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# Role for endogenous endothelin in the regulation of plasma volume and albumin escape during endotoxin shock in conscious rats

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**1** To explore the role of endogenous endothelin (ET) in the regulation of vascular functions, we studied the effects endothelin receptor blockade on blood pressure, plasma volume and albumin escape during endotoxin shock in conscious, chronically catheterized rats. Red blood cell volume and plasma volume were determined by using chromium-51-tagged erythrocytes and iodine-125-labelled albumin, respectively.

**2** Intravenous injection of lipopolysaccharide (LPS, 10 mg $kg^{-1}$) resulted in hypotension, haemoconcentration, and increased total-body albumin escape, which is reflected by a 30% reduction in plasma volume. Plasma ET-1 concentrations increased 2.1 fold and 5.4 fold at 30 and 120 min post-LPS, respectively.

**3** LPS-induced losses in plasma volume and albumin escape were significantly attenuated by pretreatment of animals with the dual $ET_A/ET_B$ receptor antagonist bosentan (17.4 $\mu$mol $kg^{-1}$, i.v. 15 min prior to LPS) or the $ET_A$ receptor antagonist FR 139317 (3.8 $\mu$mol $kg^{-1}$, i.v.) during both the immediate and delayed phases of endotoxin shock. The inhibitory actions of bosentan and FR 139317 were similar. Both antagonists augmented the hypotensive action of LPS.

**4** Administration of bosentan or FR 139317 70 min after injection of LPS also attenuated further losses in plasma volume and increases in total body and organ albumin escape rates with the exception of the lung and kidney.

**5** These results indicate a role for endogenous endothelin in mediating losses in plasma volume and albumin escape elicited by LPS predominantly through activation of $ET_A$ receptors, and suggest that by attenuating these events, $ET_A$ or dual $ET_A/ET_B$ receptor blockers may be useful agents in the therapy of septic shock.

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**Keywords:** Endothelin-1; $ET_A$ and $ET_B$ receptors; bosentan; FR 139317; vascular permeability; plasma volume; endotoxin; septic shock

**Abbreviations:** BV, blood volume; ET-1, endothelin-1; $^{125}$I-$AER_{organ}$, organ albumin escape rate; $^{125}$I-$AER_t$, total body albumin escape rate; [$^{125}$I]-HSA, $^{125}$I-labelled human serum albumin; LPS, lipopolysaccharide; LVHct, large vessel haematocrit; MABP, mean arterial blood pressure; NO, nitric oxide; PV, plasma volume; RCV, red blood cell volume

## Introduction

Dysfunction of the vascular endothelium is an early event in septic shock. Microvascular injury and endothelial dysfunction not only result in overproduction of nitric oxide (NO) and other vasodilators (Moncada & Higgs, 1993), but also enhance the synthesis/release of vasoconstrictors, including endothelin-1 (ET-1). Lipopolysaccharide (LPS) stimulates the expression of ET-1 mRNA and release of ET-1 from cultured endothelial cells (Marsden & Brenner, 1992;. Nakamura *et al*., 1991). Markedly elevated plasma ET-1 levels occur in experimental animals following injection of LPS (Sugiura *et al*., 1989; Pernow *et al*., 1990; Vemulapalli *et al*., 1991; Nakamura *et al*., 1991) and in patients with septic shock (Pittet *et al*., 1991, Weitzberg *et al*., 1991; Voerman *et al*., 1992; Takakuwa *et al*., 1994). It is still controversial whether elevated plasma ET-1 levels are markers of endothelial dysfunction/damage or mediators of the disease. Increased systemic and/or local production of ET-1 has been implicated to support blood pressure (Gardiner *et al*., 1995; Ruetten *et al*., 1996), and to mediate renal vasoconstriction and renal failure (Pernow *et al*., 1990), and pulmonary and portal hypertension in endotoxin shock (Yamamoto *et al*., 1997).

Data from our and other laboratories indicate a role for ET-1 in the regulation of plasma volume and albumin escape (Filep *et al*., 1991; 1997; Zimmerman *et al*., 1992). Injection of exogenous ET-1 evokes losses in plasma volume and promotes total-body albumin escape and augments albumin extravasation in various organs of rats (Filep *et al*., 1991; Zimmerman *et al*., 1992). These actions of ET-1 are predominantly mediated through activation of $ET_A$ receptors (Filep *et al*., 1994). Reduction of circulating blood volume and enhanced microvascular permeability are characteristic features of endotoxin shock and contribute to the progression of shock to a multiple organ dysfunction syndrome that is associated with a substantial increase in mortality (Bone, 1991; Parillo, 1993). The aim of the present study was to investigate the role of endogenous endothelin in the regulation of plasma volume and albumin escape during the early and delayed phase of endotoxin shock in conscious rats by using the selective $ET_A$ receptor antagonist FR 139317 (Sogabe *et al*., 1993) and the dual $ET_A/ET_B$ receptor antagonist bosentan (Clozel *et al*., 1994).

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## Methods

### *Materials*

Bosentan (4-*tert*-butyl-N-[6-(2 hydroxy-ethoxy)-5-(2-methoxy-phenoxy)2,2′bipyrimidin-4-yl]-benzene-sulphonamide) was a gift from Dr M. Clozel (Hoffmann LaRoche Ltd. Basel, Switzerland). FR 139317 ((R)2-[(R)-2-[[1-(hexahydro-1H-azepinyl)]-carbonyl]-amino-4-methylpentanoyl]amino-3-[3-(1-methyl-1H-indoyl)]-propionyl]amino-3-(2-pyridyl)propionic acid) was a gift from Fujisawa Pharmaceutical Co. (Osaka, Japan). Sodium-51-chromate was obtained from DuPont-NEN (Mississauga, Ontario, Canada) $^{125}$I-labelled human serum albumin ([$^{125}$I]-HSA) was from ICN Pharmaceuticals (Costa Mesa, CA, U.S.A.). Norepinephrine and *E. coli* LPS (serotype: O111:B4) were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

### *Experimental protocols*

The experiments were performed on conscious, chronically catheterized male Cr1:(WI)BR Wistar rats weighing 210–300 g (Charles River, Québec, Canada) as previously described (Filep *et al*., 1991). Mean arterial blood pressure (MABP) was monitored continuously by a blood pressure analyser (Micro-Med, Louisville, KY, U.S.A.) using a COBE CDX III pressure transducer.

On the day of the experiment, after an equilibration period, baseline cardiovascular parameters were measured for 20 min. Figure 1 depicts the experimental protocols used to study immediate (series 1) and delayed cardiovascular responses to LPS (series 2). The animals received the following intravenous injections: $^{51}$Cr-tagged rat erythrocytes (~0.5 $\mu$Ci, 1.5 ml kg$^{-1}$) (Filep, 1997; Filep *et al*., 1997); [$^{125}$I]-HSA (1 $\mu$Ci in a volume of 100 $\mu$l); saline, FR 139317, 3.8 $\mu$mol kg$^{-1}$ (2.5 mg kg$^{-1}$) or bosentan, 17.4 $\mu$mol kg$^{-1}$ (10 mg kg$^{-1}$) in a volume of 500 $\mu$l kg$^{-1}$ or LPS (10 mg kg$^{-1}$, in a 10 mg ml$^{-1}$ solution over 2 min) as indicated. In series 1 the following groups were studied: group 1 ($n=7$): saline only (control); group 2 ($n=7$): LPS; group 3 ($n=7$): bosentan plus LPS; group 4 ($n=7$): FR 139317 plus LPS; group 5 ($n=4$): bosentan plus saline; and group 6 ($n=4$): FR 139317 plus saline. In series 2, the animals were divided into five groups: group 1 ($n=7$): saline followed by LPS; group 2 ($n=7$): bosentan followed by LPS; group 3 ($n=6$): FR 139317 followed by LPS; group 4 ($n=6$): LPS followed by FR 139317 and group 5 ($n=6$): LPS followed by bosentan. Triplicate arterial blood samples (15 $\mu$l) were taken for measuring 'large vessel' haematocrit (LVHct) and $^{51}$Cr and $^{125}$I radioactivities at indicated times. LVHct was determined by a manual haematocrit reader (the coefficient of variation for triplicate samples did not exceed 2%). Immediately after the last blood sample was taken, the animals were sacrificed with an overdose of sodium pentobarbitone, and portions of selected organs were prepared for measurement of $^{51}$Cr and $^{125}$I radioactivities. In the third series of experiments, pressor responses to norepinephrine, 3.1 nmol kg$^{-1}$ (1 $\mu$g kg$^{-1}$ in a volume of 30 $\mu$l kg$^{-1}$) were compared 30 min before and 30, 60, 120 and 150 min after injection of LPS (10 mg kg$^{-1}$, $n=6$) in rats treated with bosentan (17.4 $\mu$mol kg$^{-1}$, $n=5$), FR 139317 (3.8 $\mu$mol kg$^{-1}$, $n=5$), or saline (control, $n=5$). Arterial blood (1 ml) was collected for analysis of plasma ET-1 immunoreactivity from another group of control animals ($n=6$) and at 30 and 120 min after injection of LPS (each group $n=6$). All procedure were in accordance with the Guidelines of the Canadian Council of Animal Care and were approved by the local Animal Care Committee.

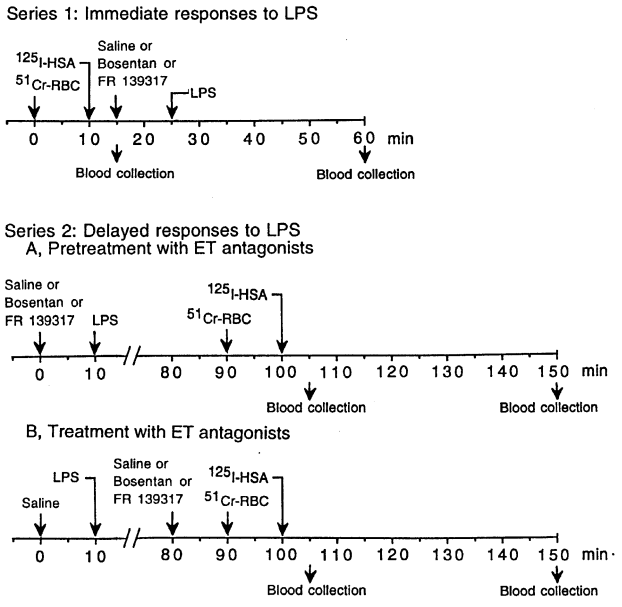


**Figure 1** Experimental protocols. $^{51}$Cr-RBC indicates $^{51}$Cr-tagged rat red blood cells; [$^{125}$I]-HSA, $^{125}$I-labelled human serum albumin.

### *Red blood cell, plasma and blood volumes*

In series 1 and 2, for the blood sample taken 5 min after injection of [$^{125}$I]-HSA, red blood cell volume (RCV), plasma volume (PV) and blood volume (BV) were determined according to the following formulas: RCV = total $^{51}$Cr activity injected × LVHct ÷ blood $^{51}$Cr activity concentration; PV = total $^{125}$I activity injected × (1-LVHct) ÷ blood $^{125}$I activity concentration; and BV = RCV + PV. The ratio of whole-body haematocrit to LVHct ($F_{cells}$ ratio) was calculated as (RCV ÷ BV) ÷ LVHct. For the blood sample taken 50 min after injection of [$^{125}$I]-HSA, the following formulas were used: BV = ($^{51}$Cr activity injected − sampling loss of $^{51}$Cr activity) ÷ blood $^{51}$Cr activity concentration) ÷ $F_{cells}$; $RCV = RCV_{first} - RCV_{lost}$, where $RCV_{first}$ and $RCV_{lost}$ are RCV measured during the first sample and RCV lost through sampling, respectively; $RCV_{lost}$ = $^{51}$Cr activity lost through sampling ÷ ($^{51}$Cr activity injected ÷ $RCV_{first}$); and PV = BV − RCV.

### *[$^{125}$I]-albumin escape rate*

The rate at which [$^{125}$I]-HSA escaped from the circulation ($^{125}$I-$AER_t$) was calculated as $^{125}$I-$AER_t$ = [(net $^{125}$I activity injected − total plasma $^{125}$I activity in the second blood sample) ÷ net $^{125}$I activity injected] ÷ 50 min × 100, where net $^{125}$I activity injected is the total $^{125}$I activity injected less the cumulative radioactivity removed from the circulation by blood sampling, and total plasma $^{125}$I activity = plasma $^{125}$I activity concentration × plasma volume at 50 min after injection of $^{125}$I-labelled albumin.

The rate at which [$^{125}$I]-HSA escaped from the circulation of each organ ($^{125}$I-$AER_{organ}$) was determined by using the formula $^{125}$I-$AER_{organ}$ = (tissue [$^{125}$I]-albumin activity ÷ net $^{125}$I activity injected) ÷ 50 min ÷ corrected organ weight × 100. Tissue [$^{125}$I]-albumin activity was calculated as the difference in total organ $^{125}$I and organ plasma [$^{125}$I]-albumin activity.

Organ plasma $^{125}$I activity is the product of organ plasma volume and plasma $^{125}$I-albumin activity concentration. Organ plasma volume was determined as organ blood volume × (1-LVHct) for heart, lung, liver and kidney, where organ haematocrit is similar to that of LVHct or as organ blood volume × [1 − ($F_{cells}$ × LVHct) for gastrointestinal tract, where the ratio of organ haematocrit to LVHct is similar to the ratio of whole body haematocrit to LVHct (Zimmerman *et al*., 1992). Organ BV was calculated as (organ $^{51}$Cr activity ÷ blood $^{51}$Cr activity concentration) for heart, lung, liver and kidney; and as (organ $^{51}$Cr activity ÷ blood $^{51}$Cr activity concentration) ÷ $F_{cells}$ for gastrointestinal tract. Organ weight was corrected by substracting estimated organ blood weight (organ BV × blood specific gravity) from wet organ weight.

### *Measurement of plasma immunoreactive ET-1*

Plasma samples were assayed with an ET-1 enzyme-linked immunosorbent assay (R&D Systems, Minneapolis, MN, U.S.A.) after extraction on a C18 Sep-Pak cartridge (Millipore, Bedford, MA, U.S.A.) as previously described (Filep *et al*., 1995). The assay has less than 1% cross-reactivity with big ET-1 and 45% and 14% cross-reactivity with ET-2 and ET-3, respectively. The intra-assay coefficient of variation was 5%. The recovery of 4 fmol exogenous ET-1 added to 1 ml normal rat plasma was 72 ± 3% ($n$ = 4). All ET-1 values were corrected for recovery.

### *Statistical analysis*

Results are expressed as mean ± s.e.mean. Results were compared by one-way ANOVA using ranks (Kruskal-Wallis test) followed by Dunn's multiple contrast hypothesis test when various treatments were compared to the same control group, or by the Wilcoxon signed rank test and Mann-Whitney U test for paired and unpaired observations, respectively. A level of $P < 0.05$ was considered significant for all tests.

## Results

### *Effects of bosentan and FR 139317 on immediate cardiovascular responses to LPS*

Injection of saline (control) neither affected MABP (Figure 2A) nor evoked detectable changes in blood volume, plasma volume and red cell volume (Table 1). Bolus IV injection of bosentan (17.4 μmol kg$^{-1}$) or FR 139317 (3.8 μmol kg$^{-1}$) does not affect these parameters, nor increases total-body albumin escape rate in conscious rats (Table 1).

Administration of LPS caused a dramatic fall in MABP within 5 min. Thereafter, MABP gradually increased and was ~75 mmHg 30 min after injection of LPS (Figure 2A). Bosentan or FR 139317-pretreatment enhanced by 9 ± 2 and 8 ± 2 mmHg the maximum decrease in MABP elicited by LPS (Figure 2A). Endotoxemia resulted in a substantial attenuation of the pressor responses to norepinephrine, which were unaffected by bosentan or FR 139317 pretreatment (Figure 2D).

LPS-induced hypotension was accompanied by a marked haemoconcentration. Haematocrit increased from 0.468 ± 0.004 to 0.573 ± 0.010 ($P < 0.01$). Plasma volume decreased by 30 ± 3 % ($P < 0.01$), whereas no changes were detected in red blood cell volume (32.9 ± 1.5 vs 32.6 ± 1.5 ml kg$^{-1}$, $P > 0.1$). Accordingly, total-body blood

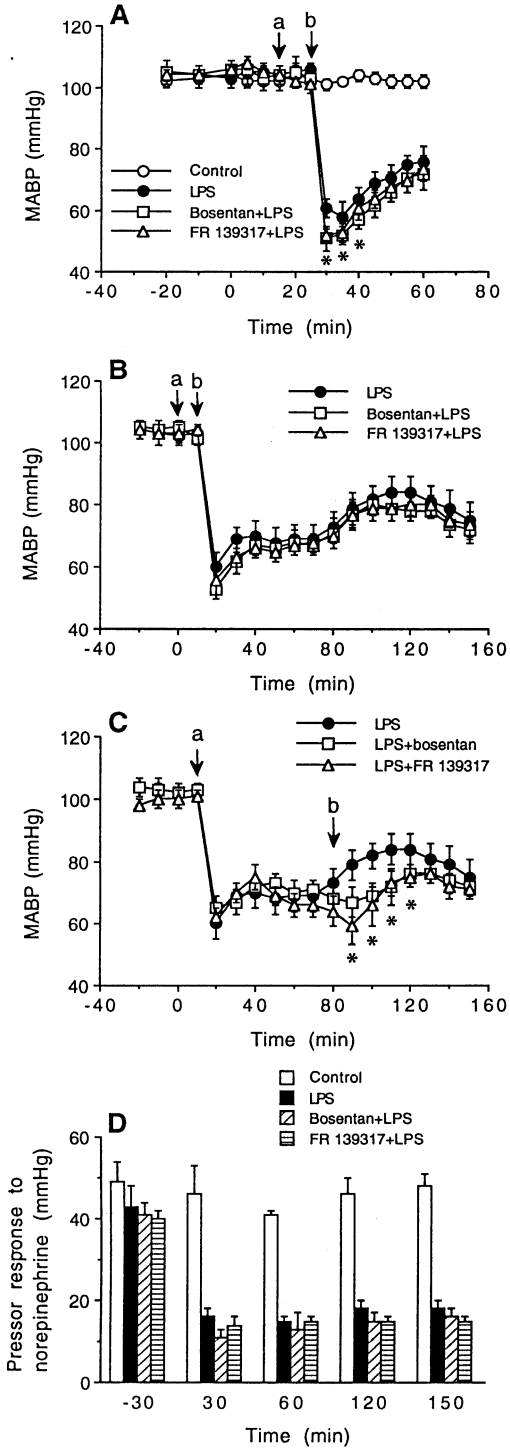


**Figure 2** Changes in mean arterial blood pressure (MABP) and in pressor responses to norepinephrine in conscious rats treated with LPS during endothelin receptor blockade. (A) Different groups of animals were injected intravenously with vehicle (saline, control), bosentan (17.4 μmol kg$^{-1}$) or FR 139317 (3.8 μmol kg$^{-1}$) at 15 min (arrow a) followed by LPS (10 mg kg$^{-1}$) or saline 10 min later (arrow b). (B) Different groups of rats received saline, bosentan or FR 139317 at time 0 (arrow a) followed by LPS 10 min later (arrow b). (C) Different groups of rats received LPS at 10 min (arrow a) followed by vehicle (saline), bosentan or FR 139317 70 min later (arrow b). (D) Pressor responses to intravenous norepinephrine (3.1 nmol kg$^{-1}$) in rats pretreated with saline (control), bosentan or FR 139317 for 10 min before injection of LPS (at time 0). Values are means ± s.e.mean. *$P < 0.05$ for both bosentan and FR 139317 compared with LPS at the same time point).

**Table 1** Mean arterial blood pressure, blood volume, plasma volume, red blood cell volume and total-body albumin escape rate in conscious rats treated with endothelin receptor antagonists

| | *MABP* (mmHg) | *Blood volume* (ml $kg^{-1}$) | *Plasma volume* (ml $kg^{-1}$) | *Red blood cell* (volume, ml $kg^{-1}$) | *Total-body albumin escape rate* % *Net* $^{125}$*I-albumin injected* |
|---|---|---|---|---|---|
| Vehicle ($n=7$) | | | | | |
| Baseline | 103±3 | 85.6±4.5 | 54.9±4.0 | 30.6±1.4 | – |
| Experimental | 102±2 | 85.5±4.7 | 55.2±4.2 | 30.3±1.4 | 11.1±0.6 |
| Bosentan ($n=4$) | | | | | |
| Baseline | 103±1 | 88.0±4.0 | 58.3±1.5 | 29.7±2.5 | – |
| Experimental | 103±1 | 87.9±4.2 | 58.3±1.8 | 29.6±2.5 | 10.4±0.7 |
| FR 139317 ($n=4$) | | | | | |
| Baseline | 102±2 | 84.0±0.7 | 55.7±1.1 | 28.3±1.1 | – |
| Experimental | 103±2 | 83.5±0.9 | 55.4±1.2 | 28.1±1.1 | 11.2±0.8 |

Measurements were made at 15 min (baseline) and 60 min (experimental) after injection $^{51}$Cr-tagged red blood cells. Different groups of rat were injected with saline (vehicle), bosentan (17.4 μmol $kg^{-1}$) or FR 139317 (3.8 μmol $kg^{-1}$) at 15 min after injection of radiolabelled red blood cells. Values are means±s.e.mean.

volume was lower in LPS-treated than in control animals (72.5±2.1 vs 85.5±4.7 ml $kg^{-1}$, $P<0.01$). LPS increased the total-body albumin escape rate on average by 253% (Figure 3) and enhanced albumin escape rate in the bronchus, liver, kidney and duodenum, but not in the pulmonary parenchyma and heart (Figure 4). Figure 3 compares the values derived from the second blood samples in the different groups. LPS-induced plasma volume losses and total-body albumin escape rate were significantly lower in animals pretreated with bosentan or FR 139317 than in rats who had received LPS alone. Both ET antagonists significantly attenuated the albumin escape rate elicited by LPS in the bronchus, liver, kidney and duodenum (Figure 4). $F_{cells}$ ratios were 0.76±0.01, 0.74±0.01, and 0.77±0.01 in rats which received LPS, bosentan plus LPS, and FR 139317 plus LPS, respectively ($P>0.1$).

### *Effects of bosentan and FR 139317 on delayed cardiovascular responses to LPS*

After the initial fall, MABP gradually increased and stabilized at ~80 mmHg from 90–120 min after LPS, and fell toward the end of the experimental period (Figure 2B). Similar trends were observed in animals pretreated with either bosentan or FR 139317, although these animals had slightly lower MABP than LPS-rats (Figure 2B).

Although haematocrit values were higher (LPS: 0.578±0.020; control: 0.465±0.016, $P<0.01$), whereas plasma volume was markedly lower at 105 min after LPS injection than in control animals (34.0±2.9 ml $kg^{-1}$ vs 55.2±4.2 ml $kg^{-1}$, $P<0.01$), during the next 45 min, hematocrit increased by 0.046±0.011 ($P<0.01$), whereas plasma volume decreased by 5.7±0.7 ml $kg^{-1}$ ($P<0.01$). Total-body albumin escape rate was 33.4±2.8% net [$^{125}$I]-HSA injected between 100 and 150 min post-LPS (Figure 5).

Blood and plasma volumes were higher, whereas haematocrit was lower in animals pretreated with bosentan than in LPS-rats at 105 min post-LPS (blood volume: 68.0±3.2 ml $kg^{-1}$ vs 58.9±3.5 ml $kg^{-1}$; plasma volume: 41.5±3.0 ml $kg^{-1}$ vs 32.2±2.6 ml $kg^{-1}$; haematocrit: 0.530±0.010 vs 0.578±0.020; all $P<0.05$). During the next 45 min, decreases in plasma volume and increases in haematocrit were significantly smaller in animals pretreated with bosentan (Figure 5) than those detected in LPS-rats (Δplasma volume: −1.3±0.8 ml $kg^{-1}$; Δhaematocrit: 0.017±0.009, both $P<0.05$). Similar changes were observed with FR 139317 (Figure 5). Total-body albumin escape rate (Figure 5) and organ albumin escape rates (Figure 6) were markedly attenuated in animals pretreated with bosentan or FR

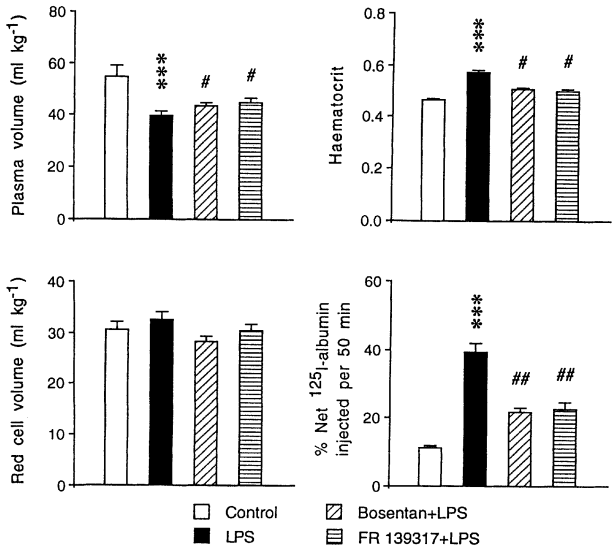


**Figure 3** Effects of bosentan (17.4 μmol $kg^{-1}$), FR 139317 (3.8 μmol $kg^{-1}$) or their vehicle (saline, control) on haematocrit, plasma volume, and red blood cell volume and total-body albumin escape during the early phase of endotoxin shock in conscious rats. The animals were pretreated with bosentan, FR 139317 or vehicle for 10 min before administration of LPS (10 mg $kg^{-1}$, i.v.). Values are mean±s.e.mean and were obtained at 50 min after injection of [$^{125}$I]-HSA. ***$P<0.001$ (compared with control), # $P<0.05$; ## $P<0.01$ (compared with LPS by Dunn's test).

139317. $F_{cells}$ ratios were 0.75±0.02, 0.74±0.01, and 0.77±0.02 at 105 min after administration of LPS in animals which were pretreated with saline, bosentan and FR 139317, respectively ($P>0.1$).

Administration of bosentan or FR 139317 70 min after the onset of endotoxemia resulted in a fall in MABP with maximum decreases of 16±3 and 18±3 mmHg, respectively (Figure 2C). Bosentan treatment partially inhibited further losses in blood and plasma volume (Δblood volume: −4.1±0.6 ml $kg^{-1}$; Δplasma volume: −4.0±0.6 ml $kg^{-1}$, both $P<0.05$) and attenuated LPS-induced total-body albumin escape (Figure 5) and albumin escape rates in the heart, liver and duodenum, but not in the bronchus, pulmonary parenchyma and kidney (Figure 6). Similar inhibition was observed with FR 139317 (Figures 5 and 6). $F_{cells}$ ratios were 0.77±0.02 and 0.77±0.02 at 105 min after administration of LPS in animals which were treated with bosentan and FR 139317, respectively.

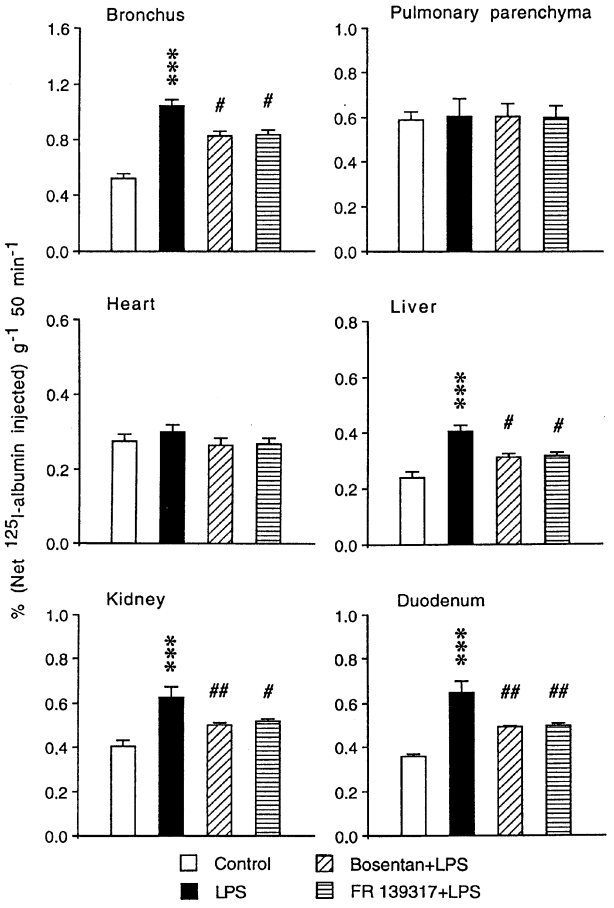


**Figure 4** Effects of bosentan (17.4 $\mu$mol kg$^{-1}$), FR 139317 (3.8 $\mu$mol kg$^{-1}$) or their vehicle (saline, control) on albumin escape rates in various vascular beds during the early phase of endotoxic shock in conscious rats. The animals were pretreated with bosentan, FR 139317 or vehicle for 10 min before administration of LPS (10 mg kg$^{-1}$, i.v.). Values are mean$\pm$s.e.mean. ***$P<0.001$ (compared with control), # $P<0.05$; ## $P<0.01$ (compared with LPS by Dunn's test).

### *LPS increases plasma immunoreactive ET-1*

Plasma immunoreactive ET-1 levels were markedly higher 30 min (6.5$\pm$0.6 pM) and 120 min after injection of LPS (16.6$\pm$1.2 pM) than in control animals (3.1$\pm$0.2 pM, $P<0.01$).

## Discussion

The present results, obtained in conscious chronically catheterized rats, indicate an important role for endogenous endothelin in mediating LPS-induced losses in plasma volume and albumin escape. Blockade of $ET_A/ET_B$ receptors by bosentan or selective inhibition of $ET_A$ receptors with FR 139317 resulted in an attenuation of losses in plasma and blood volume and albumin escape during both the early and delayed phases of endotoxin shock.

Confirming previous observations, endotoxemia resulted in a rapid and profound elevation of the plasma levels of ET-1 (Sugiura *et al*., 1989; Pernow *et al*., 1990; Nakamura *et al*., 1991; Vemulapalli *et al*., 1991). The main mechanism responsible for this rise is believed to be upregulation of ET-1 synthesis in various organs (Hemsen *et al*., 1996; Guo *et al*., 1998). The most likely cellular source for generation of ET-1 is the vascular endothelium. Mast cells, smooth muscle cells (Guo *et al*., 1998), monocytes/macrophages (Ehrenreich *et al*., 1990) and activated neutrophils (Kaw *et al*., 1992) may also contribute to the elevated plasma ET-1 levels. Plasma ET-1 may increase as a consequence of endothelial injury (Voerman *et al*., 1992; Filep *et al*., 1995). We detected very high plasma ET-1 levels at 120 min post-LPS, when endothelial cell injury may also occur (Parillo, 1993; Szabó *et al*., 1997). The polarized release of ET-1 by endothelial cells toward the basolateral side (Wagner *et al*., 1992) suggests that local concentrations of ET-1 might be even much higher than plasma levels.

The rapid release of ET-1 may function to attenuate the fall in MABP, for both bosentan and FR 139317 augmentated the LPS hypotension. These observations are consistent with previous findings using another $ET_A/ET_B$ receptor antagonist SB 209670 (Gardiner *et al*., 1995; Ruetten *et al*., 1996). Greater decreases in MABP were achieved with bosentan or FR 139317 when plasma ET-1 levels were higher, suggesting that endogenous ET-1 may be more important in supporting MABP during the delayed than the immediate phase of endotoxin shock. The similar actions of bosentan and FR 139317 point to predominant activation of $ET_A$ receptors, which are highly selective for ET-1 (Arai *et al*., 1990).

The present study documents for the first time that ET receptor blockade attenuates the marked fluid shifts from the vascular to extravascular spaces and increases albumin escape caused by LPS, thereby reducing losses in blood and plasma volume. The varying degree of inhibition of albumin escape observed with bosentan and FR 139317 in various tissues would indicate regional differences in the production of and/or sensitivity to endogenous endothelin. Comparison of the inhibitory actions of bosentan and FR 139317 revealed that these effects of endogenous endothelin are mediated predominantly *via* $ET_A$ receptors. This interpretation is supported by the findings that the $ET_B$ receptor agonist IRL 1620 is a considerably less potent agent than exogenous ET-1 in inducing albumin extravasation in the same vascular beds of rats (Filep *et al*., 1994). Interestingly, endogenous endothelin promoted albumin escape during the immediate phase despite increased production of NO by the constitutive NO synthase (Szabó *et al*., 1993), which is thought to inhibit albumin extravasation (Kubes & Granger, 1992; Filep *et al*., 1993; Kurose *et al*., 1993; Filep, 1997). Indeed, inhibition of NO production in early endotoxic shock has been reported to enhance albumin escape (Filep *et al*., 1997). Furthermore, the increased albumin escape observed following inhibition of NO synthesis in non-endotoxemic rats can in part be attributed to unmasking and amplifying the actions of endogenous endothelin (Filep, 1997) Taken together, these observations support that enhanced NO formation (through activation of endothelial constitutive NO synthase) in early endotoxaemia may protect against endothelin-mediated vascular dysfunction at this stage of shock. These results suggest a potentially important cross-talk between NO and ET-1 signalling pathways in early stages of endotoxic shock and indicate that the imbalance between NO (produced *via* constitutive NO synthase) and ET-1 is a critical determinant in the development of vascular dysfunction.

Increases in whole-body albumin escape from circulation likely reflects fluid transfer, because in most tissues convection appears to be the dominant mechanism for transmicrocirculatory transport of molecules with dimension similar to albumin (Taylor & Granger, 1984). Previous results have indicated that albumin extravasation elicited by

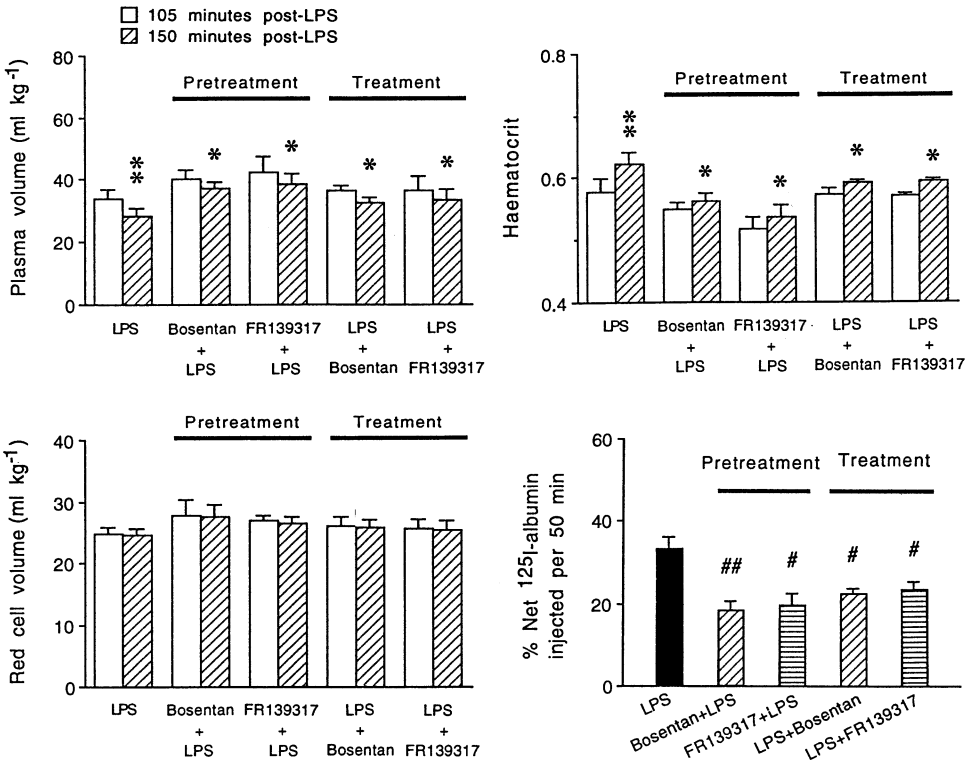


**Figure 5** Effects of bosentan (17.4 $\mu$mol kg$^{-1}$), FR 139317 (3.8 $\mu$mol kg$^{-1}$) or their vehicle (saline, control) on haematocrit, plasma volume, and red blood cell volume and total-body albumin escape during the delayed phase of endotoxic shock in conscious rats. The animals were either pretreated with bosentan, FR 139317 or vehicle for 10 min before administration of LPS (10 mg kg$^{-1}$, i.v.) (Pretreatment) or they first received saline at time 0 then LPS at 10 min and 70 min later bosentan or FR 139317 (Treatment). [$^{125}$I]-labelled human serum albumin was injected at 100 min and measurements were performed at 105 min and at 150 min after injection of LPS. Values are mean ± s.e.mean. *$P<0.05$; **$P<0.01$ (compared with measurements at 105 min in the same group), # $P<0.05$, ## $P<0.01$ (compared with LPS by Dunn's test).

ET-1 can primarily be attributed to increase in vascular permeability (Filep *et al.*, 1991, Zimmerman *et al.*, 1992; Filep, 1997). However, reduction of albumin escape by bosentan and FR 139317 might have partly been due to decreases in MABP in LPS-rats, since transmission of decreased systemic pressure to capillaries is expected to inhibit rather than to promote albumin extravasation. ET-1 is a more potent constrictor of venous than arterial vessels (Yang *et al.*, 1989). $ET_A$-like receptors predominate arterial and $ET_B$-like contractile receptors predominate on venous smooth muscle cells (Moreland *et al.*, 1992). Thus, blocking of ET-1-mediated vasoconstriction on capacitance vessels may considerably reduce microvascular hydrostatic pressure and consequently albumin extravasation. Presumably, ET-1 has tissue-specific effects on large arterioles affecting systemic vascular resistance and on small arterioles controlling capillary surface area. The relative participation of these two components of the microcirculation will likely determine the responses to ET-1 in various vascular beds. Platelet-activating factor (Filep *et al.*, 1991), priming of neutrophils to produce free radicals (Ishida *et al.*, 1990), and promotion of neutrophil-endothelial adhesion (López-Farré *et al.*, 1993; Zouki *et al.*, 1999) have been implicated as mediators of increased albumin escape elicited by ET-1. The findings that $ET_A$ receptor blockade prevents ET-1-induced adhesion of neutrophils to endothelial cells (Zouki *et al.*, 1999) are consistent with an $ET_A$ receptor-mediated increase in permeability.

The rapidly developing endothelial dysfunction contributes to the prolonged hypotension (Bone, 1991; Parillo, 1993), and leads to further reductions in blood and plasma volume and increases in albumin escape between 100 and 150 min after injection of LPS (Filep *et al.*, 1997, and the present study). These changes were partially inhibited by administration of bosentan or FR 139317 70 min after LPS. It is notable that neither bosentan nor FR 139317 treatment affected albumin escape rates in the bronchus, pulmonary parenchyma and kidney, indicating that endogenous endothelin exerts little influences on albumin escape in these organs during the delayed phase. Enhanced albumin escape in the bronchus leads to oedema formation in the airway wall and consequently to narrowing of the airways. This could contribute to the development of acute respiratory failure and therefore, to the increased mortality rate (Bone, 1991; Parillo, 1993). The present results also suggest that pulmonary oedema develops at least after 50 min of adminsitration of LPS in this model and that a critical event implicating endothelin occurs between 50 to 70 min of endotoxaemia. Since the lung contains the highest amount of NO synthase activity in normal rats (Szabó *et al.*, 1993), it could be argued that NO production protected against oedema formation (see above). At later stages of endotoxaemia, the imbalance between NO and ET-1 production might have unmasked the actions of endogenous endothelin. However, increases in lung permeability cannot be reduced when therapy with endothelin antagonists is delayed 70 min after LPS, indicating endothelin-independent mechanisms. Indeed, selective inhibition of NO production through inducible NO synthase prevented pulmonary oedema during the delayed phase of

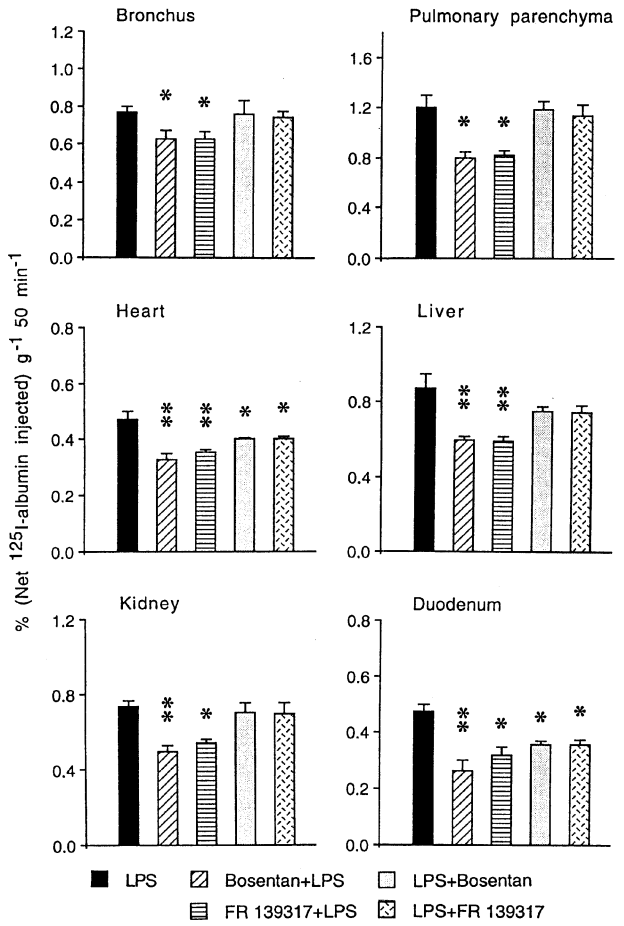


**Figure 6** Effects of bosentan (17.4 $\mu$mol kg$^{-1}$), FR 139317 (3.8 $\mu$mol kg$^{-1}$) or their vehicle (saline, control) on albumin escape rates in various vascular beds during the delayed phase of endotoxic shock in conscious rats. The animals were pretreated with bosentan, FR 139317 or vehicle for 10 min before administration of LPS (10 mg kg$^{-1}$, i.v.) or they first received saline at time 0 then LPS at 10 min and 70 min later they were treated with bosentan or FR 139317. $^{125}$I-labelled human serum albumin was injected at 100 min and the animals were killed at 150 min after injection of LPS. Values are mean$\pm$s.e.mean. *$P<0.05$; **$P<0.01$ (compared with LPS by Dunn's test).

endotoxin shock (Filep *et al.*, 1997). Furthermore, even though ET-1 may contribute to the development of pulmonary hypertension (Yamamoto *et al.*, 1997), it is not surprising that delayed treatment with endothelin receptor antagonists failed to reduce pulmonary oedema. Sudden increases in pulmonary perfusion pressure could cause structural changes (disruption and widening of endothelial junctions) in lung capillaries, leading to increases in permeability (Rippe *et al.*, 1984; Tsukimoto *et al.*, 1990). When structural damage occurs, it cannot be reversed by treatment with endothelin receptor antagonists.

The observations that neither bosentan nor FR 139317 treatment affected significantly albumin escape in the kidney during the delayed phase of endotoxemia are consistent with previous findings that $ET_A/ET_B$ receptor blockade did not affect the marked renal vasodilatation in LPS-rats (Gardiner *et al.*, 1995). Exogenous ET-1 evokes marked renal vasoconstriction in conscious rats (Gardiner *et al.*, 1994). One interpretation of these observations is that locally released ET-1 is responsible for regionally differentiated haemodynamic changes during endotoxaemia. Pernow *et al.* (1990) have reported a positive correlation between endotoxin-induced elevation of arterial plasma endothelin-like immunoreactivity and renal vascular resistance in anaesthetized pigs. Apart from differences in the experimental conditions (conscious versus anaesthetized animals) and species differences in renal sensitivity to endogenous endothelins, the reasons for this apparent discrepancy are not clear at present.

The present results raise the question of whether endogenous ET-1 would be beneficial in endotoxin shock, as suggested by Ruetten *et al.* (1996). Indeed, plasma ET-1 levels correlate positively with the severity of endotoxemia in patients (Pittet *et al.*, 1991) and are lower in survivors than in non-survivors of septic shock (Takakuwa *et al.*, 1994). Furthermore, an antisense homology box-derived peptide fragment of the human $ET_A$ receptor was found to block endotoxin shock in rats (Baranyi *et al.*, 1995). We propose that by reducing plasma volume and promoting albumin escape, endogenous endothelin contributes to the development of shock. While ET-1-mediated peripheral vasoconstriction support MABP, this may occur at the expense of transmitting increases in systemic arterial pressure to capillaries and/or local vasoconstriction, which may further aggravate losses in plasma volume and albumin escape.

As markedly elevated plasma ET-1 levels can be detected in patients with septic shock (Pittet *et al.*, 1991, Weitzberg *et al.*, 1991; Voerman *et al.*, 1992; Takakuwa *et al.*, 1994), endothelin receptor antagonism may be expected to reduce losses in plasma volume and albumin escape in patients. However, a word of caution is warranted regarding extrapolating the results from animal studies to the clinical setting. In animal models, including our rat model, the effects of endotoxin are studied, whereas many septic patients have bacteremia (Bone, 1991), which may trigger a systemic inflammatory response (Parillo, 1993). Furthermore, correction of hypovolaemia with intravenous administration of fluids leads to a hyperdynamic shock syndrome in more than 90% of septic patients (Parillo, 1993). It remains to be investigated whether the presence of systemic inflammation and/or fluid resuscitation could affect the responses to endogenous endothelin in septic shock.

In summary, the present study demonstrates a role for endogenous edothelin in mediating losses in plasma volume and albumin escape elicited by LPS and suggest that selective $ET_A$ or dual $ET_A/ET_B$ receptor antagonists may be useful in the therapy of septic shock.

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