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Endothelin-1-induced potentiation of human airway smooth muscle proliferation: an ET_A receptor-mediated phenomenon

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1 In this study the mitogenic effects in human cultured tracheal smooth muscle cells of endothelin-1 (ET-1), ET-3, and sarafotoxin S6c (S6c), the ET_B receptor-selective agonist, were explored either alone or in combination with the potent mitogen, epidermal growth factor (EGF).

2 In confluent, growth-arrested human airway smooth, neither ET-1 (0.01 nM – 1 μM) nor ET-3 (0.001 nM – 1 μM) or S6c (0.01 nM – 1 μM) induced cell proliferation, as assessed by [^{3}H]-thymidine incorporation. In contrast, EGF (1.6 pM – 16 nM) produced concentration-dependent stimulation of DNA synthesis (EC_{50} of about 0.06 nM). The maximum increase of about 60 fold above control, elicited by 16 nM EGF, was similar to that obtained with 10% foetal bovine serum (FBS). EGF (0.16 – 16 nM) also produced a concentration-dependent increase in cell counts, whereas ET-1 (1 – 100 nM) was without effect on this index of mitogenesis.

3 ET-1 (1 – 100 nM) potentiated EGF-induced proliferation of human tracheal smooth muscle cells. For example, ET-1 (100 nM), which alone was without significant effect, increased by 3.0 to 3.5 fold the mitogenic influence of EGF (0.16 nM). The potentiating effect of ET-1 on EGF-induced proliferation was antagonized by BQ-123 (3 μM), the ET_A receptor antagonist, but was unaffected by the ET_B receptor antagonist BQ-788 (10 μM).

4 Neither ET-3 (1 – 100 nM) nor S6c (1 – 100 nM) influenced the mitogenic effects of EGF (0.16 – 1.6 nM).

5 [^{125}I]-ET-1 binding studies revealed that on average the ratio of ET_A to ET_B receptors in human cultured tracheal smooth muscle cells was 35 : 65 ($\pm$3; n = 4), confirming the predominance of the ET_B receptor subtype in human airway smooth muscle.

6 These data indicate that ET-1 alone does not induce significant human airway smooth muscle cell proliferation. However, it potently potentiated mitogenesis induced by EGF, apparently via an ET_A receptor-mediated mechanism. These findings suggest that ET-1, a mediator detected in increased amounts in patients with acute asthma, may potentiate the proliferative effects of mitogens and contribute to the airway smooth muscle hyperplasia associated with chronic severe asthma.

Keywords: Human cultured airway smooth muscle; mitogenesis; airway smooth muscle proliferation; endothelin-1; endothelin-3; sarafotoxin S6c; endothelin receptor subtypes; ET_A receptor; ET_B receptor; epidermal growth factor

Introduction

The endothelins (ETs) and their receptors are present in abundance in the lung (Power *et al.*, 1989; Hemsèn *et al.*, 1990; Henry *et al.*, 1990; Nunez *et al.*, 1990; Giaid *et al.*, 1991; Goldie *et al.*, 1995). The ETs, in particular ET-1, produce a variety of biological effects in the pulmonary system including airway and pulmonary vascular smooth muscle contraction, mediator release, mucus secretion and enhanced microvascular permeability (Filep, 1993; Hay *et al.*, 1993a; Hay & Goldie, 1995). Furthermore, increased expression and levels of the ETs in some lung diseases has been demonstrated (Springall *et al.*, 1991; Stewart *et al.*, 1991; Hay *et al.*, 1993a; Sofia *et al.*, 1993; Redington *et al.*, 1995). Accordingly, it has been speculated that the ETs may play a pathophysiological role in lung diseases such as asthma and pulmonary hypertension (Springall *et al.*, 1991; Stewart *et al.*, 1991; Hay *et al.*, 1993a; Filep, 1993; Sofia *et al.*, 1993; Hay & Goldie, 1995).

ET-1 has been reported by several groups of investigators to induce vascular smooth muscle proliferation (Komuro *et al.*, 1988; Bobik *et al.*, 1990) or to be a co-mitogen, potentiating the proliferative effects of other factors (Weissberg *et al.*, 1990). Significant mitogenic effects of ET-1 in human airway smooth muscle may be an important component of the proposed pathophysiological role of this mediator in asthma, a chronic inflammatory disease which is characterized ultimately with thickening of the airway smooth muscle layer and increased airway smooth muscle mass (Dunhill, 1960; Heard & Hossain, 1973).

In airway smooth muscle cells, ET-1 caused only modest mitogenic effects in cultured tracheal smooth muscle cells from rabbits (Noveral *et al.*, 1992), guinea-pigs (Stewart *et al.*, 1994), sheep (Glassberg *et al.*, 1994) and human subjects (Tomlinson *et al.*, 1994), compared with those of serum, PDGF or epidermal growth factor (EGF). There have been no published reports on the influence of ET-1 as a co-mitogen with other growth factors in cultured airway smooth muscle cells. Accordingly, the objectives of the present study were to investigate the proliferative effects of ET-1 and ET-3 alone and in combination with EGF in human cultured airway smooth muscle. In addition, the selective ligands, sarafotoxin S6c (S6c; ET_B receptor agonist) (Williams *et al.*, 1991), BQ-123 (ET_A receptor antagonist; cyclo(-D-Asp-L-Pro-D-Val-L-Leu-D-Trp-)) (Ihara *et al.*, 1991) and BQ-788 (ET_B receptor antagonist; [*N-cis*-2,6-dimethylpiperidinocarbonyl-L-γ-methylleucyl-D-1-methoxycarbonyltryptophanyl-D-norleucine]) (Ishikawa *et al.*, 1994) were used to determine the ET receptor subtype(s) responsible for any mitogenic influences of ET-1.

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Methods

Airway smooth muscle cell culture

Human trachea was obtained from lung transplant donors in accordance with procedures approved by the University of Pennsylvania Committee on Studies Involving Human Beings. A segment of trachea just proximal to the carina was removed under sterile conditions and the trachealis muscle isolated (Panettieri *et al.*, 1989). Approximately 0.5 g of wet tissue was obtained, which was then minced, centrifuged and resuspended in 10 ml of buffer containing 0.2 mM $CaCl_2$, 640 u ml^{-1} collagenase, 1 mg ml^{-1} soybean trypsin inhibitor and 10 u ml^{-1} elastase. Enzymatic dissociation of the tissue was performed for 90 min in a shaking water bath at 37°C. The cell suspension was filtered through 105 μm Nytex mesh, and the filtrate was washed with equal volumes of Ham's cold F12 medium supplemented with 10% foetal bovine serum (FBS) (Hyclone Laboratories, Inc., Logan, UY, U.S.A.). Aliquots of the cell suspension were plated at a density of 1.0×10^4 cells cm^{-2}. Ham's F12 media was supplemented with 10% FBS, 100 u ml^{-1} penicillin, 0.1 mg ml^{-1} streptomycin, and 2.5 mg ml^{-1} amphotericin B and was replaced every 72 h. Cell counts were obtained in triplicate wells with 0.5% trypsin in 1 mM EDTA solution.

Human airway smooth muscle cells in subculture during the 10th–20th cumulative population doubling (3rd–5th passage cells) were used since the cells, over this time interval, retain native contractile protein expression, as demonstrated by immunocytochemical staining for smooth muscle actin and myosin (Panettieri *et al.*, 1989). Further, these cells express functional cell-excitation coupling systems as determined by fura-2 measurements of agonist-induced changes in cytosolic calcium (Panettieri *et al.*, 1989; Murray *et al.*, 1993).

[^{125}I]-ET-1 binding studies in human cultured tracheal smooth muscle

To assess accurately the relative proportions of specific ET_A and ET_B binding sites in the cells used in these studies, it is necessary to determine the binding maxima for these separate binding site populations. Since [^{125}I]-ET-1 binds in a psuedo-irreversible manner to its specific sites (Marsault *et al.*, 1991; Waggoner *et al.*, 1992), it is not appropriate to apply a competitive binding isotherm to derive binding maxima from data describing the concentration-dependence of specific binding. However, appropriate estimates can be derived from analyses of the time courses of [^{125}I]-ET-1 binding in the presence and absence of receptor subtype-selective antagonists (Goldie *et al.*, 1994; 1995).

In these experiments, confluent cell cultures were prepared in 24-well culture plates. The adhering cells were washed to remove culture medium, air-dried and frozen at −85°C before use. The preparations were then thawed and incubated for 10 min at 22°C in Tris HCl buffer (50 mM Tris, 100 mM NaCl; pH 7.4) containing 0.25% (w/v) bovine serum albumin and the protease inhibitor, phenylmethylsulphonyl fluoride (10 μM). Cells were then incubated in this buffer containing 0.2 nM [^{125}I]-ET-1 for 10–180 min in the presence and absence of 1 μM BQ-123, or 100 nM S6c or in the combined presence of 1 μM BQ-123 and 100 nM S6c (to assess non-specific binding). Tissue was then wiped from culture wells with glass fibre filter paper (Whatman GF/A) and radioactivity estimated in a Packard gamma counter (Model 5650). Each data point is the mean ± s.e.mean of 4 observations. DNA content was measured in 8 wells according to the methods of Hinegardner (Hinegardner, 1971).

[^{3}H]-thymidine and cell proliferation assays

Proliferation studies were performed in confluent, growth-arrested human airway smooth muscle cells. Cells were growth-arrested by incubating the cultures at day 10 in serum-free media consisting of F12 media with 5 ng ml^{-1} insulin and 5 ng ml^{-1} transferrin (Panettieri *et al.*, 1990). Confluent, growth-arrested cells were used because cells can be synchronized in G_0/G_1 phase of the cell cycle and at this baseline, minimally incorporate [^{35}S]-methionine and [^{3}H]-thymidine (Panettieri *et al.*, 1989; 1990). After 24 h in serum-free media, the cells were stimulated with either EGF, 10% FBS, varying concentrations of ET-1, ET-3 or S6c, or a combination of EGF and ET-1, ET-3 or S6c. In a separate series of experiments, the cells were pretreated with BQ-123 or BQ-788 and DNA synthesis stimulated with agonists as described above. After 18 h of stimulation with mitogen, human airway smooth muscle cells were labelled with 1.0 μCi ml^{-1} of [methyl-^{3}H]-thymidine [40–60 Ci $mmol^{-1}$] (Amersham Corp., Arlington Heights, IL, U.S.A.) for 18 h. The cells were then scraped, lysed and the protein/DNA precipitated with 12% trichloroacetic acid; the precipitant was aspirated onto glass filters, extensively washed, dried and counted (Panettieri *et al.*, 1989; 1990). [^{3}H]-thymidine incorporation experiments were performed in parallel with cell proliferation assays. Cell number was quantified by removing cells from wells with 0.5% trypsin-1 mM EDTA solution. Cell counts were then obtained from each well with a Coulter Counter (Coulter Electronics, Hialeah, FL, U.S.A.).

Statistics

The [^{3}H]-thymidine and cell count data represent means ± s.e.mean from a minimum of four wells from a minimum of four separate experiments; statistical significance was assessed by one way analysis of variance (Bonferroni-Dunn test) with $P<0.05$ regarded as significant. The proliferation assays were performed with a minimum of three different airway smooth muscle cell lines.

Drugs

ET-1, ET-3, S6c were obtained from Peninsula Laboratories (Belmont, CA, U.S.A.), American Peptide Co. (Sunnyvale, CA, U.S.A.) or Sigma Chemical Co. (St. Louis, Mo, U.S.A.), BQ-123 was from American Peptide Co. and BQ-788 was synthesized by colleagues in the Department of Medicinal Chemistry, SmithKline Beecham Pharmaceuticals. Phenymethylsulphonyl fluoride was obtained from Calbiochem (La Jolla, CA, U.S.A.), bovine serum albumin from Gibco Diagnostics (Madison, WI, U.S.A.), and EGF and Tris HCl from Sigma.

Results

[^{125}I]-ET-1 binding studies in human cultured tracheal smooth muscle

Total specific binding and residual specific binding to either ET_A (in presence of S6c) or ET_B (in presence of BQ-123) receptors increased to plateaus in a time-dependent manner such that B_{max} was reached at about 90 min and maintained thereafter (Figure 1). Both the ET_A receptor-selective ligand BQ-123 (1 μM) and the ET_B receptor-selective ligand, S6c (100 nM), significantly reduced specific binding at all time points, indicating the presence of both ET_A and ET_B binding sites in these cells. Thus, binding values, determined at 120 min (i.e. at B_{max}), in the absence and presence of these ligands, indicated that the proportion of ET_A to ET_B receptor subtypes in human cultured bronchial smooth muscle cells was 35 : 65 (±3; $n=4$).

Effects of EGF, ET-1, ET-3 or S6c on DNA synthesis

EGF (1.6 pM–16 nM) induced a concentration-dependent increase in DNA synthesis, as assessed by [^{3}H]-thymidine incorporation, in human tracheal smooth muscle cells with an EC_{50} of about 0.06 nM (Figure 2). The maximum stimulation

induced by 16 nM EGF was about 60 fold over basal (27305 ± 2539 c.p.m. per cell; basal = 437 ± 38 c.p.m. per cell) which was similar to that caused by 10% FBS (28500 ± 1500 c.p.m. per cell; $P > 0.10$ compared to 16 nM EGF).

ET-1 (0.01 nM – 1 μM), ET-3 (0.01 nM – 1 μM) or S6c (0.01 nM – 1 μM) alone had no significant effect on [^{3}H]-thymidine incorporation ($P > 0.05$; Figure 2). For example, although ET-1 (1 – 100 nM) produced an apparent approximate 2 fold increase in DNA synthesis, it was not statistically significant, or concentration-related and was not inhibited by BQ-123 (10 μM) or BQ-788 (10 μM) (data not shown). Note, the concentrations of ET-1, ET-3 and S6c used in the above experiments were those that elicited increases in cytostolic [Ca^{2+}] transients in human airway smooth muscle cells (data not shown).

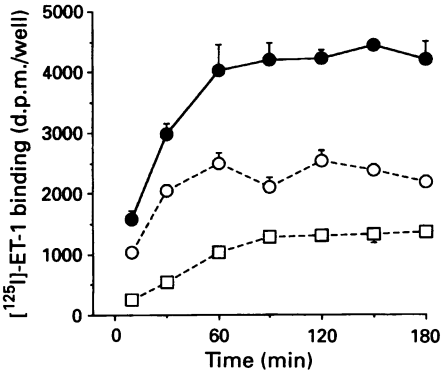

Figure 1 Time-dependence of [^{125}I]-ET-1 (0.2 nM) binding in human cultured tracheal airway smooth muscle cells (3rd – 5th passage). Total specific binding (●) is shown, as well as residual specific binding (d.p.m. mg^{-1} DNA per well) in the presence of 1 μM BQ-123 (○; i.e. binding to ET_B sites) and residual specific binding in the presence of sarafotoxin S6c (□, i.e. binding to ET_A sites). Non-specific binding (not shown) was assessed in the combined presence of 1 μM BQ 123 and 100 nM sarafotoxin S6c. Data are presented as mean ± s.e.mean (unless s.e. smaller than the size of the symbol) of 4 mean estimates.

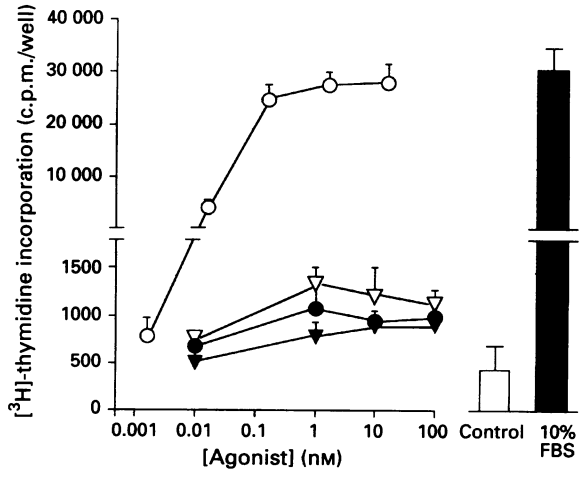

Figure 2 Effects of EGF, ET-1, ET-3 or S6c on stimulation of DNA synthesis in human cultured tracheal smooth muscle cells. Human tracheal smooth muscle cells were grown to confluence and then growth-arrested for 48 h in serum-free media. As described in Methods, [^{3}H]-thymidine incorporation was measured after 18 h stimulation with agonist. DNA synthesis was compared with that obtained from cells treated with either 10% FBS (shown in the column) or those treated with diluent alone (control). Data represents mean ± s.e.mean (unless s.e. smaller than the size of the symbol) from 10 separate experiments for all agonists with each experimental condition consisting of 6 replicate wells. A minimum of 3 different human airway smooth muscle cell lines were used in these experiments: (○) EGF; (▽) ET-1; (●) ET-3; (▼) S6c.

Effects of ET-1, ET-3 or S6c on EGF-induced proliferation

ET-1 (1 – 100 nM) augmented EGF-induced DNA synthesis. For example, ET-1 (100 nM), which alone was without effect on cell proliferation, increased [^{3}H]-thymidine incorporation by about 3.5 fold from 18,232 ± 1,904 c.p.m. cell (1.6 nM EGF alone) to 68,812 ± 3,012 (ET-1 and 1.6 nM EGF; $P < 0.05$) (Figure 3a). The magnitude of the response with the combination of ET-1 (100 nM) and EGF (1.6 nM) was greater than that induced by 10% FBS (19,832 ± 2,104 c.p.m./cell; $P < 0.03$) (Figure 3a). Similar results were obtained with 10 nM ET-1 (Figure 3a). In contrast to ET-1, ET-3 (1 – 100 nM) (Figure 3b) or S6c (1 – 100 nM) (Figure 3c) were without effect on EGF-induced DNA synthesis of human tracheal smooth muscle cells.

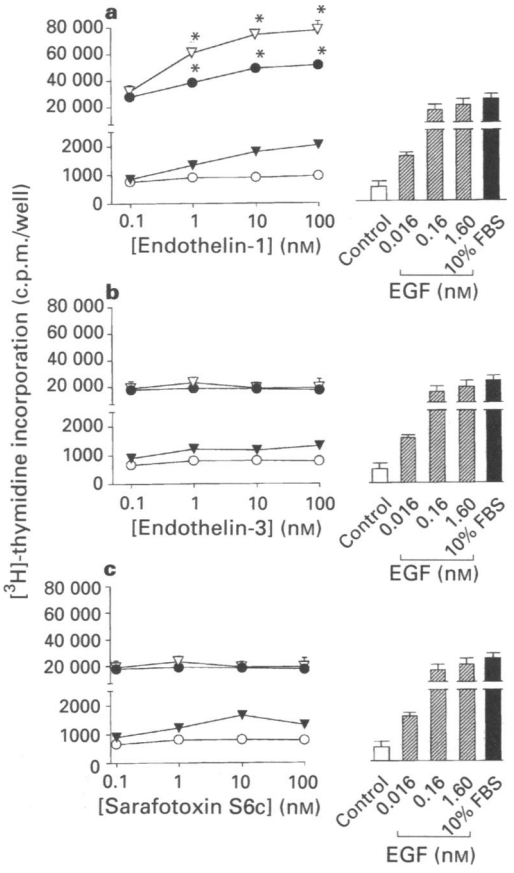

Figure 3 Effects of (a) ET-1 (0.1 – 100 nM), (b) ET-3 (0.1 – 100 nM) or (c) S6c (0.1 – 100 nM) on EGF-induced DNA synthesis in cultured human tracheal smooth muscle cells. Confluent human tracheal smooth muscle cells were growth-arrested and then stimulated with either ET-1, ET-3 or S6c alone or in combination with EGF (0.016 – 1.6 nM). [^{3}H]-thymidine incorporation was measured after 18 h stimulation with agonist. DNA synthesis was compared with that obtained from cells treated with either 10% FBS or EGF (shown in the columns) or those treated with diluent alone (control). Data represent mean ± s.e.mean (unless s.e. smaller than the size of the symbol) from 6 separate experiments with each experimental condition consisting of 6 replicate wells. A minimum of three different human airway smooth muscle cell lines were used in these experiments. (a) (○) ET-1 alone; (▼) ET-1 + 0.016 nM EGF; (●) ET-1 + 0.16 nM EGF; (▽) ET-1 + 1.6 nM EGF; (b) (○) ET-3 alone; (▼) ET-3 + 0.016 nM; (●) ET-3 + 0.16 nM EGF; (▽) ET-3 + 1.6 nM EGF; (c) (○) S6c alone; (▼) S6c + 0.016 nM EGF; (●) S6c + 0.16 nM EGF; (▽) S6c + 1.6 nM EGF. *Significant vs. EGF alone, $P < 0.05$.

Effects of BQ-123 or BQ-788 on ET-1-induced enhancement of proliferation

BQ-123 (3 μM), the ET_A receptor antagonist, abolished ET-1-induced enhancement of DNA synthesis produced by EGF (0.16 nM) (Figure 4a). In contrast, BQ-788 (10 μM), the ET_B receptor antagonist, had no effect on the augmentation of EGF (0.16 nM)-induced DNA synthesis produced by ET-1 (1–100 nM). BQ-123 (3 μM) alone (Figure 4a) or BQ-788 (10 μM) alone (Figure 4b) were without significant effect on basal cell proliferation (data not shown) or on that induced by EGF (0.16 nM) (Figure 4).

Effects of ET-1 and EGF on cell number

Since DNA synthesis may occur without cell division, we investigated, by assessing cell number, whether ET-1 augmented human tracheal smooth muscle cell proliferation induced by EGF. ET-1 alone had no effect on airway smooth muscle cell growth as compared with those cells treated with diluent alone (control) (Figure 5a). ET-1 (10 nM) augmented EGF (0.16 nM)-induced airway smooth muscle cell proliferation in a concentration-dependent manner (Figure 5b). Augmentation of the EGF (0.16 nM)-induced increase in cell number elicited by ET-1 (10 nM) was abolished by BQ-123 (3 μM) but was unaffected by BQ-788 (10 μM) (Figure 5c).

Discussion

The major findings of the present study are: (1) ET-1, ET-3 or S6c alone had minimal effects on proliferation of human tracheal smooth muscle; (2) ET-1, but not ET-3 or S6c, potentiated the mitogenic effects of EGF; (3) the potentiating effects of ET-1 were antagonized by BQ-123, the ET_A receptor antagonist, but not by BQ-788, the ET_B receptor antagonist. These results suggest that ET-1 could augment the mitogenic effects of growth factors in human airway smooth muscle via ET_A receptor activation, and may play a role in mediating human airway smooth muscle hyperplasia seen in patients with chronic severe asthma. To our knowledge this is the first report describing the synergism between ET-1 and a growth factor in cultured human airway smooth muscle cells.

The mitogenic effects of ET-1 in vascular smooth muscle have been well-documented (Komuro *et al.*, 1988; Nakaki *et*

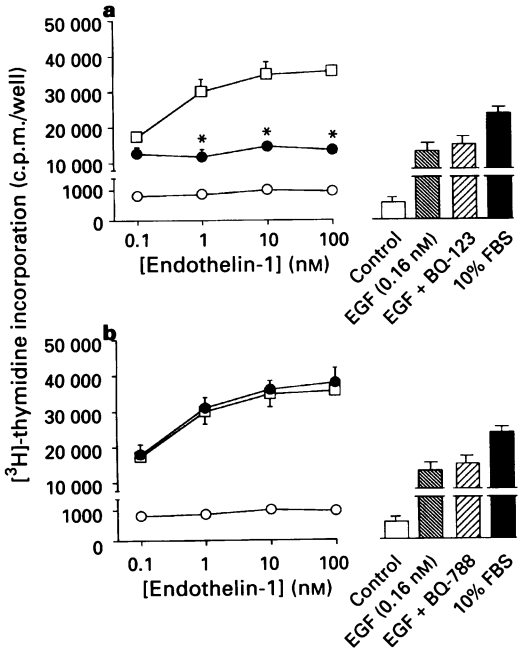

Figure 4 Effects of (a) BQ-123 (3 μM), the ET_A receptor antagonist or (b) BQ-788 (10 μM) the ET_B receptor antagonist, on augmentation of EGF-induced DNA synthesis produced by ET-1 (0.1–100 nM) in human cultured tracheal smooth muscle cells. [^{3}H]-thymidine incorporation was determined in growth-arrested human tracheal smooth muscle cells as outlined in Methods. Data represent mean ± s.e.mean (unless s.e. smaller than the size of the symbol) of (a) 6 and (b) 4 separate experiments with each experimental condition consisting of 6 replicate wells. DNA synthesis was compared with that obtained from cells treated with either 10% FBS, EGF alone or diluent alone (control) (columns). The effects of (a) BQ-123 or (b) BQ-788 alone on EGF-induced DNA synthesis is shown in the columns (a) (○) ET-1 alone; (●) ET-1 + 0.16 nM EGF; (□) ET-1 + 0.16 nM EGF + BQ-123; (b) (○) ET-1 alone; (●) ET-1 + 0.16 nM EGF; (□) ET-1 + 0.16 nM EGF + BQ-788. *Significant vs. ET-1 + EGF, $P<0.01$.

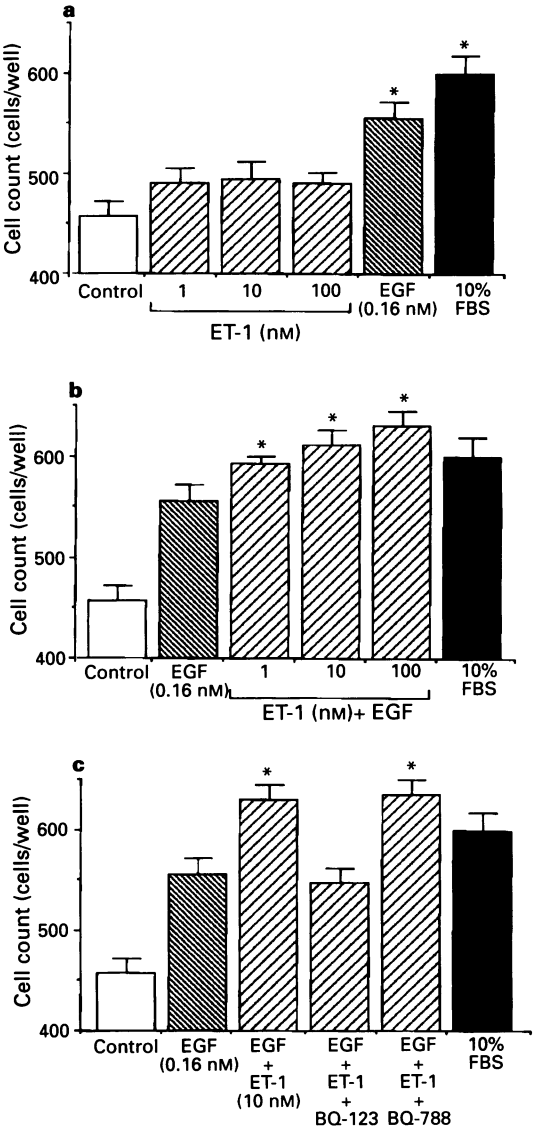

Figure 5 Effects of ET-1 (1–100 nM) alone and in combination with EGF on proliferation of human tracheal smooth muscle cells, assessed by measuring cells counts. (a) ET-1 (1–100 nM); (b) ET-1 (1–100 nM) on proliferation induced by 0.16 nM EGF; (c) BQ-123 (3 μM) or BQ-788 (10 μM) on ET-1-induced augmentation of proliferation induced by 0.16 nM EGF. Confluent, growth-arrested human tracheal smooth muscle were treated with either various concentrations of ET-1 alone or in combination with 0.16 nM EGF, in the absence and presence of BQ-123 (3 μM) or BQ-788 (10 μM) and compared with those cells treated with diluent (control) or with 10% FBS. After 18 h stimulation under appropriate conditions, cell counts per well were obtained as described in the Methods section. Data represents mean ± s.e.mean from 6 separate experiments each experiment consists of 4 replicate per experimental condition. (a) *Significant versus Control; $P<0.01$; (b) *Significant versus EGF alone; $P<0.05$; (c) *Significant versus EGF alone; $P<0.01$.

al., 1989; Bobick et al., 1990; Eguchi et al., 1992; Weissberg et al., 1990). Some recent studies have explored the proliferative effects of ET-1 in airway smooth muscle cells and demonstrated that ET-1 alone caused modest increases in growth, compared with the effects of serum, PDGF or EGF, in cultured tracheal smooth muscle cells from rabbits (Noveral et al., 1992), guinea-pigs (Stewart et al., 1994), sheep (Glassberg et al., 1994) and human subjects (Tomlinson et al., 1994). In the study in human airway smooth muscle, ET-1 produced a 2.3 fold increase in DNA synthesis (EC_{50} approximately 0.3 nM) and, in contrast to the effects of EGF, thrombin and the thromboxane-mimetic, U46619, the mitogenic activity of ET-1 was not inhibited by salbutamol (Tomlinson et al., 1994). In the present study ET-1 was without effect on proliferation, whether assessed by [^{3}H]-thymidine incorporation or cell number. There was about a 2 fold increase in [^{3}H]-thymidine incorporation induced by ET-1, but this was not statistically significant, or concentration-dependent and apparently not mediated via ET_A or ET_B receptor activation, as it was not inhibited by either BQ-123 or BQ-788. Similar small increases in DNA synthesis induced by ET-1 have been reported in cultured porcine and bovine pulmonary artery smooth muscle cells (Hassoun et al., 1992). Thus, in human and animal cultured airway smooth muscle, ET-1 on its own is at best a weak mitogen. Importantly, however, ET-1 significantly potentiated the mitogenic activity of EGF.

EGF is a potent and effective mitogen, and the increase in proliferation produced by a combination of EGF and ET-1 is greater than that elicited by 10% FBS. ET-1 has also been demonstrated to be a co-mitogen in rat vascular smooth muscle cells, potentiating proliferation induced by PDGF (Weissberg et al., 1990). If one considers that the combination of ET-1 and EGF stimulated mitogenesis to a greater extent than that induced by 10% serum, a markedly effective mitogenic stimulus, then the magnitude of the ET-1 effects on myocyte growth is impressive. Given that other contractile agonists mediate their effects by activating similar downstream signalling events as those induced by ET-1, we reason that other agonists may also augment growth factor-induced cell proliferation. Both bradykinin and leukotriene D_4 (LTD_4) potentiated EGF-induced mitogenesis (unpublished observations). These data suggest that agonist-induced augmentation of myocyte growth may occur in part, by activation of common downstream signalling events such as G protein-linked activation of phospholipase C or the subsequent increases in cytostolic calcium evoked by these agonists (Panettieri, 1995). However, the precise mechanism(s) by which ET-1 potentiates EGF-induced cell growth remains unknown.

The biological effects of the ET family of peptides are mediated via activation of membrane receptors. In the present series of experiments the selective ligands, sarafotoxin S6c (S6c; ET_B receptor agonist) (Williams et al., 1991), BQ-123 (ET_A receptor antagonist) (Ihara et al., 1991) and BQ-788 (ET_B receptor antagonist) (Ishikawa et al., 1994) were used to determine the ET receptor subtype(s) responsible for the mitogenic influences of ET-1. The potentiating effect of ET-1 on EGF-induced proliferation was antagonized by BQ-123 but not by BQ-788. Furthermore, S6c did not influence the mitogenic effects of EGF. These data would suggest strongly that the augmenting effects of ET-1 were mediated via activation of ET_A receptors. Similarly, in various systems, including human pulmonary artery (Zamora et al., 1994), rat aortic smooth muscle (Eguchi et al., 1992; Ohlstein et al., 1992) and human keratinocytes (Bagnato et al., 1995), the proliferative effects of ET-1 appear due to stimulation of the ET_A receptor subtype.

Binding, autoradiographic and functional studies have detected both ET_A and ET_B receptors in human lung (Hay & Goldie, 1995; Goldie et al., 1995). In human bronchial smooth muscle the proportion of ET_A to ET_B receptors has been calculated to be about 15%:85% (Goldie et al., 1995). The results of the present binding studies using selective ligands for these ET receptor subtypes in human cultured tracheal smooth muscle cells, confirms the predominance of ET_B receptors (65%) versus ET_A receptors (35%). The concentrations of ligands used, S6c (100 nM) and BQ-123 (1 μM), have previously been shown to inhibit maximally specific [^{125}I]-ET-1 binding in airway smooth muscle (Goldie et al., 1994; Knott et al., 1995). The quantitative difference in the relative proportions of the ET_A and ET_B receptors between the two studies may reflect regional differences between trachea and bronchus, a phenomenon which has been suggested previously in guinea-pig airways (Hay et al., 1993b). Interestingly, a further change in ET_A and ET_B receptor proportions from 15%:85% respectively in human bronchial smooth muscle to 32%:68% respectively in human lung alveolar wall (Knott et al., 1995) has been detected. Alternatively, this relatively small change in receptor subtype proportions may be a consequence of placing the tracheal airway smooth muscle cells in culture.

Contraction of human bronchial smooth muscle appears to be mediated predominantly by ET_B receptor activation (Hay et al., 1993b). Despite their presence in lower amounts, the above results suggest that activation of the ET_A subtype mediated the mitogenic effects of ET-1. In addition, there is evidence that ET_A receptors are involved in ET-1-induced mediator release in human bronchus (Hay et al., 1993c). ET_A receptors are also responsible for ET-1-induced contraction of human pulmonary artery (Hay et al., 1993b; Buchan et al., 1994).

There are several reports of increased expression and elevated levels of ET-1 in bronchoalveolar lavage fluid in airways of patients with asthma compared to control subjects (Springall et al., 1991; Stewart et al., 1991; Hay et al., 1993a; Sofia et al., 1993; Redington et al., 1995). These observations, in conjunction with the abundance of airway ET receptors (Power et al., 1989; Hemsèn et al., 1990; Henry et al., 1990; Hay et al., 1993a; Hay & Goldie, 1995) and the many potentially pertinent effects of ET-1 in several cell types in the lung (Filep, 1993; Hay et al., 1993a; Hay & Goldie, 1995), has formed the basis of the speculation that ET-1 may be important in the pathogenesis of pulmonary disorders such as asthma and pulmonary hypertension (Springall et al., 1991; Stewart et al., 1991; Hay et al., 1993a; Filep, 1993; Sofia et al., 1993; Hay & Goldie, 1995). The mitogenic effects of ET-1 in human airway smooth muscle may also play a pathophysiological role in asthma, a chronic inflammatory disease associated with thickening of the airway smooth muscle layer and increased airway smooth muscle mass (Dunhill, 1960; Heard & Hossain, 1973).

The therapeutic implications from these findings, which suggest that different subtypes of ET receptors mediate the effects of ET-1 in human lung, may be that the desired pharmacological profile for a compound in some pulmonary disorders in which ET-1 is thought to play a role, should be a combined ET_A/ET_B receptor antagonist rather than one selective for ET_A or ET_B receptors. However, more research is required to test this hypothesis, in particular the clarification of the ET receptors mediating the many effects of ET-1 in the pulmonary system. It may be that the most appropriate compound profile with regard to ET selectivity may depend on the specific pulmonary disease.

In summary, the present data indicate that, although ET-1 alone does not induce significant human airway smooth muscle cell proliferation, it potently augments mitogenesis induced by EGF. Based on the influence of the specific receptor antagonists, BQ-123 (ET_A) and BQ-788 (ET_B) and also the lack of effect of S6c, the selective ET_B agonist, the evidence suggests strongly that this phenomenon is mediated via activation of ET_A. These findings may have pathophysiological significance, since the increased levels of ET-1 found in the lungs of asthmatics might potentiate the proliferative effects of other mitogens and thereby contribute to the airway smooth muscle hyperplasia seen in this disease.

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