ELSEVIER

European Journal of Pharmacology 318 (1996) 369–376

ejp

# Effects of Ro 47-0203 and PD155080 on the plasma kinetics, receptor binding and vascular effects of endothelin in the pig

Anette Hemsén [a,*], Agnes Modin [a], Michael Wanecek [b], Rickard E. Malmström [a], Eddie Weitzberg [b]

[a] *Division of Pharmacology, Department of Physiology and Pharmacology, Karolinska Institutet, S-171 77 Stockholm, Sweden*
[b] *Department of Anaesthesiology and Intensive Care, Karolinska Hospital, Stockholm, Sweden*

Received 25 April 1996; revised 27 September 1996; accepted 8 October 1996

**Abstract**

The effects of the mixed endothelin $ET_A$/endothelin $ET_B$ receptor antagonist Ro 47-0203 (bosentan, 4-*tert*-butyl-*N*-[6-(2-hydroxyethoxy)-5-(2-methoxy-phenoxy)-2,2′-bipyrimidin-4-yl]-benzenesulfonamide) and the selective endothelin $ET_A$ receptor antagonist PD155080 (sodium 2-benzo[1,3]dioxol-5-yl-3-benzyl-4-(4-methoxy-phenyl)-4-oxobut-2-enoate) on plasma half-life and regional extraction of exogenous endothelin-1 as well as on the regional vascular effects of endothelin-1 were investigated in the pig in vivo. Bosentan but not PD155080 (5 mg/kg, i.v. bolus, both drugs) increased the arterial plasma levels of endothelin-1-like immunoreactivity. Neither of the drugs affected the plasma half-life of infused endothelin-1. In the spleen, both the extraction and vascular effects of exogenous endothelin-1 were attenuated by both bosentan and PD155080 whereas renal extraction and vascular effects in the kidney were unaffected by both drugs. In the lung, only bosentan decreased pulmonary extraction of endothelin-1. In conclusion, the bosentan-induced increase of circulating endothelin-1 seems to be related to blockade of endothelin-1 binding to endothelin $ET_B$ receptors. Blockade of these receptors does not influence the overall elimination of endothelin-1, however.

*Keywords:* Endothelin-1; Endothelin receptor antagonist; Bosentan; PD155080; Clearance; Half-life; Vascular effect

## 1. Introduction

Endothelin-1 is a vasoconstrictor peptide which was isolated from vascular endothelial cells by Yanagisawa et al. (1988). Elevated plasma levels of endothelin-1 have been found in various disorders such as myocardial infarction (Miyauchi et al., 1989), atherosclerosis (Lerman et al., 1991) and sepsis (Weitzberg et al., 1991b).

Endothelin-1 can exert its vascular effects by activating at least two different receptors, the endothelin $ET_A$ receptor which mediates constriction (Arai et al., 1990) or the endothelin $ET_B$ receptor which mediates dilatation (Sakurai et al., 1990). The existence of a third receptor, mediating constriction, has been postulated (Pollock and Opgenorth, 1993).

The rapid disappearance of endothelin-1 from plasma (half-life 1–2 min) might involve enzymatic degradation of circulating peptide or may be related to receptor binding followed by internalisation (Frelin and Guedin, 1994). A specific degrading enzyme or clearance receptor has not yet been identified, although several candidates exist.

Previous experiments show that the non-peptide endothelin antagonist bosentan (Clozel et al., 1994) decreases basal vascular resistance and increases plasma levels of endothelin-1 in control pigs (Weitzberg et al., 1994). The bosentan-induced increase of plasma endothelin-1 levels has also been demonstrated in man (Kiowski et al., 1995). It is not known whether a specific clearance receptor exists. Löffler et al. (1993) suggest that the increase in endothelin-1 levels induced by another non-selective endothelin-antagonist (Ro 46-2005) in the rat is due to displacement from endothelin $ET_B$ receptors, since the selective endothelin $ET_A$ receptor antagonists BQ123 and FR139317 did not increase endothelin-1 levels in the rat.

The aims of the present study were to examine the effects of the mixed endothelin $ET_A$/endothelin $ET_B$ receptor antagonist bosentan and the selective endothelin $ET_A$ receptor antagonist PD155080 on endothelin kinetics and regional vascular effects during endothelin-1 infusion as well as their effects on endothelin-1 receptor binding.

* Corresponding author.


*PII* S0014-2999(96)00807-2

## 2. Materials and methods

### 2.1. Surgical preparation

Domestic pigs of either sex (18–25 kg) were used. The experimental set-up has been described in earlier studies (see Weitzberg et al., 1995). The experimental protocol was approved by the Animal Studies Committee of the Karolinska Institute.

Mean arterial blood pressure was measured with a Statham P23Ac pressure transducer via a catheter inserted in a femoral artery and mean pulmonary arterial pressure was measured via a catheter in the pulmonary artery. Local blood flows were measured with ultrasonic flow probes (Transonic, Ithaca, NY, USA) which were placed around the pulmonary artery, the left renal and the splenic artery. Both the mean arterial blood pressure and the local blood flows were continuously recorded on a Grass Polygraph. Blood samples were drawn from catheters inserted in the right brachial artery, the pulmonary artery and the renal and splenic veins.

### 2.2. Experimental procedure

Ten pigs were used in the antagonist experiments, which were performed under anaesthesia. After preparation and a 60 min equilibration period following surgery the pigs were given i.v. bolus doses of phenylephrine (1 mg/kg) and neuropeptide Y (1 μg/kg) as well as 20 min infusions of endothelin-1 (Peptide Institute, Japan) 4 pmol/kg per min. After a resting period, bosentan (5 mg/kg in 20 ml saline, i.v. bolus, $n = 4$) or PD155080 (5 mg/kg in 20 ml saline, i.v. bolus, $n = 6$) was given and the same drugs were administered again, as well as a higher dose of endothelin-1, 20 pmol/kg per min. Control infusions of endothelin-1 at 20 pmol/kg per min were done in another group of 5 pigs.

#### 2.2.1. In vivo efficacy of bosentan and PD155080

In a study on subarachnoid haemorrhage-induced delayed cerebral vasospasm in rats (Zuccarello et al., 1996), bosentan or PD155080 were given orally to rats at a dose of 40 mg/kg/day for bosentan and 60 mg/kg/day for PD155080. After 6 days treatment, plasma bosentan levels were $5 \pm 3$ μM ($n = 6$) whereas plasma PD155080 levels ranged from approximately 1 to 30 μM ($n = 7$). The effect of bosentan was not significantly different from that of PD155080 at these plasma levels.

### 2.3. 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.

### 2.4. Radioimmunoassay

Radioimmunoassay for endothelin-1 was performed in a 0.1 M phosphate buffer containing 0.05 M NaCl, 0.1% $NaN_3$ and 0.1% bovine serum albumin. The samples were incubated for 2 days at 4°C with the E1 rabbit antiserum (Hemsén and Lundberg, 1991) before [$^{125}$I]endothelin-1 was added and the assay incubated for two more days. Bound and free fractions were then separated using a secondary antibody method (SacCel, IDS, Boldon, UK). The bound fractions were counted in an LKB 1249 Gamma Counter (LKB-Pharmacia, Uppsala, Sweden) and compared with known concentrations of synthetic endothelin-1. The lowest detectable amount was 0.39 fmol/tube. The cross-reactivity of the E1 antiserum was: endothelin-1, 100%; endothelin-2, 27%; endothelin-3, 8%; pBig endothelin-1, 0.03%.

### 2.5. Receptor binding studies

Spleen, lung and kidney were dissected from untreated pentobarbitone-anaesthetized pigs and placed in ice-cold saline. The tissues were then weighed, cut into small pieces and homogenised in 10 volumes of ice-cold 0.3 M sucrose containing 5 mM HEPES, pH 7.4, using a Polytron. The homogenates were centrifuged at $1000 \times g$ for 10 min at 4°C and the supernatants were decanted and further centrifuged at $10000 \times g$ for 30 min. The pellets were resuspended in 0.9% NaCl, containing 5 mM HEPES and stored in aliquots at −80°C. For binding studies, 30 μl of the membrane preparation, corresponding to 100–200 μg protein, was diluted in a physiological salt solution, pH 7.4, of the following composition in mM: NaCl 137, KCl 2.68, $MgCl_2$ 2.05, $CaCl_2$ 1.80 and HEPES 20. Before use, bovine serum albumin (0.1% or 1%, as indicated) was added.

For displacement studies, the competing ligands (endothelin-1, endothelin-3, [Ala$^{1,3,11,15}$]endothelin-1 (endothelin $ET_B$ receptor agonist), bosentan and PD155080) were added to diluted membranes (with 1% bovine serum albumin) in a concentration range of 1 pM to 1 mM (10 mM for bosentan) and incubated with [$^{125}$I]endothelin-1 for 2 h. Non-specific binding was determined in the presence of endothelin-1 at a concentration of 100 nM. Bound and free ligand were separated by a rapid high speed centrifugation, the supernatants were aspirated and the pellets were washed once with the buffer. The radioactivity in both the pellets and the supernatants was counted.

To detect the degree of binding between ligand and bovine serum albumin and its interaction with the specific binding, separate experiments were made on splenic mem-

branes with endothelin-1, bosentan and PD155080 using 0.1% bovine serum albumin. The binding experiment was otherwise performed as above.

Off-rate binding studies with bosentan were performed on splenic and renal membranes with 0.1% bovine serum albumin after an initial on-rate binding period of 20 min for the radioligand. The binding was compared with the binding obtained without addition of bosentan.

### 2.6. Drugs

Ketamine hydrochloride was from Parke-Davis (Morris Plains, NJ, USA), atropine was from Kabi-Pharmacia (Stockholm, Sweden), sodium pentobarbitone was from ACO (Stockholm, Sweden) and pancuronium bromide was from Organon (Oss, Netherlands).

Bosentan (4-*tert*-butyl-*N*-[6-(2-hydroxy-ethoxy)-5-(2-methoxy-phenoxy)-2,2′-bipyrimidin-4-yl]-benzene-sulfonamide) was a kind gift from Dr. Martine Clozel (F. Hoffmann-La Roche, Basel, Switzerland).

PD155080 (sodium 2-benzo[1,3]dioxol-5-yl-3-benzyl-4-(4-methoxy-phenyl)-4-oxobut-2-enoate) was kindly provided by Dr. Annette M. Doherty (Parke-Davis Pharmaceutical Research Division, Ann Arbor, MI, USA).

Endothelin-1, endothelin-3 and [$Ala^{1,3,11,15}$]endothelin-1 were from Peninsula Laboratories Europe (St. Helens, UK).

### 2.7. Calculations and statistics

The fractional extraction was calculated as the arteriovenous concentration difference divided by the arterial concentration. The regional vascular conductance was calculated as the local blood flow divided by mean arterial blood pressure or mean pulmonary arterial pressure where appropriate.

Statistical analysis was performed with Student's *t*-test, one-way analysis of variance or repeated measures analysis of variance as appropriate, using InStat 2.01 for Macintosh (GraphPad Software, San Diego, CA, USA).

All data are presented as means and S.E.M.

## 3. Results

### 3.1. Arterial levels of endothelin-1-like immunoreactivity

Administration of bosentan to untreated pigs caused a gradual increase of the arterial plasma levels of endothelin-like immunoreactivity, which was significant already after 2 min. After 15 min the levels of endothelin-like immunoreactivity had increased to 53.6 ± 7.8 pM from basal levels of 19.9 ± 4.8 pM ($n = 3$, $P < 0.01$, Fig. 1). The basal levels of endothelin-like immunoreactivity in another group of pigs (26.0 ± 2.1 pM) were not significantly changed 15 min after administration of PD155080 (29.1 ± 2.7 pM, $n = 6$) (Fig. 1).

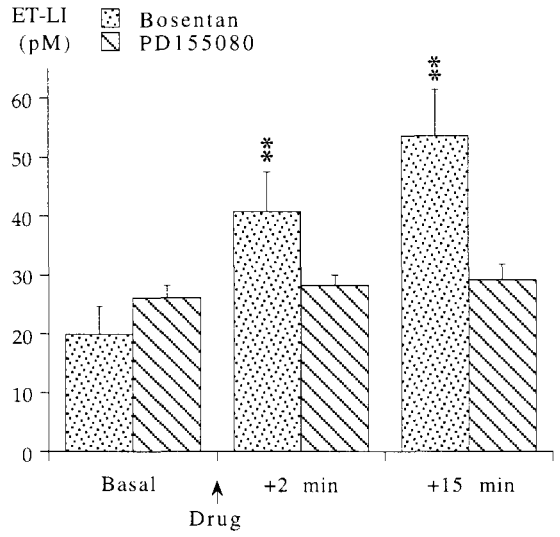


Fig. 1. Arterial plasma levels of endothelin-1-like immunoreactivity in untreated pigs before and after a bolus dose (5 mg/kg, i.v.) of bosentan (dotted bar) or PD155080 (striped bar). ** $P < 0.01$, ANOVA, $n = 3$–6.

### 3.2. Fractional extraction during endothelin-1 infusion

Twenty minutes after infusion with synthetic endothelin-1 (4 pmol/kg per min, low dose) the arterial levels of endothelin-1-like immunoreactivity were 46.0 ± 8.4 pM ($n = 4$) and extraction of endothelin-1 was observed in the spleen (35 ± 5%), kidney (46 ± 6%) and lung (36 ± 8%) (Fig. 2a). After treatment with bosentan the arterial levels of endothelin-1-like immunoreactivity were 181 ± 40 pM after the endothelin-1 infusion and the fractional extraction was reduced to −2.3 ± 7% in the spleen ($P < 0.05$) and −3.4 ± 7% in the lung ($P < 0.01$), whereas in the kidney there was no change (30 ± 9%, NS) (Fig. 2a).

In the other group of pigs infusion with the low dose of endothelin-1 caused an increase of arterial levels of endothelin-1-like immunoreactivity to 75.9 ± 11.7 pM (from basal levels of 25.8 ± 1.5 pM) and the fractional extraction was 40.2 ± 4.0% in the spleen, 45.6 ± 6.2% in the kidney and 32.0 ± 6.3% in the lung (Fig. 2b). After treatment with PD155080 the low-dose infusion increased the arterial levels of endothelin-1-like immunoreactivity to 68.2 ± 4.4 pM (from basal levels of 26.0 ± 2.1 pM) and the extraction in the spleen was reduced to 13.3 ± 6.4% ($P < 0.01$) whereas no significant changes of the extraction where observed in the kidney or the lung (48.6 ± 4.3% and 19.8 ± 6.2%, respectively) (Fig. 2b).

Infusion with a higher dose of endothelin-1 (20 pmol/kg per min) in untreated pigs increased the arterial levels of endothelin-1-like immunoreactivity to 737 ± 171 pM and abolished the extraction of endothelin-1 in the lung. The

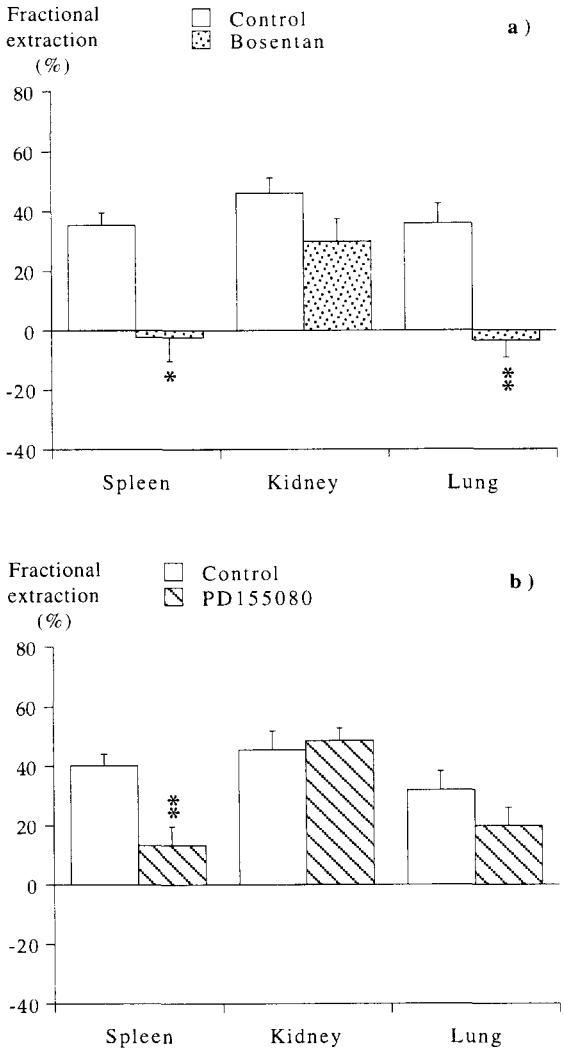


Fig. 2. (a) Fractional extraction of endothelin-1 before (open bars) and after treatment with bosentan (5 mg/kg, i.v.) (dotted bars) over the spleen, kidney and lung after a 20 min infusion of endothelin-1 at a dose of 4 pmol/kg per min. * $P < 0.05$, ** $P < 0.01$, Student's $t$-test, $n = 3$–4. (b) Fractional extraction of endothelin-1 before (open bars) and after treatment with PD155080 (5 mg/kg, i.v.) (striped bars) over the spleen, kidney and lung after a 20 min infusion of endothelin-1 at a dose of 4 pmol/kg per min. ** $P < 0.01$, Student's $t$-test, $n = 3$–4.

fractional extraction in the spleen was 58 ± 4% and in the kidney 66 ± 5% (Fig. 3). In the bosentan-treated pigs the fractional extraction in the spleen was reduced to 24 ± 8% ($P < 0.01$), whereas a smaller reduction was observed after treatment with PD155080 (47 ± 3%; $P < 0.05$). The removal over the kidney was not changed either by bosentan (59 ± 1%) or PD155080 (64 ± 4%) (Fig. 3).

### 3.3. Regional vasoconstriction during endothelin-1 infusion

The low-dose infusion of endothelin-1 (4 pmol/kg per min) decreased the vascular conductance (VC) in the spleen to 66 ± 5% of basal values (1.38 ml/min per mmHg) (Fig. 4a) and in the kidney to 80 ± 1% of basal values (1.24 ml/min per mmHg) (Fig. 4b). Pretreatment with bosentan significantly attenuated the decrease in the spleen ($P < 0.05$) (Fig. 4a) but not in the kidney (Fig. 4b).

In the other group of pigs the low-dose endothelin-1 infusion decreased splenic vascular conductance to 36 ± 6% of basal values (0.735 ± 0.102 ml/min per mmHg) (Fig. 4a) and renal vascular conductance to 80 ± 3% of basal values (0.710 ± 0.102 ml/min per mmHg) (Fig. 4b). Treatment with PD155080 significantly attenuated the decrease in the spleen ($P < 0.01$, Fig. 4a) but not in the kidney (Fig. 4b).

After the high-dose infusion of endothelin-1 (20 pmol/kg per min) to untreated pigs the splenic vascular conductance decreased to 10 ± 1% of basal values (0.621 ± 0.053 ml/min per mmHg) ($P < 0.05$) and renal vascular conductance decreased to 51 ± 11% of basal values (1.59 ± 0.34 ml/min per mmHg). In the bosentan-treated pigs the decrease of splenic vascular conductance was attenuated to 36 ± 2% of basal values (1.15 ± 0.29 ml/min per mmHg) ($P < 0.01$, Fig. 5) whereas the decrease of renal vascular conductance was unaffected (Fig. 5). Treatment with PD155080 did not change the decrease of either splenic or renal vascular conductance induced by the high-dose endothelin-1 infusion (Fig. 5).

The pulmonary vascular conductance was not significantly changed either by the low dose or by the high dose of endothelin-1 and no change was observed in the presence of bosentan or PD155080 (Fig. 4c, Fig. 5).

The decreased splenic and renal vascular conductance induced by i.v. bolus injections of phenylephrine and neuropeptide Y was not affected by prior treatment with bosentan or PD155080 (not shown).

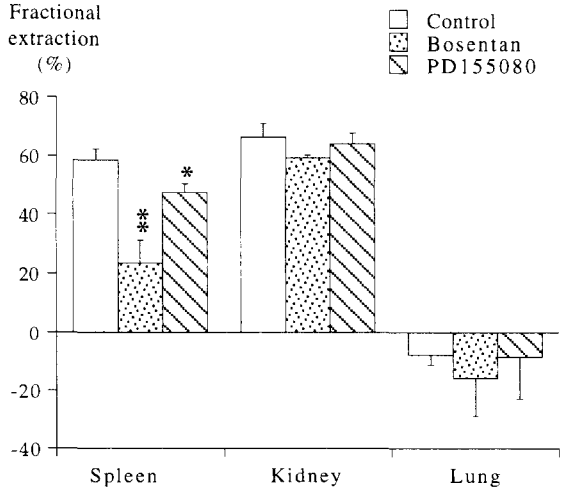


Fig. 3. (a) Fractional extraction of endothelin-1 over the spleen, kidney and lung in control pigs (open bars), pigs treated with bosentan (5 mg/kg, i.v.) (dotted bars) and pigs treated with PD155080 (5 mg/kg, i.v.) (striped bars) after a 20 min infusion of endothelin-1 at a dose of 20 pmol/kg per min. * $P < 0.05$, ** $P < 0.01$, Student's $t$-test, $n = 3$–6.

### 3.4. Plasma half-life of endothelin-1

Infusion with the high dose of endothelin-1 (20 pmol/kg per min) increased arterial levels to 737 ± 171 in untreated pigs, to 1313 ± 356 in pigs treated with bosentan and to 820 ± 132 in pigs treated with PD155080.

The plasma half-life of endothelin-1 was calculated from the fall in plasma concentration during the first 5 min after the respective infusions and was 52 ± 13 s in untreated pigs, 37 ± 2 s in bosentan-treated pigs and 60 ± 13 s in the group treated with PD155080 (not shown). The changes in plasma half-life between the groups were not significant.

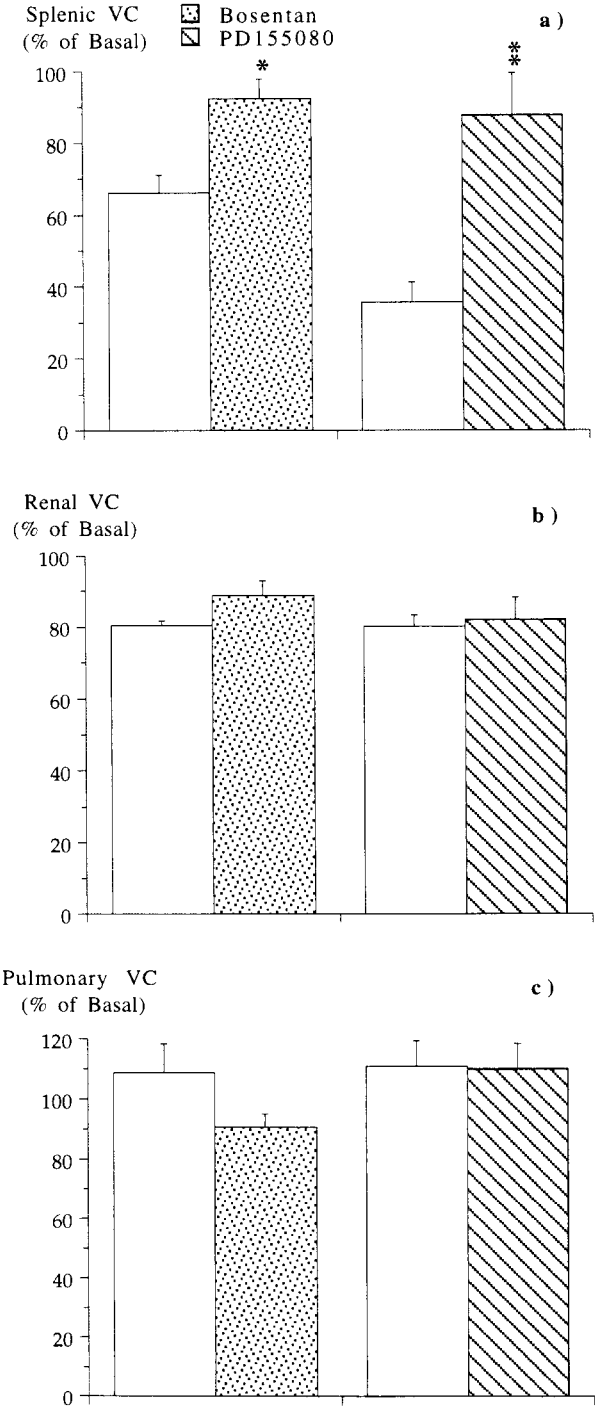


Fig. 4. Regional vascular conductance expressed as percentage of basal values in the spleen (a), kidney (b) and lung (c) after a 20 min infusion of endothelin-1 (4 pmol/kg per min) before (open bars) and after (dotted bars) treatment with bosentan (5 mg/kg, i.v.) and before (open bars) and after (striped bars) treatment with PD155080 (5 mg/kg, i.v.) (striped bars). * $P < 0.05$, ** $P < 0.01$, Student's $t$-test, $n = 4–6$.

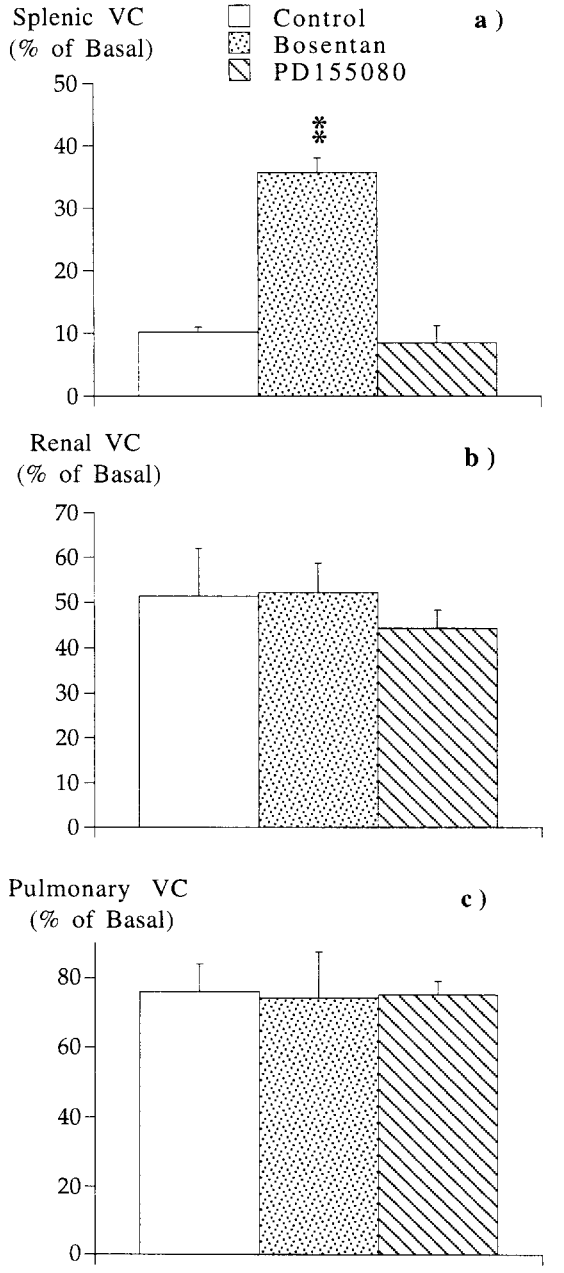


Fig. 5. Regional vascular resistance expressed as percentage of basal values in the spleen (a), kidney (b) and lung (c) after a 20 min infusion of endothelin-1 (20 pmol/kg per min) in control pigs (open bars), pigs treated with bosentan (5 mg/kg, i.v.) (dotted bars) and pigs treated with PD155080 (5 mg/kg, i.v.) (striped bars). ** $P < 0.01$, Student's $t$-test, $n = 4–6$.

### 3.5. Displacement binding studies

In the spleen the $IC_{50}$ of endothelin-1 binding was 0.34 nM, which was almost 300 times higher than for endothelin-3. The affinity of bosentan was 60 times lower than that of endothelin-1, whereas the affinity of PD155080 was 130 times lower. [$Ala^{1,3,11,15}$]Endothelin-1 was a very weak ligand in the spleen (Table 1).

In the lung the $IC_{50}$ of endothelin-1 was 0.35 nM whereas endothelin-3 had 26 times lower affinity. The affinity of bosentan, PD155080 and [$Ala^{1,3,11,15}$]endothelin-1 was much lower (Table 1).

In the kidney endothelin-1 and endothelin-3 had rather similar affinity, $IC_{50}$ 2.3 and 6.5 nM respectively. Of the drugs [$Ala^{1,3,11,15}$]endothelin-1 and bosentan had 60 and 135 times lower affinity than endothelin-1, respectively, whereas the affinity of PD155080 was much lower (Table 1).

The binding experiments in the presence of 0.1% bovine serum albumin revealed that while the $IC_{50}$ value of endothelin-1 was the same at both concentrations of bovine serum albumin, bosentan had about ten times higher affinity in the presence of 0.1% than of 1% bovine serum albumin whereas PD155080 had 3-fold higher affinity when 0.1% bovine serum albumin was used (data not shown).

### 3.6. Dissociation binding studies

The specific binding was expressed as percentage of the specific binding obtained after 80 min incubation of the membranes with the radioligand in the absence of bosentan.

In splenic membranes, the specific binding of the radioligand observed at 20 min ($52 \pm 2\%$) was reduced to $42 \pm 4\%$ 10 min after addition of bosentan. The specific binding was maintained at this level during the 60 min incubation with bosentan, whereas the specific binding obtained in the absence of bosentan increased to $100 \pm 6\%$ during the same time period (Fig. 6).

Table 1
$IC_{50}$ values (nM) for displacement of specific [$^{125}$I]endothelin-1 binding by endothelin-1 (ET-1), endothelin-3 (ET-3, [$Ala^{1,3,11,15}$]endothelin-1 ([Ala]ET-1), bosentan and PD155080

| | $IC_{50}$ (ratio) | | | | |
|---|---|---|---|---|---|
| | ET-1 | ET-3 | [Ala]ET-1 | Bosentan | PD155080 |
| Spleen | 0.34 | 95.2 | > 1000 | 19.6 | 45.2 |
| | (1) | (280) | (> 2941) | (57.6) | (133) |
| Lung | 0.35 | 9.20 | 106 | 333 | 41245 |
| | (1) | (26) | (303) | (951) | (118000) |
| Kidney | 2.3 | 6.51 | 135 | 310 | 16895 |
| | (1) | (3) | (59) | (135) | (7300) |

Each value represents the mean of 3 separate experiments performed in duplicate. The approximate ratios between the $IC_{50}$ values for the other substances and those for endothelin-1 are also given in parentheses.

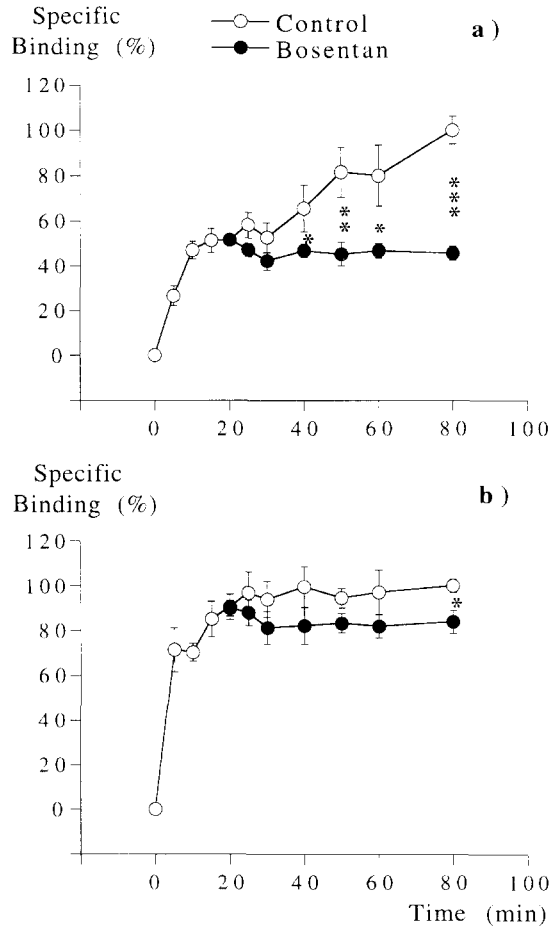


Fig. 6. Time-related binding curves of [$^{125}$I]endothelin-1 on splenic (a) and renal (b) membranes in the absence (○, $n = 4–8$) or presence (●, $n = 6–8$) of bosentan (1 mM), which was added after 20 min of incubation. The binding is expressed as percentage of the specific binding of [$^{125}$I]endothelin-1 obtained after 80 min incubation in the absence of bosentan. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, changes between groups, Student's $t$-test.

In renal membranes, the specific binding was $90 \pm 4\%$ after 20 min incubation with the radioligand, and addition of bosentan caused a small decrease to $81 \pm 7\%$ after 10 min (Fig. 6).

## 4. Discussion

Endothelin-1 is well known to have a very short half-life in the circulation both in pig (Pernow et al., 1989) and in man (Weitzberg et al., 1991a). The clearance mechanism has not yet been identified, although the contribution of endothelin-1 degrading enzymes has been postulated. Frelin and Guedin (1994) presented another hypothesis, namely that the low levels of circulating endothelin-1, and thus the clearance, depend on binding to functional receptors and internalisation of the complexes. That the mixed endothelin receptor antagonist bosentan per se causes an increase of arterial endothelin-1-like immunoreactivity in

pig (Weitzberg et al., 1994) and man (Kiowski et al., 1995) is in line with this hypothesis, and a similar phenomenon has also been shown in the rat for the endothelin antagonist Ro-46-2005 (Löffler et al., 1993).

Of the two endothelin receptor antagonists used in the present study, the mixed endothelin $ET_A$/endothelin $ET_B$ receptor antagonist bosentan increased arterial levels of endothelin-1-like immunoreactivity, whereas no such increase was observed for the selective endothelin $ET_A$ receptor antagonist PD155080.

In the spleen, both bosentan and PD155080 inhibited splenic extraction of exogenous endothelin-1 as well as the endothelin-1-induced splenic vasoconstriction. These results indicate that blockade of functional splenic endothelin $ET_A$ receptors does not cause the increase of plasma endothelin-1. Furthermore, since the plasma half-life of endothelin-1 was unaffected by both bosentan and PD155080, splenic binding sites seem not to be important for the overall clearance of endothelin-1.

In the lung, in which vasodilatory endothelin $ET_B$ receptors are present (Hemsén et al., 1991), bosentan reduced the fractional extraction of endothelin-1 during the low-dose infusion, whereas PD155080 did not significantly change the extraction, which suggests that blockade of pulmonary binding sites may account for the increase of circulating endothelin-1 after bosentan. In this, as in earlier studies, fractional extraction of endothelin-1 over the lung was only observed during the low-dose infusion (4 pmol/kg per min) and not during infusion with the higher dose (20 pmol/kg per min) indicating a saturable removal mechanism in this vascular bed.

In the kidney, endothelin-1 extraction was observed at both doses and was not inhibited by either drug. Since PD155080 was unable to attenuate the renal vasoconstriction this effect may be mediated by constricting endothelin $ET_B$ receptors. That receptor antagonists are ineffective on renal clearance sites may have many reasons, e.g. degradation of antagonist or inefficient competition due to presence of large concentrations of endogenous endothelin-1.

Thus, if receptor blockade is involved in the bosentan-induced plasma increase of endothelin-1, the best candidate for this receptor would be the dilatory endothelin $ET_B$ receptor, which is located on the endothelial cell and would have the best opportunity to bind and inactivate circulating endothelin-1 before it reaches the smooth muscle cells and the constricting receptors. However, blockade of other binding sites which may not be accessible to circulating endothelin-1 cannot be excluded.

The selectivity of bosentan is supported by receptor binding data showing that bosentan has 10-fold higher affinity for endothelin-1 receptors in the spleen compared to the receptor populations in the kidney and lung. This difference is most likely related to the predominance of endothelin $ET_A$ receptors in the pig spleen, while endothelin $ET_B$ receptors predominate in the kidney (Hemsén et al., 1991). Bosentan also has antagonistic effects at endothelin $ET_B$ receptors, although with a 20–30-fold lower affinity than at endothelin $ET_A$ receptors (Clozel et al., 1994). However, when trying to find a correlation between functional effects and clearance of endothelin-1 in vivo after antagonist treatment and the distribution of endothelin receptor subtypes in different tissues in vitro, one should keep in mind that all expressed receptors are not accessible to i.v. administered endothelin-1.

It is also worth noting that bosentan had a 10-fold higher affinity in the presence of 0.1% than of 1% bovine serum albumin, indicating that serum protein binding could play a crucial part for the in vivo potency of bosentan. Also PD155080 had higher affinity (3-fold) in the presence of 0.1% bovine serum albumin.

The off-rate binding studies show that bosentan was not able to displace already bound endothelin-1 to any major extent, although further binding of the radioligand was inhibited, suggesting that the inhibitory effect of bosentan is on receptor level.

In conclusion, the bosentan-induced increase of circulating endothelin-1 seems to be related to blockade of the binding of endothelin-1 to endothelin $ET_B$ receptors. Whether these receptors are functional or are specific clearance receptors remains to be established. Receptors involved in decreasing plasma levels of endothelin-1 do not seem to be important for the overall elimination of endothelin-1, however.

## Acknowledgements

We are grateful to Ms. Margareta Stensdotter and Mrs. Carina Nihlén for expert technical assistance. The present study was supported by Åke Wiberg's Foundation, King Gustav V:s and Queen Victoria Foundation, and the Swedish Society of Medicine (i 705.0).

## References

Arai, H., S. Hori, I. Aramori, H. Ohkubo and S. Nakanishi, 1990, Cloning and expression of a cDNA encoding an endothelin receptor, Nature 348, 730.

Clozel, M., V. Breu, G.A. Gray, B. Kalina, B.-M. Löffler, K. Burri, J.-M. Cassal, G. Hirth, M. Müller, W. Neidhart and H. Ramuz, 1994, Pharmacological characterization of bosentan, a new potent orally active nonpeptide endothelin receptor antagonist, J. Pharmacol. Exp. Ther. 270, 228.

Frelin, C. and D. Guedin, 1994, Why are circulating levels of endothelin-1 so low?, Cardiovasc. Res. 28, 1613.

Hemsén, A. and J.M. Lundberg, 1991, Presence of endothelin-1 and endothelin-3 in peripheral tissues and central nervous system of the pig, Regul. Pept. 36, 71.

Hemsén, A., O. Larsson and J.M. Lundberg, 1991, Characteristics of endothelin A and B binding sites and their vascular effects in pig peripheral tissues, Eur. J. Pharmacol. 208, 313.

Kiowski, W., G. Sütsch, P. Hunziker, P. Müller, J. Kim, E. Oechslin, R. Schmitt, R. Jones and O. Bertel, 1995, Evidence for endothelin-1-mediated vasoconstriction in severe chronic heart failure, Lancet 346, 732.

Lerman, A., B.S. Edwards, J.W. Hallett, D.M. Heublein, S.M. Sandberg and J.C. Burnett Jr., 1991, Circulating and tissue endothelin immunoreactivity in advanced atherosclerosis, New Engl. J. Med. 325, 997.

Löffler, B.-M., V. Breu and M. Clozel, 1993, Effect of different endothelin receptor antagonists and of the novel non peptide antagonist Ro-46-2005 on endothelin levels in rat plasma, FEBS Lett. 333, 108.

Miyauchi, T., M. Yanagisawa, T. Tomizawa, Y. Sugishita, N. Suzuki, M. Fujino, R. Ajisaka, K. Goto and T. Masaki, 1989, Increased plasma concentrations of endothelin-1 and big endothelin-1 in acute myocardial infarction, Lancet ii, 53.

Pernow, J., A. Hemsén and J.M. Lundberg, 1989, Tissue specific distribution, clearance and vascular effects of endothelin in the pig, Biochem. Biophys. Res. Commun. 161, 647.

Pollock, D.M. and T.J. Opgenorth, 1993, Evidence for endothelin-induced renal vasoconstriction independent of $endothelin_A$ receptor activation, Am. J. Physiol. 264, R222.

Sakurai, T., M. Yanagisawa, Y. Takuwa, H. Miyazaki, S. Kimura, K. Goto and T. Masaki, 1990, Cloning of a cDNA encoding a non-isopeptide-selective subtype of the endothelin receptor, Nature 348, 732.

Weitzberg, E., G. Ahlborg and J.M. Lundberg, 1991a, Long-lasting vasoconstriction and efficient regional extraction of endothelin-1 in human splanchnic and renal tissues, Biochem. Biophys. Res. Commun. 180, 1298.

Weitzberg, E., J.M. Lundberg and A. Rudehill, 1991b, Elevated plasma levels of endothelin in patients with sepsis syndrome, Circ. Shock 33, 222.

Weitzberg, E., A. Rudehill, A. Modin and J.M. Lundberg, 1994, Porcine intrinsic pulmonary and systemic vascular tone is endothelin-dependent, Acta Physiol. Scand. 152, 433.

Weitzberg, E., J.M. Lundberg and A. Rudehill, 1995, Inhibitory effects of diclofenac on the endotoxin shock response in relation to endothelin turnover in the pig, Acta Anaesthesiol. Scand. 39, 50.

Yanagisawa, M., H. Kurihara, S. Kimura, Y. Tomobe, M. Kobayashi, Y. Mitsui, Y. Yazaki, K. Goto and T. Masaki, 1988, A novel potent vasoconstrictor peptide produced by vascular endothelial cells, Nature 332, 411.

Zuccarello, M., G.B. Soattin, A.I. Lewis, V. Breu, H. Hallak and R.M. Rapoport, 1996, Prevention of subarachnoid hemmorrhage-induced cerebral vasospasm by oral administration of endothelin receptor antagonists, J. Neurosurg. 84, 503.