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Mediation of endothelin-1-induced inhibition of platelet aggregation via the ET_B receptor

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1 The effects of FR139317 (ET_A antagonist) or PD145065 (non-selective ET_A/ET_B antagonist) on endothelin-1 (ET-1)-induced changes in blood pressure and inhibition of *ex vivo* platelet aggregation were investigated in the anaesthetized rabbit.

2 ET-1 (1 nmol kg^{-1}, i.a. bolus) caused a sustained increase in mean arterial pressure (MAP) (peak increase 47 ± 5 mmHg, $n = 8$). Intravenous infusion of FR139317 at 0.2 ($n = 4$) or 0.6 mg kg^{-1} min^{-1} ($n = 4$) inhibited the ET-1 pressor response by 83 or 89%, respectively. Infusion of PD145065 at 0.2 ($n = 4$) or 0.6 mg kg^{-1} min^{-1} ($n = 4$) inhibited the ET-1-induced increase in MAP by 79 or 75%, respectively.

3 The transient depressor response (− 16 ± 3 mmHg) which preceded the rise in blood pressure induced by ET-1 (1 nmol kg^{-1}, i.a., $n = 8$) was enhanced by an intravenous infusion of FR139317 (0.6 mg kg^{-1} min^{-1}) to − 35 ± 5 mmHg ($P < 0.05$, $n = 4$). This enhancement was abolished by indomethacin (5 mg kg^{-1}, i.v.) pretreatment (− 17 ± 1 mmHg, $n = 4$). PD145065 (0.2 mg kg^{-1} min^{-1}, i.v.) attenuated the ET-1-induced fall in blood pressure to − 9 ± 1 mmHg ($n = 4$), while a higher dose of this antagonist (0.6 mg kg^{-1} min^{-1}, i.v.) completely abolished the ET-1-mediated depressor response.

4 ET-1 (1 nmol kg^{-1}, $n = 8$) inhibited *ex vivo* platelet aggregation by 96% at 5 min after injection of the peptide. FR139317 (0.2 or 0.6 mg kg^{-1} min^{-1}, i.v.) or PD145065 (0.2 mg kg^{-1} min^{-1}, i.v.) did not affect the inhibition of *ex vivo* platelet aggregation in response to ET-1. In contrast, intravenous infusion of PD145065 (0.6 mg kg^{-1} min^{-1}) abolished the anti-aggregatory effects of ET-1.

5 Thus, FR139317 inhibits the pressor, but not the depressor actions of ET-1 and has no effect on the ET-1-induced inhibition of *ex vivo* platelet aggregation. In contrast, PD145065 antagonizes the pressor and depressor responses to ET-1 and abolishes the anti-aggregatory effects of the peptide.

6 These results strongly suggest that ET-1-induced vasoconstriction in the anaesthetized rabbit is primarily mediated via the ET_A receptor while the depressor and antiaggregatory actions of ET-1 are due to activation of the ET_B receptor.

Keywords: Endothelin; prostacyclin; ET_A antagonist; ET_A/ET_B antagonist; *ex vivo* platelet aggregation

Introduction

Endothelin-1 (ET-1) is a potent 21 amino acid vasoconstrictor peptide produced by vascular endothelial cells (Yanagisawa *et al.*, 1988). ET-1 stimulates the release of a variety of endothelium-derived vasodilator autacoids including prostacyclin (PGI_2), prostaglandin E_2 and nitric oxide (NO) (De Nucci *et al.*, 1988; Rae *et al.*, 1989; Warner *et al.*, 1989a,b; see Thiemermann, 1991). The release of PGI_2 into the circulation ameliorates the pressor effects brought about by systemic application of ET-1 (de Nucci *et al.*, 1988; Thiemermann *et al.*, 1990) and inhibits platelet aggregation when measured *ex vivo* (Thiemermann *et al.*, 1989) and *in vivo* (Herman *et al.*, 1989; Thiemermann *et al.*, 1990).

At present two endothelin receptors have been cloned and expressed, namely ET_A and ET_B. Each receptor contains seven trans-membrane domains and shows remarkable similarity to the rhodopsin receptor and other G protein coupled receptors (see Webb, 1991). The ET_A receptor is highly selective for ET-1 and is characterized by the rank order of binding affinities: ET-1 > sarafotoxin 6b > ET-3 (Arai *et al.*, 1990), while the ET_B receptor is non-isopeptide selective (ET-1 = ET-3) (Sakurai *et al.*, 1990). The discovery of distinct endothelin receptors has prompted the development of selective endothelin receptor antagonists, two of which (FR 139317 and PD145065) have been used in this study. FR 139317 is a potent, highly specific ET_A antagonist *in vitro* and *in vivo* (Sogabe *et al.*, 1992). PD145065 is a non-selective ET_A/ET_B receptor antagonist (Cody *et al.*, 1993).

Intravenous injection of ET-1 in several species including man (see Thiemermann, 1991) causes a transient vasodilatation followed by a sustained vasoconstriction. The vasoconstrictor/pressor effects of ET-1 are mainly due to activation of the ET_A receptor, for they are attenuated by selective ET_A receptor antagonists *in vitro* and *in vivo* (Ihara *et al.*, 1991; McMurdo *et al.*, 1993). The ET-1 induced fall in blood pressure is not inhibited by ET_A receptor antagonists, suggesting that this response may be mediated via the ET_B receptor (Ihara *et al.*, 1991; McMurdo *et al.*, 1993). However, the receptor(s) through which ET-1 stimulates the release of PGI_2 and, hence inhibits platelet aggregation, have not been characterized, although the non-selectivity to the endothelins (Lidbury *et al.*, 1990) suggests it is an ET_B type.

Here, we investigate the effects of FR139317 and PD 145065 on blood pressure changes induced by ET-1 in the anaesthetized rabbit. In addition, we have studied the effects of these antagonists on ET-1-induced inhibition of *ex vivo* platelet aggregation.

Methods

Surgical procedure

Male New Zealand White rabbits (2–3 kg) were premedicated with Hypnorm (fentanyl citrate at 0.1 ml kg^{-1} and fluanisone at 10 mg ml^{-1}, i.m.). General anaesthesia was then induced with sodium pentobarbitone (30 mg kg^{-1}, i.v.) and anaesthesia was maintained with supplementary doses of

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sodium pentobarbitone as required. The rabbits were intubated and ventilated with room air from a Harvard ventilator at 36–40 strokes min^{-1} and a tidal volume of 6 ml kg^{-1}. Body temperature was monitored and maintained at 38 ± 1°C by means of a rectal probe thermometer attached to a homeothermic blanket control unit (Harvard, Edenbridge, Kent). The left femoral vein was cannulated for drug administration. The left femoral artery was cannulated for the measurement of blood pressure from which mean arterial pressure (MAP) and heart rate (HR) were derived. Another catheter was placed in the left ventricle via the right common carotid artery for measurement of left ventricular pressure (LVP) and injection of ET-1.

Platelet aggregation ex vivo

Ex vivo platelet aggregation was measured as previously described (Thiemermann *et al.*, 1988). Briefly, arterial blood samples were collected from the left femoral artery into 3.15% tri-sodium citrate (9:1 v/v) and immediately centrifuged at 1400 *g* (4000 r.p.m.) for 20 s (Heraeus, Biofuge 15) to produce platelet rich plasma (PRP). The blood was further centrifuged at 14900 *g* (12000 r.p.m.) for 1 min to obtain platelet poor plasma (PPP). Platelet aggregation was studied with a dual channel aggregometer (Payton) calibrated using PRP (0%) and PPP (100%) with respect to the degree of light transmission. Aliquots of PRP (0.4 ml) were added to siliconised cuvettes, warmed to 37°C and stirred at 1000 r.p.m. After incubation for 30 s, a sub-maximal dose of adenosine-5'-diphosphate (ADP; 2 μg ml^{-1}) was added and the extent of aggregation measured as peak increase in light transmission.

The inhibition of platelet aggregation induced by ET-1 (1 nmol kg^{-1}) was calculated from peak increase in light transmission observed over a 4 min period after addition of a sub-maximal dose (70–80% maximum response) of ADP, as compared to that of control.

Experimental design

After surgery, all animals were allowed to stabilize for 20 min before the first arterial blood sample was withdrawn. Antagonists (FR139317 or PD145065) were administered as an infusion of 0.2 or 0.6 mg kg^{-1} min^{-1} (preceded by loading doses of 1 or 3 mg kg^{-1}, respectively) for 30 min prior to and continued for a further 30 min after a bolus injection of ET-1 (1 nmol kg^{-1}, intra-ventricular). In some experiments, indomethacin (5 mg kg^{-1}, i.v. bolus) was given 20 min before ET-1 injection. *Ex vivo* platelet aggregation was measured 5 min before antagonist infusion, 5 min before ET-1 injection (control) and 1, 5, 15 and 30 min after injection of ET-1.

Materials

Hypnorm was bought from Janssen Pharmaceutical Co., (Oxford, UK). Sodium pentobarbitone (Sagatal) was purchased from May and Baker (Dagenham, Essex, UK). Adenosine-5'- diphosphate, indomethacin and dimethylsulphoxide were obtained from Sigma Chem. Co., (Poole, Dorset, UK). Tri-sodium citrate was bought from B.D.H. Chem. Co., (Essex, UK). ET-1 was supplied by Scientific Marketing Associates (London, UK) and was reconstituted in 0.1% acetic acid and diluted in 0.9% w/v saline containing 1% w/v bovine serum albumin and 0.06% sodium bicarbonate. FR 139317 ((**R**)-2[(**R**)-2-[(**S**)-2-[[1-(hexahydro-1H-azepinyl)] carbonyl] amino-4-methylpentanoyl]amino-3[3-(1-methyl-1H-indolyl]propionyl] amino-3-(2-pyridyl)proprionic acid) and PD 145065 [Ac-(D-5H-dibenzyl[a,d] cyclo-heptene-10,11-dihydroglycine-L-Leu-L-Asp-L-Ile-L-Ile-L-Trp)] were provided by Dr Annette M. Doherty and Dr Wayne L. Cody of the Medicinal Chemistry Department at Parke-Davis Pharmaceutical Research Division of Warner-Lambert Co. (Ann Arbor, U.S.A.). The antagonists were dissolved in 20% dimethylsulphoxide. Aliquots of ET-1 and of the ET antagonists were stored frozen (– 20°C) until use.

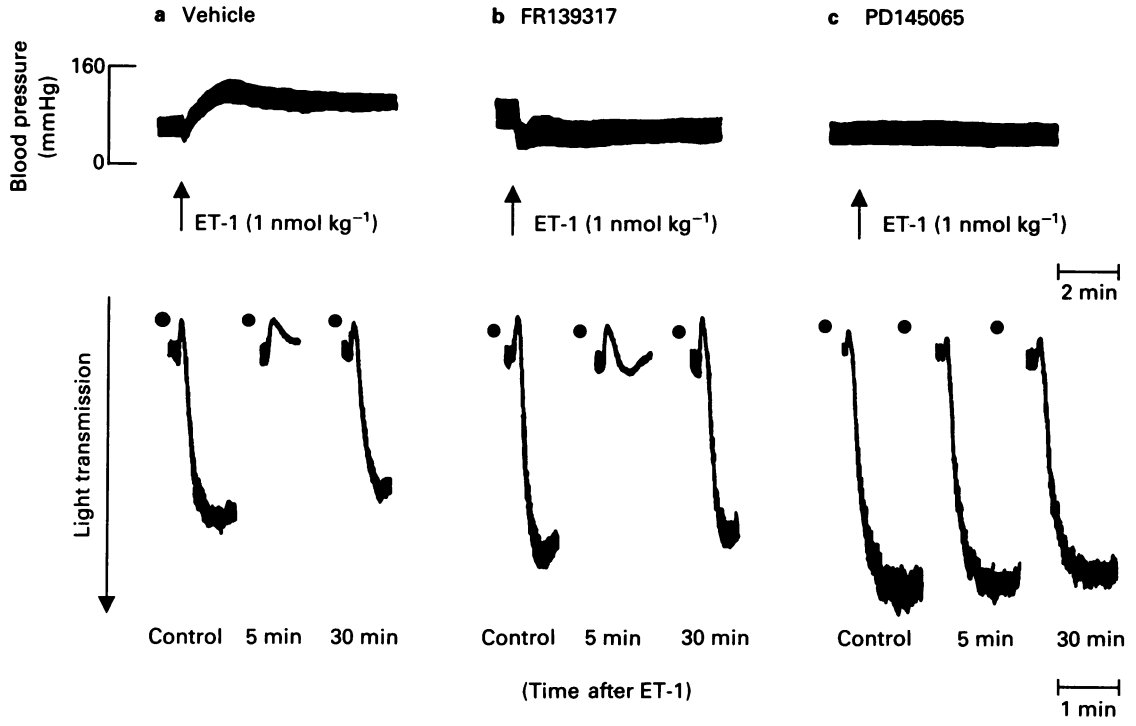

Figure 1 The figure shows 3 representative traces comparing endothelin-1 (ET-1, 1 nmol kg^{-1}, i.a., bolus)-induced changes in blood pressure (upper panel) and *ex vivo* platelet aggregation (lower panel) in rabbits treated with (a) ET-1 at 30 min after the start of an infusion of vehicle (20% DMSO) for 60 min; (b) ET-1 at 30 min after the start of an infusion of FR139317 (loading dose of 3 mg kg^{-1} then 0.6 mg kg^{-1} min^{-1} for 60 min, i.v.) and (c) ET-1 at 30 min after the start of an infusion of PD145065 (loading dose of 3 mg kg^{-1} then 0.6 mg kg^{-1} min^{-1} for 60 min, i.v.). The solid circle signifies an injection of ADP (2 μg ml^{-1}).

Statistical analysis

All values in the figures and text are expressed as mean ± s.e.mean of n observations. Statistical evaluation of the data was by Student's t test for unpaired determinations. A P value of <0.05 was considered significant.

Results

Mean resting values for MAP were 59 ± 1 mmHg and for HR were 246 ± 4 beats min^{-1} ($n = 24$). ET-1 (1 nmol kg^{-1}, i.a., bolus) caused an initial transient depressor response (− 16 ± 3 mmHg, $n = 8$) which was succeeded by a sustained increase in MAP (peak: 47 ± 5 mmHg) ($n = 8$, Figure 1a, Figure 2 and Figure 3). There was no change in ADP-induced platelet aggregation over a period of 30 min in rabbits treated with vehicle ($n = 3$). ET-1 (1 nmol kg^{-1}, i.a., bolus) caused a 96 ± 2% inhibition of *ex vivo* platelet aggregation at 5 min after injection which returned towards control levels within 30 min (15 ± 8% inhibition, $n = 8$) (Figure 1a and Figure 4).

The effect of FR139317 on haemodynamics and platelet aggregation

FR139317 (0.2 mg kg^{-1} min^{-1}, i.v.) had no significant effect on the ET-1 depressor response ($n = 4$, Figure 2b). However, a higher dose of FR139317 (0.6 mg kg^{-1} min^{-1}, i.v.) enhanced the ET-1-induced fall in MAP from − 16 ± 3 mmHg to − 35 ± 5 mmHg ($n = 4$, $P<0.05$; Figure 1b and Figure 2b). The fall in blood pressure remained at control levels (− 17 ± 1 mmHg) after pretreatment with indomethacin (5 mg kg^{-1}, i.v., bolus; $n = 4$). Infusions of FR139317 at 0.2 or 0.6 mg kg^{-1} min^{-1} significantly attenuated the pressor response produced by ET-1 from 47 ± 5 mmHg (control) to 8 ± 2 mmHg or 5 ± 4 mmHg, respectively ($n = 4$, $P<0.05$; Figure 1b and Figure 2a). In contrast, inhibition of *ex vivo* platelet aggregation in response to ET-1 (control: 96 ± 2% at 5 min after injection) was not affected by FR139317 (0.2 mg kg^{-1} min^{-1}: 92 ± 5%; 0.6 mg kg^{-1} min^{-1}: 92 ± 3%) (Figure 1b and Figure 4). Pretreatment with indomethacin (5 mg kg^{-1}, i.v. bolus) in the presence of FR139317 (0.6 mg kg^{-1} min^{-1}, i.v.) attenuated the ET-1-induced inhibition of platelet aggregation (18 ± 3%; $n = 4$).

The effect of PD145065 on haemodynamics and platelet aggregation

PD145065 (0.2 mg kg^{-1} min^{-1}, i.v.) reduced (by 47%) the ET-1-induced fall in blood pressure ($n = 4$, Figure 3b). The fall in MAP produced by ET-1 was abolished by pretreat-

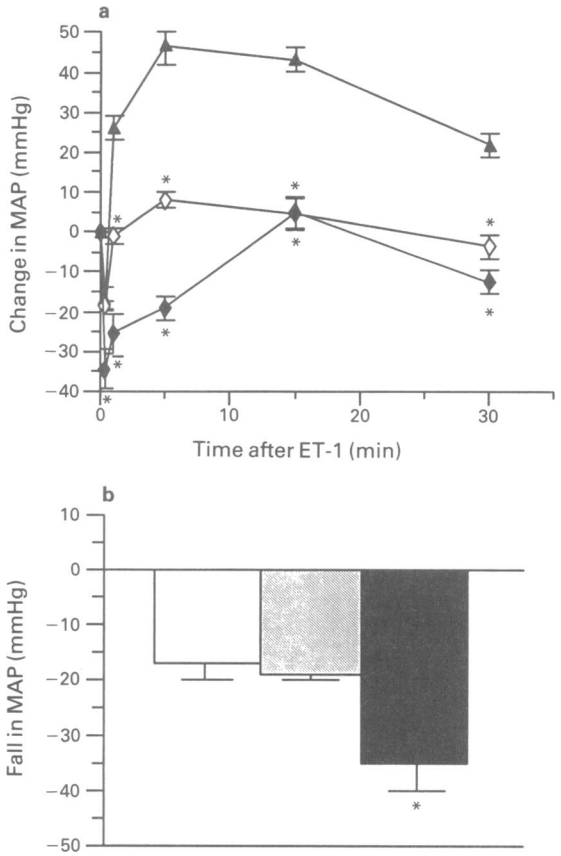

Figure 2 (a) Time course of the effect of endothelin-1 (ET-1, 1 nmol kg^{-1}, i.a., bolus given at time = 0 min) on mean arterial pressure (MAP) in the anaesthetized rabbit. ET-1 was given 30 min after the start of a 60 min infusion of either vehicle (▲, $n = 8$), FR139317 (loading dose of 1 mg kg^{-1} then 0.2 mg kg^{-1} min^{-1}, i.v., ◇, $n = 4$) or FR139317 (loading dose of 3 mg kg^{-1} then 0.6 mg kg^{-1} min^{-1}, i.v., ◆, $n = 4$). (b) Depicted are the ET-1 induced falls in MAP in rabbits given either vehicle (open column, $n = 8$), FR139317 (loading dose of 1 mg kg^{-1} then 0.2 mg kg^{-1} min^{-1}, i.v., hatched column, $n = 4$) or FR139317 (loading dose of 3 mg kg^{-1} then 0.6 mg kg^{-1} min^{-1}, i.v., solid column, $n = 4$). Data are expressed as mean ± s.e.mean of n observations. $^*P<0.05$ when compared to vehicle control.

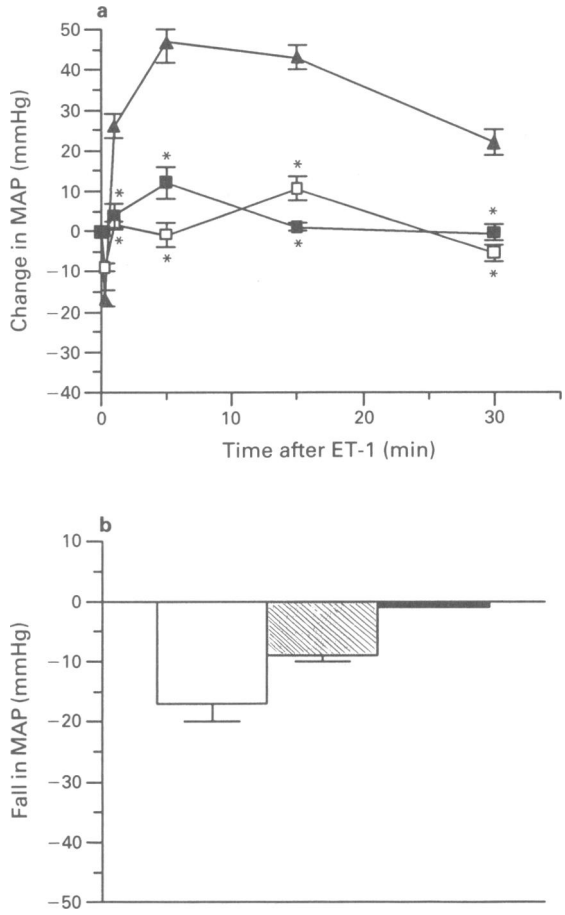

Figure 3 (a) Time course of the effect of endothelin-1 (ET-1, 1 nmol kg^{-1}, i.a., bolus given at time = 0 min) on mean arterial pressure (MAP) in the anaesthetized rabbit. ET-1 was given 30 min after the start of a 60 min infusion of either vehicle (▲, $n = 8$), PD145065 (loading dose of 1 mg kg^{-1} then 0.2 mg kg^{-1} min^{-1}, i.v., □, $n = 4$) or PD145065 (loading dose of 3 mg kg^{-1} then 0.6 mg kg^{-1} min^{-1}, i.v., ■, $n = 4$). (b) Depicted are the ET-1-induced falls in MAP in rabbits given either vehicle (open column, $n = 8$), PD145065 (loading dose of 1 mg kg^{-1} then 0.2 mg kg^{-1} min^{-1}, i.v., hatched column, $n = 4$) or PD145065 (3 mg kg^{-1} then 0.6 mg kg^{-1} min^{-1}, i.v., solid column, $n = 4$). Data are expressed as mean ± s.e.mean of n observations. $^*P<0.05$ when compared to vehicle control.

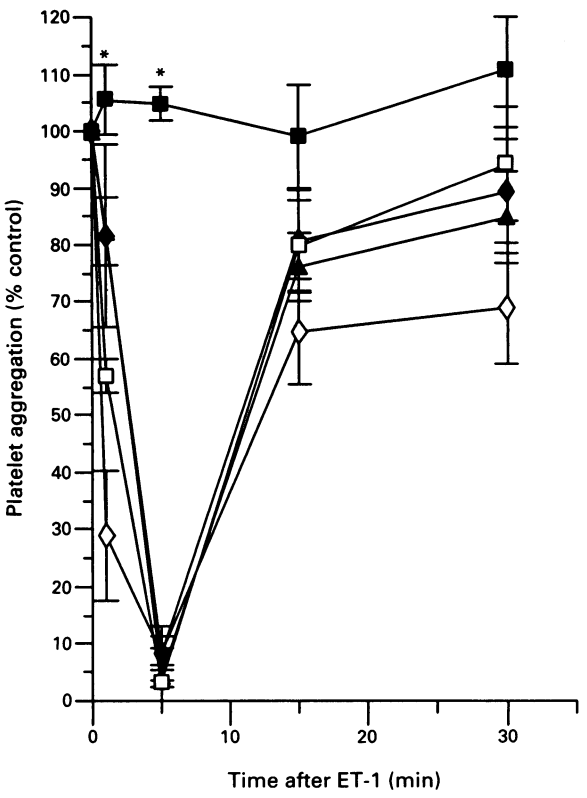

Figure 4 Time course of the effects of endothelin-1 (ET-1, 1 nmol kg^{-1}, i.a., bolus) on *ex vivo* platelet aggregation induced by ADP (2 $\mu g\,ml^{-1}$) in anaesthetized rabbits given either vehicle (▲, $n = 8$), FR139317 (loading dose of 1 $mg\,kg^{-1}$ then 0.2 $mg\,kg^{-1}\,min^{-1}$, i.v., ◇, $n = 4$), FR139317 (loading dose of 3 $mg\,kg^{-1}$ then 0.6 $mg\,kg^{-1}\,min^{-1}$, i.v., ◆, $n = 4$), PD145065 (loading dose of 1 $mg\,kg^{-1}$ then 0.2 $mg\,kg^{-1}\,min^{-1}$, i.v., □, $n = 4$) or PD145065 (loading dose of 3 $mg\,kg^{-1}$ then 0.6 $mg\,kg^{-1}\,min^{-1}$, i.v., ■, $n = 4$). Data are expressed as mean ± s.e.mean of n observations. $^*P < 0.05$ when compared to vehicle control.

ment with a higher dose (0.6 $mg\,kg^{-1}\,min^{-1}$, i.v.) of this antagonist (Figure 1c and Figure 3b). The peak pressor effect of ET-1 was significantly reduced from 47 ± 5 mmHg (control) to 10 ± 3 mmHg or 12 ± 4 mmHg by PD145065 (0.2 or 0.6 $mg\,kg^{-1}\,min^{-1}$, i.v.) respectively, ($n = 4$, Figure 3a). PD 145065 (0.2 $mg\,kg^{-1}\,min^{-1}$, i.v.) had no effect on ET-1-induced inhibition of *ex vivo* platelet aggregation (Figure 4). In contrast, pretreatment with PD145065 (0.6 $mg\,kg^{-1}\,min^{-1}$, i.v.) abolished the anti-aggregatory effects of ET-1 ($n = 4$, Figure 1c and Figure 4).

Discussion

This study demonstrates that the non-selective ET_A/ET_B receptor antagonist PD145065, but not the selective ET_A receptor antagonist FR139317, abolished the ET-1-induced inhibition of *ex vivo* platelet aggregation in the anaesthetized rabbit. In addition, PD145065 abolished the transient fall in blood pressure produced by ET-1 and largely attenuated the ET-1-induced pressor response. FR139317 also strongly attenuated the pressor response to ET-1, but in contrast to PD145065 enhanced the vasodilator response to the peptide.

Intravenous injection of ET-1 is associated with a release of PGI_2 which ameliorates the vasoconstrictor/pressor effects of the peptide and inhibits platelet aggregation (Thiemermann *et al.*, 1988; 1990; Herman *et al.*, 1989; Lidbury *et al.*, 1990). A variety of pressor agents including angiotensin II, vasopressin, noradrenaline and the thromboxane A_2-mimetic, U46619, stimulate vascular PGI_2 synthesis (see: Thiemermann, 1991) suggesting that PGI_2 release may occur as a defence mechanism in response to elevation of arterial blood pressure. However, this study demonstrates that the ET_A receptor antagonist, FR139137, attenuates the rise in blood pressure produced by ET-1 in the anaesthetized rabbit without affecting the ET-1-induced inhibition of *ex vivo* platelet aggregation. Thus, it seems unlikely that the ET-1-induced release of PGI_2 occurs as a response to elevated blood pressure, but rather is a direct receptor-mediated event.

Which receptor, then, mediates the stimulation by ET-1 of endogenous PGI_2 formation? The finding that the ET_A receptor antagonist BQ-123 markedly reduced the release of PGI_2 in response to ET-1 in rat isolated perfused lungs, suggested that this effect of ET-1 in the rat is due to selective activation of ET_A receptors (D'Orleans-Juste *et al.*, 1992). However, we demonstrate here that the non-selective ET_A/ET_B antagonist PD145065, but not the ET_A antagonist FR139137, abolished the inhibition of *ex vivo* platelet aggregation produced by ET-1 in the anaesthetized rabbit. These findings clearly demonstrate that ET-1-stimulated release of PGI_2 in the anaesthetized rabbit is not due to activation of the ET_A receptor. The finding that the inhibition of *ex vivo* platelet aggregation in response to ET-1 was abolished by the non-selective ET_A/ET_B receptor antagonist, PD145065, strongly suggests that the ET-1-induced release of PGI_2 and, hence, inhibition of platelet aggregation in the anaesthetized rabbit is due to activation of the ET_B receptor. PD145065 (1 $mg\,ml^{-1}$) did not influence the inhibition of platelet aggregation produced by PGI_2 (10 $ng\,ml^{-1}$) *in vitro* (unpublished observation). Thus, it is unlikely that PD145065 abolishes the antiaggregatory effects of PGI_2 by acting at the platelet prostaglandin receptor.

The hypothesis that the vasodilator effects of ET-1, which are partly mediated by the release of NO and PGI_2 (see Thiemermann, 1991), are due to activation of the ET_B receptor (Ihara *et al.*, 1991) is strongly supported by the finding that PD145065 abolishes the transient vasodilator response to ET-1. In contrast, FR139317 enhanced the ET-1 induced depressor response probably by inhibiting the vasoconstrictor (ET_A) without affecting the vasodilator (ET_B) properties of the peptide. The finding that this augmentation of the depressor response to ET-1 in the presence of FR139317 was largely, but not completely, inhibited by indomethacin supports the view that the ET-1-induced vasodilatation is mediated by the release of prostanoids, principally PGI_2, and to a lesser degree by NO (Whittle *et al.*, 1989; Fozzard & Part, 1992).

There is increasing evidence pointing to the existence of multiple ET receptor subtypes (Emori *et al.*, 1990; Samson *et al.*, 1990; Fukuroda *et al.*, 1992; Harrison *et al.*, 1992). The hypothesis that ET_A receptors exclusively mediate ET-1-induced vasoconstriction was recently challenged by findings demonstrating that activation of non-ET_A (ET_B ?) receptors causes vasoconstriction (Williams *et al.*, 1991; Bigaud & Pelton, 1992; Cristol *et al.*, 1993). The notion that the contraction of vascular and non-vascular smooth muscle is not solely due to activation of the ET_A receptor is also supported by *in vitro* studies demonstrating that the selective ET_A antagonist BQ-123 does not affect ET-1-induced contractions in the guinea-pig bronchus, rabbit pulmonary artery and rat stomach strip (Hay, 1992; Warner *et al.*, 1992). Moreover, BQ-123 does not completely inhibit the pressor responses to ET-1, big ET-1, sarafotoxin 6b or sarafotoxin 6c in the anaesthetized, ganglion-blocked rat (McMurdo *et al.*, 1993). The results of the present study suggest that the ET-1 pressor response in the anaesthetized rabbit is mediated almost entirely via the ET_A receptor. FR139317 (ET_A antagonist) and PD145065 (ET_A/ET_B antagonist) were equipotent at inhibiting the ET-1-induced rise in blood pressure. Thus, it is unlikely that a regional, ET_B-mediated vasoconstriction substantially contributes to the pressor response produced by ET-1 in the anaesthetized rabbit. These findings also suggest that there are species-related differences in the ET-receptors

responsible for mediating vasoconstriction and PGI_2 release (D'Orleans-Juste *et al.*, 1992; in this study).

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