Contractile activity of big endothelin-1 on the human isolated bronchus

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1 We have studied the contractile activity of the 39 amino acid precursor of endothelin-1 (ET-1), big endothelin-1 (big ET-1), on human isolated bronchi. The contribution of the metalloproteases, neutral endopeptidase (NEP) and angiotensin converting enzyme (ACE), in the presence or absence of the epithelium lining, by use of specific inhibitors, was also evaluated on the effects of big ET-1.

2 Big ET-1 elicited a potent contraction of human isolated bronchus. The $-\log EC_{50}$ value for big ET-1 was 7.53 ± 0.08 ($n = 11$) and E_{max} $78.5 \pm 3.8\%$ (% of ACh 3 mM).

3 Incubation of human isolated bronchi with the NEP inhibitor phosphoramidon (10^{-5} M) induced a rightward shift of the concentration-response curve induced by big ET-1 (10^{-9} M to 3×10^{-7} M). Similar results were observed when human bronchi were incubated with thiorphan (10^{-5} M), but the shift to the right was significantly less ($P < 0.01$) than that observed in the case of phosphoramidon (-0.35 ± 0.05 vs -0.67 ± 0.07 log unit).

4 The two inhibitors of angiotensin I converting enzyme (ACE), captopril or enalapril diacid, did not affect the concentration-response curve for contraction induced by big ET-1.

5 When the epithelium was removed, a leftward shift of the concentration-response curve of big ET-1 (10^{-9} M to 3×10^{-7} M) was observed. Incubation of human isolated bronchi with phosphoramidon or thiorphan (10^{-5} M) or with enalapril diacid or captopril did not modify the leftward shift of the concentration-response curve for big ET-1 after epithelium removal.

6 These results suggest that big ET-1 elicits potent contractile activity in the human isolated bronchus and that its effect is the consequence of the conversion to ET-1 by a phosphoramidon-sensitive metalloprotease which, although different from NEP and ACE, appears to be similar to the endothelin-converting enzyme (ECE) described in other studies in animals.

Keywords: Big-Endothelin (Big ET-1); human isolated airways; metalloproteases; phosphoramidon; thiorphan

Introduction

The 21-amino acids peptide, endothelin (ET-1), is one of the most potent contractile agents of guinea-pig and human airway smooth muscle *in vitro* (Uchida *et al.*, 1988; Maggi *et al.*, 1989; Hay, 1989; Advenier *et al.*, 1990; Sarrià *et al.*, 1990). Furthermore, ET-1 administered either intravenously or by aerosol in the guinea-pig *in vivo* induces a marked and sustained bronchoconstriction (Payne & Whittle, 1988; Macquin-Mavier *et al.*, 1989; Lagente *et al.*, 1989), suggesting a role for endothelin in airways dysfunction.

By analysis of the nucleotide sequence of the cDNA encoding for ET-1, Yanagisawa *et al.* (1988) proposed that the vasoactive peptide endothelin (ET-1) is produced by a 39-amino-acid precursor named big endothelin (big ET-1). The vasoconstrictor and bronchoconstrictor activities of big ET-1 are much lower than those of ET-1, and conversion of the precursor appears to be essential for the pathophysiological actions of ET-1 (Kimura *et al.*, 1989; Fukuroda *et al.*, 1990; D'Orléans-Juste *et al.*, 1991; Pons *et al.*, 1992). ET-1 is formed from big ET-1 via a single cleavage between amino acids Trp-21 and Val-22. Therefore, the C-terminal fragment (22–39), as well as big ET-1 and ET-1, are present in the culture medium of endothelial cells (Emori *et al.*, 1989; Sawamura *et al.*, 1989). However, the enzyme responsible for this processing has not yet been identified precisely, but it seems to be a metalloprotease and has been called ET-converting enzyme (ECE) (Sawamura *et al.*, 1991; McMahon *et al.*, 1991). Vascular endothelium cells possess such a metalloprotease potentially responsible for the metabolism of big ET-1 into ET-1 via a single cleavage, and this enzyme

appears to be sensitive to phosphoramidon (Matsumura *et al.*, 1990a; McMahon *et al.*, 1991). It has recently been demonstrated that phosphoramidon suppresses the hypertensive (Matsumura *et al.*, 1990b; McMahon *et al.*, 1991) effects and modulates the bronchoconstrictor (Fukuroda *et al.*, 1990; Pons *et al.*, 1992) effects of big ET-1 in rats and guinea-pigs respectively.

The ET-converting enzyme appears to be different from other metalloproteases, especially the angiotensin I converting enzyme (ACE) and the neutral endopeptidase 3.4.24.11 (NEP) which are widely distributed and involved in the metabolism of a large variety of hormonal, neuromodulator and neurotransmitter peptides (Erdős, 1984; Turner *et al.*, 1985). In human lung tissues, ACE is localized at the luminal surface of the endothelium, whereas NEP appears to be concentrated within the epithelial cells (Johnson *et al.*, 1985; Naline *et al.*, 1989) and participates in the metabolism of ET-1 in the human and guinea-pig bronchus (Advenier *et al.*, 1990; Candenas *et al.*, 1992).

This led us to study the contractile activity of big ET-1 on the human isolated bronchus. The contribution of the metalloproteases NEP and ACE in the presence or absence of the epithelium lining (by use of specific inhibitors) were also evaluated on the effects of big ET-1.

Methods

Human bronchial tissue preparation

Bronchial tissues were removed from 19 patients with lung cancer at the time of the surgical operation (18 men and 1 women; mean age, 58.4 ± 3.4 years; 12 squamous cell car-

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cinomas, 4 oat cell carcinomas and 3 adenocarcinomas). Just after resection, segments of human bronchi with an inner diameter of 3 to 5 mm were taken as far away as possible from the malignancy. They were placed in oxygenated Krebs-Henseleit solution (composition, mM: NaCl 119, KCl 4.7, CaCl₂ 2.5, KH₂PO₄ 1.2, MgSO₄ 1.2, NaHCO₃ 25.0 and glucose 11.7) at 4°C and stored overnight at 4°C. After removal of the adhering fat and connective tissue, two to eight adjacent rings from the same bronchus were prepared and suspended in organ baths (10 ml) under an initial tension of 2.5 g in Krebs-Henseleit solution, bubbled with 95% O₂:5% CO₂ and maintained at 37°C. The epithelium of some rings was removed by gently rubbing the luminal surface (over both smooth muscle and cartilage areas) with a cotton tipped applicator (Naline *et al.*, 1989). Changes of force of contraction were measured isometrically with strain gauge amplifiers and I.O.S.-Moise 3 recorder system (Dei Lierre, Mitry Mory, France).

Protocol

Each experiment began with contraction of the bronchial strips to maximal contractile force with acetylcholine (ACh, 3 mM), then maximal relaxation was induced with theophylline (3 mM). During the next 120 min, the tissues were washed every 15 min, and the resting tension was adjusted from 2 to 2.5 g, which has been found to be optimal for recording contractions in such tissues (Advenier *et al.*, 1986; Naline *et al.*, 1989). Thereafter, cumulative concentration-response curves to either ET-1 or big ET-1 were constructed by applying increasing concentrations at 15 to 20 min intervals in logarithmic increments. Acetylcholine (3 mM) was added at the end of the concentration-response curve to determine the maximal response of the preparation. In some experiments, phosphoramidon, thiophan, captopril or enalapril diacid (MK 422) at a final concentration of 10⁻⁵ M, were added to the bath 30 min before the contractile agents. In all experiments, only one concentration-response curve to ET-1 or big ET-1 was recorded in each strip. Concentrations of big ET-1 up to 3 × 10⁻⁷ M were not tested because of the limited availability and the cost of the peptide.

The data are expressed in terms of -log EC₅₀ for potency and E_{max} for efficacy. -log EC₅₀ values were derived graphically from the log concentration-effect curves and defined as the negative log of the drug concentration that caused 50% of maximal effect of ACh 3 mM. The maximal effect (E_{max}) was calculated as the maximal increase in tone induced by ET-1 or big ET-1 and expressed as a percentage of the maximum tension induced by ACh 3 mM.

The maximal contractile force obtained with acetylcholine (3 mM) was not affected by thiophan, phosphoramidon, captopril and enalapril diacid.

Statistical evaluation of data

Statistical analysis of the results was performed by analysis of variance and with Student's *t* test for paired or unpaired data. All values in the text and tables are expressed as mean ± s.e.mean.

Drugs

Drugs used were: endothelin-1 (ET-1) (human, porcine) and big endothelin-1 (big ET-1) (human) (Peptide Institute Inc, Osaka, Japan), acetylcholine dihydrochloride (PCH, Paris, France), thiophan (Sigma, St Louis, U.S.A.), phosphoramidon [N-(alpha-L-rhamnopyranosyloxy-hydroxy-phosphinyl)-L-leucyl-L-tryptophan] (Sigma), captopril (Squibb, Paris, France), enalapril diacid (MK 422, Merck, Paris, France). All agents were dissolved in distilled water. As with the other drugs, these solutions were diluted with Krebs solution.

Results

Contratile activity of big endothelin-1

Big ET-1 elicited a potent concentration-dependent contraction of the human isolated bronchus. The -log EC₅₀ value for big ET-1 was 7.53 ± 0.08 (*n* = 11) and E_{max} 78.5 ± 3.8% (% of E_{max} for ACh). The big ET-1-induced contraction appeared similar to that of ET-1 (-log EC₅₀ = 7.64 ± 0.13, E_{max} = 81.7 ± 4.8, *n* = 11) (Figure 1).

Effect of enzymatic inhibitors on the contractile activity of big endothelin-1

Incubation of human isolated bronchi with phosphoramidon (10⁻⁵ M) induced a rightward shift of the concentration-response curve induced by big ET-1 (10⁻⁹ M to 3 × 10⁻⁷ M) (Figure 2a). The displacement of the concentration-response curve was significant as demonstrated by the difference between the -log EC₅₀ values before and after incubation with phosphoramidon (-0.67 ± 0.07 log unit, *P* < 0.001) (Table 1). Similar results were observed when human bronchi were incubated with thiophan (10⁻⁵ M) (Figure 2b).

Figure 1 Response of the human isolated bronchus to endothelin-1 (ET-1, ●) or big endothelin-1 (big ET-1, ○). Points represent means; vertical bars represent s.e.mean. *n* = 11.

Figure 2 Response of the human isolated bronchus to big endothelin-1 (big ET-1) in the absence (○) or presence (●) of (a) phosphoramidon (10⁻⁵ M), (b) thiophan (10⁻⁵ M), (c) captopril (10⁻⁵ M) and (d) enalapril diacid (MK 422) (10⁻⁵ M). Points represent means; vertical bars represent s.e.mean. *n* = 5 to 9.

Table 1 Effect of various enzyme inhibitors of neutral endopeptidase (phosphoramidon, thiorphan) or angiotensin converting enzyme (captopril, enalapril diacid) on big endothelin-1 (big ET-1) induced contraction of human isolated bronchi

Inhibitors	n	Big ET-1 ($-\log EC_{50}$)		Shift of the C/R curves (log unit)
		Pre-drug	Post-drug	
Phosphoramidon 10^{-5} M	9	7.51 ± 0.09	6.84 ± 0.12	$-0.67 \pm 0.07^*$
Thiorphan 10^{-5} M	5	7.40 ± 0.12	7.05 ± 0.11	$-0.35 \pm 0.05^*$
Captopril 10^{-5} M	5	7.53 ± 0.07	7.54 ± 0.05	0.01 ± 0.06 NS
Enalapril diacid 10^{-5} M	8	7.45 ± 0.10	7.38 ± 0.08	-0.07 ± 0.11 NS

n = number of experiments.

Statistical significance of the shift of the C/R curves are shown as: $*P < 0.001$ and NS: not significant.

Table 2 Effect of various enzyme inhibitors on big endothelin-1 (big ET-1)-induced contraction of human isolated bronchi in the presence (+E) or absence (-E) of epithelium ($-\log EC_{50}$ values)

Inhibitors	n	Big ET-1 ($-\log EC_{50}$)		Shift of the C/R curves (log unit)
		+E	-E	
Control	6	7.54 ± 0.14	7.94 ± 0.13	$0.40 \pm 0.05^*$
Phosphoramidon 10^{-5} M	5	6.81 ± 0.20	7.34 ± 0.17	$0.53 \pm 0.08\$$
Enalapril diacid 10^{-5} M	5	7.31 ± 0.05	7.79 ± 0.13	$0.48 \pm 0.15^\dagger$
Thiorphan 10^{-5} M	4	7.14 ± 0.09	7.55 ± 0.05	$0.41 \pm 0.12^\dagger$

n = number of experiments.

Statistical significance of the shift of the C/R curves are shown as: $*P < 0.001$; $\$P < 0.01$ and $^\dagger P < 0.05$.

Although significant ($P < 0.001$), the shift to the right of the concentration-response curve was significantly less ($P < 0.01$) than that observed with phosphoramidon (-0.35 ± 0.05 vs -0.67 ± 0.07 log unit) (Table 1).

In contrast, the two inhibitors of angiotensin I converting enzyme (ACE), captopril or enalapril diacid, did not affect the concentration-response curve induced by big ET-1 (Figure 2c and d) and consequently no significant difference was noted between the $-\log EC_{50}$ values before and after incubation with captopril or enalapril diacid (Table 1).

Effect of epithelium removal on the contractile activity of big endothelin-1

When epithelium was removed, a leftward shift of the concentration-response curve of big ET-1 (10^{-9} M to 3×10^{-7} M) was observed (Figure 3a). Indeed, the difference in $-\log EC_{50}$ values in the presence (7.54 ± 0.14) or absence (7.94 ± 0.13) of the epithelium was 0.40 ± 0.05 ($P < 0.001$, $n = 6$) (Table 2).

After incubation of human isolated bronchi with phosphoramidon (10^{-5} M), the same leftward shift (0.53 ± 0.08 , $P < 0.01$, $n = 5$) of the concentration-response curve of big ET-1 was also observed after epithelium removal, suggesting an epithelium-independent effect for the activity of phosphoramidon (Figure 3a, Table 2).

Similar results were observed when the metalloprotease inhibitors thiorphan (10^{-5} M) or enalapril diacid (10^{-5} M) were used under the same conditions (0.41 ± 0.12 , $P < 0.05$, $n = 4$ and 0.48 ± 0.15 , $P < 0.05$, $n = 5$) (Figure 3b, Table 2).

Discussion

The vasoactive peptide endothelin (ET-1) has been shown to elicit potent contractile effect on airway smooth muscle *in vitro* in guinea-pig and rat trachea (Uchida *et al.*, 1988; Maggi *et al.*, 1989; Turner *et al.*, 1989; Sarria *et al.*, 1990). On human airways, Uchida *et al.* (1988) and Advenier *et al.* (1990) also described a potent contractile activity of ET-1.

The present data demonstrate that the 39-amino acid precursor of ET-1, big endothelin-1 (big ET-1), also elicits

potent contractile activity in human isolated bronchi. In our preparations, the big ET-1-induced contraction was similar to that of ET-1. Nevertheless, ET-1 was more potent at lower concentrations (10^{-12} to 10^{-9} M) than big ET-1. These data also confirm that, in comparison to big ET-1, ET-1 produced a potent contraction of the human isolated bronchus that proceeded in two steps of different intrinsic activity (Advenier *et al.*, 1990).

These results contrast with those found in rat isolated aorta, where the vasoconstrictor activity of big ET-1 is much lower (about 1/55) than that of ET-1 (Kashiwabara *et al.*, 1989; Kimura *et al.*, 1989). When injected intravenously into ganglion-blocked anaesthetized rats, big ET-1 produces a large pressor response (Kashiwabara *et al.*, 1989). This would suggest that (1) big ET-1 itself has a direct but weak vasoconstrictor activity accounting for the low *in vitro* effect and (2) the conversion of big ET-1 to ET-1 by an endothelin converting enzyme may occur *in vivo* to induce the pressor response, although the response to big ET-1 differs somewhat from the response to ET-1 itself (McMahon *et al.*, 1991).

Figure 3 Effect of epithelium removal on the contractile response of the human isolated bronchus to big endothelin-1 (big ET-1) in the absence or presence of (a) phosphoramidon (10^{-5} M) and (b) enalapril diacid (MK 422) (10^{-5} M). Experiments with epithelium: control (○), phosphoramidon or enalapril diacid (●). Experiments after epithelium removal: control (□), phosphoramidon or enalapril diacid (■). Points represent means; vertical bars represent s.e.mean. $n = 4$ to 6.

This also indicates that big ET-1 was rapidly and almost totally converted into ET-1 in our preparation since the concentration-response curves observed with both peptides were not significantly different over the mid to upper concentration ranges. Similarly, a rapid generation and degradation of ET-1-like activity was also noted in human polymorphonuclear leukocytes (Sessa *et al.*, 1991).

Previous results showed that a metalloprotease called ET-converting enzyme (ECE) is involved in the conversion of big ET-1 to ET-1. Moreover, the protease inhibitor phosphoramidon is able to inhibit the intracellular conversion of big ET-1 to ET-1 in endothelial cells (Ikegawa *et al.*, 1990; Matsumura *et al.*, 1990a; Sawamura *et al.*, 1991; McMahon *et al.*, 1991) and to reduce the hypertensive and bronchoconstrictor effects of big ET-1 in rat and guinea-pig (Matsumura *et al.*, 1990b; Fukuroda *et al.*, 1990; D'Orléans-Juste *et al.*, 1991; McMahon *et al.*, 1991; Pons *et al.*, 1992). McMahon *et al.* (1991) also reported that in a rabbit lung membrane preparation, ECE activity was blocked by phosphoramidon and 1,10-phenanthroline in a concentration-dependent manner. Nevertheless, ECE seems to be different from neutral endopeptidase, although sensitive to phosphoramidon. In ganglion-blocked anaesthetized rats, thiorphan dose-dependently inhibited the pressor response to big ET-1, but was substantially less potent than phosphoramidon (McMahon *et al.*, 1991), even though both compounds inhibited totally NEP activity at the same concentration of 10^{-5} M (Turner *et al.*, 1985). Our results in human bronchus showed that a similar phenomenon can be observed, since it is suggested that the conversion of big ET-1 to ET-1 is under the influence of a phosphoramidon-sensitive metalloprotease and that this enzyme is also different from neutral endopeptidase (NEP), since thiorphan is less efficient than phosphoramidon in reducing the activity of big ET-1 on human isolated bronchi. This possibility has recently been strengthened by the observation that cultured vascular smooth muscle cells contain two types of metalloproteases, which convert big ET-1 to ET-1 and one of them is insensitive to phosphoramidon (Matsumura *et al.*, 1991).

However, it is not possible at this time to specify where ECE is functional in bronchial smooth muscle or near the endothelial cells of bronchial vessels where ECE was originally localized. The present data also confirm that the angiotensin-converting enzyme (ACE) is not involved in the conversion of big ET-1 to ET-1 in human pulmonary tissue, since selective inhibitors of ACE such as captopril and enalapril diacid, have no effects on the contractile activity of big ET-1.

The data obtained in the preparation of human airways after epithelium removal, also suggest the existence of a specific enzyme different from NEP and responsible for the degradation of big ET-1. It has been shown that bronchial epithelium largely modulates the contractile or relaxant activities of various agents. Several hypotheses have been put forward to explain the modulatory role of epithelium. The first hypothesis is to consider epithelium as a diffusion barrier, the removal of which facilitates access of substances to submucosal structures, including smooth muscle, resulting in an increased responsiveness to drug (Small *et al.*, 1990; Iriarte *et al.*, 1990). The second hypothesis is to consider that the epithelium may act as a site of loss (i.e. site of meta-

bolism), limiting the interaction of the agent with its receptor on smooth muscle by reducing its concentration in proportion to the epithelium-dependent rate of degradation. The arguments for this kind of explanation are mainly based on the comparison of the concentration-response curves in the absence or presence of enzyme inhibitors, such as thiorphan or phosphoramidon, which hydrolyse substance P and vasoactive intestinal peptide (VIP) (Devillier *et al.*, 1988; Farmer & Togo, 1990) transport inhibitors (dipyridamole for adenosine) (Advenier *et al.*, 1988). The third hypothesis is that the airway epithelium may release one or more inhibitory factors called epithelium-derived inhibitory factor(s) (or EpDIF) which are able to down-regulate airway responsiveness. The nature of the EpDIF released seems to vary with species and spasmogen agents. It might be a prostanoid, as shown by the effect of cyclo-oxygenase inhibitors on the concentration-response curve to cholinomimetics, leukotrienes and peptides in the guinea-pig isolated trachea or rabbit isolated bronchus (Flavahan *et al.*, 1985; Tschirhart & Landry, 1986), but the release of a non-prostanoid inhibitory factor has been suggested by Fernandes & Goldie (1990).

In the human bronchus, Candenas *et al.* (1992) have found that removal of the epithelium potentiated ET-1-induced contraction. This was shown by a leftward shift of the dose-response curve similar to that observed after incubation of the bronchus with phosphoramidon. This suggests that the neutral endopeptidase plays a role in the degradation and inactivation of ET-1 in the bronchopulmonary system *in vitro* and *in vivo* (Hay, 1989; Boichot *et al.*, 1991; Candenas *et al.*, 1992). Therefore, inhibition of the neutral endopeptidase by phosphoramidon leads to potentiation of the contractile activity of ET-1. On the other hand, in the presence of phosphoramidon, removal of the epithelium does not induce an additional leftward shift of the concentration-response curve to ET-1, suggesting that only epithelium plays a role in the metabolism of this peptide (Candenas *et al.*, 1992).

The situation was markedly different when big ET-1 was used. Removal of the epithelium elicited, as in the case of ET-1, a leftward shift of the dose-response curve to big ET-1, resulting in potentiation of the contractile activity of big ET-1; but in the absence of epithelium, pretreatment with phosphoramidon induced a rightward shift of the concentration-response curve to big ET-1. Metabolic activity of the epithelium cannot be retained and confirms that the enzyme involved in the conversion of big ET-1 to ET-1 is different from that responsible for the degradation of ET-1 (NEP). In contrast, as bronchial epithelium removal potentiates the contractile activity of big ET-1, it is possible to regard the epithelium as a diffusion barrier. The release of a cyclo-oxygenase-derived epithelial mediator under the influence of big ET-1 is not suggested, since indomethacin (3×10^{-6} M) did not modify the concentration-response curve to big ET-1 in the human isolated bronchus (shift of the concentration-response curve: $+0.09 \pm 0.07$ log unit, $n = 4$, unpublished data).

In conclusion, this study shows that in the human isolated bronchus, big ET-1 elicits potent contractile activity, and that this effect is the consequence of the conversion to ET-1 by a phosphoramidon-sensitive metalloprotease which is different from NEP and ACE, but appears to be similar to the ECE described in other studies in animals.

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