British Journal of Pharmacology (1996) 118, 617–626

Bosentan-improved cardiopulmonary vascular performance and increased plasma levels of endothelin-1 in porcine endotoxin shock

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1 To evaluate the possible contribution of endothelin-1 (ET-1) to the pathophysiology of porcine septic shock, the non-peptide, mixed ET-receptor antagonist, bosentan (RO 47–0203) was administered (5 mg kg^{-1}, i.v.) 30 min before infusion of lipopolysaccharide (LPS) (*E. coli.*, serotype 0111:B4) (15 $\mu g\ kg^{-1}\ h^{-1}$) and at 3.5 h of endotoxaemia in six anaesthetized and mechanically ventilated pigs. Six other pigs served as controls and received only LPS infusion. Pulmonary and systemic haemodynamics as well as splenic, renal and intestinal blood flows were measured continuously. Release and synthesis of ET-1 and Big ET-1 were also measured.

2 Only three of the six pigs in the control group survived 3 h of LPS infusion while in the bosentan-treated group all six pigs were alive at that time. A biphasic increase in mean pulmonary arterial pressure (MPAP) and pulmonary vascular resistance (PVR) was seen in control pigs. Pretreatment with bosentan did not influence the first peak but markedly attenuated the second, more prolonged increase in MPAP and PVR. The second dose of bosentan completely restored these parameters to pre-LPS levels. The LPS-induced changes in mean arterial blood pressure, heart rate and systemic vascular resistance were similar in both groups, while cardiac output (CO) was significantly higher in the bosentan-treated group. The second bosentan dose increased CO and splenic and intestinal blood flow without further lowering of blood pressure.

3 Bosentan caused an increase of the basal arterial plasma levels of ET-1-like immunoreactivity (LI), from 16.8 ± 1.3 pM to 49.6 ± 10.0 pM ($n=6$, $P<0.01$). However, the rate of the increase of ET-1 levels during the LPS infusion was not affected by bosentan. Repeated administration of bosentan during LPS infusion caused an additional increase of ET-1-LI levels. Neither the basal levels of Big ET-LI nor the LPS induced 8 fold increase in Big ET-LI were changed by bosentan. The level of preproET-1 mRNA in the lung was increased about 3 fold after 4.5 h of LPS treatment. This elevation was not influenced by bosentan.

4 From these studies using bosentan, a non-peptide, selective and mixed ET-receptor antagonist, we conclude that during LPS-induced shock bosentan can abolish the late phase pulmonary hypertension and improve cardiac output as well as increase blood flow to the splenic and intestinal vascular beds without causing a further decrease in mean arterial blood pressure. Further investigations in the clinical setting are needed to evaluate the use of ET-receptor antagonists, such as bosentan, in treatment of septic shock.

Keywords: Endothelin; big endothelin; endothelin receptor antagonist; bosentan; lipopolysaccharide; septic shock; pulmonary hypertension; intestinal blood flow; splenic blood flow; noradrenaline

Introduction

The endothelium-derived peptide endothelin-1 (ET-1) is the most potent vasoconstrictor known to date (Yanagisawa *et al.*, 1988). ET-1 belongs to a family of peptides also including ET-2 and ET-3 of which ET-1 is thought to be the most widely distributed. Two different ET-receptors have been identified and cloned; the ET_A-receptor on the vascular smooth muscle cell, mediating vasoconstriction (Arai *et al.*, 1990) and the ET_B-receptor localized on the endothelium, mediating vasodilatation via release of prostacyclin and/or nitric oxide (De Nucci *et al.*, 1988; Sakurai *et al.*, 1990). Recent data indicate that there is a subtype of the ET_B-receptor localized on the vascular smooth muscle cell, mediating vasoconstriction (Clozel *et al.*, 1992; Moreland *et al.*, 1992). Increased plasma levels of ET-1 have been found in various disorders such as myocardial infarction (Miyauchi *et al.*, 1989), cardiogenic shock (Cernacek & Stewart, 1989) and atherosclerosis (Lerman *et al.*, 1991). Also in experimental endotoxin models and in human septic shock a 3–4 fold elevation of plasma levels of ET-1 are seen (Pernow *et al.*, 1989a; Weitzberg *et al.*, 1991b) which are in accordance with *in vitro* studies showing that ET-1 production from cultured endothelial cells is stimulated by lipopolysaccharide (LPS) (Sugiura *et al.*, 1989) and tumour necrosis factor-α (TNFα) (Maemura *et al.*, 1992), mediators with a central role in sepsis. However, it remains to be determined whether ET-1 has any pathophysiological role or is just a marker of disease. Exogenous ET-1 given intravenously to healthy subjects induces long-lasting vasoconstriction in the pulmonary, renal and splanchnic vascular beds as well as a reduction in cardiac output (Weitzberg *et al.*, 1991a; 1993a) which partly resembles the features of clinical sepsis (see Parker & Parrillo, 1983; Bone, 1991).

Previous experiments show that the non-peptide ET antagonist, bosentan (Clozel *et al.*, 1994) decreases basal vascular resistance and increases plasma levels of ET-1 in control pigs (Weitzberg *et al.*, 1994).

The aim of the present study was to investigate the participation of ET-1 in the cardiovascular changes seen in LPS-induced shock, by use of the mixed non-peptide competitive

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ET-receptor antagonist, bosentan. Furthermore, we have investigated the effects of bosentan on ET-1 release and synthesis during LPS-induced shock.

Parts of these results have been published before, in a preliminary form (Weitzberg *et al.*, 1994; Lundberg *et al.*, 1995).

Methods

Surgical preparation

The experimental protocol was approved by the Animal Studies Committee of the Karolinska Institute. Domestic pigs of either sex (18–25 kg body weight) were fasted overnight and premedicated with ketamine hydrochloride (20 mg kg^{-1}) and atropine (0.02 mg kg^{-1}) injected intramuscularily. Anaesthesia was induced by an intravenous bolus injection of sodium pentobarbitone (20 mg kg^{-1}) and skeletal muscle relaxation was achieved by use of pancuronium bromide (0.2 mg kg^{-1}). After tracheostomy the pigs were mechanically ventilated with a volume-regulated ventilator (Servo ventilator 900, Siemens-Elema, Sweden) with a mixture of air and oxygen (250 ml kg^{-1} min^{-1} FiO_2: 0.30). Anaesthesia and muscle relaxation were maintained by continuous infusion of sodium pentobarbitone (3 mg kg^{-1} h^{-1}) and pancuronium bromide (0.5 mg kg^{-1} h^{-1}) through a catheter inserted via a femoral vein into the inferior caval vein. This catheter was also used for infusion of Ringer solution with glucose (250 ml h^{-1}) and heparin (200 iu kg^{-1} h^{-1}) to prevent blood clotting in the catheters. An arterial catheter was inserted via a femoral artery for measurement of mean arterial blood pressure (MABP) and heart rate with a Statham P23Ac pressure transducer; responses were recorded continuously on a Grass Polygraph (model 7B). For measurement of cardiac output a left sided thoracotomy was performed and an ultrasonic flow probe (Transonic Inc., Ithaca, NY, U.S.A.) was placed around the pulmonary artery. After opening of the abdomen via a left subcostal incision, additional ultrasonic flow probes were placed around the left renal artery, the splenic artery and the superior mesenteric artery for measurement of local blood flows. The flow probes were connected to Transonic flowmeters (T202-s) and blood flows were continuously recorded on the Grass Polygraph. A catheter was inserted into the left ureter for estimation of urine production. A triple-lumen balloon-tipped catheter was placed in the pulmonary artery via a femoral vein to allow measurements of mean pulmonary artery pressure (MPAP), pulmonary capillary wedge pressure (PCWP) and right atrial pressure (RAP). A catheter was inserted in a brachial artery for collection of arterial blood. Blood gases were measured regularly by means of an automatic blood gas analyzer (IL 1302, Metric AB, Solna, Sweden).

Arterial plasma levels of noradrenaline were analyzed by cation exchange high performance liquid chromatography (h.p.l.c.) with electrochemical detection (see Hjemdahl, 1987).

Experimental protocol

Twelve pigs were challenged with a continuous infusion of LPS (*E. coli*, serotype 0111:B4) (15 μg kg^{-1} h^{-1}). Six of these pigs received pretreatment with bosentan (5 mg kg^{-1}, i.v. bolus in 10 ml of saline) 30 min prior to the start of LPS infusion. These pigs were also given a second dose of bosentan at 3.5 h of endotoxaemia. Haemodynamic parameters were measured and recorded continuously. Arterial blood samples were taken at basal state (−30 min), 30 min after bosentan (0 min), and at 60, 120 and 180 min of LPS infusion for determination of ET-1, Big ET-1, noradrenaline and blood gases. Urine was collected during 60 min before and 30 min after bosentan injection and at 60, 120 and 180 min of LPS infusion. After the experiments the surviving animals were killed with an overdose of sodium pentobarbitone and tissue specimens were rapidly dissected out and frozen on dry ice. Additional tissue samples were obtained from untreated (although anaesthetized) pigs.

Sample handling

Collected blood samples were mixed with EDTA (final concentration 10 mM), chilled in an ice-water bath and centrifuged at 4°C. Plasma aliquots of 1 ml were pipetted off, frozen and stored at −20°C. Before analysis, the samples were extracted with 2 ml acid ethanol. After centrifugation the supernatants were decanted and dried in a 54°C waterbath under a nitrogen stream.

Production of antisera

Antisera against porcine (p) Big ET-1(1–39) were raised in New Zealand white rabbits. Synthetic pBig ET-1 (Peptide Institute, Japan) was coupled to bovine serum albumin (BSA, fraction V, Sigma, St. Louis, MO, U.S.A.) using the carbodiimide method (Goodfriend *et al.*, 1964). Conjugated pBig ET-1 (300 μg) was emulsified with Freund's complete adjuvant, giving a final volume of 3 ml. The mixture was then administered to three rabbits (B16 to B18) via two injections, one in each hindleg. Three booster injections with conjugated pBig ET-1 emulsified with Freund's incomplete adjuvant were given at 5 weeks intervals, the first dose containing 100 μg and the last two containing 50 μg of pBig ET-1. Blood samples were obtained 10–14 days after each booster injection and the antisera titres against pBig ET-1 were tested with radioimmunoassay (RIA). After the final booster dose the rabbits were anaesthetized with pentobarbitone and terminally bled. Antiserum B17 (terminal bleeding) was chosen for radioimmunoassay (RIA) due to its slightly lower detection limit at a titre of 1:3000 (i.e. the final dilution when both sample and radioligand has been added).

Radiolabelling of pBigET-1

One mCi Bolton and Hunter Reagent (Amersham International plc, Little Chalfont, Bucks, U.K.) was dried in a stream of nitrogen and 10 μg pBig ET-1(1–39) in 50 μl 0.1 M sodium borate buffer, pH 8.3, was added and incubated overnight at 4°C; 200 μl 0.1 M sodium phosphate buffer, pH 7.4, was added and the sample was then separated by reverse phase h.p.l.c. and eluted as below. Fractions of 1 ml were collected. Two fractions, 31 and 33, showed specific binding to the B17 antiserum and were stored frozen in aliquots for subsequent use in the RIA.

Radioimmunoassay

RIA for pBig ET-1 was performed in a 0.1 M phosphate buffer containing 0.05 M NaCl, 0.1% NaN_3 and 0.1% BSA. The samples were incubated for three days at 4°C with the B17 rabbit antiserum (final dilution 1:3000) before [^{125}I]-pBig ET-1 was added and the assay incubated for one more day. Bound and free fractions were then separated by a secondary antibody method (SacCel, IDS Ltd, Boldon, U.K.). The bound fractions were counted in a LKB 1249 Gamma Counter (LKB-Pharmacia, Uppsala, Sweden) and compared with known concentrations of synthetic pBig ET-1. The detection limit (given as the lowest detectable amount, with 95% confidence) was 1.6 fmol per tube. The crossreactivity of the B17 antiserum was: pBig ET-1 (1–39), 100%; pBig ET-1 (22–39), 37%; ET-1, <0.008%.

RIA for ET-1 was performed essentially as above except that the E1 antiserum (see Hemsén, 1991) and [^{125}I]-ET-1 (Amersham) were used as antiserum and tracer, respectively, and that the assay was incubated for 2+2 days. The lowest detectable amount was 0.39 fmol per tube. The crossreactivity of the E1 antiserum was: ET-1, 100%; ET-2, 27%; ET-3, 8%; pBig ET-1, 0.14%.

High performance liquid chromatography

For characterization of the immunoreactivity, the extracted samples were separated on a reverse phase h.p.l.c.-column

(SuperPac Cartridge, 4.0 × 250 mm, 5 μm, Pharmacia, Sweden) which was packed with Pep-S. The eluent, a 40 min linear gradient of 20–50% acetonitrile in 0.1% trifluoroacetic acid, was delivered at a flow rate of 1.0 ml min^{-1}. Fractions of 0.5 ml were collected, evaporated in a vacuum centrifuge (Savant Instruments Inc., Farmingdale, NY, U.S.A.) and stored at −20°C for subsequent analysis with RIA. Synthetic ET-1, ET-3, pBig ET-1 (1–39) and pBig ET- 1 (22–39) were used as standards in separate runs or together. The positions of the standards were determined by RIA for ET-1 and Big ET-1, respectively.

Preparation of ET-1 cDNA probe

Total RNA was isolated from porcine lung by a single extraction with an acid guanidinium thiocyanate-phenol-chloroform mixture as described by Chomzynski & Sacchi (1987). Selection of polyadenylated (PolyA$^+$) RNA was made by oligo(dT) chromatography. cDNA was generated using 5 μg poly(A$^+$) RNA as template by reverse transcription using murine Moloney leukaemia virus reverse transcriptase (Pharmacia). After incubation, the cDNA was purified on a Qiagen tip-20 (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Then 4 μl of the diluted cDNA was amplified using two specific ET-1 primers (forward primer: 5′-CCG AAT TCG TCA ACA CTC CAG AAC ACA T, backward primer: 5′-CGG AAT TCT CCT GTG CCA GTC TGC AGT A; (Scandinavian Diagnostic Services (SDS), Falkenberg, Sweden) containing cleavage sites for EcoRI, and 2.5 u Taq DNA polymerase (SDS). PCR was performed for 36 cycles (94°C for 1 min, 46°C for 1.5 min, 72°C for 1.5 min; final extension 72°C for 15 min). The PCR product was cleaved out with Eco RI (Amersham) and ligated with T4 DNA ligase (Amersham) into the Bluescript KS+ vector (Stratagene, La Jolla, CA, U.S.A.) which was then transformed to competent *E. coli* cells (XL-1, Stratagene). The culture was grown overnight at 37°C and spread on agar plates (containing 1.5% agar, 0.2% ampicillin, 0.5% IPTG and 0.2% X-gal) for blue-white selection. After incubation overnight at 37°C, colonies were picked and the plasmids were isolated by standard procedures. After cleavage with Eco RI the samples were separated by gel electrophoresis and the preparations containing inserts were subjected to dideoxy sequencing (T7 Sequencing Kit, Pharmacia). The sequence of the insert which was chosen for large scale plasmid preparation corresponded 100% with the nucleotide sequence 220–762 of the published porcine preproendothelin-1 (Itoh *et al.*, 1988). After plasmid isolation and enzymatic cleavage, the product was separated by gel electrophoresis and the insert was eluted into 0.7% LM agarose and further purified with phenol/chloroform extraction.

Before use in hybridization, the 533 bp probe was labelled with α[^{32}P]-dCTP (3000 Ci $mmol^{-1}$, Amersham) by nick translation.

Northern blot

Poly A$^+$ RNA was isolated as above from lungs from untreated pigs, pigs treated with LPS and pigs treated with both LPS and bosentan. The mRNA (10 μg) was separated by formaldehyde/agarose gel electrophoresis and blotted onto a Hybond-N (Amersham) nylon membrane with capillary blotting. The mRNA was fixed using u.v. crosslinking (Spectronics Corporation, Westbury, NY, U.S.A.). The filter was prehybridized in a hybridization oven (Techne, Cambridge, U.K.) for at least 15 min at 65°C in 10 ml of RapidHyb buffer (Amersham). An aliquot of the buffer was then removed and mixed with the ^{32}P-labelled ET-1 cDNA probe and added to the filter. Hybridization was performed overnight at 65°C. The filter was then washed with 50 ml of 2 × SSC + 0.1% SDS for 20 min at room temperature once and then washed three times with 0.2 × SSC + 0.1% SDS at 65°C for 15 min each. The membrane was then exposed to Hyperfilm β-max (Amersham) for 3–5 days at −80°C. As control for the amount of loaded mRNA, the filter was rehybridized with a ^{32}P-labelled GAPDH cDNA probe. The films were developed and the optical density was measured with an image analyzing system (Imaging Research Inc., St. Catharines, Ontario, Canada).

Drugs

Ketamine hydrochloride was from Parke-Davis, Morris Plains, NJ, U.S.A.; atropine was from Kabi-Pharmacia, Stockholm, Sweden; sodium pentobarbitone was from ACO, Stockholm, Sweden and pancuronium bromide was from Organon, Oss, Netherlands.

E. coli LPS, serotype 0111:B4 was from Sigma, St. Louis, MO, U.S.A. Bosentan (4-tert-butyl-N-[6-(2-hydroxy-ethoxy)-5-(2-methoxy-phenoxy)-2,2′-bipyrimidin-4-yl]-benzene-sulphonamide) was a kind gift from Dr Martine Clozel, F. Hoffman La Roche Ltd., Basel, Switzerland.

Data analysis

Systemic vascular resistance (SVR), pulmonary vascular resistance (PVR), oxygen delivery (DO_2), whole body arterial-venous oxygen difference (a-v O_2) and total body oxygen consumption (VO_2) were calculated according to standard formulae. Regional vascular resistances (VR) in the renal, splenic and intestinal vascular beds were calculated as MABP divided by the local blood flow.

For changes within each group of pigs, Wilcoxon's signed rank test was used, except at the end of LPS infusion in the bosentan-treated group, when only four pigs were alive; then Student's *t* test was used. Comparison between groups was done with Mann-Whitney U test. A *P* value of <0.05 was taken as significant. Data are presented as mean ± s.e.mean. Since only three of the six pigs in the control group survived 3 h of LPS infusion the statistical comparison between the groups was made only up to 2.5 h of LPS, when 5 of six pigs in the control group still were alive. All six pigs in the bosentan group were alive at 3 h of LPS. Four pigs in this group survived 210 min of LPS and were subjected to a second dose of bosentan.

Results

Effects of bosentan on intrinsic cardiovascular tone

Basal MABP and MPAP were reduced by 20 ± 2% (from 130 ± 6 mmHg to 103 ± 7 mmHg, $P<0.05$) and 22 ± 4% (23.8 ± 3.0 mmHg to 17.5 ± 1.5 mmHg, $P<0.05$), respectively, 30 min after bosentan; in fact a small but significant reduction was observed after only 5 min ($P<0.05$, not shown). Heart rate increased by 15 ± 6% (from 142 ± 6 beats min^{-1} to 163 ± 7 beats min^{-1}, $P<0.05$) after bosentan and stroke volume decreased by 18 ± 2% (from 11 ± 1 ml to 9 ± 1 ml, $P<0.05$), which resulted in an unchanged cardiac output. Of the vascular beds examined, the splenic circulation was the most sensitive to bosentan and splenic blood flow increased by 58 ± 28% (from 136 ± 31 to 192 ± 31 ml min^{-1}, $P<0.02$), while renal and intestinal blood flows were unchanged (Table 1). Since MABP decreased, significant decreases of regional VR were observed in both the splenic, renal and intestinal circulations; splenic VR decreased from basal levels of 1.38 ± 0.44 mmHg ml^{-1} min to 0.61 ± 0.10 mmHg ml^{-1} min ($P<0.05$), renal VR decreased from 1.23 ± 0.11 mmHg ml^{-1} min to 0.96 ± 0.04 mmHg ml^{-1} min ($P<0.05$) and intestinal VR decreased from 0.39 ± 0.04 mmHg ml^{-1} min to 0.28 ± 0.02 mmHg ml^{-1} min ($P<0.05$).

Urine production from the left kidney increased from 13 ± 2 ml h^{-1} to 23 ± 5 ml h^{-1} (71 ± 30%, $P<0.05$) after bosentan injection while it was stable in the control group.

Table 1 Regional blood flows (BF) in the spleen, kidney and intestine during lipopolysaccharide (LPS) infusion (15μg kg^{-1} h^{-1}) in control (n = 2-6) and bosentan-treated groups (n = 6)

				LPS infusion		
Unit	*Group*	*Basal*	*30 min after bosentan*	*60 min*	*120 min*	*180 min*
Splenic BF	Control	128 ± 15	120 ± 12	32.8 ± 8.6#	20.2 ± 4.1#	28.0 ± 3.8
(ml min^{-1})	Bosentan	136 ± 31	192 ± 31#	98.3 ± 23*	44.8 ± 18#	53.7 ± 16
Renal BF	Control	102 ± 20	103 ± 10	93.8 ± 18	66.7 ± 21	112 ± 12
(ml min^{-1})	Bosentan	110 ± 9	107 ± 6	127 ± 17	100 ± 18	101 ± 17
Intestinal BF	Control	282 ± 37	273 ± 28	195 ± 24#	230 ± 15	211 ± 57
(ml min^{-1})	Bosentan	354 ± 36	377 ± 27*	344 ± 71*	376 ± 46*	370 ± 75

# $P<0.05$ for intragroup changes compared to basal values and * $P<0.05$ for differences between groups.

LPS infusion

In the control group only three out of six pigs survived 180 min of LPS infusion. One pig died after 150 min and two pigs after 165 min. In the bosentan pretreated group all six pigs were alive after 180 min. Two pigs died after 195 min and four pigs were still alive at 270 min, after which they were killed with an overdose of sodium pentobarbitone.

Pulmonary haemodynamics during LPS infusion

LPS infusion in control pigs induced a biphasic increase in both MPAP and PVR with a significant elevation after only 15 min ($P<0.01$, Figure 1). In the bosentan pretreated pigs, the initial peak in MPAP and PVR was similar to that in controls whereas the second, more prolonged increase was markedly reduced by bosentan ($P<0.05$, Figure 1). In these pigs, MPAP and PVR were relatively stable after 3 h of LPS infusion and were not significantly higher than the levels seen before the initial dose of bosentan.

Systemic haemodynamics during LPS infusion

In the control pigs, MABP started to decline after 30 min of LPS and reached its nadir at 75 min after which it was stable at this low level ($P<0.01$, Figure 2a). The LPS effect in the bosentan group was similar, although starting at a lower level. Heart rate increased in a similar way in both groups (Figure 2b). Cardiac output declined up to 90 min of LPS infusion in the control group ($P<0.05$) while in the bosentan group, cardiac output was higher than in controls and even showed a tendency to increase (Figure 2c). The LPS-induced changes of SVR were the same in both groups, although absolute levels were lower in the bosentan group ($P<0.05$, Figure 2d).

Regional haemodynamics during LPS infusion

In the control group, the LPS infusion caused a large decrease of splenic blood flow (83 ± 9% after 2 h, Table 1). There was also a significant decrease of intestinal blood flow (29 ± 10% after 1 h, Table 1) whereas only a small decrease was observed in the kidney (35 ± 15% after 2 h, Table 1). In the bosentan-treated group splenic blood flows decreased in a similar way as in the control group, whereas no significant change of intestinal or renal blood flow was observed after 1 h (Table 1).

Splenic VR was lower in the bosentan group during the LPS infusion although the rate of increase in VR was the same in both groups (Figure 3a). There was no statistically significant difference between the groups in renal VR during LPS infusion but the bosentan-treated pigs had a tendency to lower and more stable levels (Figure 3b). An initial short increase in intestinal VR was noted in both groups; this was followed by a modest decrease compared to basal levels. The absolute levels were lower in the bosentan pretreated pigs ($P<0.05$, Figure 3c).

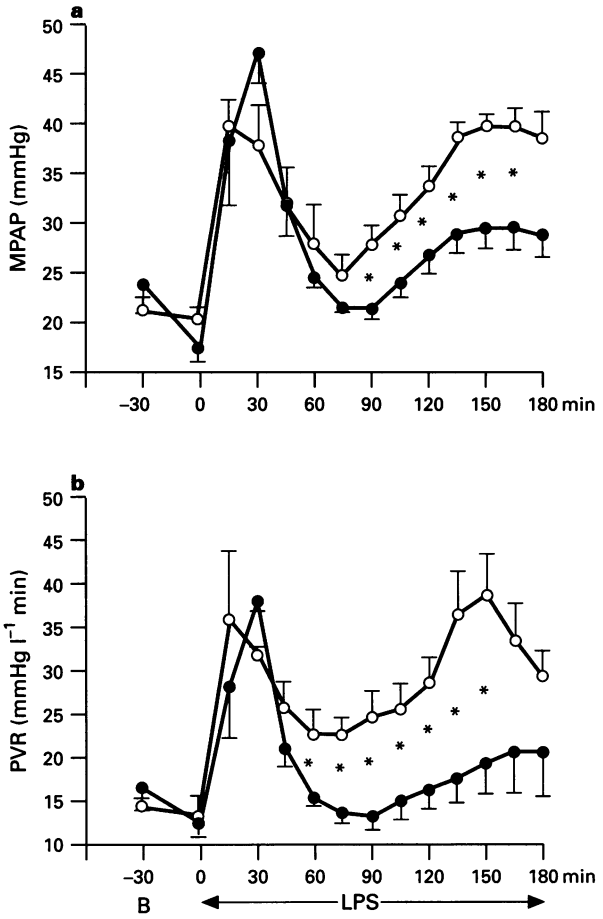

Figure 1 Mean pulmonary arterial pressure (MPAP) (a) and pulmonary vascular resistance (PVR) (b) before and during an i.v. infusion of lipopolysaccharide (LPS, *E. coli*, serotype 0111:B4; 15 μg kg^{-1} h^{-1}) in control pigs (○, n = 3–6) and in pigs pretreated with bosentan (B, 5 mg kg^{-1}, ●, n = 6) 30 min prior to the start of LPS infusion. * $P<0.05$ for differences between groups.

Second dose of bosentan during LPS infusion

After 3.5 h of LPS infusion a second dose of bosentan was given to the bosentan pretreated pigs and cardiovascular parameters were observed for another 60 min. MPAP went down to levels (22 ± 2 mmHg) that were similar to the basal values (24 ± 3 mmHg) and only slightly higher than the values seen after the initial bosentan injection (18 ± 1 mmHg, Figure 4a). The decrease in PVR was even more marked, with a reduction to levels (12 ± 2 mmHg ml^{-1} min) similar to those seen after the initial dose of bosentan (13 ± 2 mmHg ml^{-1} min, Figure 4b).

There was no further decrement in the already low MABP upon the second injection of bosentan (Figure 4c) and heart

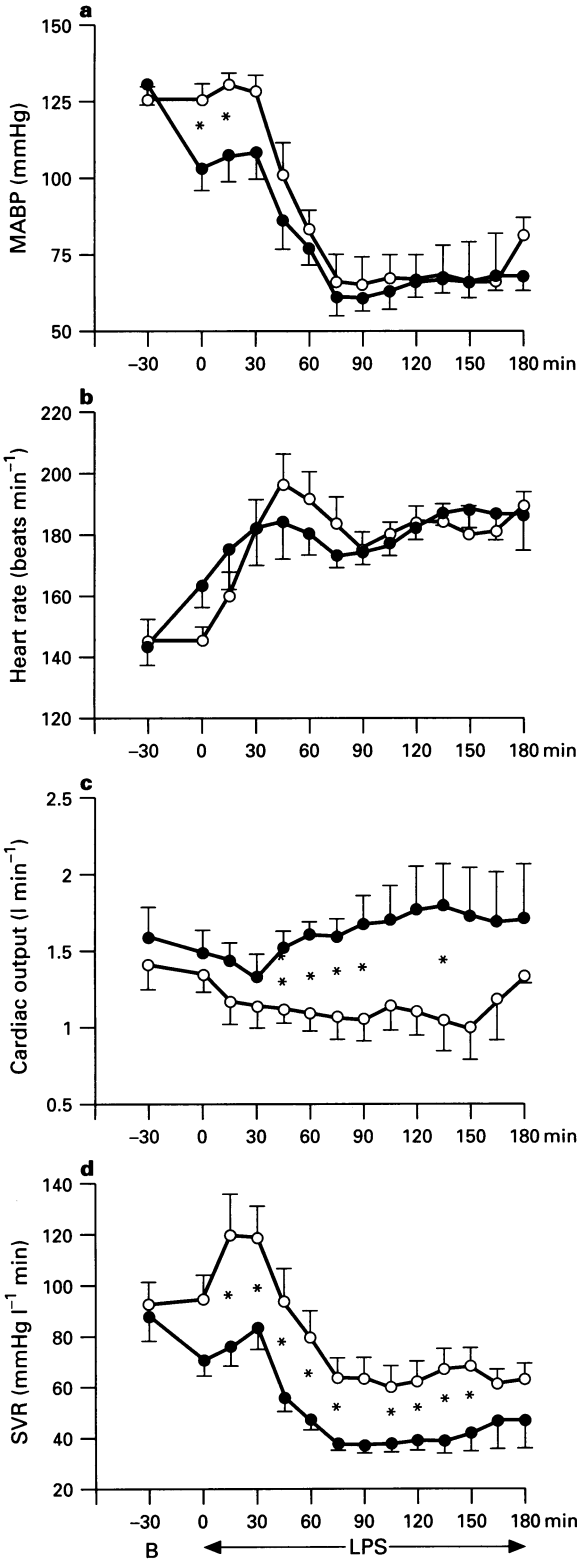

Figure 2 Mean arterial blood pressure (MABP) (a), heart rate (b), cardiac output (c) and systemic vascular resistance (SVR) (d) before and during an i.v. infusion of lipopolysaccharide (LPS, *E. coli*, serotype 0111:B4; 15 μg kg^{-1} h^{-1}) in control pigs (○, $n=3-6$) and in pigs pretreated with bosentan (B, 5 mg kg^{-1}) (●, $n=6$) 30 min prior to the start of LPS infusion. *$P<0.05$ for differences between the groups.

rate did not change significantly (not shown). On the other hand, an increased cardiac output (22±5%, from 1.45±0,04 to 1,77±0,10 l min^{-1} $P<0.05$, Figure 4d) was observed together with a decrease in SVR (20±6%, from 44.7±4.7 to 35.5±2.8 mmHg ml^{-1} min, $P<0.05$, Figure 4e).

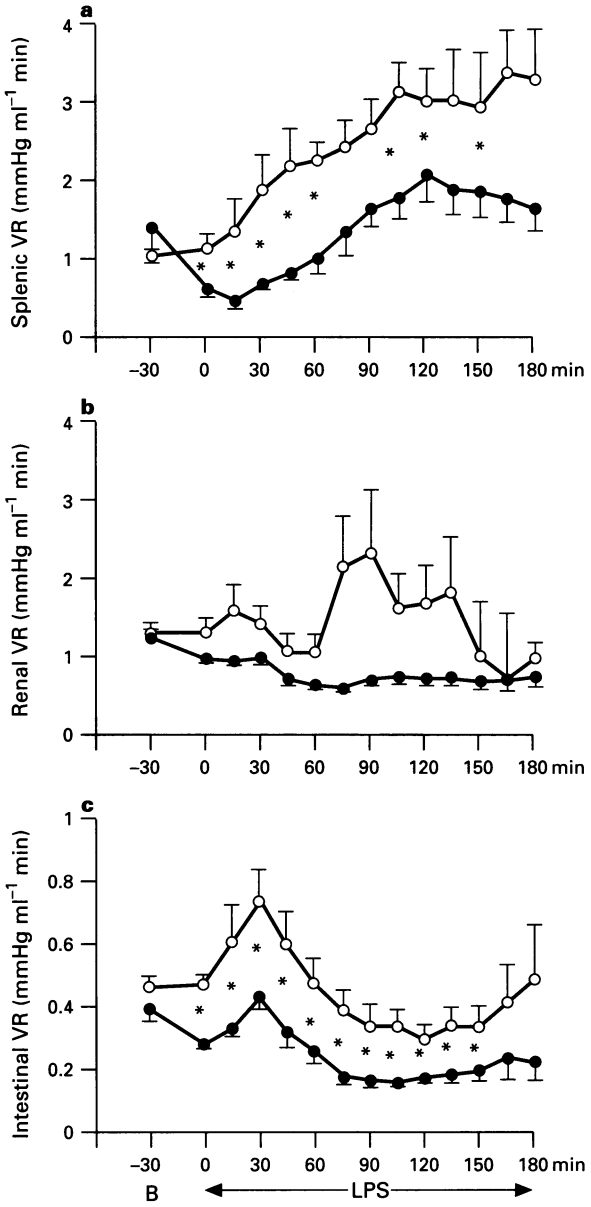

Figure 3 Splenic vascular resistance (VR) (a), renal VR (b) and intestinal VR (c) before and during an i.v. infusion of lipopolysaccharide (LPS, *E. coli*, serotype 0111:B4; 15 μg kg^{-1} h^{-1}) in control pigs (○, $n=3-6$) and in pigs pretreated with bosentan (B, 5 mg kg^{-1}, ●, $n=6$) 30 min prior to the start of LPS infusion. *$P<0.05$ for differences between the groups.

Blood flows in the spleen and the intestine were significantly increased by the second dose of bosentan while renal blood flow was unchanged (Table 2). As with the initial bosentan injection, the splenic vascular bed showed the strongest response to the second dose, with an almost 50% reduction in splenic VR (from 1.50±0.50 to 0.78±0.14 mmHg ml^{-1} min, $P<0.05$, Figure 4f). The values 60 min after the second dose of bosentan were similar to those 30 min after the initial injection. Intestinal VR was slightly reduced (Figure 4h) but renal VR was unaffected by the second dose of bosentan (Figure 4g).

Urine production, blood gases, oxygen delivery and arterial noradrenaline levels

During LPS infusion, urine production fell markedly without any significant differences between the groups (not shown) although there was a tendency towards higher urine output in the bosentan group at 60 min of LPS. The second dose of bosentan did not significantly increase urine production (not shown).

Haematocrit values were unchanged in both groups.

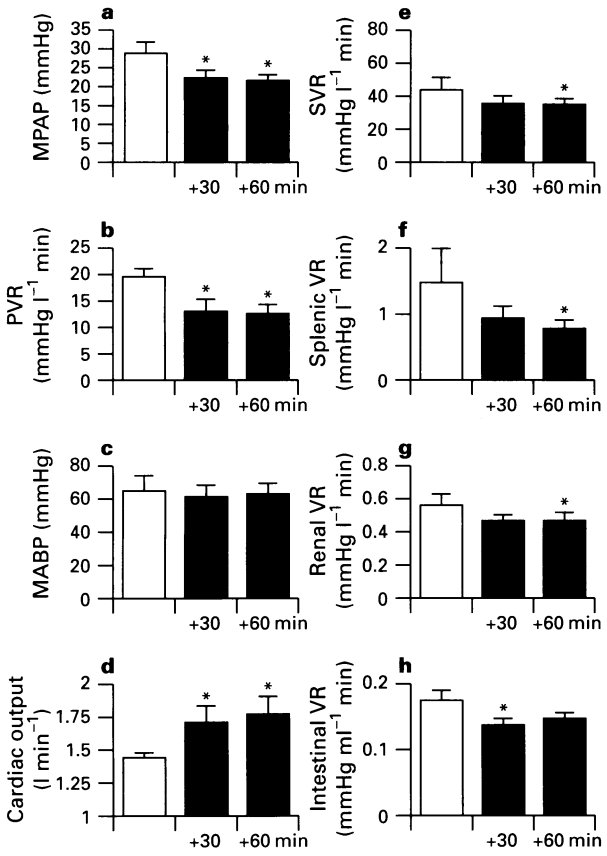

Figure 4 Mean pulmonary arterial pressure (MPAP) (a), pulmonary vascular resistance (PVR) (b), mean arterial blood pressure (MABP) (c), cardiac output (d), systemic vascular resistance (SVR) (e), splenic VR (f), renal VR (g) and intestinal VR (h) in four pigs given a second dose of bosentan (5 mg kg^{-1}, i.v.) after 210 min of an i.v. infusion of lipopolysaccharide (LPS, *E. coli*, serotype 0111:B4; 15 $\mu g\,kg^{-1}\,h^{-1}$). The initial dose of bosentan was given prior to LPS infusion (240 min earlier). Open bar shows values before the second dose and filled bars show the cardiovascular response after 30 and 60 min. $*P<0.05$ compared to values at 210 min of LPS infusion.

Table 2 Splenic, renal and intestinal blood flows (ml min^{-1}) in four pigs given a second dose of bosentan (5 mg kg^{-1}, i.v.) after 210 min of an i.v. infusion of lipopolysaccharide (LPS, *E. coli*, 15 $\mu g\,kg^{-1}\,h^{-1}$)

Blood flow (ml min^{-1})	*Basal*	*+30 min*	*+60 min*
Splenic	74 ± 36	81 ± 29	102 ± 30*
Renal	119 ± 18	132 ± 12	133 ± 10
Intestinal	378 ± 51	436 ± 45**	424 ± 52*

* $P<0.05$ compared to basal value; ** $P<0.01$ compared to basal values.

Blood gases and oxygen utilization are shown in Table 3. Bosentan induced a small but significant decrease in arterial oxygen saturation (SaO_2) and arterial oxygen partial pressure (PaO_2) before induction of LPS infusion ($P<0.05$) and SaO_2 and PaO_2 decreased in both groups upon LPS. Arterial PCO_2 increased markedly in both groups during LPS infusion and as a sign of acidosis, arterial pH and base excess decreased in both groups during endotoxaemia with lower values in the control pigs, but the differences did not reach statistical significance.

VO_2 did not change during endotoxaemia and there was no difference between the groups. As DO_2 was slightly higher in the bosentan group the $avDO_2$ was lower than in the controls.

Upon the second dose of bosentan during LPS blood gas parameters were unchanged except for base excess which significantly increased from -4.2 ± 1.6 mmol l^{-1} to -1 ± 1.3 mmol l^{-1} ($P<0.05$) after 60 min.

Arterial plasma noradrenaline markedly increased from 1.3 ± 0.3 and 2.1 ± 0.7 nmol l^{-1} in the control and bosentan group, respectively, to 36 ± 20 and 21 ± 11 nmol l^{-1} at 3 h of LPS infusion with a tendency for higher values in the control group.

Arterial levels of ET-1-LI and Big ET-1-LI

Bosentan (5 mg kg^{-1}) increased basal ET-1-LI levels from 16.8 ± 1.3 to 49.6 ± 10.0 pM ($n=6$), $P<0.05$ (Figure 5a). The LPS infusion caused a gradual increase in arterial plasma levels of ET-LI in the control group, from basal levels of 9.58 ± 0.8 pM ($n=5$) to 39.7 ± 6.4 pM ($n=5$, $P<0.01$) after 3 h of infusion (Figure 5a). In pigs pretreated with bosentan, and thus with three-fold higher arterial levels of ET-1-LI, the rate of increase during LPS infusion was the same as in pigs not treated with bosentan (Figure 5a). A second bolus dose of bosentan given after 3.5 h of LPS infusion caused an additional twofold increase of the ET-1-LI levels, to 139 ± 26 pM ($P<0.05$) 30 min after the injection (Figure 5a).

LPS also caused a concomitant elevation of arterial plasma levels of Big ET-LI, from basal levels of 41.9 ± 15 pM ($n=5$) to 333 ± 78 pM ($n=5$, $P<0.01$) after 3 h of infusion (Figure 5b). Bosentan treatment did not change the basal levels of Big ET-LI or the LPS-induced increase of Big ET-LI even upon repeated bosentan administration (Figure 5b).

Characterization with h.p.l.c.

Characterization of the immunoreactive material in arterial plasma obtained from the bosentan-treated pigs during infusion of LPS was performed with reverse phase h.p.l.c. followed by RIA analysis. The ET-1 immunoreactivity eluted as one major peak in the same position as synthetic ET-1 (fraction 46) and a smaller peak in the position of ET-3 (fraction 39) (Figure 6a). Most of the Big ET-1 immunoreactive material eluted slightly ahead of synthetic Big ET-1(1–39) whereas smaller amounts eluted exactly in the position of Big ET-1 (1–39) and synthetic Big ET-1(22–39) (Figure 6b).

Northern blot

Basal levels of ET-1 mRNA could be detected in lungs from untreated (although anaesthetized) pigs. In lungs dissected out after 5 h of LPS infusion the levels of ET-1 mRNA were significantly increased ($P<0.01$) whereas additional treatment with bosentan did not change this increase (Figure 7).

Discussion

The present study describes the effects of bosentan, a non-peptide mixed ET-receptor antagonist on the cardiovascular and metabolic changes during LPS-induced porcine shock as well as on release and synthesis of ET-1 and Big ET-1.

Bosentan was given 30 min before initiation of LPS infusion. This was done to enable observation of any effects on intrinsic vascular tone but also to study the importance of ET-1 in the early phases of the cardiovascular changes seen in this porcine endotoxin shock model. The decrease in intrinsic MABP and MPAP that was observed without a concomitant reduction in cardiac output indicates a reduction in vascular tone in the systemic and pulmonary circulation. It is possible that the increase in heart rate upon bosentan treatment was a baro-reflex response due to peripheral vasodilatation.

The splenic vascular bed showed the greatest response to bosentan in the basal state compared with the renal and intestinal vasculature. This difference in response to bosentan is most likely due to the predominance of ET_A-receptors in the pig spleen in contrast to the kidney, where ET_B-receptors

Table 3 Arterial blood gases, oxygen delivery (DO_2), arterio-venous oxygen extraction ($avDO_2$) and oxygen consumption (VO_2) in pigs receiving a lipopolysaccharide (LPS) infusion (15 μg kg^{-1} h^{-1}); bosentan (5mg kg^{-1} i.v.) was given 30 min before start of LPS

				LPS infusion		
Unit	*Group*	*Basal*	*30 min after bosentan/NaCl*	*60 min*	*120 min*	*180 min*
Pao_2 (kPa)	Control	12.7 ± 0.8	13.3 ± 0.8	10.2 ± 0.4#	11.4 ± 0.4#	9.3 ± 0.6
	Bosentan	14.3 ± 1.7	12.7 ± 1.5#	10.9 ± 0.7	10.2 ± 0.5	9.4 ± 0.9#
$Paco_2$ (kPa)	Control	5.4 ± 0.4	5.5 ± 0.5	6.2 ± 0.6	6.7 ± 0.7#	8.4 ± 1.3
	Bosentan	5.4 ± 0.4	5.4 ± 0.3	5.8 ± 0.3	6.4 ± 0.4	7.0 ± 0.6#
pH	Control	7.34 ± 0.02	7.33 ± 0.03	7.27 ± 0.03#	7.14 ± 0.05#	7.15 ± 0.06
	Bosentan	7.37 ± 0.02	7.38 ± 0.03	7.34 ± 0.03	7.26 ± 0.05	7.22 ± 0.06#
Base excess (mmol l^{-1})	Control	−0.7 ± 0.9	−1.1 ± 1.5	−2.6 ± 1.0	−8.6 ± 2.4#	−5.0 ± 2.1
	Bosentan	−0.1 ± 1.4	−0.4 ± 1.1	−1.7 ± 1.6	−3.3 ± 2.1#	−4.6 ± 1.8
DO_2 (ml min^{-1})	Control	225 ± 27	200 ± 13	175 ± 23	160 ± 34#	182 ± 31
	Bosentan	238 ± 29	217 ± 26#	234 ± 178	227 ± 23	213 ± 33
$avDO_2$ (ml l^{-1})	Control	48 ± 6	46 ± 7	56 ± 8*	57 ± 9	59 ± 5
	Bosentan	42 ± 6	46 ± 5	39 ± 4	38 ± 6	42 ± 4
VO_2 (ml min^{-1})	Control	64 ± 3	59 ± 4	59 ± 4	59 ± 5	79 ± 7
	Bosentan	61 ± 4	67 ± 3#	62 ± 2	66 ± 5	79 ± 11

#$P<0.05$ for intragroup changes compared to basal values and *$P<0.05$ for differences between the groups.

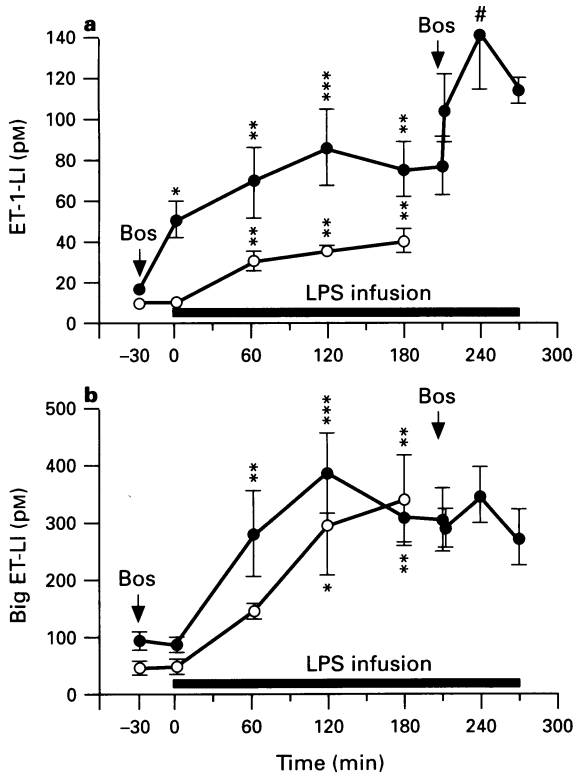

Figure 5 Arterial plasma levels of ET-1-LI (a) and Big ET-LI (b) in control pigs (○–○, $n=5$) and pigs treated with bosentan (●–●, two bolus doses given at arrows, $n=6$) during a continuous infusion of LPS (15 μg kg^{-1} hr^{-1}). *$P<0.05$, **$P<0.01$ for changes from basal levels and #$P<0.05$ for changes from levels before the second dose of bosentan, ANOVA, $n=5-6$.

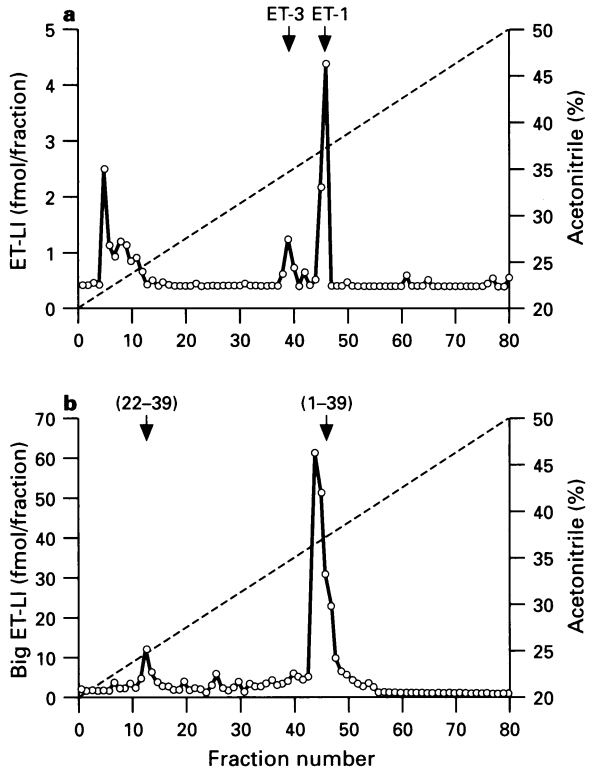

Figure 6 Reverse phase h.p.l.c. chromatograms of arterial plasma obtained from pigs treated with bosentan (5 mg kg^{-1}, i.v.) after 3 h of LPS infusion. The collected fractions were evaporated and subsequently analyzed with RIA using antiserum E1 (a), which crossreacts 100% with ET-1 and 0.14% with pBig ET-1, or antiserum B17 (b), which crossreacts 100% with pBig ET-1 (1–39), 35% with pBig ET-1 (22–39) and <0.008% with ET-1. Arrows show the positions of synthetic ET-1, ET-3, pBig ET-1 (1–39) and pBig ET-1 (22–39).

predominate (Hemsén, 1991). Also in man, ET_B-receptors are the main subtype in the kidney (Karet *et al.*, 1993). Bosentan has a 20–30 fold higher potency for the ET_A- than for the ET_B-receptor (Clozel *et al.*, 1994) and with the modest dose of bosentan chosen in the present study there might have been only a very low antagonistic effect on the ET_B-receptors. In the rat (Clozel *et al.*, 1994; Gardiner *et al.*, 1994) bosentan did not affect basal MABP. This species difference could be due to the higher intrinsic ET-1 production in the pig, reflected by higher plasma ET-1 levels compared with the rat (Löffler *et al.*, 1993). This may not be the only explanation since endogenous generation of ET-1 in healthy human subjects seems to contribute to basal vascular tone although basal plasma levels of ET-1 in man are lower than in the pig (Haynes & Webb, 1994). The vascular effects of bosentan in the pig were rapid and after only 5 min a significant decrease was seen in MABP and MPAP.

Interestingly, bosentan markedly increased basal urine production without affecting renal blood flow. The role of ET-1 in diuresis is not fully elucidated; ET-1 decreases glomerular filtration rate (Badr *et al.*, 1989; Katoh *et al.*, 1990) and reduces the ultrafiltration coefficient (K_f) in the glomeruli (Badr *et al.*, 1989; Kon *et al.*, 1989). On the other hand ET-1 infusion to rats increases diuresis (Oishi *et al.*, 1991) and ET-1 seems to inhibit arginine vasopressin-induced water diuresis; it is sug-

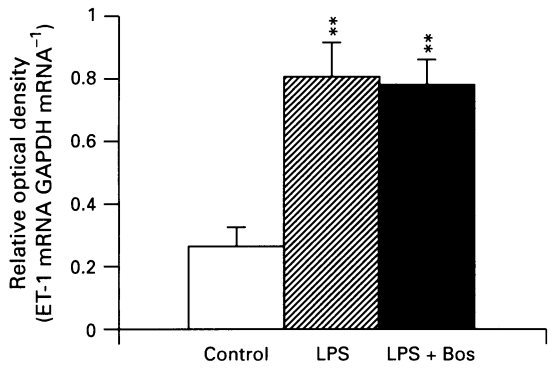

Figure 7 Densitometric analysis of Northern blot using an image analyzing system. The Northern blot was made on 10 μg Poly-A$^+$RNA from lungs of control pigs (open column), pigs treated with LPS (hatched column) and pigs treated with bosentan and LPS (solid column), $n=3$ of each group. The optical density of the preproET-1 mRNA signal was compared to the GAPDH mRNA signal. $^{**}P<0.01$, changes from basal levels, ANOVA.

gested that the latter effect is mediated via the ET_B-receptor subtype (Woodcock & Land, 1992). It is possible that in the pig, the relatively high basal ET-1 levels (Pernow *et al.*, 1989b) mediate intrinsic antidiuresis. Another explanation may be that bosentan, with a 20–30 fold higher affinity for the ET_A receptors than for the ET_B type, displaces ET-1, which may then act on renal ET_B-receptors, inducing diuresis. Unpublished pharmacological characterization has revealed that bosentan under the present *in vivo* conditions mainly has an ET_A profile, i.e. it inhibits exogenous ET-1 vasoconstriction in the spleen but not in the kidney. A small but significant reduction in SaO_2 and PaO_2 was observed upon bosentan treatment. This finding is not unexpected after intravenous administration of a pulmonary vasodilator, which also dilates blood vessels in the lung that are not ventilated, resulting in an increased intrapulmonary shunt. These effects are seen with other pulmonary vasodilators such as prostacyclin and nitroglycerin (Radermacher *et al.*, 1989; 1990).

In the present porcine endotoxin shock model as well as in other species, when LPS is infused intravenously there is a reproducible, biphasic increase in MPAP and PVR (Hardie & Olson, 1987; Leeper-Woodford *et al.*, 1991). The first peak is cyclo-oxygenase-dependent, and probably thromboxane A_2 mediated, since it is abolished by cyclo-oxygenase inhibitors, such as diclofenac (Weitzberg *et al.*, 1995), but also by selective thromboxane inhibitors (Svartholm *et al.*, 1989). The cause of the second peak is probably more multifactorial, including increased extravasal pressure and intravascular obliteration but also active vasoconstriction (see Rinaldo & Rogers, 1982; Zapol *et al.*, 1985). Bosentan did not affect the initial peak but markedly reduced the second, more prolonged increase in MPAP and PVR, indicating participation of ET-1 in the late phase pulmonary hypertension in this model. Moreover, the second increase in MPAP and PVR in this experimental setting, coincides in time with elevation of plasma ET-1-LI levels. Lung tissue has relatively high concentrations of ET-1 both in pig and man (Hemsén, 1991) and both increased plasma levels and pulmonary ET-1 release are seen in patients with pulmonary hypertension (Stewart *et al.*, 1991), indicating that the lung is a target organ for ET-1.

ET-1 is well known to have a very short half life in the circulation both in pig (Pernow *et al.*, 1989b) and in man (Weitzberg *et al.*, 1991a). The clearance mechanism has not yet been identified, although the contribution of ET-1 degrading enzymes has been postulated. Frelin & Guedin (1994) presented another hypothesis, namely that the low levels of circulating ET-1, and thus the clearance, is dependent on binding to functional receptors and internalization of the complexes. The present results showing that the ET receptor antagonist bosentan *per se* causes an increase of arterial ET-1-LI in the pig, are partly in line with this hypothesis, and a similar phenomenon has also been shown in the rat for the ET antagonist Ro-46-2005 (Löffler *et al.*, 1993). It has been shown that ET-1 may induce its own synthesis in rat-cultured vascular smooth muscle cells (Hahn *et al.*, 1990) and the presence of autoregulatory receptors causing the increased ET-1 levels has been postulated. However, since bosentan did not increase the levels of Big ET-1-LI or cause any further increase of the LPS induced elevation of preproET-1 mRNA, the ability of bosentan to increase the basal ET-1-LI levels most probably depends on displacement of ET-1 from tissue binding sites. This also suggests that ET-1 is released and exerts regulatory vascular effects under basal conditions in these anaesthetized pigs. The second dose of bosentan caused a further increase of plasma ET-1-LI levels, indicating that the first dose no longer had full antagonistic effect, which may be due to both the increased production of ET-1 and metabolism of bosentan. To obtain a continuous blockade with bosentan, administration with repetitive doses or infusion is recommended.

The LPS-induced increase of plasma Big ET-1 and ET-1-LI levels is related to an induced synthesis of ET-1 as shown by the large increase in preproET-1 mRNA in the lung after LPS infusion. Studies with cultured endothelial cells have demonstrated that both LPS and TNF_α are potent stimulants of ET-1 production (Sugiura *et al.*, 1989; Ohta *et al.*, 1990). During the LPS infusion there was an eight fold increase in arterial levels of Big ET-1-LI, which should be compared with the four fold increase of ET-1-LI levels. Since Big ET-1-LI apparently is released concomitantly with ET-1, but has a longer half life in the circulation (Hemsén, 1991c) it may be easier and more accurate to measure Big ET-1 levels as a marker for activity of the ET-1 system. The slight shift of the Big ET immunoreactivity ahead of synthetic Big ET-1 (1–39) in the h.p.l.c. chromatogram may be due to oxidation, as has previously been shown for ET-1 (Hemsén, 1991b). In the present chromatogram, the peak in the position of Big ET-1 (1–39) only results in a broadening of the major peak, which elutes two fractions ahead, whereas in other chromatograms the peak of Big ET-1 (1–39) is more distinct (not shown). Furthermore, the present results indicate that Big ET-1 exerts its effects through conversion to ET-1 and does not itself act on ET receptors, since the Big ET-1-LI levels were not affected by bosentan, which has been shown also to antagonize the vasoconstrictor effects of Big ET-1 (Clozel *et al.*, 1994).

The second dose of bosentan at 3.5 h of LPS completely restored MPAP and PVR to levels seen before LPS infusion. Firstly, this suggests that ET-1 participates in the development of pulmonary hypertension in this model and that a major part of this hypertension is due to vasoconstriction. Secondly, it is obvious that the initial dose of bosentan, given 4 h earlier, no longer had full antagonistic effect which is reasonable since production of ET-1 during LPS infusion is markedly increased (Pernow *et al.*, 1989a). Therefore, a continuous infusion of bosentan in this setting may be more appropriate.

Interestingly, MABP during endotoxaemia was not lower in the bosentan treated pigs than in controls and the already low MABP at 3.5 h was not further reduced by the second dose of bosentan. An explanation for this may be that the increased cardiac output compensates for a systemic vasodilatation or that mediators other than ET-1 are more important in blood pressure control during endotoxaemia. Nitric oxide and prostacyclin are two candidates suggested for the vasodilatation seen in sepsis (see Bone, 1991; Moncada *et al.*, 1991). Cardiac output was higher in the bosentan group compared to controls but there was no difference in heart rate, which increased in both groups in a similar way. This means that it is not merely a reflex increase in heart rate that is responsible for the improved cardiac output in the bosentan-treated pigs but rather an effect on coronary arteries or directly on cardiac contractility. Another explanation for the increase in cardiac output could be the reduction in afterload (i.e. the fall in SVR).

One of the main problems in septic shock is maldistribution of blood flow and inadequate oxygen supply to the tissues.

Increasing regional blood flow to organs like the intestines and the kidneys is central in treatment of septic shock. Both splenic and intestinal VR were lower in the bosentan pretreated pigs, although the LPS-induced changes were similar to those in the control group. Upon the second dose of bosentan, vascular resistance was further decreased in the splenic and the intestinal circulation but not in the kidney, with the most marked response in the spleen. Hence, bosentan improved local organ blood flow during endotoxaemia in the present pig model, a property which may be beneficial.

Oxygen content in arterial blood was similar in both groups, a-v O_2 was higher in the control pigs which may indicate a poorer perfusion in parts of the body. This is supported by a tendency to lower arterial pH and lower base excess. VO_2 was similar in both groups, indicating a similar metabolic oxygen need. DO_2 was only slightly higher in the bosentan group. These data taken together show that bosentan does not cause a significantly deterioration of arterial oxygenation during endotoxaemia and that it may marginally improve oxygen delivery to the tissue by increasing cardiac output and regional blood flow.

As a marker of sympathetic nervous system activation, noradrenaline markedly increased in both groups during development of septic shock. In a similar porcine LPS model we could reduce the increased plasma levels of noradrenaline by pretreatment with diclofenac or nitric oxide inhalation, both interventions that reduce pulmonary hypertension in the pigs (Weitzberg *et al.*, 1993b; 1995). Also in the present study the bosentan pretreated pigs showed a tendency to lower noradrenaline levels during LPS infusion although the difference between the groups was not statistically significant.

From the results of this study using bosentan, a non-peptide, selective and mixed ET-receptor antagonist, we conclude that during LPS-induced shock bosentan can abolish the late phase pulmonary hypertension and improve cardiac output as well as increase blood flow to the splenic and intestinal vascular beds without further decreasing mean arterial blood pressure. Mixed or subtype selective ET-receptor antagonists could be new pharmacological tools to ameliorate the negative cardiovascular changes seen in septic shock.

We are most grateful to Dr Martine Clozel (F. Hoffman La Roche Ltd.) for the generous gift of bosentan. We are also grateful to Ms Margareta Stensdotter and Mrs Carina Nihlén for expert technical assistance. The present study was supported by the Swedish Medical Research Council (14X-6554), the Swedish Society of Medicine, the Laerdal Foundation and the Karolinska Institute.

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(Received June 30, 1995
Revised February 8, 1996
Accepted Februaru 15, 1996)