.) in 96-well plates. Binding was detected by a peroxidase-conjugated polyclonal anti-mouse TNFα antibody with tetramethylbenzidine used as a

substrate. Following acidification (sulphuric acid, 0.5 M final) the absorbance of each well was measured at 450 nm with a Molecular microplate reader (Anthos Labtec Instruments, Richmond, CA, U.S.A.).

For the determination of IFNγ in serum samples (100 μl), a Cytoscreen rat IFN-γ ELISA kit (Biosource International, Camarillo, CA, U.S.A.) was used. Binding was detected by a biotin-conjugated monoclonal anti-rat IFNγ antibody using streptavidin as a substrate. The absorbance of each well (96-well plate) was measured at 450 nm.

For the detection of ET-1, serum samples (200 μl) were acidified with 50 μl HCl (2 M) and centrifuged (10,000 *g* for 5 min) before being loaded onto Amprep (500 mg C 18, Amersham Life Science, Little Chalfont, Bucks., U.K.) columns pre-equilibrated with methanol (2 ml) and water (2 ml). After washing with water (5 ml) and 0.1% trifluoroacetic acid, the columns were eluted with 80% methanol + 0.1% trifluoroacetic acid (2 ml) and the eluent dried down under N_2. Samples were reconstituted and assayed with an ET-1 ELISA system (Biotrak, Amersham Life Science). Assay plates (96-well) were read as above.

Materials

Bacterial lipopolysaccharide (*E. coli* serotype 0.127:B8), trifluoroactic acid, hydrochloric acid, sulphuric acid, sulphanilamide, naphthylethylenediamide, phosphoric acid and noradrenaline bitartate were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). Sodium thiopentone (Intraval) was obtained from May and Baker Ltd. (Dagenham, Essex, U.K.). The disodium salt of SB 209670 ([(±)-(1S,2R,3S)-3-(2-carboxymethoxy-4-methoxyphenyl)-1-(3,4-methylenedioxyphenyl)-5-(prop-1-yloxy) indane-2-carboxylic acid]) was a generous gift from Dr E. Ohlstein (SmithKline Beecham Pharmaceuticals, King of Prussia, PA, U.S.A.), which was dissolved in saline (0.9% sodium chloride, w/v) and stored at −20°C.

Statistical evaluation

All values in the figures and the 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, if appropriate, by a Bonferoni's test was used to compare means between groups. Students unpaired *t* test was used to compare means between groups A *P* value of less than 0.05 was considered to be statistically significant.

Results

Effects of SB 209670 on the pressor and depressor effects elicited by intravenous injection of exogenous ET-1 in anaesthetized rats

In rats pretreated with vehicle for SB 209670, repeated injections of ET-1 (0.3 nmol kg^{-1}, i.v.) 2 and 4 h after vehicle injection caused transient falls in MAP of 11±3 mmHg and 14±2 mmHg, respectively. The transient vasodilator responses caused by injections of ET-1 at 120 and 240 min were followed by rises in MAP of 15±3 mmHg and 17± 2 mmHg ($n=7$), respectively. Pretreatment of a separate group of animals with SB 209670 (3 mg kg^{-1}, i.v., $n=6$) attenuated the vasodilator response elicited by subsequent injections of ET-1 at 2 and 4 h to 2±1 mmHg ($P<0.05$) and 5±2 mmHg ($P<0.05$), respectively. In these animals, SB 209670 also reduced the pressor response elicited by subsequent injections of ET-1 at 2 and 4 h to 2±1 mmHg ($P<0.05$) and 3±1 mmHg ($P<0.05$).

Time-course of the release into the serum of ET-1, TNF-α and IFNγ caused by endotoxaemia

Injection of endotoxin (10 mg kg^{-1}, i.v.) resulted, within 60 min, in a substantial increase in the serum levels of ET-1, reaching a maximum at 120 min after injection of LPS ($P<0.05$, $n=6$; Figure 1). This increase in the serum level of ET-1 was accompanied by an increase in the serum levels of TNF-α. The increase in the serum level of TNF-α caused by LPS was maximal 60 min ($P<0.05$, $n=6$) after injection of LPS and returned to baseline levels within 180 min (Figure 1). After 120 min there was a sustained increase in the serum level of IFN-γ ($n=6$), reaching a maximum of more than 2500 pg ml^{-1} at 300 min.

Effects of the non-selective ET_A/ET_B receptor antagonist, SB 209670, on the circulatory failure caused by endotoxaemia

Baseline values of MAP or heart rate were not significantly different between any of the experimental groups studied (Figure 2). In rats without endotoxaemia which were pretreated with vehicle for SB 209670, there was no significant alteration in MAP or heart rate (Figure 2). Moreover, pretreatment of rats (without endotoxaemia) with SB 209670 did

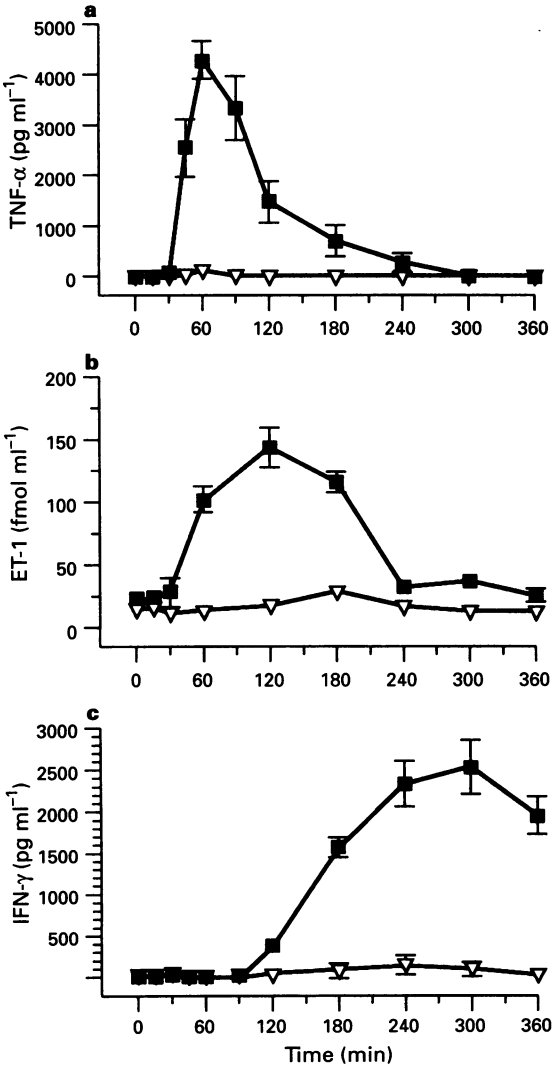

Figure 1 Time-course of the release into the plasma of (a) tumour necrosis factor-α (TNF-α), (b) endothelin-1 (ET-1), and (c) interferon-γ (IFNγ) in rats treated with *E. coli* lipopolysaccharide (■, 10 mg kg^{-1}, i.v., $n=6-9$) or vehicle (△, $n=3-6$). Data are expressed as mean±s.e.mean of *n* observations.

not result in any significant change in MAP or heart rate (Figure 2).

In contrast, injection of LPS (10 mg kg^{-1}, i.v.) resulted in a rapid, but transient fall of MAP of approximately 40 mmHg within 10 min ($P<0.05$, $n=15$; Figure 2a). The MAP values of rats treated with LPS remained above 95 mmHg from 60 to 240 min, and fell significantly towards the end of the experimental period at 360 min (Figure 2a). Thus, the MAP values of LPS-rats were significantly lower than the ones measured in rats which received vehicle (rather than LPS) between 10–360 min after injection of LPS ($P<0.05$, Figure 2a). Pretreatment of LPS-rats with a bolus injection of SB 209670 (3 mg kg^{-1}, i.v. at 15 min prior to LPS) significantly augmented the hypotension caused by endotoxin (Figure 2a). Similarly, continuous infusion of the non-selective ET-receptor antagonist (3 mg kg^{-1} i.v., plus 100 $\mu g\ kg^{-1}\ min^{-1}$ starting 15 min prior to LPS and continued throughout the experiment) markedly augmented the fall in MAP elicited by endotoxin (Figure 2a).

Endotoxaemia for 360 min resulted in a time-dependent increase in heart rate (Figure 2b). This tachycardia was more pronounced in LPS-rats which were pretreated with either a bolus injection of SB 209670 or with an infusion of the non-selective ET-receptor antagonist (Figure 2b).

Effects of the non-selective ET_A/ET_B receptor antagonist, SB 209670, on the vascular hyporeactivity to noradrenaline caused by endotoxaemia

The mean baseline values for the pressor response to NA (1 $\mu g\ kg^{-1}$, i.v.) ranged from 29 ± 3 to 36 ± 3 mmHg and were not significantly different between any of the experimental groups studied. In animals without endotoxaemia, injection of neither vehicle (for SB 209670) nor SB 209670 had any significant effect on the pressor responses elicited by NA (Figure 3).

Endotoxaemia resulted in a rapid, biphasic attenuation of the pressor responses elicited by NA ($P<0.05$, $n=15$, Figure 3). This vascular hyporeactivity was more pronounced in LPS-rats which were pretreated with either a bolus injection of SB 209670 or with an infusion of the non-selective ET-receptor antagonist (Figure 3).

Effects of the non-selective ET_A/ET_B receptor antagonist, SB 209670, on survival in rats with endotoxic shock

Although all control animals treated with LPS (10 mg kg^{-1} i.v.) survived for 240 min, 6 out of 21 animals subjected to endotoxaemia for 6 h died. Pretreatment of LPS-rats with SB 209670 (3 mg kg^{-1}, i.v. at 15 min prior to LPS) reduced the time to the onset of death so that the 6 h-survival declined from 71% (control) to 30% ($P<0.05$; Figure 4). Similarly, continuous infusion of SB 209670 (3 mg kg^{-1}, i.v. prior to LPS plus 100 $\mu g\ kg^{-1}\ min^{-1}$) reduced the 6 h-survival of LPS-rats to 13%.

Effects of the non-selective ET_A/ET_B receptor antagonist, SB 209670, on the renal and liver dysfunction caused by endotoxaemia

As more than 70% of animal treated with SB 209670 died within 4 and 6 h after injection of LPS, the effect of the non-selective ET_A/ET_B receptor antagonist on organ function was assessed in a separate set of experiments in which the period of endotoxaemia was limited to 4 h. Endotoxaemia for 4 h resulted in rises in serum urea and creatinine. Pretreatment of LPS-rats with SB 209670 (3 mg kg^{-1}, i.v. 15 min prior to LPS) had no effect on the rise in serum urea, but significantly augmented the increase in serum levels of creatinine. Four hours of endotoxaemia caused only moderate (if any) increases in the serum levels of bilirubin, GPT and GOT. Pretreatment of LPS-rats with SB 209670, however, was associated with a pro-

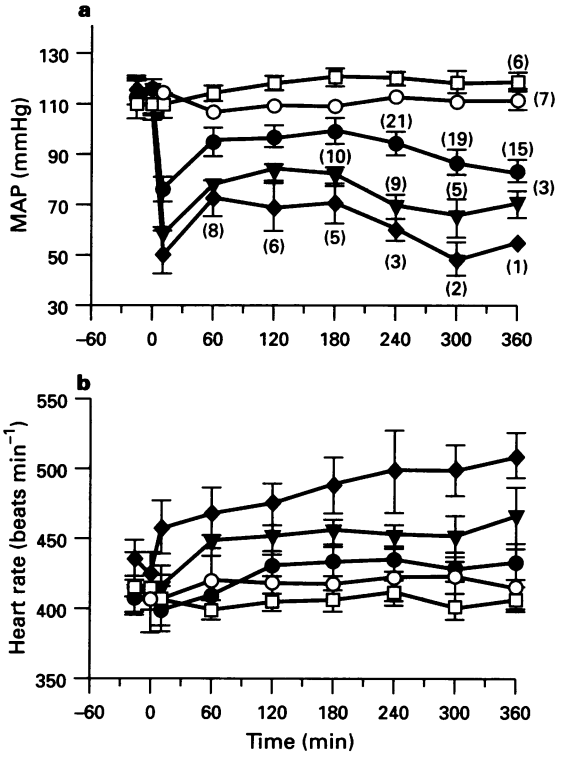

Figure 2 Effects of the non-selective ET_A/ET_B receptor antagonist, SB 209670, on the circulatory failure in endotoxic shock in the anaesthetized rat. Depicted are the changes in (a) mean arterial blood pressure (MAP) and (b) Heart rate in rats which had received (i) vehicle (saline) for LPS and vehicle (saline) for SB 209670 (□, $n=6$), (ii) vehicle for LPS plus SB 209670 (3 mg kg^{-1} i.v., ○, $n=7$), (iii) *E. coli* lipopolysaccharide (10 mg kg^{-1}, i.v.) and were treated with bolus injections of vehicle for SB 209670 (0.6 ml $kg^{-1}\ h^{-1}$ of saline, ●, $n=21$), (iv) LPS plus SB 209670 (3 mg kg^{-1} i.v., 15 min prior to LPS followed by i.v. infusion of 100 $\mu g\ kg^{-1}\ min^{-1}$; ◆, $n=8$) or (v) LPS (as above) plus SB 209670 (SB/LPS, 3 mg kg^{-1} i.v., 15 min prior to LPS, ▲, $n=10$). Data are expressed as mean±s.e.mean of n observations. The number of animals alive at a respective time point is shown in parentheses. MAP and heart rate in SB 209670 treated LPS-rats were significantly lower ($P<0.05$) at time 10 min, 60, 120, 180, 240 and 300 min after LPS when compared to LPS-control rats.

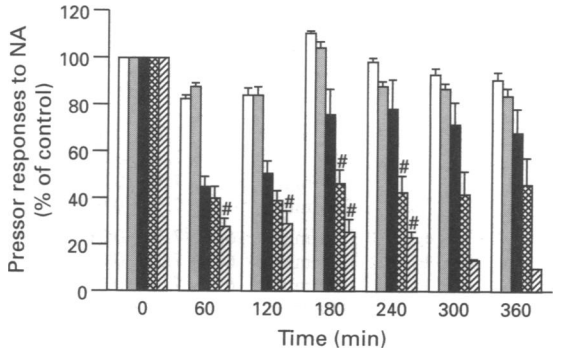

Figure 3 Effects of the non-selective ET_A/ET_B receptor antagonist, SB 209670, on the pressor responses to noradrenaline in endotoxic shock in the anaesthetised rat. These pressor responses to noradrenaline were measured in rats which had received (i) vehicle (saline) for LPS and vehicle (saline) for SB 209670 (open columns, $n=6$), (ii) vehicle for LPS plus SB 209670 (0.6 ml $kg^{-1}\ h^{-1}$ of saline, dotted columns, $n=21$), (iii) LPS alone (10 mg kg^{-1} i.v., black columns, $n=15$), (iv) LPS plus SB 209670 (3 mg kg^{-1} i.v. 15 min prior to LPS followed by i.v. infusion of 100 $\mu g\ kg^{-1}\ min^{-1}$; hatched columns, $n=8$), or (v) LPS (as above) plus SB 209670 (3 mg kg^{-1}, i.v., crossed columns, $n=10$) 15 min prior to LPS. Data are expressed as mean±s.e.mean of n observations. $^{\#}P<0.05$ represents significant differences when compared to LPS-controls at the same time point.

nounced increase in the serum levels of bilirubin, GPT and GOT. Although endotoxaemia resulted in a pronounced increase in the serum levels of CK and nitrite, pretreatment of LPS-rats with SB 209670 had no effect on this rise in the serum levels of CK or nitrite (Figure 5).

Effects of the non-selective ET_A/ET_B receptor antagonist, SB 209670, on acid-base balance and blood gases in endotoxaemia

Injection of LPS (10 mg kg^{-1}, i.v.) caused within 15 min an acute metabolic acidosis as indicated by the falls in pH, HCO_3^- and base excess (see Table 1). The fall in pH, but not the falls in HCO_3^- and base excess, returned to baseline at 240 min. In addition, endotoxaemia for 240min caused a significant fall in $PaCO_2$ (Table 1). Pretreatment of LPS-rats with SB 209670 (3 mg kg^{-1}, i.v. bolus) significantly augmented the falls in HCO_3^- and base excess as well as the fall in $PaCO_2$ at 15 and 60 min (Table 1). Please note that no significant alternation in any of the above parameters was observed in rats without endotoxaemia treated with either vehicle for LPS or vehicle for SB 209670 (Table 1).

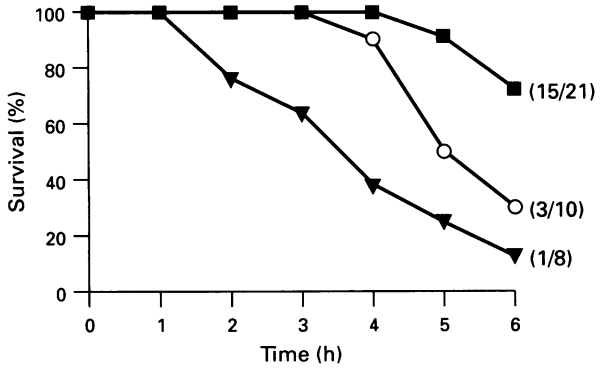

Figure 4 SB 209670 reduced the 6 h-survival of rats treated with *E. coli* lipopolysaccharide (LPS). Different groups of LPS-rats had received (15 min prior to LPS) infusions of either vehicle for SB 209670 (saline, 0.6 ml kg^{-1} h^{-1}, ■, $n=21$) or SB 209670 (3 mg kg^{-1} plus 100 μg kg^{-1} min^{-1}, ▲, $n=8$), or a bolus injection of SB 209670 (3 mg kg^{-1}, i.v., ○, $n=10$).

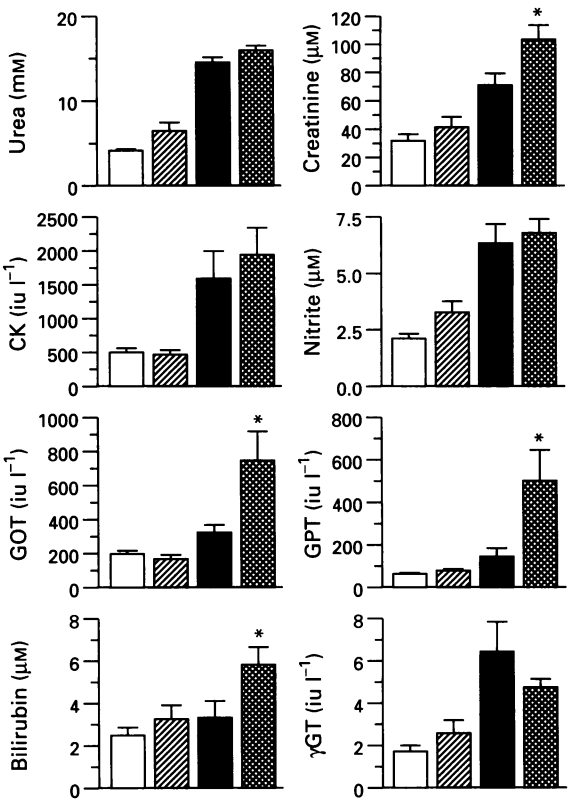

Figure 5 Effects of the non-selective ET_A/ET_B receptor antagonist, SB 209670, on the LPS-induced increases in plasma concentrations of urea, creatinine, creatinine kinase (CK), nitrite, glutamate-oxalate-transferase (GOT), glutamate-pyruvate-transferase (GPT), bilirubin and gamma-glutamyl transferase (γGT). These enzymes activities were measured in plasma obtained from rats which had received (i) vehicle (saline) for LPS and vehicle (saline) for SB 209670 (open columns, $n=6$), (ii) vehicle LPS plus SB 209670 (3 mg kg^{-1}, i.v., hatched columns, $n=7$), (iii) *E. coli* lipopolysaccharide (LPS, 10 mg kg^{-1}, i.v.) for 240 min and were treated with bolus injections of vehicle for SB 209670 (LPS, 0.6 ml kg^{-1} h^{-1} of saline, solid columns, $n=8$), or (iv) LPS (as above) plus SB 209670 (3 mg kg^{-1} i.v. bolus injection, $n=8$) 15 min prior to LPS. Data are expressed as mean ± s.e.mean of n observations. $^*P<0.05$ represents significant increase in enzyme activity in LPS-rats treated with SB 209670 when compared to LPS-control.

Table 1 Effects of SB 209670 on blood gases and acid-base balance in rats with endotoxic shock

Treatment					Pa_{O_2}	Pa_{CO_2}	tCO_2	HCO_3^-	*BE*
LPS	*SB 209670*		n	*pH*	(mmHg)	(mmHg)	(mmol l^{-1})	(mmol l^{-1})	(mmol l^{-1})
–	–	0 min	7	7.34±0.01	69.3±1.8	47.5±0.4	30.3±0.6	28.8±0.6	2.2±0.6
–	–	15 min	7	7.35±0.01	70.2±2.1	47.9±0.3	30.2±0.3	28.6±0.3	2.1±0.4
–	–	60 min	7	7.35±0.01	70.3±2.6	46.1±0.9	29.1±0.9	27.5±0.8	1.4±0.7
–	–	240 min	7	7.38±0.01#	81.7±3.8#	40.9±1.9#	27.7±1.1	26.4±1.1	1.9±0.8
–	+	0 min	6	7.35±0.01	69.8±1.3	48.9±0.6	28.9±0.7	28.2±0.7	1.7±0.5
–	+	15 min	6	7.35±0.01	70.1±1.7	48.3±0.7	28.2±0.6	28.5±0.5	1.8±0.8
–	+	60 min	6	7.36±0.01	73.1±2.2	45.6±1.1	27.1±0.8	27.9±1.2	1.4±0.4
–	+	240 min	6	7.37±0.01	79.7±3.1#	41.9±1.7#	27.9±1.8	26.7±1.7	1.6±1.3
+	–	0 min	8	7.34±0.01	68.9±1.3	48.8±0.8	30.9±0.8	29.1±0.7	1.5±0.8
+	–	15 min	8	7.29±0.01#	78.7±2.0#	46.5±1.4	25.7±1.2#	24.2±1.1#	–3.0±1.2#
+	–	60 min	8	7.31±0.02#	79.8±2.6#	42.9±1.9#	23.8+1.4#	22.4±1.3#	–3.9±1.1#
+	–	240 min	8	7.35±0.02	79.9±3.7#	34.0±1.3#	30.2±1.0#	19.6±1.0#	–4.8±1.1#
+	+	0 min	8	7.33±0.01	68.3±1.7	47.6±1.6	28.1±0.6	27.9±0.5	1.1±0.4
+	+	15 min	8	7.29±0.01#	74.4±1.8#	34.3±1.4**#	18.9±0.7**#	17.9±0.6**#	–6.8±0.8**#
+	+	60 min	8	7.30±0.02	74.6±1.1#	31.0±2.4**#	18.0±0.8**#	16.8±1.0**#	–7.2±0.7*#
+	+	240 min	8	7.34±0.02	70.4±4.7	34.6±4.8#	18.0±0.6#	15.5±1.5#	–7.5±2.0#

Data are expressed as mean ± s.e.mean of n observation. $^*P<0.05$ and $^{**}P<0.01$ represents significant differences when compared to LPS-controls at the same time point. $\#P<0.05$ when compared to time 0 min within the same group of animals. Abbreviations: arterial oxygen tension, Pa_{O_2}; arterial carbon dioxide tension, Pa_{CO_2}; total carbon dioxide, tCO_2; standard bicarbonate, HCO_3^-, base excess, BE Lipopolysaccharide (LPS, 10 mg kg^{-1} i.v.) was given at time 0; SB 209670 (3 mg kg^{-1} i.v. bolus) was given 15 min prior to LPS.

Discussion

This study demonstrates that the non-selective ET_A/ET_B receptor antagonist SB 209670 augments the hypotension and the vascular hyporeactivity to noradrenaline caused by endotoxaemia in the anaesthetized rat. We also demonstrate that the blockade of ET_A and ET_B receptors with SB 209670 augments the serum levels of creatinine, GTP, GOT as well as bilirubin. The rises in the serum levels of these biochemical markers are likely to reflect progressive organ dysfunction or failure. For instance, the criteria for the definition of organ failure in patients with septic shock (see Baue, 1993, for review) state that a greater than 2 fold rise in serum bilirubin is indicative of the development of liver failure, while rises in the serum levels of GPT and GOT (less specific) suggest liver cell injury (see Baue, 1993). Interestingly, endotoxaemia for 240 min in the anaesthetized rat (this study) did not result in significant rises in the serum levels of bilirubin, GPT or GOT, and, hence, liver failure or injury. In contrast, pretreatment of LPS-rats with SB 209670 resulted in significant elevations in the serum levels of bilirubin (2 fold), GOT (3 fold) and GTP (5 fold) suggesting the development of an impairment of liver function (bilirubin) and cell integrity (GPT, GOT). Moreover, the finding that the ratio of GOT to GPT (De Ritis ratio) is greater than 1, is indicative of a severe hepatocellular injury.

In addition, the criteria for the definition of organ failure of sepsis (see Baue, 1993, for review) state that a 2 fold increase in serum creatinine (regardless of polyuria or oliguria) indicates acute renal failure. In our model, endotoxaemia for 240 min alone results in a nearly 2 fold increase in serum creatinine suggesting that acute renal failure has developed. We also demonstrate that treatment of LPS-rats with SB 209670 results in a further rise in serum creatinine from 70 μM to 100 μM. Although the inverse relationship between serum creatinine and glomerular filtration rate is non-linear (see Levey *et al.*, 1991), it is likely that, at least at this level of impaired renal function, the observed rise in creatinine caused by SB 209670 in rats with endotoxaemia equates with a more severe fall in glomerular filtration rate.

Endotoxaemia also resulted (within 15 min) in falls in pH, HCO_3^- and base excess and, hence, a metabolic acidosis. The continuous fall in $PaCO_2$ observed during the remainder of the experiment was associated with a normalization of pH suggesting that the acute lactate acidosis was largely compensated for by an increase in respiration rate (fall in $PaCO_2$ and HCO_3^-). Interestingly, pretreatment of rats with SB 209670 augmented the degree of the metabolic acidosis caused by endotoxin. The metabolic acidosis associated with shock is due to tissue hypoxia secondary to an impairment of tissue oxygen extraction. Most notably, tissue oxygen extraction in shock is dependent on oxygen delivery (supply-dependency of tissue oxygenation) and, hence blood pressure and cardiac output. Thus, we propose that the increase in the degree of organ dysfunction caused by the ET-receptor antagonist is secondary to the observed fall in blood pressure and, hence, organ perfusion.

Although the rapid release of ET-1 may serve to attenuate the fall in blood pressure associated with endotoxaemia (beneficial effect of ET-1), excessive rises in the serum levels of ET-1 for longer periods are also associated with an excessive vasoconstriction in some vascular beds (harmful effects of ET-1). For instance, the decrease in microvascular blood flow caused by injection of live *E. coli* bacteria into anaesthetized rats is attenuated by pretreatment of animals with a monoclonal antibody to ET-1 (Wilson *et al.*, 1993). Similarly, the ET_A receptor antagonist, BQ-485, attenuates the decrease in gastric mucosa blood flow and subsequently the injury to the gastric mucosa caused by endotoxic shock in the rat (Kitajima *et al.*, 1994). The delayed and sustained increase in pulmonary arterial pressure associated with endotoxaemia in the pig is also attenuated by bosentan, a non-selective ET_A/ET_B receptor antagonist (Weitzberg and Lundberg, personal communication). The finding that bosentan had no effect on blood pressure, but caused a significant increase in cardiac output (and, hence, a fall in peripheral vascular resistance) lends support to our hypothesis that the release of endogenous ET-1 in endotoxaemia attenuates the fall in peripheral vascular resistance. Indeed, while this manuscript was under review, Gardiner *et al.* (1995) reported that treatment of conscious, chronically-instrumented rats with SB 209670 also enhanced the hypotension caused by infusion of LPS for 24 h. This effect of SB 209670 was associated with a conversion of mesenteric vasoconstriction to vasodilatation as well as an augmentation of the hindquarters vasodilatation elicited by endotoxaemia (Gardiner *et al.*, 1995).

What, then, is the mechanism by which endotoxaemia leads to this rapid elevation of the serum levels of ET-1? There is increasing evidence that proinflammatory cytokines such as TNF-α stimulate the biosynthesis of ET-1 (Ham *et al.*, 1994; Klemm *et al.*, 1995). In addition to proinflammatory cytokines, other factors may contribute to the enhanced formation of ET-1 in endotoxaemia. Indeed, activation of the sympathetic nervous system by cold stress (Fyhrquist *et al.*, 1990) or passive upright tilt (Kaufmann *et al.*, 1991) increases the circulating levels of ET-1 in humans subjects. Moreover, endotoxaemia leads to rapid increases in the serum levels of adrenaline, angiotensin II, and vasopressin, all of which stimulate the synthesis of ET-1 from endothelial cells in culture (Emori *et al.*, 1989). Clearly, shear stress (Yoshizumi *et al.*, 1989) or stretch (McArthur *et al.*, 1994) cause the release of ET-1 from endothelial cells in culture. Although the vascular endothelium is the most likely source for the generation of ET-1 in endotoxaemia, it should be noted that mast cells, smooth muscle cells, monocytes or particularly macrophages (Ehrenreich *et al.*, 1990) also have the ability to generate ET-1. In addition, activation of neutrophils leads to the generation of proteases such as cathepsin G, which in turn converts big ET-1 to ET-1 (Kaw *et al.*, 1992).

In conclusion, this study demonstrates that blockade of ET_A and ET_B receptors with SB 209670 augments *(i)* the circulatory failure (hypotension, vascular hyporeactivity to noradrenaline), *(ii)*, the degree of renal dysfunction, and *(iii)* the metabolic acidosis caused by endotoxaemia in the rat. In addition, treatment of LPS-rats with SB 209670 resulted in the development of hepatocellular injury and liver dysfunction, which did not occur (within 4 h) in rats treated with LPS alone. Finally, SB 209670 reduced the 6 h-survival of rats with endotoxaemia. The increase in the severity of the organ dysfunction and metabolic acidosis caused by SB 209670 is likely to be due to a reduction in perfusion pressure. Thus, the release of endogenous ET-1 in endotoxaemia constitute a double-edged sword in that ET-1 counteracts the severe hypotension and fall in peripheral vascular resistance (beneficial effect), and also may cause an excessive vasoconstriction in some vascular beds (harmful effect).

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