Characterization of receptors mediating vascular responses to endothelin-1 in the conscious rat

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1 The present study has determined the receptors mediating the vascular responses (pressor and depressor actions and vascular permeability effect) to endothelin-1 (ET-1) in the conscious rat by using the novel non-peptide ET_A/ET_B receptor antagonist, bosentan (Ro 47-0203, 4-*tert*-butyl-N-[6-(2 hydroxyethoxy)-5-(2-methoxy-phenoxy)-2,2'-bipyrimidine-4-yl]benzene-sulphonamide), the ET_A receptor-selective antagonist, FR 139317 and the ET_B receptor-selective peptide agonist, IRL 1620.

2 Bolus injection of ET-1 (1 nmol kg⁻¹, i.v.) resulted in a prolonged pressor effect (maximum increase in mean arterial blood pressure (MABP) was 47 ± 3 mmHg, *n* = 6) preceded by a transient depressor response (maximum decrease in MABP was 17 ± 1 mmHg). Both these responses were inhibited by bosentan (1–20 mg kg⁻¹, i.v. bolus) in a dose-dependent manner. The maximum inhibition of ET-induced depressor and pressor responses did not exceed 53 and 87%, respectively. FR 139317 (2.5 mg kg⁻¹, i.v.) attenuated the pressor response to ET-1 by 75% without affecting the depressor response. Furthermore, FR 139317, but not bosentan, prolonged the depressor action of ET-1. Corresponding to changes in blood pressure, a small transient tachycardia (Δheart rate 15 ± 5 beats min⁻¹) followed by a sustained bradycardia (Δheart rate –48 ± 10 beats min⁻¹, *n* = 6) was observed following injection of 1 nmol kg⁻¹ ET-1. FR 139317 and bosentan (10 mg kg⁻¹) inhibited ET-1-induced bradycardia by 79% and 71%, respectively. ET-1-induced tachycardia was significantly attenuated by bosentan, but not FR 139317.

3 The ET_B receptor agonist, IRL 1620 (0.1–2 nmol kg⁻¹, i.v.) produced biphasic dose-dependent changes in MABP with an initial transient fall followed by a prolonged pressor action. The maximum decrease and increase in MABP were 11 ± 2 and 19 ± 3 mmHg, respectively (*n* = 5). These changes in MABP were accompanied by a transient tachycardia (Δheart rate 9 ± 3 beats min⁻¹) and prolonged bradycardia (Δheart rate –17 ± 11 beats min⁻¹), respectively. Pretreatment of the animals with FR 139317 (2.5 mg kg⁻¹, i.v.) did not affect IRL 1620 (1 nmol kg⁻¹)-induced changes in MABP and heart rate, whereas both the depressor and pressor actions of IRL 1620 and the accompanying tachycardia and bradycardia were almost completely inhibited by bosentan (10 mg kg⁻¹).

4 ET-1 (1 nmol kg⁻¹) enhanced albumin extravasation in the upper and lower bronchi, spleen, kidney, stomach and duodenum (up to 246%) as measured by the extravasation of Evans blue dye. FR 139317 (2.5 mg kg⁻¹) completely inhibited ET-1-induced protein extravasation in the stomach and duodenum, whereas 40–75% inhibition was observed in the other vascular beds studied. The permeability effect of ET-1 was almost completely inhibited by bosentan (10 mg kg⁻¹) in all vascular beds studied.

5 IRL 1620 (0.4 or 1 nmol kg⁻¹, i.v.) enhanced albumin extravasation (up to 219%) in the upper and lower bronchi, spleen and kidney in a dose-dependent manner. Unlike ET-1, IRL 1620 failed to increase albumin extravasation in the stomach and duodenum.

6 The present study demonstrates in the conscious rat that ET_A and ET_B receptors are responsible for mediating the majority of the pressor response to ET-1 and suggest that a small component of the ET-1 pressor response might be mediated via a non-ET_A, non-ET_B receptor, whereas ET_B and perhaps a non-ET_A, non-ET_B receptor may mediate the depressor action of ET-1. Furthermore, the ET-1 induced albumin extravasation is mediated solely via ET_A receptors in the stomach and duodenum, whereas both ET_A and ET_B receptors are involved in the permeability effect of ET-1 in the bronchial, splenic and renal vascular beds.

Keywords: Endothelin-1; ET_A and ET_B receptors; vascular effects; vascular permeability; bosentan; FR 139317; IRL 1620; airways; gastrointestinal tract; kidney

Introduction

An increasing body of evidence supports a role for endothelin-1 (ET-1) in the regulation of cardiovascular functions including systemic and local vascular tone, cardiac output (see Simonson & Dunn, 1991; Rubanyi & Parker Botelho, 1991) and microvascular permeability (Filep *et al.*, 1991; Zimmerman *et al.*, 1992). Receptor binding studies, functional data and cross-desensitization experiments implied the existence of multiple endothelin receptor subtypes (see

Sakurai *et al.*, 1992; Sokolovsky, 1992), which may mediate the cardiovascular actions of ET-1.

To date, two mammalian ET receptor subtypes, designated ET_A and ET_B, have been cloned, sequenced and characterized (Arai *et al.*, 1990; Sakurai *et al.*, 1990). The ET_A receptor is considered isopeptide-selective, since it binds ET-1 and ET-2 more avidly than ET-3. In contrast, the ET_B receptor binds all three ET isopeptides with a similar affinity and is considered non-isopeptide-selective. Recently, an ET-3-specific receptor (termed ET_C) was also cloned from *Xenopus melanophores* (Karne *et al.*, 1993). However, it remains to be established

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whether this amphibian ET_C receptor has a mammalian counterpart. Activation of ET_A receptors is implicated in mediating the vasoconstrictor and pressor actions of ET-1, whereas it has been suggested that ET_B receptors mediate vasodilatation and depressor responses to ET-1 (Takayanagi *et al.*, 1991; Ihara *et al.*, 1992). However, these concepts have been challenged as recent studies have demonstrated that complete inhibition of the pressor effect of ET-1 cannot be achieved by the putative ET_A receptor antagonists, BQ-123 (Ihara *et al.*, 1992; Filep *et al.*, 1992; 1993b; McMurdo *et al.*, 1993) and FR 139317 (Bird & Waldron, 1993; Sogabe *et al.*, 1993) both in conscious and anaesthetized rats. The pressor effect of the ET_B receptor-selective agonists, sarafotoxin S6c and [Ala^{1,3,11,15}]endothelin-1 has also been described in pithed and conscious rats (Gardiner *et al.*, 1990; Douglas & Hiley, 1991; Williams *et al.*, 1991; Clozel *et al.*, 1992). Furthermore, recent studies on isolated blood vessels provided evidence for ET_B-mediated vasoconstriction (Bigaud & Pelton, 1992; Clozel *et al.*, 1992; Moreland *et al.*, 1992; Sumner *et al.*, 1992; LaDouceur *et al.*, 1993) and for the involvement of a novel, non-ET_A, non-ET_B receptor in mediating the coronary vasoconstrictor action of ET-1 (Harrison *et al.*, 1992). We have shown recently that ET-1-induced albumin extravasation can be partially inhibited by BQ-123 in selected vascular beds of conscious rats (Filep *et al.*, 1993b). These findings suggest that a component of the permeability effect of ET-1 is not mediated via the ET_A receptor. Recent observations raised the possibility that BQ-123 may also antagonize a non-ET_A, non-ET_B receptor as it has been reported to show a preferential antagonistic effect on the ET-3 over the ET-1 twitch response in the rat vas deferens (Eglezos *et al.*, 1993).

The aim of the present experiments was to characterize the ET receptor subtypes which mediate the vascular (haemodynamic and albumin extravasation) responses to ET-1 in the conscious rat by using a novel, non-peptide ET_A/ET_B receptor antagonist, bosentan (Ro 47-0203, 4-*tert*-butyl-N-[6-(2-hydroxy-ethoxy)-5-(2-methoxy-phenoxy)-2,2'-bipyrimidin-4-yl]-benzene-sulphonamide) (Clozel *et al.*, 1994), the modified tripeptide ET_A receptor-selective antagonist, FR 139317 ((R)-2-[(R)-2-[(S)-2-1-(hexahydro-1*H*-azepinyl)] carbonyl]amino-4-methylpentanoyl] amino-3-[-3-(1-methyl-1*H*-indoyl)] propionyl]amino-3-(2-pyridyl)propionic acid) (Aramori *et al.*, 1993; Sogabe *et al.*, 1993) and the ET_B receptor-selective agonist, IRL 1620 (Suc-[Glu⁹, Ala^{11,15}]endothelin-1(18-21)) (Takai *et al.*, 1992).

Methods

Preparation of the animals

The experiments were performed on conscious, chronically catheterized male Wistar rats weighing 210–280 g. The animals were housed in individual metabolic cages. During anaesthesia (ketamine, 75 mg kg⁻¹ and sodium pentobarbitone, 15 mg kg⁻¹), catheters were implanted into the abdominal aorta and vena cava as described previously (Filep & Fejes-Tóth, 1986). The animals were allowed to recover completely for at least four days following the surgical procedures. During the experiments the animals could move freely and had free access to food and water. Mean arterial blood pressure (MABP) and heart rate were monitored continuously by a blood pressure analyser (Micro-Med, Louisville, KY, U.S.A.) using a COBE CDX III pressure transducer.

Experimental protocols

In the first series of experiments, following an equilibration period of 1 h, animals were given ET-1 or IRL 1620 (0.1, 0.4, 1, 1.3 or 2 nmol kg⁻¹, i.v., bolus) in a volume of 5 µl per 100 g body weight, followed by 30 µl 0.9% w/v NaCl. Some animals were given bosentan (1, 3, 10 or 20 mg kg⁻¹, i.v.) 10

min before injection of ET-1 (1 nmol kg⁻¹). In a separate set of experiments, IRL 1620 (1 nmol kg⁻¹, i.v.) was injected following pretreatment of the animals with FR 139317 (2.5 mg kg⁻¹, i.v.) or bosentan (10 mg kg⁻¹, i.v.). On any one day each animal received only one dose of ET-1 or IRL 1620. At least 2 days were allowed to elapse between different experiments on the same rat. Both the order in which ET-1 or IRL-1620 were injected and the doses within each dose-response curve was randomized. In another set of experiments, the specificity of bosentan as an ET receptor antagonist was assessed by comparing changes in MABP elicited by i.v. bolus injections of noradrenaline (15 nmol kg⁻¹), arginine vasopressin (40 µg kg⁻¹), acetylcholine (8 nmol kg⁻¹) or platelet-activating factor (1.9 nmol kg⁻¹) in the absence and presence of bosentan (10 mg kg⁻¹, i.v., 10 min prior to vasoactive agents.)

In subsequent experiments to measure albumin extravasation, Evans blue dye which binds to plasma albumin (Rawson, 1943) was used as a marker. Previous studies have demonstrated close correlation for the extravasation of Evans blue dye and radioiodinated serum albumin (Udaka *et al.*, 1970; Rogers *et al.*, 1989). Animals were pretreated with FR 139317 (2.5 mg kg⁻¹ i.v., bolus) for 5 min, bosentan (10 mg kg⁻¹, i.v., bolus) for 10 min or 0.9% NaCl before injection of ET-1 (1 nmol kg⁻¹, i.v.) and Evans blue dye (20 mg kg⁻¹). In a separate series of experiments IRL 1620 (0.4 or 1 nmol kg⁻¹) was administered i.v. together with Evans blue dye. Ten min after injection of Evans blue with ET-1 or IRL 1620, the animals were anaesthetized with sodium pentobarbitone (50 mg kg⁻¹, i.v.) and perfused with 50 ml 0.9% w/v NaCl solution through a catheter inserted into the abdominal aorta to remove the excess intravascular dye. The perfusion was completed by 5 min. Portions of the trachea, upper bronchi (airways extending from the bifurcation of the trachea to its entry to parenchyma), lower bronchi (defined as major airways surrounded by pulmonary parenchyma that could be easily dissected without magnification), liver, spleen, pancreas, right kidney, stomach and duodenum were then excised and weighed. The tissue content of Evans blue dye was then measured by spectrophotometry following extraction with formamide as described previously (Filep *et al.*, 1991) and expressed as µg dye g⁻¹ dry tissue weight, to avoid underestimation of changes due to plasma fluid extravasation.

Drugs and chemicals

ET-1 and IRL 1620 were synthesized in our laboratories by solid-phase methodology (Germain *et al.*, 1993). The purity of the preparations were greater than 97% as measured by high performance liquid chromatography. ET-1 and IRL 1620 were dissolved in distilled water and stored at -20°C. On the day of the experiments an aliquot was removed and diluted further with 0.9% w/v NaCl. Bosentan, (Ro 47-0203 as the sodium salt, Hoffmann-La Roche Ltd, Basel, Switzerland) was dissolved in distilled water containing 300 mM glucose. FR 139317 (Fujisawa Pharmaceutical Co., Osaka, Japan), acetylcholine chloride, noradrenaline hydrochloride, arginine vasopressin (Grade VI, 100 U ml⁻¹, Sigma Chemical Co., St. Louis, MO, U.S.A.) and platelet-activating factor (1-O-hexadecyl-2-O-acetyl-sn-glycero-3-phosphorylcholine, Bachem, Bubendorf, Switzerland) were dissolved in 0.9% w/v NaCl. Evans blue dye was purchased from Sigma Chemical Co.

Statistical analysis

Results are expressed as mean values ± s.e.mean. Statistical evaluation of the data was performed by one-way analysis of variance using ranks (Kruskal-Wallis test) followed by Dunn's multiple contrast hypothesis test (Dunn, 1964), when various treatments were compared to the same control; or by Wilcoxon signed rank test and Mann-Whitney U test, for paired

and unpaired observations, respectively. A P value of <0.05 was considered significant for all tests.

Results

Baseline values for MABP and heart rate in conscious rats were 103 ± 1 mmHg and 340 ± 5 beats min^{-1} ($n = 78$), respectively. These values were unaffected by vehicle, FR 139317 and bosentan at any of the doses employed in this study.

Effects of bosentan on the pressor and depressor responses to endothelin-1

Preliminary experiments showed that bosentan (10 mg kg^{-1} , i.v.) was an effective inhibitor when given 10 min prior to ET-1 (1 nmol kg^{-1} , i.v., bolus, $n = 4$). Since a pretreatment greater than 10 min did not enhance further the inhibitory action of bosentan, this was used in all subsequent experiments.

As well established, ET-1 (1 nmol kg^{-1}) produced a sustained pressor effect preceded by a transient depressor action (Figure 1). The depressor response lasted for 30–50 s. Maximum increases in MABP was observed within 3–4 min after injection of the peptide. Corresponding to the changes in MABP, a small transient tachycardia (Δ heart rate: 15 ± 5 beats min^{-1} , $n = 6$) was followed by a sustained bradycardia (Δ heart rate -48 ± 10 beats min^{-1} , $n = 6$).

Both the depressor and pressor effects of ET-1 were attenuated by bosentan in a dose-dependent manner (Figure 1). However, complete inhibition of the ET-1-induced depressor and pressor responses after bosentan treatment was not observed. The peak depressor and pressor effects of ET-1 were decreased on average by 53 and 87%, respectively, following bosentan (10 mg kg^{-1}) (Figure 1). ET-1-induced tachycardia and bradycardia were also markedly reduced by bosentan (Δ heart rate were 6 ± 2 and -14 ± 7 beats min^{-1} , respectively, $n = 6$, $P < 0.05$ compared to ET-1). Bosentan at a dose of 20 mg kg^{-1} did not cause a significantly greater reduction in either the maximum decrease or maximum increase in MABP elicited by ET-1 (1 nmol kg^{-1}) than 10 mg kg^{-1} (Figure 1). Bosentan, up to a dose of 20 mg kg^{-1} , did not prolong the depressor action of ET-1. For example, the duration of ET-1 (1 nmol kg^{-1})-induced hypotension was 35–60 s in the presence of 10 mg kg^{-1} bosentan.

The inhibitory action of bosentan was qualitatively and quantitatively different from that of FR 139317. The depres-

sor response to 1 mg kg^{-1} ET-1 was unaffected by FR 139317, 2.5 mg kg^{-1} (maximum decreases in MABP were 17 ± 1 mmHg and 20 ± 3 mmHg in the absence and presence of FR 139317, respectively, $n = 6$, $P > 0.05$). On the other hand, the peak pressor response to ET-1 was decreased to 12 ± 2 mmHg ($n = 6$, $P < 0.01$) by 2.5 mg kg^{-1} FR 139317. Increasing the dose of FR 139317 to 10 mg kg^{-1} did not cause further inhibition of the pressor effect of ET-1. The maximum inhibition of the pressor effect of ET-1 that can be achieved with bosentan was greater than that observed following FR 139317 treatment (ET-1 (1 nmol kg^{-1})-induced increases in MABP were 12 ± 2 mmHg ($n = 6$) and 6 ± 1 mmHg ($n = 6$) in FR 139317 and bosentan-treated animals, respectively, $P < 0.02$). FR 139317 did not affect ET-1-induced transient tachycardia (Δ heart rate 12 ± 2 beats min^{-1} ($n = 6$) $P > 0.05$ compared to ET-1), whereas it inhibited ET-1-induced bradycardia (Δ heart rate -10 ± 3 beats min^{-1} ($n = 6$) $P < 0.05$ compared to ET-1).

Bosentan (10 mg kg^{-1}) neither affected significantly the pressor responses to 15 nmol kg^{-1} noradrenaline (maximum increases in MABP were 41 ± 3 and 41 ± 2 mmHg in the absence and presence of bosentan, respectively, $n = 4$) or $40 \text{ } \mu\text{g kg}^{-1}$ arginine vasopressin (Δ MABP 39 ± 3 and 38 ± 2 mmHg in the absence and presence of bosentan, respectively, $n = 4$) nor modified the depressor responses elicited by 8 nmol kg^{-1} acetylcholine (maximum decreases in MABP were 29 ± 2 and 31 ± 1 mmHg in the absence and presence of bosentan, respectively, $n = 3$) or 1.9 nmol kg^{-1} platelet-activating factor (Δ MABP -37 ± 3 and -36 ± 3 mmHg in the absence and presence of bosentan, respectively, $n = 3$).

Effects of bosentan and FR 139317 on IRL 1620-induced changes in blood pressure

Bolus i.v. injection of the ET_B receptor agonist, IRL 1620 (0.1 – 2 nmol kg^{-1}) produced biphasic, dose-dependent

Figure 1 Dose-dependent inhibition by bosentan of the peak depressor (open columns) and pressor responses (hatched columns) to endothelin-1 1 nmol kg^{-1} , in conscious rats. Bosentan or its vehicle (300 mM glucose) was injected i.v. 10 min before administration of ET-1. Baseline MABP was 102 ± 2 mmHg ($n = 6$). Values are means with s.e.mean for 4–6 separate experiments. * $P < 0.05$; ** $P < 0.01$ (compared to control (vehicle) by Dunn's multiple contrast hypothesis tests).

Figure 2 Peak depressor and pressor responses observed following i.v. injection of endothelin-1 (○) or IRL 1620 (●) in conscious rats. Baseline MABP was 103 ± 2 mmHg ($n = 11$). Values are means with s.e.mean for 3–6 separate experiments. * $P < 0.05$; ** $P < 0.01$ (compared to vehicle by Dunn's multiple contrast hypothesis test).

changes in MABP with a transient fall followed by a prolonged pressor action. The duration of the depressor action of IRL 1620 was longer than that of equimolar doses of ET-1. For example, the depressor response to 1 nmol kg⁻¹ IRL 1620 lasted for 50–110 s. Maximum decreases and increases in MABP were observed following 1 nmol kg⁻¹ IRL 1620 (Figure 2). Corresponding to the changes in MABP, IRL 1620 (1 nmol kg⁻¹) evoked a slight, transient tachycardia (Δ heart rate 9 ± 3 beats min⁻¹, $n = 5$) followed by a prolonged bradycardia (Δ heart rate -17 ± 11 beats min⁻¹, $n = 5$). Since at the dose of 2 nmol kg⁻¹, ET-1 caused severe bleeding from the orbital sinus, higher doses were not tested and consequently the maximum effects of ET-1 on MABP were not precisely determined. The apparent maximum decrease in MABP that could be evoked by ET-1 was 2 fold greater than that elicited by IRL 1620 (Figure 2). Similarly, the apparent maximum increase in MABP that can be evoked by ET-1 was 2.8 fold greater than that observed following IRL 1620 (Figure 2). Pretreatment of the animals with FR 139317 (2.5 mg kg⁻¹, i.v. 5 min prior to IRL 1620) did not affect IRL 1620 (1 nmol kg⁻¹)-induced changes in MABP, whereas both the depressor and pressor responses to IRL 1620 were almost completely inhibited by bosentan (10 mg kg⁻¹) (Figure 3).

Effects of bosentan and FR 139317 on endothelin-1-induced Evans blue extravasation

ET-1 at 1 nmol kg⁻¹ significantly enhanced albumin extravasation in the upper and lower bronchi (Figure 4), spleen, kidney, stomach and duodenum (Figure 5), whereas no significant changes were observed in the trachea, pulmonary parenchyma, liver and pancreas (data not shown). FR 139317 (2.5 mg kg⁻¹ i.v. 5 min prior to ET-1) reduced tissue Evans blue content on average by 56, 72, 40, 75, 96 and 100% in the upper and lower bronchi, spleen, kidney, stomach and duodenum, respectively (Figures 4 and 5). Pretreatment of the animals with the ET_A/ET_B receptor antagonist, bosentan (10 mg kg⁻¹, i.v. bolus, 10 min prior to ET-1) also protected against ET-1-induced albumin extravasation in the same vascular beds. ET-1-induced Evans blue accumulation was reduced on average by 88, 96, 89, 91, 100 and 100% in the upper and lower bronchi, spleen, kidney, stomach and duodenum, respectively in the presence of bosentan (Figures 4 and 5). Neither FR 139317 nor bosentan by itself affected significantly extravasation of Evans blue dye in the vascular beds studied (Figures 4 and 5).

Figure 3 Effects of FR 139317 and bosentan on the peak depressor (open columns) and pressor responses (hatched columns) to IRL 1620 in conscious rats. FR 139317 (2.5 mg kg⁻¹) or bosentan (10 mg kg⁻¹) were injected i.v. 10 or 5 min before administration of IRL 1620 (1 nmol kg⁻¹), respectively. Baseline MABP were 102 ± 2 mmHg ($n = 5$), 101 ± 2 mmHg ($n = 5$) and 98 ± 3 mmHg in control, FR 139317 and bosentan-treated animals, respectively. Values are means with s.e.mean. * $P < 0.05$; ** $P < 0.01$ (compared to IRL 1620 by Mann-Whitney's U test).

IRL 1620-induced albumin extravasation

Injection of 1 nmol kg⁻¹ IRL 1620 increased tissue Evans blue content in the upper and lower bronchi on average by 45 and 119%, respectively (Figure 4), whereas no significant changes were observed in the trachea and pulmonary parenchyma (Table 1). IRL 1620 injection also produced increases of 45 and 70% in albumin extravasation in the spleen and kidney (Figure 5). In these vascular beds, the effects of IRL 1620 appeared to be dose-related (Table 1). Unlike ET-1, IRL 1620 failed to increase albumin extravasation in the stomach and duodenum (Figure 5). IRL 1620-induced changes in tissue Evans blue dye content were almost completely prevented by bosentan (10 mg kg⁻¹) in all vascular beds studied (Table 1).

Discussion

It is well documented that injection of ET-1 evokes an initial transient depressor response followed by a prolonged pressor response both in anaesthetized and conscious rats (Yanagisawa *et al.*, 1988; De Nucci *et al.*, 1988; Gardiner *et al.*, 1989; Le Monnier de Gouville *et al.*, 1989), albeit quantitative differences exist in the magnitude of the responses. These changes in MABP are thought to be mediated via at least two different ET receptors. In order to characterize these receptors, we have studied the inhibitory effect of bosentan, a non-peptide, orally active ET_A/ET_B receptor antagonist (Clozel *et al.*, 1994) and the ET_A receptor-selective antagonist FR 139317 (Aramori *et al.*, 1993; Sogabe *et al.*, 1993) on the depressor and pressor actions of ET-1 and the

Figure 4 Effects of FR 139317 and bosentan on endothelin-1-induced albumin extravasation in rat airways. The animals were injected i.v. with 0.9% w/v NaCl (C), endothelin-1 (ET, 1 nmol kg⁻¹), FR 139317 (FR, 2.5 mg kg⁻¹), FR 139317 (2.5 mg kg⁻¹, 5 min prior to ET-1) plus ET-1 (1 nmol kg⁻¹), bosentan (Bos, 10 mg kg⁻¹), bosentan (10 mg kg⁻¹ 10 min prior to ET-1) plus ET-1 (1 nmol kg⁻¹) or IRL 1620 (1 nmol kg⁻¹) together with Evans blue dye (20 mg kg⁻¹). The rats were anaesthetized and perfused with 0.9% w/v NaCl 10 min after injection of Evans blue dye. The permeability measurement was made 15 min after injection of the dye. Values are means with s.e.mean for 6 experiments. * $P < 0.05$; ** $P < 0.01$ (compared to control); ♦ $P < 0.05$; ♦♦ $P < 0.01$ (compared to ET-1).

Figure 5 Effects of FR 139317 and bosentan on endothelin-1-induced albumin extravasation in selected vascular beds of conscious rats. The animals were injected i.v. with 0.9% w/v NaCl (C), ET-1 (ET, 1 nmol kg⁻¹), FR 139317 (FR, 2.5 mg kg⁻¹), FR 139317 (2.5 mg kg⁻¹, 5 min prior to ET-1) plus ET-1 (1 nmol kg⁻¹), bosentan (Bos, 10 mg kg⁻¹), bosentan (10 mg kg⁻¹, 10 min prior to ET-1) plus ET-1 (1 nmol kg⁻¹) or IRL 1620 (1 nmol kg⁻¹) together with Evans blue dye (20 mg kg⁻¹). The rats were anaesthetized and perfused with 0.9% w/v NaCl 10 min after injection of the dye. The permeability measurement was made 15 min after injection of the dye. Values are means with s.e.mean for 6 experiments. **P* < 0.05; ***P* < 0.01 (compared to control); ♦*P* < 0.05; ♦♦*P* < 0.01 (compared to ET-1).

Table 1 Effect of bosentan on IRL 1620-induced albumin extravasation in conscious rats

	Control (<i>n</i> = 6)	IRL 1620 (nmol kg ⁻¹) 0.4 (<i>n</i> = 4)	1 (<i>n</i> = 6)	Bosentan (10 mg kg ⁻¹) plus IRL 1620 (1 nmol kg ⁻¹) (<i>n</i> = 4)
Albumin extravasation (µg Evans blue dye per g dry tissue weight)				
Trachea	55 ± 5	51 ± 9	57 ± 8	54 ± 7
Upper bronchi	49 ± 3	61 ± 2*	71 ± 10*	51 ± 3*
Lower bronchi	52 ± 3	71 ± 8*	114 ± 16**	61 ± 3*
Pulmonary parenchyma	73 ± 3	72 ± 9	90 ± 10	76 ± 6
Liver	39 ± 6	40 ± 3	55 ± 11	44 ± 5
Spleen	84 ± 9	113 ± 10	122 ± 12*	78 ± 7*
Pancreas	32 ± 6	53 ± 5	52 ± 9	44 ± 7
Kidney	50 ± 3	74 ± 2*	85 ± 13**	52 ± 2*
Stomach	55 ± 3	44 ± 3	45 ± 6	47 ± 5
Duodenum	70 ± 7	76 ± 6	73 ± 10	73 ± 6

Values are means ± s.e.mean. The animals were given a bolus i.v. injection of bosentan or its vehicle (distilled water containing 300 mM glucose) 10 min before i.v. administration of IRL 1620 or its vehicle (0.9% w/v NaCl) together with Evans blue dye (20 mg kg⁻¹). Ten min later, the rats were anaesthetized and perfused with 0.9% w/v NaCl. The permeability measurement was made 15 min after injection of the dye. **P* < 0.05; ***P* < 0.01 (compared to control by Dunn's multiple contrast hypothesis test), **P* < 0.05 (compared to animals receiving 1 nmol kg⁻¹ IRL 1620).

ET_B receptor-selective agonist, IRL 1620 (Takai *et al.*, 1992). Bosentan has been shown to antagonise competitively the specific binding of ET-1 on ET_A receptors (human umbilical vein vascular smooth muscle cells) and on ET_B receptors (microsomal membranes from human placenta) with *K_i* values of 4.7 nM and 95 nM, respectively (Clozel *et al.*, 1994). The present findings that bosentan neither affected the pressor effect of noradrenaline and arginine vasopressin, nor modified the depressor effect of acetylcholine and platelet-activating factor, indicate that it is a specific ET receptor

antagonist *in vivo*. IRL 1620 has *K_i* values of 1.9 µM and 16 pM for the ET_A and ET_B receptors, respectively (Takai *et al.*, 1992). FR 139317 has been reported to be 7000 times more potent in inhibiting the binding of ET-1 to ET_A than ET_B receptors *in vitro* (Aramori *et al.*, 1993). Since FR 139317 did not affect the depressor action of ET-1 (see below) and failed to modify IRL 1620-induced changes in MABP, it might be assumed that at the dose used, FR 139317 does not interfere with ET_B receptors *in vivo*.

The depressor action of ET-1 is thought to be mediated via

ET_B receptors (Saeki *et al.*, 1991; Douglas & Hiley, 1991). Effects involving ET_B receptors would presumably be resistant to inhibition by an ET_A receptor-selective antagonist. Indeed, FR 139317 did not affect the maximum decrease in MABP elicited by ET-1 (Sogabe *et al.*, 1993; Bird & Waldon, 1993 and the present study), but significantly prolonged the duration of the depressor response. This is most likely the consequence of suppression of the ET_A-mediated vasoconstrictor activity. This prolonged depressor response in the presence of FR 139317 may indicate that ET-1 induces sustained release of endogenous vasodilators, which, in turn, ameliorate the pressor effect of the peptide. One of these vasodilators is likely to be nitric oxide as inhibition of its formation leads to potentiation of the pressor action of ET-1 in conscious rats (Filep *et al.*, 1993a). On the other hand, the ET_A/ET_B antagonist, bosentan attenuated ET-1-induced decreases in MABP in a dose-dependent fashion. However, the maximal inhibition reached a plateau at approximately 50%. Thus, there is a component of the depressor action of ET-1 which cannot be inhibited by bosentan, and, therefore, may not be mediated via the ET_B receptor. The finding that the maximum decrease in MABP that can be achieved with IRL 1620 was about 50% of the apparent peak depressor response to ET-1 would lend further support to this notion. The difference in efficacy of ET-1 and IRL 1620 might reflect the partial agonist activity of IRL 1620 at ET_B vasorelaxant receptors *in vivo*. However, the finding that IRL 1620 at equimolar concentrations to ET-1 caused a more protracted vasodilatation response in perfused lungs of neonatal pigs (Pinheiro & Malik, 1993) would argue against this explanation. Alternatively, it is possible that more than one subtype of ET_B receptor, which is insensitive to bosentan, mediates the depressor action of ET-1. This hypothesis should, however, be tempered by the facts that the two ET_B receptor subtypes yet described have been implicated in mediating vasodilatation and vasoconstriction, respectively (Sokolovsky *et al.*, 1992), and that bosentan has similar K_i values for the ET_B receptors mediating vasoconstriction and endothelium-dependent relaxation *in vitro* (Clozel *et al.*, 1994).

Pretreatment of the animals with FR 139317 markedly inhibited the pressor effect of ET-1. However, the degree of inhibition did not exceed 75% (Sogabe *et al.*, 1993 and the present study). These results are comparable to the 70–75% inhibition of the ET-1-induced pressor response observed after bolus injection of BQ-123 both in anaesthetized (McMurdo *et al.*, 1993) and conscious rats (Ihara *et al.*, 1992; Filep *et al.*, 1992; 1993b). In contrast, complete inhibition of the pressor action of ET-1 has been reported following infusion of BQ-123 in the anaesthetized rats (Pollock & Opgenorth, 1993). However, it is likely that the depressor response observed in this latter study following infusion of BQ-123 to control animals might have contributed to the apparent complete inhibition of the pressor action of ET-1. In the conscious rat, bolus injection of BQ-123 had no effect on blood pressure (Ihara *et al.*, 1992; Filep *et al.*, 1992; 1993b). The present study shows that about 90% inhibition of the ET-1 pressor response could be achieved with bosentan compared to the reduction of only 75% observed following ET_A receptor blockade. The ET_B receptor agonist, IRL 1620 can also evoke pressor responses in the conscious rat (Webb *et al.*, 1992 and the present study). Under our experimental conditions, the maximum increase in MABP that can be achieved with IRL 1620 did not exceed 36% of the apparent maximum increase elicited by ET-1. The pressor response to IRL 1620 was not modified by FR 139317, whereas it can be effectively inhibited by bosentan. These findings provide direct evidence for the notion that a portion of the pressor action of ET-1 is mediated through ET_B receptors. Previous studies have also reported a rise in MABP following injection of the supposedly ET_B receptor-selective agonist, sarafotoxin 6c in the pithed rat (Williams *et al.*, 1991; Clozel *et al.*, 1992). However, BQ-123 has been reported to inhibit the pressor action of sarafotoxin 6c

(McMurdo *et al.*, 1993), indicating that the sarafotoxin 6c-induced pressor response is not entirely mediated via the ET_B receptor as suggested previously (Williams *et al.*, 1991). It is noteworthy that ET-1 produced a small, 3–8 mmHg increase in MABP in the presence of bosentan, suggesting the involvement of a third, non-ET_A, non-ET_B receptor subtype in the generation of the pressor response. This receptor may be similar to that which has been described in the pig coronary circulation (Harrison *et al.*, 1992).

The transient fall and prolonged increase in MABP elicited by either ET-1 and IRL 1620 were accompanied by a transient increase (reflex tachycardia) and a prolonged decrease in heart rate (reflex bradycardia). The observations that both FR 139317 and bosentan markedly attenuated, but did not abolish, ET-1-induced bradycardia, coupled with the findings that bosentan, but not FR 139317, significantly reduced, but did not abolish ET-1-induced transient tachycardia suggest that changes in heart rate were secondary to changes in MABP (i.e. attenuation of the ET-1 depressor or pressor responses resulted in amelioration of the accompanying tachycardia or bradycardia, respectively).

Although ET-1 is present in low concentrations in normal rat plasma (Shirakami *et al.*, 1991; Löffler *et al.*, 1992), it is unclear whether circulating ET-1 has physiological functions on its own. As neither FR 139317, nor bosentan alone produced changes in MABP and albumin extravasation, it seems unlikely that circulating ET-1 could influence blood pressure and/or modulate albumin extravasation through ET_A and/or ET_B receptors under physiological conditions.

We have reported previously that BQ-123 completely inhibited ET-1-induced albumin extravasation in the stomach and duodenum, whereas it caused partial inhibition in the upper and lower bronchi, kidney and spleen (Filep *et al.*, 1993b). In the present experiments, qualitatively and quantitatively similar inhibition was achieved with FR 139317. In contrast, bosentan almost completely inhibited accumulation of Evans blue dye in all vascular beds studied. These findings indicate that ET-1-induced albumin extravasation in the stomach and duodenum is mediated via ET_A receptors, whereas activation of both ET_A and ET_B receptors is responsible for the permeability effect of ET-1 in the upper and lower bronchi, spleen and kidney. Additional evidence supporting a role for ET_B receptors in these latter vascular beds is derived from the experiments with IRL 1620. IRL 1620 mimicked the effect of ET-1 on albumin extravasation in the upper and lower bronchi, spleen and kidney, albeit, on a molar basis, it appeared to be less potent than ET-1. On the other hand, unlike ET-1, IRL 1620 did not enhance albumin extravasation in the stomach and duodenum, suggesting regional differences in the expression of ET_B receptors.

Previous studies from our laboratories have demonstrated that the effect of ET-1 on albumin extravasation is not simply a consequence of changes in systemic blood pressure (Filep *et al.*, 1991; 1993a,b), but, as with other mediators, it can be attributed to formation of interendothelial cell gaps in the venules (Grega *et al.*, 1986). ET-1 may induce gap formation directly via ET_A and/or ET_B receptors or through the release of secondary mediators including platelet-activating factor (Filep *et al.*, 1991). Since ET-1-induced accumulation of Evans blue dye in the stomach and duodenum can be completely inhibited by platelet-activating factor receptor antagonists (Filep *et al.*, 1991) as well as by FR 139317, it might be concluded that ET-1 enhances formation of platelet-activating factor via ET_A receptors in the microvasculature. Accordingly, the degree of inhibition of ET-1-induced albumin extravasation observed following ET_A or platelet-activating factor receptor blockade appears to be similar in the large airways, kidney and spleen (Filep *et al.*, 1991 and the present study). The observation that ET-1 is a more potent constrictor of venous than arterial vessels (Yang *et al.*, 1989; Warner, 1990) coupled with the findings that ET_A-like contractile receptors predominate on arterial preparations and ET_B-like contractile receptors on venous

smooth muscle (Moreland *et al.*, 1992) suggest that activation of ET_B receptors may lead to elevation of transcapillary pressure. This would facilitate albumin extravasation provided that interendothelial cell gaps are already formed.

In conclusion, the present study has demonstrated that the majority of the pressor response to ET-1 can be attributed to activation of ET_A and ET_B receptors, and a small component of the ET-1 pressor response might be mediated via activation of a non-ET_A, non-ET_B receptor, whereas ET_B and perhaps a third type of non-ET_A, non-ET_B receptor may mediate the depressor action of ET-1 in conscious rats. The present data also show differences in the ET receptor sub-

types mediating the permeability effect of ET-1 in different vascular beds: activation of ET_A receptors appears to be the sole mechanism by which ET-1 enhances albumin extravasation in the stomach and duodenum, whereas both ET_A and ET_B receptors mediate albumin extravasation in the bronchial, splenic and renal vascular beds.

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