British Journal of Pharmacology (2002) 136, 819–828

www.nature.com/bjp

Pressor and pulmonary responses to ET-1(1–31) in guinea-pigs

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1 Endothelin-1(1–31) (ET-1(1–31); 0.25 to 4 nmol kg^{-1}; i.v.) induced, in the guinea-pig, graded increases in MAP and an indomethacin-sensitive enhancement of pulmonary insufflation pressure (PIP). At all doses, ET-1(1–31) induced a monophasic pressor response, except at 4 nmol kg^{-1}, which caused a rapid and transient response (first phase: over first 10 min after injection) followed by a more slowly-developing and sustained (second phase: between 10 and 45 min after injection) increase in MAP. ET-1(1–31) was 4 to 10 fold less potent than ET-1 on PIP responses.

2 Phosphoramidon (5 and 10 mg kg^{-1}) reduced both pressor and PIP effects of ET-1(1–31). Thiorphan (0.25 and 2.5 mg kg^{-1}) did not affect the pressor responses to ET-1(1–31) although its PIP effects were markedly reduced by the NEP inhibitor. A selective endothelin-converting enzyme (ECE) inhibitor, CGS 35066 (1 mg kg^{-1}), significantly reduced the second phase pressor response and increase in PIP triggered by ET-1(1–31).

3 The second (but not the first) pressor phase of ET-1(1–31) (4 nmol kg^{-1}) was markedly reduced by BQ-123 (selective ET_A antagonist), whereas the increase of PIP was significantly reduced by BQ-788 (selective ET_B antagonist). Co-administration of BQ-123 plus BQ-788 abolished ET-1(1–31)-induced increase in PIP, but blockade of the second pressor phase afforded by BQ-123 was now reversed.

4 In guinea-pig isolated perfused lungs, ET-1(1–31) (50 nM) induced the release of prostacyclin and thromboxane A_2, which was inhibited by BQ-788 (5 nM) or thiorphan (25 μM), but not BQ-123 (1 μM).

5 These results suggest that ET-1(1–31) enhances MAP. Its sustained, but not transient, pressor effects are mediated *via* ET_A receptor activation. Furthermore, ET-1(1–31) increases airway resistance *in vivo* and triggers prostacyclin and thromboxane A_2 release from perfused lungs predominantly *via* ET_B receptor activation. ET-1(1–31) failed to display any selectivity of action towards either ET_A or ET_B receptors in these models.

6 We suggest that, in order to raise MAP, ET-1(1–31) requires conversion to ET-1, predominantly by ECE and to a lesser extent neutral endopeptidase 24.11, whereas the reverse holds true regarding its pharmacological effects in airways.

British Journal of Pharmacology (2002) **136,** 819–828

Keywords: Mean arterial blood pressure; pulmonary insufflation pressure; eicosanoid; endothelin-converting enzyme; neutral endopeptidase; ET receptors; ET-1(1–31)

Abbreviations: big ET-1, big endothelin-1; COX, cyclo-oxygenase; DMSO, dimethyl sulphoxide; ECE, endothelin-converting enzyme; ET_A, endothelin A; ET_B, endothelin B; ET-1, endothelin-1; ET-1(1–31), endothelin-1(1–31); MAP, mean arterial blood pressure; NEP, neutral endopeptidase 24.11; PGD_2, prostaglandin D_2; PGE_1, prostaglandin E_1; PGE_2, prostaglandin E_2; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; PGI_2, prostacyclin; PIP, pulmonary insufflation pressure; PR, phosphoramidon; TP, thiorphan; TxA_2, thromboxane A_2; TxB_2, thromboxane B_2; 6-Keto-$PGF_{1\alpha}$, 6-Keto-prostaglandin $F_{1\alpha}$

Introduction

Endothelin-1 (ET-1) is a 21 amino acid peptide which exhibits potent vasoconstrictor and hypertensive effects, alongside various other physiological actions (for reviews, see Rubanyi & Polokoff, 1994; Miyauchi & Masaki, 1999). This peptide is generated from the 38 amino acid precursor, big ET-1, through cleavage of the Trp^{21}-Val^{22} bond by an ET-converting enzyme (ECE) and produces its effects *via* stimulation of two specific G-protein-coupled receptors, namely ET_A and ET_B. Until recently, the ET peptide family was believed to encompass only two additional members, ET-2 and ET-3, both highly homologous and formed *via* the same analogous pathways as ET-1. Nevertheless, Nakano *et al*. (1997) reported that human mast cell chymase cleaves the Tyr^{31}-Gly^{32} bond of big ET-1 to yield ET-1(1–31), a novel potent contractile peptide of both vascular and non-vascular smooth muscle tissues. It has been suggested that ET-1(1–31) must be converted to ET-1 *via* the neutral endopeptidase 24-11 (NEP) to induce its pharmacological effects (Hayasaki-Kajiwara *et al*., 1999).

In several *in vitro* assays, ET-1(1–31) has been shown to be slightly less potent than ET-1 in triggering contractions of

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rat trachea (Nakano *et al*., 1997) and aorta, porcine coronary arteries (Kishi *et al*., 1998) or human coronary artery smooth muscle cells (Inui *et al*., 1999). Like ET-1, ET-1(1–31) can also stimulate vascular smooth muscle cell proliferation *via* a PKC-dependent ERK1/2 activation-mediated mechanism, as well as nitric oxide release from endothelial cells (Yoshizumi *et al*., 1998; Niwa *et al*., 2000). Some studies have shown that ET-1(1–31) acts as a selective ET_A receptor agonist in rat cultured zona glomerulosa cells (Mazzocchi *et al*., 2000; Rebuffat *et al*., 2001). Furthermore, others found that its smooth muscle contractile effects on the porcine coronary artery and nitric oxide-releasing actions on endothelial cells are amenable to blockade by selective ET_A and ET_B receptor antagonists, respectively (Nagata *et al*., 2000; Niwa *et al*., 2000).

Curiously, since the discovery of ET-1(1–31), there has been no report on its *in vivo* pharmacology. We have therefore attempted to characterize, in well-documented pharmacological models, the pressor, bronchoconstrictive and pulmonary eicosanoid-releasing properties of this peptide in the guinea-pig (D'Orléans-Juste *et al*., 1991, 1994; Gratton *et al*., 1995; Lewis *et al*., 1999). In these models, we have shown that the pressor effects of ET-1 were solely mediated by ET_A receptors, whereas both the enhancement of pulmonary insufflation pressure (PIP) *in vivo* and the thromboxane A_2 (TxA_2) release from guinea-pig isolated perfused lungs were entirely dependent on ET_B receptor activation (D'Orléans-Juste *et al*., 1994; Lewis *et al*., 1999).

Moreover, several groups including our own have previously characterized a phosphoramidon-sensitive ECE-dependent pharmacological effect of big ET-1 and -2 in the anaesthetized guinea-pig (Fukuroda *et al*., 1990; Pons *et al*., 1992; Gratton *et al*., 1995). In the human bronchial smooth muscle, the intracellular calcium-increasing properties of ET-1(1–31) are abolished by both phosphoramidon (PR) and thiorphan (TP), suggesting that the peptide must be hydrolyzed by NEP to ET-1 *in vitro* (Hayasaki-Kajiwara *et al*., 1999).

In view of the above considerations, we have addressed in the present study the putative contribution of the phosphoramidon-sensitive ECE- (Xu *et al*., 1994) and NEP-mediated conversion pathways, as well as the endothelin receptors involved in the pharmacological effects of ET-1(1–31) *in vivo*, in the anaesthetised guinea-pig. We also examined the potential involvement of eicosanoids in the pulmonary effects of ET-1(1–31) *in vivo*, as well as its ability to trigger release of these mediators from isolated perfused lungs.

Methods

In vivo *experiments*

Duncan–Hartley guinea-pigs (250–350 g; Charles River, St-Constant, Québec, Canada) of either sex were anaesthetized with ketamine/xylazine (87/13 mg kg^{-1}, i.m.). As *in vivo* experiments lasted no longer than 120 min, maintenance doses of anaesthetics were not required. Polyethylene catheters (PE-50) were inserted into the left external jugular vein and right carotid artery for drug administration and recording of mean arterial blood pressure (MAP) and heart rate, respectively. Another cannula (PE-240) was then inserted into the trachea following tracheostomy to facilitate respiration. A blood pressure analyser (Micro-Med, Louisville, KY, U.S.A.) was used to monitor MAP and heart rate. The data were recorded at fixed 60 s time intervals throughout the entire experiment by an automated computer data acquisition system (Digi-Med® System Integrator™, Model 200, Micro-Med) linked to a Compaq Prolinea 4/33 computer.

After surgery, spontaneous breathing was suppressed with succinylcholine (5 mg kg^{-1}, s.c.) and each animal was connected, through the tracheal cannula, to a rodent respirator (Model 683, Harvard Apparatus Inc., Saint-Laurent, Québec, Canada) and ventilated (6 ml kg^{-1}, 60 strokes min^{-1}). For recording of bronchoconstrictor responses, PIP was monitored continuously with a pressure transducer (Statham, Model P23AC) connected to a side arm of the tracheal cannula and coupled to a Grass polygraph (Model 7D), according to the method of Konzett & Rössler (1940). After 45 min of stabilization, the pharmacological responses to the various agonists were monitored for at least 45 min following their administration.

For sake of clarity, the biphasic pressor response to the highest dose of ET-1(1–31) was identified as having two distinct phases: a first phase with a rapid onset (within the first 10 min following injection) and a second phase with a slower onset (10–45 min following injection) and more sustained. Data shown in Figures 3 to 7 illustrate the maximal changes (Delta) in MAP or PIP from baseline induced by each dose of agonist. All baseline values are reported in the appropriate Results section.

Each animal received a single dose of only one of the following peptides: ET-1 (0.025 to 1 nmol kg^{-1}, i.v.), ET-1(1–31) (0.025 to 4 nmol kg^{-1}, i.v.) or big ET-1 (5 nmol kg^{-1}, i.v.). In some animals, treatment was preceded by an i.v. injection of either the ECE-NEP inhibitor, PR (Matsumura *et al*., 1990; 5 or 10 mg kg^{-1}), the selective NEP inhibitor, TP (Roques *et al*., 1980; 0.25 and 2.5 mg kg^{-1}), the selective ECE inhibitor, CGS 35066 (Jeng *et al*., 2000; 1 mg kg^{-1}), the selective ET_B receptor antagonist, BQ-788 (Ishikawa *et al*., 1994; 0.25 mg kg^{-1}), the selective ET_A antagonist, BQ-123 (Ihara *et al*., 1992; 2.5 mg kg^{-1}), the non-selective cyclo-oxygenase inhibitor, indomethacin (10 or 20 mg kg^{-1}), or the corresponding vehicle. All pretreatments were given 5 min before peptide injection, except indomethacin and CGS 35066, which were administered 30 min beforehand. In all cases, the effects of the inhibitors and antagonists were determined against the maximal MAP and PIP responses. The vehicles used (phosphate-buffered saline (PBS), PBS-DMSO 5–10%, PBS-Trizma base (0.2 M) 50% or $NaHCO_3$ (0.25 M)) were systematically tested and found not to alter either basal or agonist-induced changes in MAP or PIP values. Table 1 describes the different agonists, inhibitors and antagonists used in this study as well as the appropriate references for their respective dose ranges.

Measurements of eicosanoids from isolated perfused guinea-pig lungs

Guinea-pigs with the above-mentioned specifications were killed by cervical dislocation. Following thoracotomy, the pulmonary artery was cannulated for perfusion of the pulmonary circulation with a heparinized (100 units ml^{-1})

Table 1 Pharmacological agents and dose/concentration regimens

Pharmacological agents	*Dose/concentration range* In vivo	In vitro	*Pretreatment time period* In vivo	In vitro	*Effect/Action*	*Reference*
ET-1(1–31)	0.025–4 nmol kg^{-1}	5 and 50 nM	–	–	Pressor and bronchoconstrictive effects and release of eicosanoids	
ET-1	0.025–1 nmol kg^{-1}	5 nM	–	–	Pressor and bronchoconstrictive effects and release of eicosanoids	D'Orléans-Juste *et al.* (1991)
big ET-1	5 nmol kg^{-1}	100 nM	–	–	Pressor and bronchoconstrictive effects and release of eicosanoids	D'Orléans-Juste *et al.* (1991)
Phosphoramidon	5 and 10 mg kg^{-1}		5 min	–	ECE/NEP inhibitor	D'Orléans-Juste *et al.* (1991); Gratton *et al.* (1995)
Thiorphan	0.25 and 2.5 mg kg^{-1}	25 μM	5 min	60 min	NEP inhibitor	Roques *et al.* (1980) Pons *et al.* (1992)
CGS 35066	1 mg kg^{-1}		30 min	–	ECE inhibitor	Jeng *et al.* (2000)
Indomethacin	10 and 20 mg kg^{-1}		30 min	–	COX inhibitor	Lewis *et al.* (1999)
BQ-123	2.5 mg kg^{-1}	1 μM	5 min	15 min	ET_A antagonist	Noguchi *et al.* (1993)
BQ-788	0.25 mg kg^{-1}	5 and 10 nM	5 min	15 min	ET_B antagonist	D'Orléans-Juste *et al.* (1994); Lewis *et al.* (1999)

Krebs' solution of the following composition (mM): NaCl, 118; KCl, 4.7; KH_2PO_4, 1.2; $MgSO_4 \cdot 7H_2O$, 1.2; $CaCl_2 \cdot 6H_2O$, 2.5; $NaHCO_3$, 25 and glucose, 5.5. The lungs were then removed *en bloc* and immediately suspended in a heated chamber (37°C) and continuously perfused (5 ml min^{-1}) with an oxygenated (95% O_2, 5% CO_2) Krebs' solution. The lungs were left to stabilize for 45 min before a 3 min i.a. infusion of either ET-1 (5 nM), ET-1(1–31) (5 or 50 nM) or big ET-1 (100 nM). In a second series of experiments, one of the agonists was administered following infusion of either endothelin receptor antagonists, BQ-123 (1 μM, 15 min) or BQ-788 (5 or 10 nM, 15 min), or the NEP inhibitor, TP (25 μM, 60 min). Concentrations of the different drugs were chosen according to a previously reported study (D'Orléans-Juste *et al.*, 1994). The effluent from the lungs was collected (1 min samples) before, during and after infusion of the various agents. The samples were stored at −20°C until determination of their levels of stable hydrolytic metabolites of prostacyclin (6-Keto-prostaglandin $F_{1\alpha}$ (6-Keto-$PGF_{1\alpha}$)) and (TxA_2) (thromboxane B_2 (TxB_2)) by radioimmunoassay (Salmon, 1978).

Drugs

ET-1, human big ET-1 and BQ-788 (N,cis-2,6-dimethylpiperidinocarbonyl-L-γ-methylleucyl-D-l-methoxycarbonyltryptophanyl-D-norleucine; Ishikawa *et al.*, 1994) were purchased from American Peptide Company Inc. (Sunnyvale, CA, U.S.A.). BQ-123 (cyclic(D-Trp-D-Asp-Pro-D-Val-Leu); Ihara *et al.*, 1992) was synthesized in our laboratory by Dr Witold Neugebauer. ET-1(1–31) and PR were purchased from Peptides International (Louisville, KY, U.S.A.). CGS 35066 ((S)-3-Dibenzofuran-3-yl-2-(phosphomethylamino)-proprionic acid) was kindly provided by Dr A. Jeng (Novartis, Summit, NJ, U.S.A.). TP, indomethacin, 6-Keto-$PGF_{1\alpha}$, TxB_2 6-Keto-$PGF_{1\alpha}$ and TxB_2 antisera were obtained from Sigma (St Louis, MO, U.S.A.). Tritiated 6-Keto-$PGF_{1\alpha}$ and TxB_2 were purchased from Amersham (Oakville, Ontario, Canada). The 6-Keto-$PGF_{1\alpha}$ antiserum has 100% cross-reactivity with authentic 6-Keto-$PGF_{1\alpha}$, 23% with prostaglandin E_1 (PGE_1), 4% with prostaglandin E_2 (PGE_2), 7% with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and less than 1% with TxB_2. The TxB_2 antiserum has a 100% cross-reactivity with authentic TxB_2, less than 2% cross-reactivity with prostaglandin D_2 (PGD_2) and $PGF_{1\alpha}$, and less than 0.1% cross-reactivity with 6-Keto-$PGF_{1\alpha}$, PGE_1 and PGE_2. Both 6-Keto-$PGF_{1\alpha}$ and TxB_2 radioimmunoassays have a detection limit of 0.4 ng ml^{-1}. The eicosanoid antisera used in the present study did not cross-react with any of the agonists or inhibitors used. ET-1(1–31) and TP were dissolved in PBS–DMSO 10%, BQ-788, BQ-123 in PBS–DMSO 5%, indomethacin in PBS-Trizma base (0.2 M) 50% and CGS 35066 in $NaHCO_3$ (0.25 M). All other agents were dissolved in PBS.

Data analysis

Results are shown as mean ± s.e.mean for n experiments. Since the various responses to ET-1 studied were very poorly reversible, animals or perfused organs were not used as their own controls for the *in vivo* and *in vitro* experiments with inhibitors or antagonists. Statistical comparisons were made using the Student's t-test or by ANOVA followed by the Dunnett's test for multiple comparisons where specified. Differences in which $P<0.05$ were considered significant.

Ethics

The care of animals and all of the research protocols conformed to the guiding principles for animal experimentation, as enunciated by the Canadian Council on Animal Care and approved by the Ethical Committee on Animal Research of the Université de Sherbrooke.

Results

Effects of ET-1(1–31) on MAP and PIP

The average basal MAP and PIP values established in the anaesthetized guinea-pig were determined at 47.8 ± 0.6 mmHg and 3.0 ± 0.1 mmHg, respectively ($n=140$).

Figure 1 compares the time course of the pressor effects of ET-1(1–31) (4 nmol kg^{-1}), ET-1 (1 nmol kg^{-1}) and big ET-1

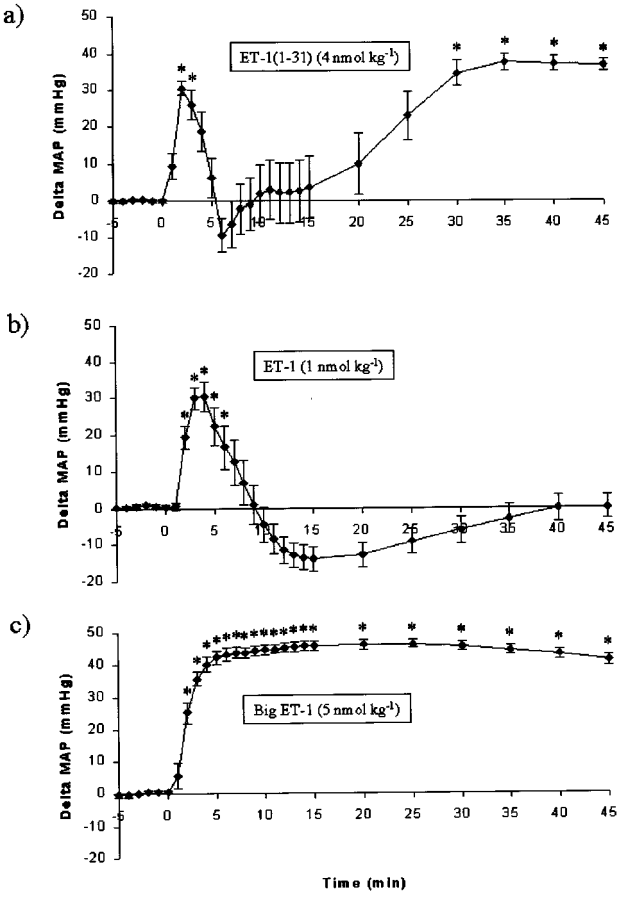

Figure 1 Time course profile of the variation in MAP induced by i.v. injection of ET-1(1–31) (4 nmol kg^{-1}) (**a**), ET-1 (1 nmol kg^{-1}) (**b**) and big ET-1 (5 nmol kg^{-1}) (**c**) in the anaesthetized guinea-pig. Each point represents the mean ± s.e.mean of 13, 10 and 6 experiments for ET-1(1–31), ET-1 and big ET-1, respectively. $^*P<0.05$ relative to baseline value using the ANOVA followed by the Dunnett's test.

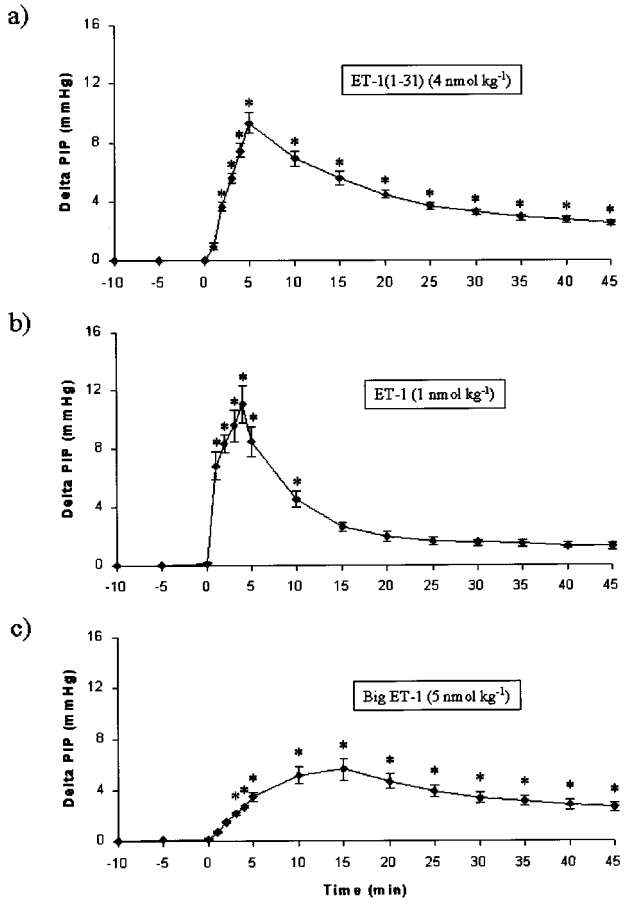

Figure 2 Time course profile of the variation in PIP induced by i.v. injection of ET-1(1–31) (4 nmol kg^{-1}) (**a**), ET-1 (1 nmol kg^{-1}) (**b**) and big ET-1 (5 nmol kg^{-1}) (**c**) in the anaesthetized guinea-pig. Each point represents the mean ± s.e.mean of 13, 10 and 6 experiments for ET-1(1–31), ET-1 and big ET-1, respectively. $^*P<0.05$ relative to baseline value using the ANOVA followed by the Dunnett's test.

(5 nmol kg^{-1}). Characteristically, ET-1(1–31) causes a biphasic change in MAP comprised of an early first phase pressor effect, which is followed by a much more sustained response (second phase) (Figure 1a). Furthermore, ET-1 induces a transient (Figure 1b) and big ET-1 a sustained (Figure 1c) increase in MAP in a monophasic fashion. On the other hand, ET-1(1–31), ET-1 and big ET-1 all induced monophasic increases in PIP (Figure 2).

In another series of experiments, the maximal responses of ET-1(1–31) and ET-1 at increasing doses were used to construct dose-response curves. Those curves represented in Figure 3 confirm that ET-1(1–31) is as potent as ET-1 to induce an initial increase in MAP. In contrast, ET-1(1–31) induces a more sustained Phase 2 pressor response only at the highest dose used in the present study. Furthermore, ET-1(1–31) is less potent than ET-1 to induce PIP increases. Higher doses of ET-1 (2.5 nmol kg^{-1}) and ET-1(1–31) (5 nmol kg^{-1}) induced death within less than 15 min of administration ($n=2$ for each).

This particular biphasic response to ET-1(1–31) (4 nmol kg^{-1}) has been compared to ET-1 and big ET-1 (1 and 5 nmol kg^{-1}, respectively) for the following *in vivo* studies.

Contribution of NEP and ECE in changes in MAP and PIP induced by ET-1(1–31)

Phosphoramidon (5 and 10 mg kg^{-1}), a dual ECE/NEP inhibitor, reduced in a dose-dependent fashion both the first and second phases of the pressor response to ET-1(1–31) and the monophasic pressor response to big ET-1 (5 nmol kg^{-1}) (Figure 4a, b). In contrast, the selective ECE inhibitor, CGS 35066 (1 mg kg^{-1}), did not reduce the first phase yet significantly inhibited the second phase of the pressor response to ET-1(1–31), as well as the pressor response to big ET-1 (Figure 4a, b). Thiorphan (0.25 and 2.5 mg kg^{-1}) was without effect on the responses to ET-1(1–31) and big ET-1 (Figure 4a, b). In a last series of control experiments, the pressor effect of ET-1 (1 nmol kg^{-1}) was not altered by either phosphoramidon, thiorphan nor CGS 35066 (Figure 4c).

Figure 5 illustrates that phosphoramidon (5 and 10 mg kg^{-1}), thiorphan (0.25 and 2.5 mg kg^{-1}) and CGS 35066 (1 mg kg^{-1}) significantly reduced the increase of PIP induced by ET-1(1–31) and big ET-1, without affecting the increase in airway resistance to ET-1. Interestingly, thiorphan at a 10-fold lower dose (0.25 mg kg^{-1}), was still able to

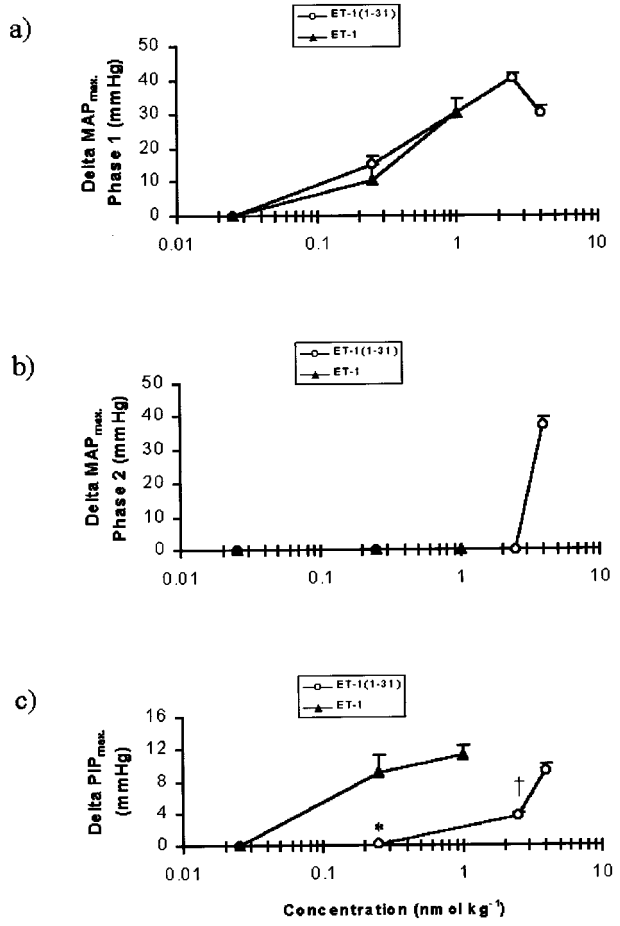

Figure 3 Dose-response curves for the maximum increases in MAP (first phase in **a**, second phase in **b**) and PIP (**c**) triggered by i.v. injection of ET-1(1–31) or ET-1 in the anaesthetized guinea-pig. Each point represents the mean±s.e.mean of 4–13 and 4–10 experiments for ET-1(1–31) and ET-1, respectively. $*P<0.05$ relative to ET-1 at the same dose and $\dagger P<0.05$ relative to the highest dose of ET-1, using the Student's *t*-test.

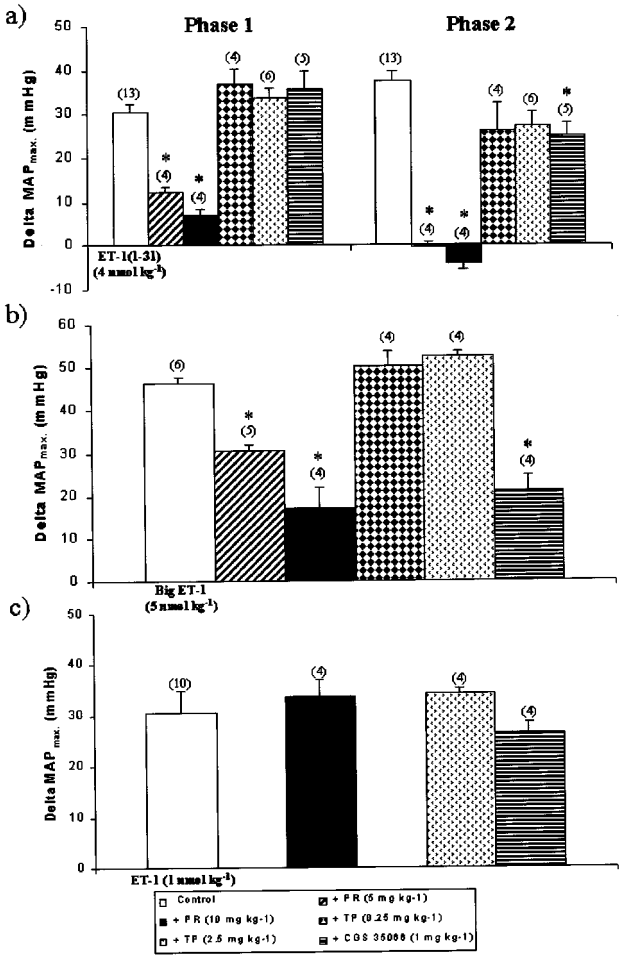

Figure 4 Effects of phosphoramidon (PR, 5 or 10 mg kg^{-1}), thiorphan (TP, 0.25 or 2.5 mg kg^{-1}) and CGS 35066 (1 mg kg^{-1}) on the maximum increases in MAP triggered by i.v. injection of ET-1(1–31) (4 nmol kg^{-1}) (**a**), big ET-1 (5 nmol kg^{-1}) (**b**) or ET-1 (1 nmol kg^{-1}) (**c**), in the anaesthetized guinea-pig. Each bar represents the mean±s.e.mean of *n* experiments as indicated in brackets. $*P<0.05$ relative to corresponding control value using the ANOVA followed by the Dunnett's test.

significantly affect the increase of PIP induced by ET-1(1–31) and big ET-1 in the anaesthetized guinea-pig (Figure 5).

Contribution of ET_A and ET_B receptors towards the pressor and PIP-increasing effects of ET-1(1–31)

The second phase of the pressor response to ET-1(1–31) (4 nmol kg^{-1}), but not its first phase nor its effects on PIP, was markedly reduced by the selective ET_A receptor antagonist BQ-123 at 2.5 mg kg^{-1} (Figure 6a). Furthermore the same antagonist also reduced the haemodynamic response to ET-1 (by less than 40% however) (Figure 6a). In contrast, the selective ET_B receptor antagonist BQ-788 (0.25 mg kg^{-1}) was without effect on the pressor response to ET-1(1–31). Interestingly, co-administration of both antagonists reversed the blockade of ET-1(1–31) induced pressor response afforded by the sole administration of BQ-123 (Figure 6a). On the other hand, the selective ET_B receptor antagonist alone partially blocked the PIP increases induced by ET-1(1–31) and more efficiently the response to ET-1. Interestingly, co-administration of both antagonists is necessary to abolish the PIP increases triggered by ET-1(1–31) (Figure 6b).

Contribution of eicosanoids to the changes in MAP and PIP induced by ET-1(1–31)

In another series of experiments, indomethacin (10 or 20 mg kg^{-1}, i.v.) failed to inhibit the transient (first phase) or sustained (second phase) pressor effects induced by ET-1(1–31) or the response to ET-1 (Figure 7a). As a matter of fact, the lower dose of indomethacin actually potentiated the first phase of the increase in MAP induced by that peptide (Figure 7a). In contrast, the cyclo-oxygenase inhibitor reduced by more than 50% the increase in PIP triggered by ET-1(1–31) and abolished that of ET-1 (Figure 7b).

Role of ET_A and ET_B receptors as well as of NEP in the eicosanoid-releasing properties of ET-1(1–31)

In a series of *in vitro* experiments, ET-1(1–31) was found to trigger the release of both TxB_2 and 6-Keto-$PGF_{1\alpha}$, albeit at a potency 10-fold lower than that of ET-1 (Figure 8a, b). Furthermore, TP (25 μM) fully abrogated the eicosanoid-

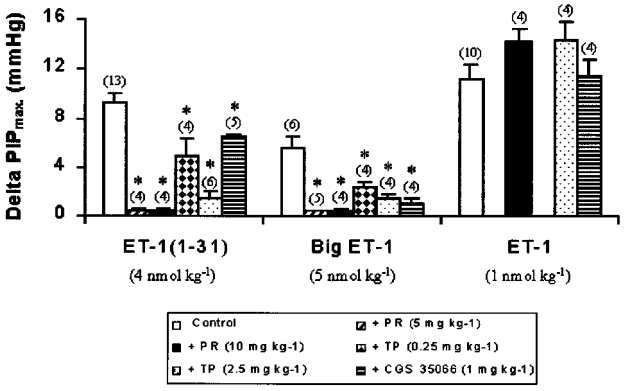

Figure 5 Effects of phosphoramidon (PR, 5 or 10 mg kg^{-1}), thiorphan (TP, 0.25 or 2.5 mg kg^{-1}) and CGS 35066 (1 mg kg^{-1}) on the maximum increases in PIP triggered by i.v. injection of ET-1(1–31) (4 nmol kg^{-1}), big ET-1 (5 nmol kg^{-1}) or ET-1 (1 nmol kg^{-1}), in the anaesthetized guinea-pig. Each bar represents the mean±s.e.mean of n experiments as indicated in brackets. *$P<0.05$ relative to corresponding control value using the ANOVA followed by the Dunnett's test.

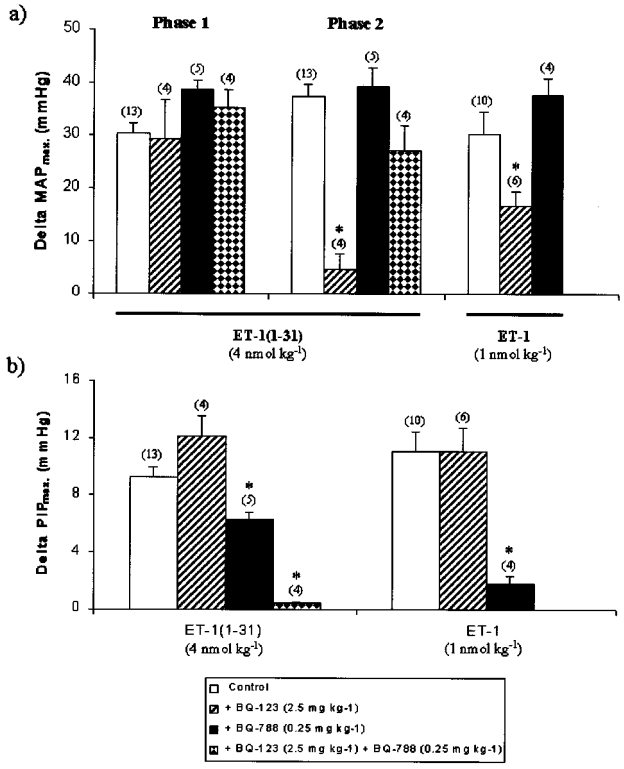

Figure 6 Effects of BQ-123 (2.5 mg kg^{-1}) and BQ-788 (0.25 mg kg^{-1}) on the maximum increases in MAP (**a**) and PIP (**b**) triggered by i.v. injection of ET-1(1–31) (4 nmol kg^{-1}) or ET-1 (1 nmol kg^{-1}) in the anaesthetized guinea-pig. The antagonists were administered i.v. either alone or in combination, 5 min prior to each agonist injection. Each bar represents the mean±s.e.mean of n experiments as indicated in brackets. *$P<0.05$ relative to corresponding control value using the ANOVA followed by the Dunnett's test.

releasing effects of ET-1(1–31) and also big ET-1 in the guinea-pig perfused lung (Figure 8c,d). We have previously shown (D'Orléans-Juste *et al*., 1991) that phosphoramidon

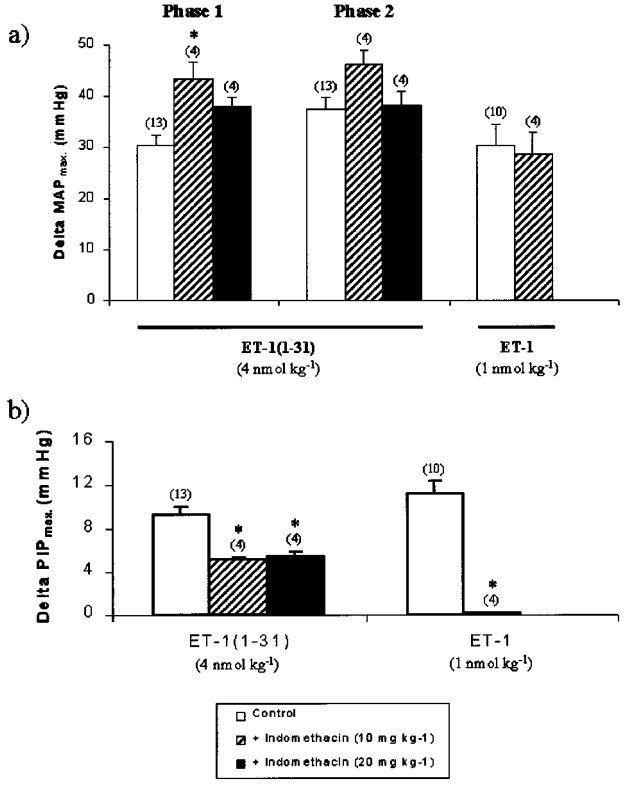

Figure 7 Effects of indomethacin (10 or 20 mg kg^{-1}) on the maximum increases in MAP (**a**) and PIP (**b**) triggered by i.v. injection of ET-1(1–31) (4 nmol kg^{-1}) or ET-1 (1 nmol kg^{-1}) in the anaesthetized guinea-pig. The cyclo-oxygenase inhibitor was administered i.v. 30 min prior to each agonist injection. Each bar represents the mean±s.e.mean of n experiments as indicated in brackets. *$P<0.05$ relative to corresponding control value using the ANOVA followed by the Dunnett's test.

abolishes the release of TxB_2, as well as that of 6-Keto-$PGF_{1\alpha}$ triggered by big ET-1, in the same preparation.

Finally, a 15 min pre-treatment with BQ-123 (1 μM) did not affect the release of TxB_2 nor that of 6-Keto-$PGF_{1\alpha}$ triggered by ET-1(1–31) or ET-1 (Figure 9). In contrast, BQ-788 (5 and 10 nM) significantly reduced in a concentration-dependent fashion the release of both eicosanoids induced by ET-1(1–31) or ET-1 (Figure 9).

Discussion

The results of the current study show that exogenous ET-1(1–31), a new member of the ET family formed by alternative cleavage of big ET-1 by mast cell chymase (Nakano *et al*., 1997), induces dose-dependent increases in MAP and PIP in the anaesthetized guinea-pig. Moreover, we have found that both these *in vivo* effects of ET-1(1–31) appear to depend, to varying extents, on its processing by ECE and/or NEP and activation of ET_A and/or ET_B receptors and are either mediated or modulated by COX-derived eicosanoids.

ET-1(1–31) was found to cause, at higher doses (4 nmol kg^{-1}), a biphasic pressor effect comprising an earlier (transient) first phase followed by a delayed (sustained) second phase, allied to a monophasic increase in PIP in the

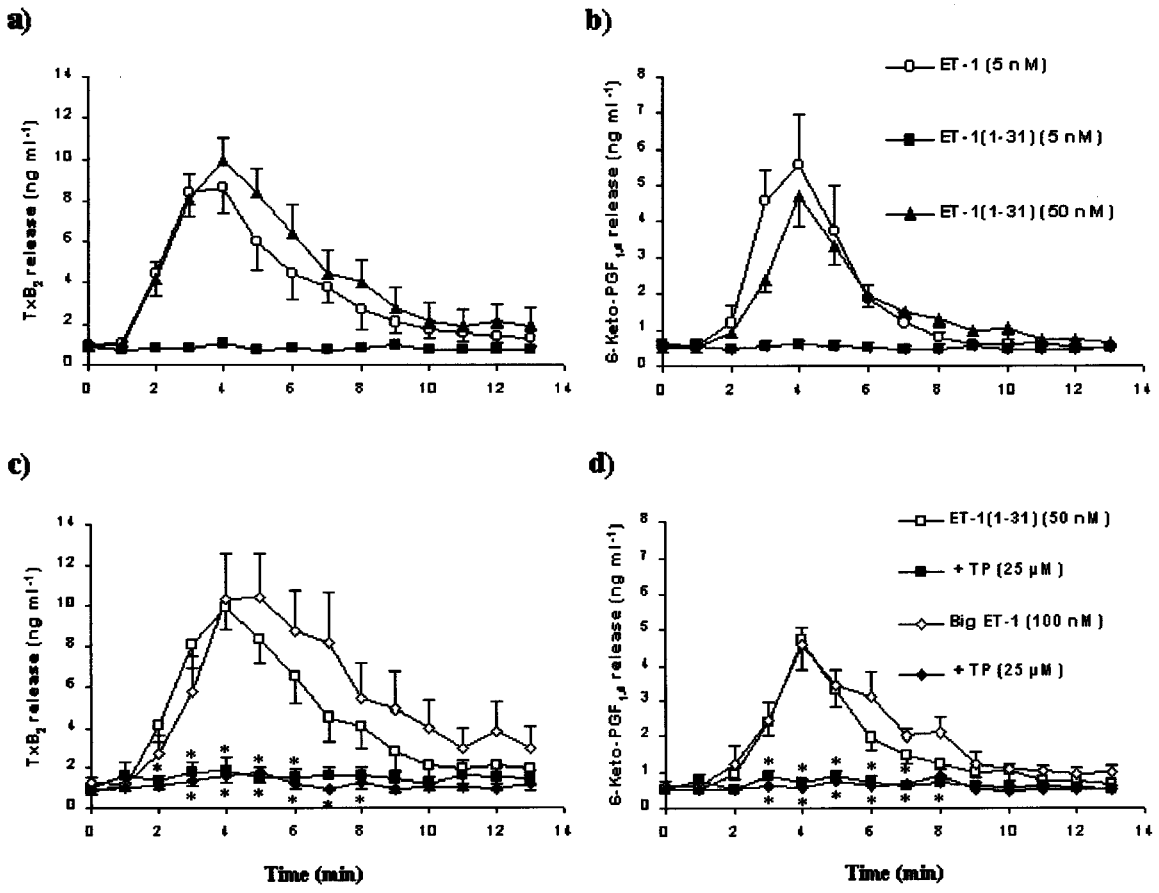

Figure 8 Effects of ET-1(1–31) (5 or 50 nM), ET-1 (5 nM) and big ET-1 (100 nM) on the release of thromboxane B_2 (**a** and **c**) and 6-Keto-$PGF_{1\alpha}$ (**b** and **d**) from the guinea-pig isolated and perfused lung. **c** and **d** also show that the release of either eicosanoid triggered by ET-1(1–31) or big ET-1 is abolished by prior incubation with thiorphan (TP, 25 μM). Each point represents the mean ± s.e.mean of at least seven different experiments. $P<0.05$ relative to corresponding control value using the Student's *t*-test.

guinea-pig. Although both ET-1(1–31) and ET-1 are clearly equipotent (on a molar basis) at transiently increasing MAP, ET-1(1–31) is about 4–10 fold less potent than ET-1 at increasing PIP. Considering that the actions of ET-1(1–31), unlike those of ET-1, are reduced by pretreatment with blockers of ECE and/or NEP (as will be discussed in more detail in the following paragraph), it seems likely that the former peptide must be processed enzymatically in order to act. We suggest that this biotransformation proceeds at a more effective rate in the systemic circulation to increase MAP (even if, ultimately, this may be found to occur in systemic extra-luminal vascular compartments), than in the pulmonary circulation (or in extravascular sites in the lungs) to raise PIP.

The pressor and PIP-increasing actions of ET-1(1–31), like those of big ET-1, are both sensitive to blockade by phosphoramidon, a dual ECE/NEP inhibitor (Matsumura *et al*., 1990). In contrast, we found that thiorphan, a selective NEP inhibitor (Noble *et al*., 1999), exerted distinct influences on ET-1(1–31)-induced increases in MAP and PIP. Whereas thiorphan modestly reduced the magnitude of the second phase of the peptide's pressor effect, it markedly and dose-dependently inhibited its effect on PIP. In addition, thiorphan also abolished the eicosanoid-releasing effect of ET-1(1–31) in isolated perfused lungs. This later finding corroborates the report of Hayasaki-Kajiwara *et al*. (1999), showing that ET-1(1–31)-induced changes in intracellular calcium concentration in human cultured bronchial smooth muscle cells are sensitive to blockade by NEP inhibitors. Altogether, these results would favour the view that NEP plays a more important role than ECE in bringing about the pulmonary effects of ET-1(1–31) (as well as in those of big ET-1), whereas the peptide's pressor effects seems to depend more on its conversion by ECE than NEP. Previous studies have also shown that the bronchoconstrictive effects of big ET-1 in the guinea-pig *in vivo* (Pons *et al*., 1992), as well as its contractile effect in isolated parenchymal strips (Battistini *et al*., 1995; Lebel *et al*., 1996) involves its conversion by a NEP-mediated (i.e. thiorphan-sensitive) pathway.

Nonetheless, as phosphoramidon causes dual ECE/NEP blockade and thiorphan, by selectively blocking NEP, also protects ET-1 from degradation, it is difficult to draw firm conclusions regarding the relative contributions of both enzymes in ET-1(1–31) conversion based solely on the differential susceptibility of the peptide's effects to modification by either compound. We attempted to circumvent this problem by testing the influence of a potent and selective ECE inhibitor, CGS 35066 (Jeng *et al*., 2000), on the effects of ET-1(1–31) (and ET-1) *in vivo*. This compound (at 1 mg kg^{-1}) was found to selectively reduce, but by no means

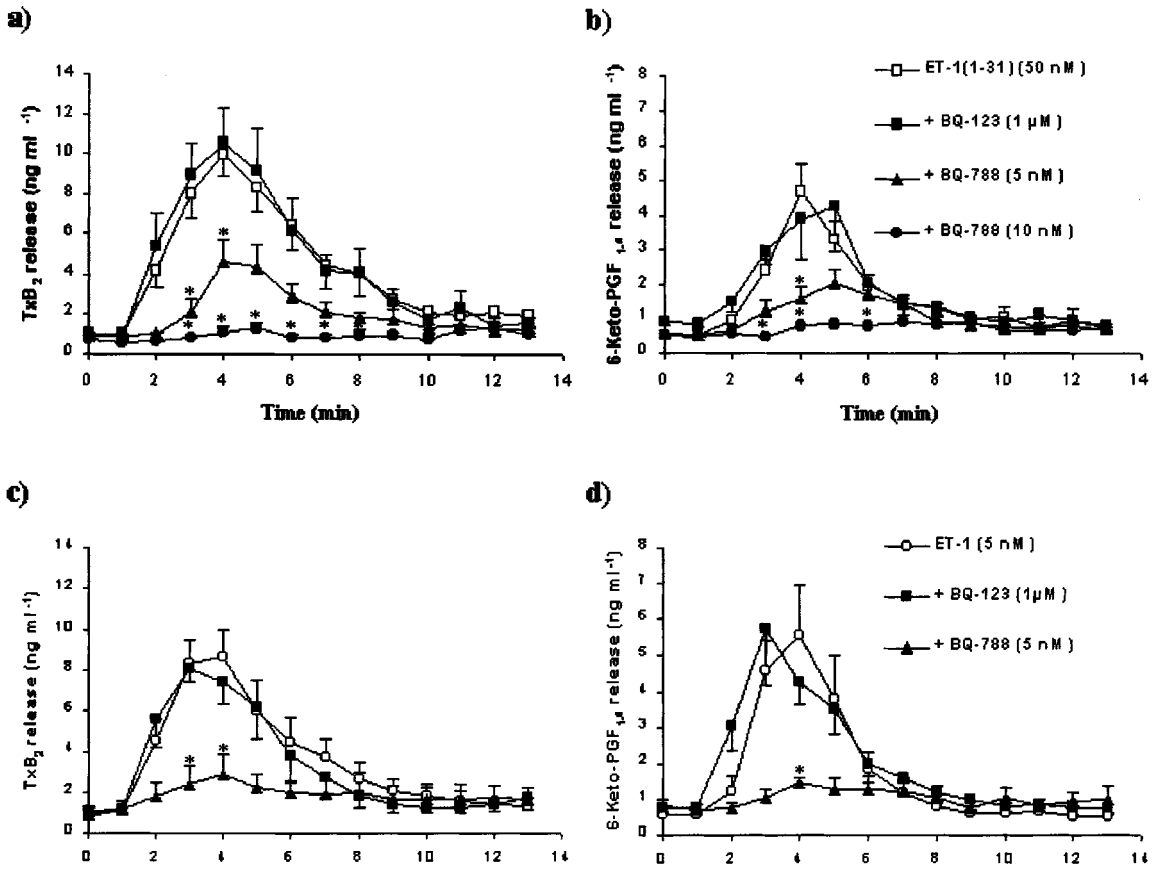

Figure 9 Influence of BQ-123 (selective ET_A receptor antagonist, 1 μM) and BQ-788 (selective ET_B receptor antagonist, 5 and 10 nM) on the release of thromboxane B_2 (**a** and **c**) and 6-Keto-$PGF_{1\alpha}$ (**b** and **d**) induced by ET-1(1–31) (50 nM, in **a** and **b**) or ET-1 (5 nM, **c** and **d**) in the guinea-pig isolated and perfused lung. Each point represents the mean ± s.e.mean of at least seven different experiments. $^*P<0.05$ when compared to the corresponding control value using ANOVA followed by the Dunnett's test.

abolish, both the second phase of the pressor response and the PIP-increasing effects of ET-1(1–31), without modifying the effects of ET-1. These results thus confirm significant contributions of ECE towards the pressor and PIP-increasing effects of ET-1(1–31). Unfortunately however, as the 100-fold selectivity of CGS 35066 towards inhibition of ECE over NEP, seen *in vitro*, is lost *in vivo* at doses higher than 1 mg kg^{-1} (Jeng *et al*., 2000; Trapani *et al*., 2000), we were unable to accurately determine to what extent conversion of ET-1(1–31) *via* the ECE pathway is responsible for the peptide's effects on MAP and PIP in the guinea-pig, relative to NEP.

On the other hand, with the use of selective antagonists for ET_A and ET_B receptors (BQ-123 and BQ-788, respectively), we demonstrated here that the sustained (second phase) pressor response to ET-1(1–31) is solely dependent on ET_A receptors. In contrast, the transient (first phase) pressor response to ET-1(1–31) was unaffected by the selective ET_A antagonist, as well as by the selective ET_B antagonist.

Considering that indomethacin potentiated the first phase pressor response to ET-1(1–31), we suggest that the peptide may transiently trigger the release of vasodilatatory eicosanoids in the systemic circulation, which in turn limit the first phase of the pressor response. Indeed, we have previously demonstrated that vasodilatatory eicosanoids effectively modulate the pressor effects of ET-1 or selective ET_B receptor agonists in the guinea-pig (Lewis *et al*., 1999). In addition, the resistance of the transient pressor response to ET-1(1–31) to inhibition by BQ-123 and the poor efficacy of the ET_A antagonist against ET-1 as described in the current study, are in agreement with Noguchi *et al*. (1993) who observed similar results with ET-1 in the anaesthetized guinea-pig. Interestingly, in our hands, simultaneous blockade of both ET_A and ET_B receptors partially reversed the intensity of blockade of the second phase pressor response to ET-1(1–31) seen with the ET_A receptor antagonist alone. This particular result reveals that ET-1(1–31) may also generate, *via* ET_B receptor activation, endogeneous inhibitors of the sustained component (second phase) of its pressor action. We have previously shown that a similar phenomenon occurs with respect to the influence of selective versus mixed (i.e. combined ET_A/ET_B receptor blockade) ET receptor antagonists against ET-1-induced vasoconstriction in the rabbit perfused kidney (Maurice *et al*., 1997).

In contrast to the pressor effect of ET-1(1–31), the PIP response predominantly involves the activation of ET_B receptors, as it was reduced by about 45% by BQ-788, but was unaffected by BQ-123. Here again, we wished to confirm that the same receptor population responsible for the bronchoconstrictive properties of ET-1(1–31) would also generate the release of thromboxane A_2 from the pulmonary circulation, as previously demonstrated for ET-1 (Lewis *et*

al., 1999). Our results clearly show that the eicosanoid-releasing properties of ET-1(1–31) are solely dependent on the activation of ET_B receptors in the isolated perfused lungs of the guinea-pig. On the other hand, unlike ET-1, ET-1(1–31) seems to also trigger a residual bronchoconstrictive effect which depends on eicosanoids, as revealed by the significant (about 50% of the total response) indomethacin-insensitive component of the PIP response. Interestingly, this finding, allied to the fact that the ET-1(1–31)-induced increase in PIP was unaffected by BQ-123 alone but abolished by co-administration of BQ-123 plus BQ-788, demonstrates that the ET_A receptor-mediated component of the PIP response to this peptide only appears when the ET_B receptor-mediated generation of COX-derived eicosanoids is blocked. The need to block both receptor types in order to fully abrogate ET-1-induced constriction in guinea-pig airways has been previously reported (Nagase *et al*., 1995). Thus, both receptor types are involved in the bronchoconstrictive properties of ET-1(1–31) in guinea-pig airways.

Previous studies have advocated that ET-1(1–31) displays selectivity of action towards either ET_A (Mazzocchi *et al*., 2000; Rebuffat *et al*., 2001) or ET_B receptors (Niwa *et al*., 2000). Our findings demonstrate, however, that both receptor types are implicated in the MAP and PIP increasing effects of this peptide in the guinea-pig *in vivo*. Rather, they add further support to the report of Goldie *et al*. (2000), showing that ET-1(1–31) contracts rat isolated airways via activation of both ET_A and ET_B receptors.

In summary, the current study, which to our knowledge is the first on this peptide to be carried out *in vivo*, reports that ET-1(1–31) displays significant pressor and eicosanoid-dependent bronchoconstrictive properties in the anaesthetized guinea-pig. Both ET_A and ET_B receptors are involved in these *in vivo* effects of the peptide, which also depend critically, but to varying extents, on conversion of ET-1(1–31) to an active metabolite, most likely ET-1, but this remains to be confirmed.

The authors gratefully acknowledge Helen Morin for her secretarial assistance and Doctor Arco Jeng for providing us with the CGS 35066. This project was financially supported by the Canadian Institutes for Health Research (MT-12889 and R-13272) and the Heart and Stroke Foundation of Québec. P D'Orléans-Juste and GA Rae are Senior Scholars of the Fonds de la recherche en santé du Québec and the Brazilian National Research Council (CNPq), respectively.

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(Received April 3, 2002
Accepted April 3, 2002)