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Contraction to big endothelin-1, big endothelin-2 and big endothelin-3, and endothelin-converting enzyme inhibition in human isolated bronchi

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1 All three endothelin precursor peptides, i.e. big endothelin-1 (big ET-1), big endothelin-2 (big ET-2) and big endothelin-3 (big ET-3), produced contractile responses in human isolated bronchi, demonstrating the presence of functional endothelin-converting enzyme (ECE) in this tissue.

2 The maximal contractile responses were equal to 108.4±8.0% (0.1 μM big ET-1; $n=4$), 85.2±11.8% (0.1 μM big ET-2; $n=7$) and 43.0±7.2% (0.1 μM big ET-3; $n=5$) of the reference response to acetylcholine (1 mM).

3 The response to big ET-1 (0.1 μM), but not endothelin-1 (ET-1, 0.1 μM), was diminished after overnight storage of the tissue at 4°C, demonstrating instability of the enzyme.

4 The responses to all three big-endothelins were significantly inhibited, by the ECE inhibitors CGS 26393 and CGS 26303, in a concentration-related manner.

5 The responses to the mature peptides ET-1, endothelin-2 (ET-2), and endothelin-3 (ET-3) were unaffected by CGS 26393 and CGS 26303.

6 Phosphoramidon (10 μM) also produced an inhibition of the response to big ET-1 that was equivalent to that produced by CGS 26393 (10 μM). Combination of CGS 26393 (10 μM) and phosphoramidon (10 μM) did not produce an additive inhibition.

7 These results demonstrate the presence of functional ECE for all three big endothelins in human bronchus and inhibition of the enzyme by newly developed orally active ECE inhibitors, as well as phosphoramidon.

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Keywords: Big endothelin; endothelin; human isolated airways; endothelin-converting enzyme; CGS 26393; CGS 26303; phosphoramidon

Abbreviations: AUC, area under the curve; big ET-1, big endothelin-1; big ET-2, big endothelin-2; big ET-3, big endothelin-3; ECE, endothelin converting enzyme; ET-1, endothelin-1; ET-2, endothelin-2; ET-3, endothelin-3; NEP, neutral endopeptidase

Introduction

The endothelins (ETs) are a family of three 21 amino acid peptides, each encoded by a separate gene. The ETs are potent bronchoconstrictors and have been shown to be present in, and released from, various human airway cells. All three forms of endothelin have contractile activity in human isolated airways although there are differences in potency among the isoforms (Advenier *et al*., 1990; McKay *et al*., 1991). Each isoform is produced from a unique inactive precursor peptide, termed big ET-1, big ET-2 or big ET-3, *via* catalysis by endothelin-converting enzymes. The enzymes are metalloproteases as they are sensitive to phosphoramidon. ECE-1 and ECE-2 have been shown to convert big ET-1 more efficiently than the other big ETs (Xu *et al*., 1994; Emoto & Yanagisawa, 1995). In an immunohistochemical study, ECE-1 was localized primarily to the epithelium of human airway tissue, with small amounts also localized to airway and vascular smooth muscle (Saleh *et al*., 1997). This report was consistent with the previous demonstration by Advenier *et al*. (1992) that big ET-1 contracted human isolated airways, which had indicated that a converting enzyme was present within the airway wall.

The potential of ECE inhibitors as pharmacological therapeutic agents has promoted the development and release of a number of compounds. CGS 26393 and CGS 26303 are two recently developed non-peptide dual ECE and neutral endopeptidase (NEP) inhibitors, the former being an orally active prodrug. These inhibitors have equivalent activity on ECE-1 and ECE-2 (Battistini & Botting, 1995) and inhibit bronchoconstriction induced by big ET-1 in the perfused rat lung (Held *et al*., 1997). While contraction in response to big ET-1 has been demonstrated in human isolated airways, there are no reports of the effects of the other precursor peptides, big ET-2 and big ET-3, nor the effect of the newly developed ECE inhibitors in human airways. In this study, we therefore examined the effects of all three big-ETs on human bronchus, as well as the effect of inhibition of ECE on these responses, and on responses to the three mature forms of ET. Finally, we have compared the effects of inhibition with CGS 26393 and phosphoramidon on the contractile response to big ET-1.

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Methods

Human bronchial tissue preparation

All studies were performed on human lung obtained at resection or at transplant in accordance with methods approved of by the Human Ethical Review Committee of the University of Sydney. Bronchi measuring 3–5 mm internal diameter were dissected from the lung and cut into rings of 5 mm length. The bronchial rings were mounted between stainless steel hooks in 5 ml jacketed tissue baths containing Krebs-Henseleit solution (composition in mM): NaCl 118.4, KCl 4.7, $CaCl_2.2H_2O$ 2.5, $MgSO_4.7H_2O$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25.0 and D-glucose 11.1, maintained at 37°C and continuously bubbled with carbogen (a mixture of 5% carbon dioxide and 95% oxygen). Care was taken to ensure that no damage was done to the epithelium during preparation and mounting of the bronchial rings. The tissue baths were washed with hydrochloric acid and treated with silicon fluid each week to prevent adherence of peptides to the glass. The upper stainless steel hook was attached to a Grass FT03 isometric force transducer which was connected to a MacLab analogue to digital electronic recording system running 'Chart' version 3.5 (ADI Instruments, Sydney, Australia) which was calibrated prior to each experiment. A 1–2 g preload was applied to the rings depending upon size, and the tissues were allowed to equilibrate over a 1 h period during which the tissue baths were flushed with fresh carbogenated Krebs-Henseleit solution at 15 min intervals. Following the equilibration period, a reference response to 1 mM acetylcholine was obtained in every tissue before use in the following series of experiments.

Response to big ET-1 and ET-1 in stored and unstored tissues

In this series of experiments, four bronchial rings from each patient were used, two rings were used immediately after being obtained from the operating theatre (the tissues were mounted in organ baths within 120 min of removal of the lung from the patient) and the remaining two rings were used after overnight storage (for a period of 16 h, as is our usual practice) in carbogen saturated Krebs-Henseleit solution which was maintained at 4°C. After the reference response to acetylcholine had been obtained in each tissue, the tissues were rinsed with Krebs-Henseleit solution until stable baseline tone was regained. Big ET-1 (0.1 μM) was then added to the tissues and the response assessed at 2 min intervals over a 90 min period (during which time a plateau was attained). A second series of experiments was performed as above; in this series however, ET-1 (at 0.1 μM) was used in place of big ET-1 in four additional tissues from each patient.

Responses to big ET-1, big ET-2 and big ET-3 with and without ECE inhibition

In these series of experiments, eight bronchial rings from each patient were used immediately after receipt of the lung from the operating theatres. The rings were formed into four pairs of tissues and the reference response to 1 mM acetylcholine was obtained. The tissues were washed until baseline tone was regained and the tissues were then incubated in CGS 26393 at 0 (vehicle alone), 1, 10 or 100 μM (one pair was used for each concentration). After a 30 min incubation period, 1 mM acetylcholine was added to each bath and the maximal response recorded. In separate series of experiments, big ET-1 (0.1 μM), big ET-2 (0.1 μM) and big ET-3 (0.1 μM) were used in place of the second addition of acetylcholine and the response was monitored at 2 min intervals for 90 min. In a further series, CGS 26393 was replaced by CGS 26303 (also at 0, 1, 10 and 100 μM).

Response to ET-1, ET-2 and ET-3 with and without ECE inhibition

Four tissues from each patient were used in each experiment in these series, again the tissues were not stored. The tissues were paired and after the reference response to 1 mM acetylcholine had been elicited and the tone had returned to baseline after washing, one tissue from each pair was incubated in CGS 26393 at 100 μM for 30 min. The remaining tissue from each pair was incubated in vehicle for 30 min before the addition of ET-1 (0.1 μM) to all four tissues and the response monitored and recorded as above, for 90 min. In separate series, these experiments were repeated with ET-2 and ET-3 instead of ET-1, and CGS 26303 (100 μM) instead of CGS 26393.

Response to big ET-1 in the presence and absence of CGS 26393 and/or phosphoramidon

In a final series of experiments, four pairs of tissues from each patient were used immediately following surgery. The reference response to 1 mM acetylcholine was obtained as above and the tissues washed until baseline tone was regained. One pair of tissues was incubated in CGS 26393 (10 μM), one pair in phosphoramidon (10 μM), one pair in the presence of both CGS 26393 (10 μM) and phosphoramidon (10 μM) and the fourth pair of tissues in vehicle alone. After 30 min, big ET-1 (0.1 μM) was added to each tissue and the response recorded as above, for 90 min.

Drugs used

Big ET-1 (porcine, 1–39), big ET-2 (human, 1–38), big ET-3 (human, 1–41 amide) and ET-2 (human, canine) were purchased from The Peptide Institute (Osaka, Japan). ET-1 (human) and ET-3 (human, rat) were obtained from Auspep (Melbourne, Australia). Acetylcholine and phosphoramidon [N-(α-L-rhamnopyranosyloxy-hydroxy-phosphinyl)-L-leucyl-L-tryptophan] were from Sigma (St Louis, MO, U.S.A.). CGS 26393 [diphenyl ((S)-2-biphenyl-4-yl-1-(1*H*-tetrazol-5-yl)-ethylamino-methyl)phosphonate] and CGS 26303 [(s)-2-biphenyl-4-yl-1-(1*H*-tetrazol-5-yl)-ethylamino-methyl phosphonic acid] were kind gifts from Dr Arco Jeng (Novartis, Summit, NJ, U.S.A.). Silicon coating (Repelcoat) for the tissue baths was obtained from BDH (Sydney, Australia) as were the salts used for preparation of the Krebs-Henseleit solution. The big ETs, ET-2 and phosphoramidon were reconstituted in distilled water, while ET-1 and ET-3 were prepared in 0.1 M acetic acid. Stock solutions of CGS 26303 were made in 0.5 M sodium bicarbonate solution and CGS 26393 in 0.01 M sodium hydroxide solution. All dilutions were made with Krebs-Henseleit solution and were prepared on the day of experiments. The appropriate vehicle was used for each control tissue when an inhibitor was being tested.

Statistical evaluation of data

Contraction measured as mg force developed at each 2 min period in response to the big ETs and the ETs was

expressed as percentage of the reference response to acetylcholine. A curve was then drawn for each tissue plotting contraction generated at each 2 min time point over the 90 min monitoring period. The area under each curve (AUC) was then calculated using the trapezoid rule. The responses for duplicate tissues were averaged to give a single value for contraction at each 2 min interval and for the area under the curve, for each concentration of CGS 26393 or CGS 26303 and for phosphoramidon alone or in combination with CGS 26393, as well as for control tissues from each patient. For the initial experiments examining the effect of the inhibitors on the response to acetylcholine, the response to acetylcholine in the presence of CGS 26393 was expressed as a percentage of the reference response to acetylcholine. The average values for tissues from each patient in each series were then used to obtain a mean and standard error of the mean (s.e.mean) value to give an overall response for each intervention and for the respective controls. Overall mean curves were then generated using this data. Differences between the contraction curves in the presence and absence of the various concentrations and combinations of the inhibitors were analysed using ANOVA and Fisher's PLSD (*post hoc* test) at 95% significance (ANOVA). Paired Student's *t*-tests were used to compare the magnitude of the maximum contractile responses and the areas under the curves for responses to ET-1, ET-2 and ET-3 in the presence and absence of inhibitor and responses to big ET-1 and ET-1 in stored and unstored tissues. Differences were again considered statistically significant if the P value was less than 0.05.

Results

Response to big ET-1 and ET-1 in stored and unstored tissues

The contractile response to big ET-1 developed within 6 min of addition of the peptide to the tissue baths and reached a plateau by 80 min (Figure 1a). Contraction developed in tissues that were used immediately after lung resection as well as in tissues that had been stored overnight in carbogen-saturated Krebs-Henseleit solution at 4°C. The response to big ET-1 was however, significantly greater in tissues that had not been stored. In this series, the maximal response to 0.1 μM big endothelin-1 was $118.4 \pm 9.7\%$ of the reference response to acetylcholine when tissues were used immediately, and $95.8 \pm 11.4\%$ ($P<0.05$ paired *t*-test, $n=6$) after storage. There was no difference in the magnitude of the response to acetylcholine in both groups of tissues (data not shown). The area under the contraction curve also decreased significantly from 8080 ± 724 to 6797 ± 957 units ($P<0.05$ paired *t*-test, $n=6$) after tissue storage. Conversely, the response to the mature peptide, ET-1 was equivalent in tissues used immediately ($Tmax_{(Ach)}$: $97.4 \pm 11.8\%$, AUC: 7191 ± 928 units, $n=4$) and after overnight storage ($Tmax_{(Ach)}$: $101.6 \pm 12.1\%$, AUC: 7177 ± 953 units). As it was therefore likely, that ECE activity was decreased by tissue storage, all other experimentation in this study was carried out immediately after lung resection.

Response to big ET-1, big ET-2 and big ET-3 with and without ECE inhibition

Contraction of human bronchus also occurred in response to big ET-2 and big ET-3. As with big ET-1, the contractile response to big ET-2 and big ET-3 was usually initiated within 6 min of addition of the peptide to the tissue bath (Figure 1b,c). Similarly, plateau of the contractile response to ET-2 was seen after 60–70 min. The response to big ET-3 was more variable between tissues from different patients and only began to plateau at 90 min. The maximal contractile response to 0.1 μM big ET-1 within the 90 min period was greater than that to the other big ETs, reaching 127% of the maximal response produced by 0.1 μM big ET-2 and 250% of the maximal response to 0.1 μM big ET-3 (Table 1).

CGS 26393 had no direct effect on the baseline tone of the airway tissue and no significant effect on the response to acetylcholine in human airways. The mean maximal response to acetylcholine was $118 \pm 5\%$ in tissues incubated in vehicle and 120 ± 4, 114 ± 4 and $109 \pm 6\%$ ($n=9$, $P>0.05$) in the presence of 1, 10 and 100 μM CGS 26393 respectively.

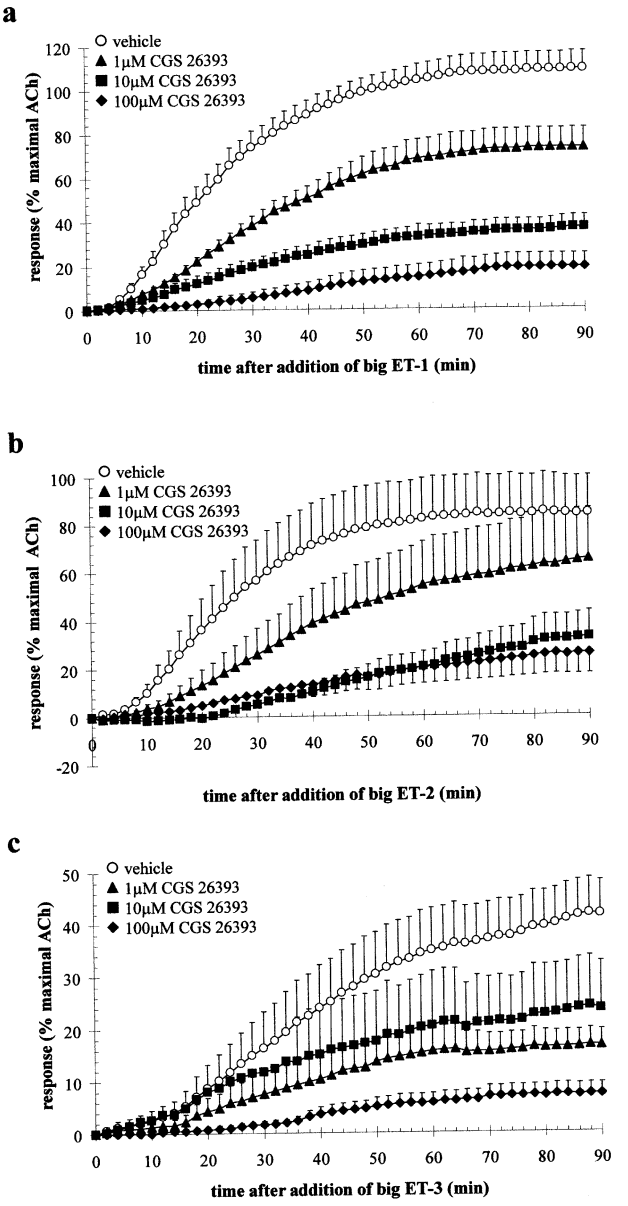

Figure 1 The mean response to (a) 0.1 μM big ET-1 ($n=4$), (b) 0.1 μM big ET-2 ($n=6$) and (c) 0.1 μM big ET-3 ($n=5$) in human isolated airways used immediately after surgical resection. Contractile responses in the presence and absence of CGS 26393 are expressed as a percentage of the reference response to 1 mM acetylcholine, and s.e.mean values are shown as vertical bars.

Table 1 The effect of CGS 26393 on the response to the three big ETs and three ETs (all at 0.1 μM) in human bronchus used immediately after surgical resection

		CGS 2693			
		0 μM	*1 μM*	*10 μM*	*100 μM*
big endothelin-1 ($n=4$)	Tmax(%)	108.4±8.0	67.1±141*	35.6±5.1*†	18.8±6.2*†
	AUC (units)	7007±426	4301±483*	2129±342*†	873±314*†
endothelin-1 ($n=3$)	Tmax	102.8±7.1	–	–	108.1±6.4
	AUC (units)	6777±265	–	–	6970±425
big endothelin-2 ($n=7$)	Tmax	85.2±11.8	63.2±14.7	29.0±8.4*†	25.7±5.9*†
	AUC (units)	5227±893	3023±846*	1072±317*†	1067±305*†
endothelin-2 ($n=5$)	Tmax	76.9±7.6	–	–	79.1±5.7
	AUC (units)	5492±541	–	–	5266±288
big endothelin-3 ($n=5$)	Tmax	43.0±7.2	18.3±4.4*	23.4±9.8*	8.0±2.1*
	AUC (units)	2143±541	927±287	1312±674	329±96*
endothelin-3 ($n=3$)	Tmax	101.5±6.7	–	–	115.0±17.3
	AUC (units)	7002±1064	–	–	7872±1215

Statistically significant differences (assessed by ANOVA and Fisher's PLSD *post hoc* test) are indicated as follows: $*P<0.05$ significantly different from responses in the absence of CGS 26393, and $\dagger P<0.05$ significantly different from response in presence of 1 μM CGS 26393.

Pretreatment of the tissues with CGS 26393 did however, result in a significant concentration-related decrease in the maximal response, as well as the area under the contraction curve to big ET-1 (Table 1, Figure 1a). The maximal response to 0.1 μM big ET-1 was decreased by 38% in the presence of 1 μM, by 67% in the presence of 10 μM and by 83% in the presence of 100 μM CGS 26393. The areas under the curves were decreased by similar proportion *viz*; 39, 70 and 88%, respectively.

As with big ET-1, the magnitude of the response to big ET-2 was also attenuated in a concentration-related manner by CGS 26393 (Table 1, Figure 1b). The maximal response to 0.1 μM big ET-2 was decreased by 26% in the presence of 1 μM, by 66% in the presence of 10 μM and by 70% in the presence of 100 μM CGS 26393. There was a transient relaxation response to big ET-2 in tissues from five of the seven patients used in this series of experiments. This relaxation response was generally of very low magnitude (less than 5% of the reference response to acetylcholine in tissue from four patients), and was of 4–36 min duration before onset of the contractile response.

Big ET-3 also induced a contractile response in human bronchus (Table 1, Figure 1c). This response was also decreased in the presence of CGS 26393 at all three concentrations examined. The maximal response to 0.1 μM big ET-3 was decreased by 57% in the presence of 1 μM, by 43% in the presence of 10 μM and by 81% in the presence of 100 μM CGS 26393. In the case of this peptide however, this inhibition was not concentration-related as the magnitude of the response to big ET-3 was equivalent in the presence of all three concentrations of CGS 26393.

CGS 26303 (1–100 μM) also inhibited the contractile response to big endothelin-1. The inhibitory effect was however different to that for CGS 26393, as inhibition was maximal with 10 μM CGS 26303. The maximal response to big ET-1 was 94.8±8.2% $Tmax_{(Ach)}$, and this was reduced to 71.1±7.6, 41.6±6.1 and 42.9±6.4% in the presence of 1, 10 and 100 μM CGS 26303, respectively ($n=7$, $P<0.05$, ANOVA in each case). The area under the contraction curve (5849±650 units) was similarly decreased, and the inhibitory effects of 10 μM (AUC: 2307±414 units) and 100 μM CGS 26303 (AUC: 2023±362 units) were equivalent. A similar concentration-related effect on the contractile response to big ET-2 was produced by pretreatment of the tissues with 1, 10 and 100 μM CGS 26303 (data not shown).

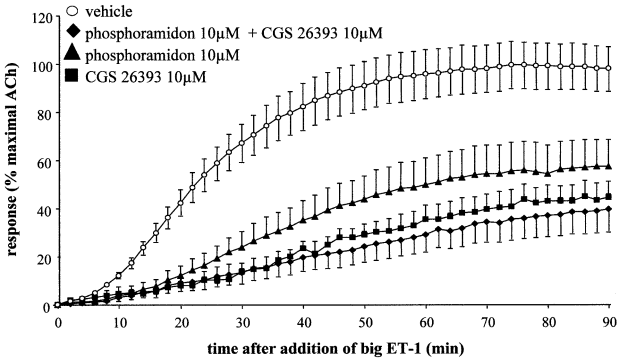

Figure 2 Comparison of the effects of phosphoramidon and CGS 26393 used alone or in combination on the mean response to 0.1 μM big ET-1 in human airways used immediately after surgical resection. Mean contractile responses for five experiments are expressed as a percentage of the reference response to 1 mM acetylcholine, and s.e.mean values are shown as vertical bars.

Response to ET-1, ET-2 and ET-3 with and without ECE inhibition

Neither the time for initiation, nor the duration of the contractile responses to ET-1, ET-2 or ET-3 were affected by the presence of 100 μM CGS 26393 (the highest concentration examined in all experiments). Similarly, the maximal responses and the areas under the contraction curves for ET-1 and ET-2 were equivalent when generated in the presence or absence of CGS 26393 (Table 1). There were slight, but not statistically significant increases in both the maximum response to ET-3 (increase of 13%) and the mean area under the ET-3 contraction curve (increase of 12%) in tissues incubated in CGS 26393 (Table 1).

As was the case with CGS 26393, CGS 26303 did not alter the response to 0.1 μM ET-1. In this series of experiments, the maximum response to ET-1 was 135.2±22.8% in the presence and 120.3±15.3% ($n=4$) in the absence of 100 μM CGS 26303. There was also no significant difference in the maximum response or area under the contraction curve to ET-2 generated with or without prior incubation in 100 μM CGS 26303 (data not shown).

Response to big ET-1 in the presence and absence of CGS 26393 and phosphoramidon

Contraction to big ET-1 was attenuated in the presence of the neutral endopeptidase inhibitor phosphoramidon (Figure 2). The maximal response to 0.1 μM big ET-1 and the area under the big ET-1 contraction curve were not significantly different (using ANOVA, $n=5$) when generated in the presence of 10 μM CGS 26393 alone ($Tmax_{(Ach)}$: $48.2\pm7.3\%$, AUC: 2137 ± 308 units), 10 μM phosphoramidon alone ($Tmax_{(Ach)}$: $58.9\pm11.7\%$, AUC: 3058 ± 698 units), or when both inhibitors were used in combination ($Tmax_{(Ach)}$: $40.4\pm9.4\%$, AUC: 1782 ± 447 units).

Discussion

Advenier *et al*. (1992) have previously demonstrated contraction of human bronchi in response to big ET-1, one of the three ET precursor peptides. In the present study, the magnitude of the response to big ET-1 was equivalent to that obtained in response to ET-1 indicating complete conversion of inactive big ET-1 to ET-1. This is consistent with results obtained in guinea-pig bronchi (Noguchi *et al*., 1991) as well as the earlier study in human bronchi (Advenier *et al*., 1992). In the present study, in addition to demonstrating conversion of big ET-1, we have shown that conversion was decreased after storage of the tissue at 4°C in oxygenated buffer, a method routinely employed by investigators using human tissue. As contraction to ET-1 was unaltered by storage of tissue under the same conditions, this finding indicates significant diminution of ECE activity with time. In a study characterizing ECE activity in rat lung homogenates, Wu-Wong *et al*. (1990) found a marked decrease in protease activity toward ET-1 with storage of the homogenate at 4°C for a period of 6 days. While that study was not designed to measure ECE activity over time, it is in keeping with our present finding of decreased enzymic activity within airway tissue over time.

Big ET-2 also produced contraction of human isolated bronchus. This is the first demonstration of such a response. Contraction to big ET-2 has previously been demonstrated in monkey trachea (Takai *et al*., 1998) and guinea-pig bronchus (Lebel *et al*., 1995) suggesting the existence of an ECE within airway tissue capable of cleaving big ET-2 to ET-2. In the present study, as with big ET-1, the maximal response to big ET-2 was equivalent to that of ET-2, suggesting full conversion of this precursor peptide in human bronchi. Such a finding suggests that the ECE which is present within human bronchi, may have equal specificity for big ET-1 and big ET-2 or that there are multiple enzymes within this tissue, at least one of which has affinity for big ET-2. The converting enzyme within the epithelia from guinea-pig lung has also been shown to be equally efficient at generating ET-1 and ET-2 from the respective precursor peptides (Laporte & Sirois, 1997). This suggests that ECE within airway tissue of certain species including man, may have this characteristic. The presence of more than one converting enzyme within airway tissue is an equally plausible possibility. Sawamura *et al*. (1993) have reported the presence of two distinct enzymes in porcine lung membranes and Aubert *et al*. (1998) have produced data indicating the presence of ECE subtypes in human lung epithelial cells in culture. The presence of more than one enzyme was also suggested as the explanation for disparate responses to the precursor peptides in the guinea-pig lung (Gratton *et al*., 1995). In addition, Saleh *et al*. (1997) have demonstrated the presence of immunoreactive ECE on more than one cell type within human airways raising the possibility of enzymes with differing selective activity at different sites within the airway.

The results presented here have also demonstrated the production of a contractile response to big ET-3 in human isolated airways. Contraction of airway tissue to big ET-3 has not previously been reported in any tissue from any species. In guinea-pig bronchus, Lebel *et al*. (1995) obtained no response to big ET-3 but elicited concentration-dependent responses to ET-3. These prior results therefore demonstrated the presence of receptors for ET-3 in guinea-pig bronchus, but an absence of converting enzyme activity for big ET-3. This finding was consistent with the subsequent report of absence of conversion of big ET-3 by Clara cells isolated from the guinea-pig (Laporte & Sirois, 1997). The site of cleavage of big ET-1 and big ET-2 to form active ET-1 and ET-2, is the $Trp^{21}-Val^{22}$ bond within each peptide. The contractile response to big ET-3 in the present study therefore demonstrates the presence of an ECE in human airways with activity at the $Trp^{21}-Ile^{22}$ bond of big ET-3, resulting in the production of active ET-3.

The dual ECE and NEP inhibitor CGS 26393 significantly inhibited the response to all three ET precursor peptides in human airways. Moreover, pretreatment of tissues with all concentrations of the inhibitor had no effect upon the response to acetylcholine, and the highest concentration of the inhibitor had no effect upon the response to ET-1, ET-2 or ET-3. This suggests that the effect of this inhibitor was attributable to specific inhibition of the ECE within human airways. Big ET-1 induced bronchoconstriction in the rat lung (Held *et al*., 1997) and ET-1 production from guinea-pig airway cells (Pelletier *et al*., 1998) have previously been shown to be sensitive to inhibition by CGS 26303. The present study demonstrates that this inhibitor is also effective against big ET-induced contraction of human bronchus.

CGS 26393 is a diphenyl prodrug and the inhibitory activity produced by CGS 26393 demonstrated in this study therefore suggests that CGS 26393 was converted to an active form in these experiments. If chemical conversion of CGS 26393 did occur, it is likely that this was by hydrolyzation of one phenyl group after dilution in the vehicle (sodium hydroxide), possibly followed by removal of the other phenyl group after addition of the inhibitor to the Krebs-Henseleit solution.

Inhibition of the response to big ET-1 and big ET-2 was concentration-related, with very little contraction being induced in the presence of 100 μM of CGS 26393 – the highest concentration examined. This finding is consistent with studies of the pressor response to big ET-1 in rats where the inhibitor was also found to have a concentration-related effect after oral administration (Trapiani *et al*., 1995). Similarly, very little contraction to big ET-3 was evidenced in the presence of the highest concentration of CGS 26393. In the case of this peptide however, the response in the presence of 10 μM of the inhibitor was equivalent to that evoked in the presence of 1 μM CGS 26393, suggesting that the effect of the inhibitor was not strictly concentration-related for big ET-3. While it is possible, that CGS 26393 has a different pharmacological profile against the ECE for big ET-3 in human airways, this result is more likely to have been influenced, in part, by an alteration in the metabolism of the ET-3 produced by cleavage of the precursor peptide. We have previously shown that the response to ET-3, but not that to ET-1 in human bronchus, is significantly enhanced in the presence of phosphoramidon (McKay *et al*., 1992). CGS 26393 is a dual inhibitor, with activity against NEP (as with phosphoramidon), as well as ECE. It is possible therefore, that the metabolism of ET-3 by NEP was decreased along with inhibition of the production of ET-3 by ECE.

Mitigating against such a conclusion however, was the absence of significant potentiation of the response to ET-3 in the presence of CGS 26393 in this study. Our previous study with ET-3 was carried out with overnight storage of the tissue, which was not done in the present study. In addition, the response to ET-3 in the present study was of a much greater magnitude than that we have previously observed in human bronchi. These factors combine to make a conclusion as to precisely what effect CGS 26393 has on the degradation of ET-3, difficult. The simultaneous inhibition of ECE and NEP and the effects of each on the production and degradation of ET-3, may however, explain the absence of a clear concentration-related effect of CGS 26393 against big ET-3 in human bronchi.

In the present study, the contractile response to big ET-1 in human bronchus was not different when evoked in the presence of phosphoramidon and CGS 26393, either alone or in combination. Epithelial removal and the use of various enzyme inhibitors enabled Advenier *et al*. (1992) to conclude that ECE within human bronchi, while sensitive to phosphoramidon, was different to NEP neprilysin. Moreover, Aubert *et al*. (1998) have shown that ECE in human lung epithelia is unaffected by specific NEP inhibition by thiorphan, but is sensitive to phosphoramidon. As CGS 26393 is a dual ECE and NEP inhibitor, and as ECE has been shown to be sensitive to phosphoramidon, the results of the present study to not allow us to make any definitive conclusions as to the identity of the ECE in human bronchus or if this enzyme is different to NEP neprilysin. Our demonstration that the responses to ET-1, ET-2 and ET-3 were not affected by CGS 26393, may however, provide some evidence in this regard. Previous reports have shown enhanced responses to the ETs in human airways (Candenas *et al*., 1992) with inhibition of NEP and that all three ETs are substrates for this enzyme (Vijayaghavan *et al*., 1990). The absence of an effect of CGS 26393 on the response to the mature ETs in the present study combined with these previous reports is therefore consistent with the proposition that human airway ECE is different from NEP neprilysin.

We have therefore, demonstrated functional ECE for all three big ETs in human bronchi. The enzyme activity is attenuated with time. This finding has implications for the interpretation of other studies of endothelin-converting enzyme activity and enzyme inhibitor activity. Further, ECE activity in human airways was inhibited in a concentration-related manner by CGS 26393, which had no effect upon the response to the mature ETs. The demonstration of ECE inhibition by an orally active compound in this study, suggests possible therapeutic utility of ECE inhibitors in respiratory conditions involving excessive endothelin production.

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