Relationship between endothelin-1 binding site densities and constrictor activities in human and animal airway smooth muscle

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1 Endothelin-1 (ET-1) binding site densities and constrictor activities were compared in airway smooth muscle preparations of human, guinea-pig, rat and mouse.

2 The mean contractile response to 0.3 μM ET-1 (measured as the % maximum response to 10 μM carbachol, % $C_{\text{max}} \pm \text{s.e.mean}$) and the mean concentration of ET-1 producing 30% C_{max} (95% confidence limits) were respectively; $85.9 \pm 5.4\%$ and 3.4 nM (2.4–5.0) for mouse trachea ($n = 11$), $88.8 \pm 4.7\%$ and 18.2 nM (11.2–25.2) for rat trachea ($n = 6$), $71.0 \pm 7.1\%$ and 35.2 nM (5.4–231) for human bronchus ($n = 3$), and $32.3 \pm 3.0\%$ and 241 nM (125–460) for guinea-pig trachea ($n = 6$).

3 Light microscopic autoradiography revealed specific [¹²⁵I]-ET-1 binding sites localized to the smooth muscle band, with very low levels of binding associated with cartilage, submucosal and epithelial cells.

4 Quantitative autoradiographic analyses of the concentration-dependence of specific [¹²⁵I]-ET-1 binding (0.1–2 nM) to smooth muscle revealed similar dissociation constants but markedly different specific binding site densities for the various animal species. The order of densities of specific [¹²⁵I]-ET-1 binding sites was rat trachea ($69.0 \pm 11.2 \text{ amol mm}^{-2}$) > human bronchus ($42.7 \pm 17.5 \text{ amol mm}^{-2}$) > mouse trachea ($28.7 \pm 2.6 \text{ amol mm}^{-2}$) > guinea-pig trachea ($8.3 \pm 1.8 \text{ amol mm}^{-2}$).

5 A positive relationship between [¹²⁵I]-ET-1 binding site density and ET-1 constrictor activity was observed in airway smooth muscle preparations from rat, human and guinea-pig. The greater sensitivity of mouse trachea to the constrictor actions of ET-1 was not dependent on the release of cyclo-oxygenase- or epithelium-derived constrictor substances, but may have been due to an inter-species difference in the receptor-effector system for ET-1.

Introduction

Endothelin-1 (ET-1) is a 21-amino acid peptide derived from vascular endothelial cells under hypoxic conditions that exerts significant effects on the vasculature including contraction and mitosis of vascular smooth muscle cells (Yanagisawa *et al.*, 1988; Hirata *et al.*, 1989). In addition to the pronounced actions of ET-1 on the circulatory system, it is becoming increasingly apparent that ET-1 has prominent roles in other organs, especially the lung. Whole body autoradiographic studies have revealed that following intravenous administration of radiolabelled ET-1 to rats, the lungs became highly enriched with ET-1, suggesting that this organ may be a major target for ET-1 action (Neuser *et al.*, 1989). Indeed, quantitative autoradiographic studies have identified specific binding sites for ET-1 on airway smooth muscle and alveolar septa (Power *et al.*, 1989; Turner *et al.*, 1989). Furthermore, both *in vitro* (Uchida *et al.*, 1988; Maggi *et al.*, 1989) and *in vivo* (Payne & Whittle, 1988; Braquet *et al.*, 1989; Matsuse *et al.*, 1989) studies have revealed that ET-1 is a potent bronchoconstrictor of airway smooth muscle.

Although ET-1 has been shown to constrict airway smooth muscle, marked inter-species and within species differences exist with respect to ET-1 constrictor potency (Uchida *et al.*, 1988; Maggi *et al.*, 1989; Hay, 1989; Turner *et al.*, 1989; Secrest & Cohen, 1989). Whether these potency differences are due to variations in ET-1 receptor density or to different post-receptor events is not known. In the current study, we have investigated the relationship between ET-1 receptor density and *in vitro* constrictor activity in airway smooth muscle preparations from several animal species, including man.

Methods

Functional studies

Male Wistar rats (8 wks, 200–250 g), CBA/CaH mice (8–10 wks) and SR/C Tricolor guinea-pigs (400–500 g) were killed

by cervical dislocation. Trachea and thoracic aorta were rapidly excised, cleaned of connective tissue and cut into segments (mouse trachea, 2–3 mm long; all other tissues, 3–4 mm long). Segments of human isolated bronchi were obtained from macroscopically normal areas of lung resected for lung carcinoma (3 male patients, ex-smokers, 44, 69 and 74 years), stored overnight at 4°C and used the following day. Isolated tissue preparations were suspended under 0.5 g resting tension (except rat aorta, 1.0 g) in Sigmacoat-treated organ baths containing 3 ml of Krebs-bicarbonate solution at 37°C, bubbled with 5% CO₂ in O₂. Changes in smooth muscle tension were recorded on a Model 7D Polygraph via FT03 force displacement transducers (Grass Instruments). Preparations were allowed to equilibrate for at least 45 min before any drug addition.

Airway preparations

The viability of airway preparations was assessed by measuring contractile responses to 0.3 μM carbachol. Following a 30 min washout period, a cumulative concentration-effect curve was completed to carbachol (10 nM–10 μM). Tissues were washed for 45 min and a concentration-effect curve obtained to ET-1 (0.1 nM–0.3 μM). In some experiments, airway epithelium was removed by rubbing with a cotton wool-covered needle. Successful removal of epithelium was verified histologically. In experiments examining the effects of indomethacin, tissues were exposed to 5 μM indomethacin for 30 min prior to, as well as during, the completion of a concentration-effect curve.

Vascular preparations

The viability of the arterial preparations was tested by addition of 0.5 μM phenylephrine. Following a 30 min washout period, cumulative concentration-effect curves were completed to phenylephrine (10 nM–10 μM) and then ET-1 (0.1 nM–0.3 μM). A 45 min washout period was used between concentration-effect curves. In some experiments, the vascular endothelium

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was removed intentionally by gently rubbing the intimal surface of the artery with a thin wire. Endothelial integrity was assessed pharmacologically. The presence of a functional endothelium was confirmed by the ability of 1 and 10 μM carbachol to induce relaxation of arteries constricted with 0.5 μM phenylephrine.

Autoradiography

Preparation of autoradiographs Segments of trachea (4 mm long) from mice, rats and guinea-pigs and of human bronchus were submerged in Macrodex (6% dextran 70 in 5% glucose) and frozen by immersion in isopentane, quenched with liquid nitrogen. Serial transverse frozen sections (10 μm thick) were cut at -20°C and thaw-mounted onto gelatin chrom-alum-coated glass slides. Slide-mounted tissue sections were pre-incubated for 2×10 min in buffer (50 mM Tris-HCl, 100 mM NaCl, 0.25% bovine serum albumin, 10 μM phenylmethylsulphonylfluoride, pH 7.4) and then incubated at 22°C for 60 min in buffer containing [^{125}I]-ET-1 (0.1 nM–2 nM). Nonspecific binding was determined with 1 μM unlabelled ET-1. Slides were washed for 2×10 min in buffer and rinsed briefly in H_2O at 4°C . Sections were dried rapidly in a stream of cold dry air. Emulsion-coated coverslips (Kodak NTB-2) were attached with cyanoacrylate adhesive to one end of the slide and exposed for 6 days at 4°C . The emulsion was developed (Kodak Dektol, 1:1, 3 min), rinsed for 15 s in dilute acetic acid (2%) containing hardener (Ilford) and fixed (Ilford Hypam, 1:4, 2.75 min). Sections were stained with haematoxylin, dehydrated in ethanol, cleared with xylene and mounted (Depex, BDH) for light microscopy.

Quantitative analysis of autoradiographs Autoradiographic grain densities were determined by the automated grain detection and grain counting system described previously (Henry *et al.*, 1990). Briefly, autoradiographic slides were viewed with a video camera (National solid state, black and white) attached to a photomicroscope (Olympus BH-2; 100 $\times$ oil immersion objective lens, Zeiss Planapo). Images were processed by the MD-20 image analysis system (Flinders Imaging, Adelaide, Australia) and then analyzed for autoradiographic grains with a series of assembler algorithms based on the method of Reep & Creegan (1988). In these experiments, 5 separate fields were viewed from each tissue section and triplicate total and non-specific slides were analyzed at each [^{125}I]-ET-1 concentration. The emulsion background grain density was measured over non-tissue areas within the lumen of airway tissues and determined from analysis of three fields for each slide.

Radioactive standards Radiolabelled [^{125}I]-fibrinogen (specific activity 100 $\mu\text{Ci ml}^{-1}$, 0.8 mg ml^{-1} , 0–40 μl) was mixed with a concentrated solution of unlabelled fibrinogen (30.96 mg ml^{-1} , Red Cross Blood Transfusion Service, 260–300 μl). The 300 μl mixture was pipetted into a cylindrical mould containing thrombin (20 μl , 1000 units ml^{-1}) and allowed to clot for 1 to 2 h. The clot was removed from the mould, fixed in buffered formaline saline for 24 h and processed into paraffin blocks. Microtome sections (10 μm) were then cut. The radioactivity of alternate sections was determined by gamma radiation counting. Remaining sections were mounted on glass slides, dewaxed, rehydrated, dried and apposed to NTB-2 emulsion-coated slides. Emulsion was exposed, fixed and developed as described above for the autoradiographs. The slide-mounted sections of [^{125}I]-fibrinogen standards were stained with toluidine blue and their cross-sectional area determined by the MD-20 image analysis system. Autoradiographic grain densities were determined as outlined above and the relationship between the autoradiographic grain density and section radioactivity determined. By this procedure, a linear relationship between section radioactivity and section autoradiographic grain density was observed for autoradiographic grain densities of up to 300 grains $1000 \mu\text{m}^{-2}$.

Drugs

Drugs used were: endothelin-1 (porcine/human), [^{125}I]-endothelin-1 (Auspep, Melbourne, Australia), carbamylcholine chloride (carbachol), phenylephrine hydrochloride, indomethacin, bovine serum albumin (Sigma Chemical Company, St. Louis, U.S.A.), phenylmethylsulphonylfluoride (Calbiochem, La Jolla, U.S.A.), [^{125}I]-fibrinogen (Amersham, Sydney, Australia), thrombin (Armour Pharmaceutical Company, Kankakee, U.S.A.). The composition of the Krebs-bicarbonate solution was (in mM): NaCl 117, KCl 5.36, NaHCO_3 25.0, KH_2PO_4 1.03, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.57, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 2.5 and glucose 11.1.

Analysis of results

ET-1-induced contractions of airway and vascular smooth muscle preparations were expressed in terms of the maximum tissue response to carbachol (10 μM) and phenylephrine (10 μM), respectively. Agonist EC_{30} and EC_{50} values were calculated by interpolation of concentration-effect curves and are expressed as the geometric mean with associated 95% confidence limits. All other functional data are presented as the arithmetic mean $\pm$ s.e.mean.

Estimates of the dissociation constant (K_d) and maximum binding capacity (B_{max}) of specific [^{125}I]-ET-1 binding to airway smooth muscle were obtained for each individual animal from non-linear regression analysis of data fitted to a one-site binding model and from Scatchard analysis. Group B_{max} values are expressed as arithmetic mean $\pm$ s.e.mean and group K_d values presented as the geometric means (95% confidence limits) from 3 animals. Differences between means were established by Student's two-tailed, paired or non-paired *t* test, as appropriate, and were considered significant if $P < 0.05$.

Results

Functional studies

Airway smooth muscle preparations ET-1 produced concentration-dependent contraction of airway smooth muscle preparations isolated from humans, guinea-pigs, rats and mice (Figure 1). No relaxation responses were observed

Figure 1 Representative concentration-effect curves to endothelin-1 (ET-1) in (a) mouse trachea, (b) rat trachea, (c) human bronchus and (d) guinea-pig trachea. ET-1 (nM) was added at the points indicated (Δ), in incremental doses.

over the concentration-range examined (0.1 nM–0.3 μ M). ET-1-induced contractions of human bronchus, guinea-pig trachea and rat trachea were invariably slow in onset, taking 5 to 10 min to reach a plateau. In contrast, ET-1-induced contractions of mouse isolated trachea occurred more rapidly, in general taking no more than 3 min to reach a plateau (Figure 1). Over the concentration-range studied (0.1 nM–0.3 μ M), it was not possible to obtain maximal contractile responses to ET-1 in rat trachea, guinea-pig trachea or human bronchus (Figure 1 and 2a). Accordingly, ET-1-induced contractions were expressed as a percentage of the maximal contractile response to 10 μ M carbachol ($C_{\max}$) rather than to 0.3 μ M ET-1.

Mouse isolated trachea was significantly more sensitive to ET-1 than airways preparations from the other animal species tested (Figure 2a). In mouse isolated trachea, maximally effective concentrations of ET-1 (0.3 μ M) produced, on average, $85.9 \pm 5.4\%$ $C_{\max}$ ($n = 11$ animals) and the mean concentration of ET-1 producing 30% $C_{\max}$ was 3.4 nM (95% confidence limits, 2.4–5.0 nM). Rat tracheal and human bronchial preparations also produced greater than 70% $C_{\max}$ in response to 0.3 μ M ET-1, although mean concentrations producing 30% $C_{\max}$ were respectively, 4.9 and 10.4 fold higher than the mean concentration producing this level of response in mouse trachea. Guinea-pig trachea was the least sensitive of the airway preparations exposed to ET-1, producing only $32.2 \pm 3.0\%$ $C_{\max}$ at the highest concentration of ET-1, (i.e. 0.3 μ M). Thus, the concentration of ET-1 producing 30% $C_{\max}$ in guinea-pig trachea was on average, 71 fold higher than that observed in mouse trachea.

In contrast to the marked inter-species differences observed in airway smooth muscle responsiveness to ET-1, (Figure 2a), EC_{50} values to carbachol differed by less than 2.5 fold between airways preparations from human, guinea-pig, rat and mouse (Figure 2b). Indeed, the guinea-pig trachea which

Figure 2 Mean cumulative concentration-effect curves to (a) endothelin-1 (ET-1) and (b) carbachol in mouse trachea (●), rat trachea (○), human bronchus (■) and guinea-pig trachea (□). Each curve represents the mean of 6–14 preparations from 3–11 animals; vertical bars show s.e.mean.

was the least responsive of the preparations to ET-1 was the most sensitive to carbachol.

In the presence of the cyclo-oxygenase inhibitor indomethacin (5 μ M), both mouse and guinea-pig trachea were significantly more responsive to ET-1 ($P < 0.05$, Figure 3a,b). For example, the concentrations of ET-1 producing 30% $C_{\max}$ were 2.1 and 2.2 fold lower in indomethacin-treated preparations of mouse and guinea-pig trachea, respectively than in paired control preparations. The constrictor responses of mouse trachea to carbachol were also slightly potentiated in the presence of indomethacin (1.4 fold difference in EC_{50} values, $P < 0.05$, Figure 3a). In rat trachea, indomethacin had no significant effect on responses to ET-1 or carbachol (Figure 3c).

Removal of the tracheal epithelium had no significant effect on the responsiveness of mouse trachea to the constrictor actions of ET-1 or carbachol ($n = 4$, data not shown).

Vascular smooth muscle preparations In accord with previously published reports (D'Orleans-Juste *et al.*, 1989; Eglen *et al.*, 1989), ET-1 was a potent constrictor of rat isolated aorta (Figure 4). The EC_{50} value of 2.4 nM (1.7–3.3, $n = 12$) observed for ET-1 in endothelium-intact rat aorta preparations (Figure 4) was nearly 12 times lower than the EC_{50} value of 28.3 nM (21.6–37.2, $n = 6$) observed in rat trachea (Figure 2a). Conversely, whereas mouse trachea was highly

Figure 3 Effect of 5 μ M indomethacin (closed symbols) on mean concentration-effect curves to endothelin-1 (ET-1, squares) and carbachol (circles) in (a) mouse trachea ($n = 7$ animals), (b) guinea-pig trachea ($n = 5$) and (c) rat trachea ($n = 5$). Control responses in paired preparations (open symbols) are also shown. Vertical bars represent s.e.mean.

Figure 4 Mean cumulative concentration-effect curves to endothelin-1 (ET-1) in endothelium-intact (○) and paired endothelium-denuded (●) mouse isolated aortic preparations. Responses of endothelium-intact rat aortic preparations are also shown (▲). Each curve represents the mean responses of preparations from 4 animals; vertical bars show s.e.mean.

responsive to the constrictor actions of ET-1 (Figure 2a), mouse aorta was only marginally responsive (Figure 4). In endothelium-intact mouse aortic preparations, the highest concentration of ET-1 used (0.3 μ M) induced only $20.2 \pm 5.8\%$ ($n = 4$) of the maximum response to phenylephrine. In mouse aortic preparations denuded of vascular endothelium, ET-1-induced contractions were also small ($11.7 \pm 4.6\%$, $n = 4$).

Autoradiography

Light microscopic autoradiography revealed very high densities of specific [¹²⁵I]-ET-1 binding to human bronchial smooth muscle (Figure 5). Moderate levels of specific binding were also associated with vascular elements; however, only very low levels of binding were associated with the cartilage, submucosal and epithelial cells. A similar profile of specific [¹²⁵I]-ET-1 binding was observed in tracheal sections from mouse (Figure 6), rat and guinea-pig.

Quantitative autoradiography

Specific [¹²⁵I]-ET-1 binding to airway smooth muscle was identified in each of the animal species examined (Figure 7). Specific [¹²⁵I]-ET-1 binding was saturable and involved a single class of non-interacting binding sites, as determined from Scatchard analysis. No significant inter-species differences were detected for dissociation constants (K_d) of specific [¹²⁵I]-ET-1 binding, which ranged from 0.11 nM (0.04–0.28 nM, $n = 3$) for guinea-pig tracheal smooth muscle to 0.43 nM (0.087–2.1 nM, $n = 3$) for rat tracheal smooth muscle. However marked inter-species differences were observed for estimates of specific [¹²⁵I]-ET-1 binding site density (B_{max}). The highest B_{max} value was found in rat tracheal smooth muscle (69.0 ± 11.2 amol mm⁻², $n = 3$) and the lowest in guinea-pig tracheal smooth muscle (8.3 ± 1.8 amol mm⁻², $n = 3$). The density of specific [¹²⁵I]-ET-1 binding sites in human bronchial smooth muscle was 42.7 ± 17.5 amol mm⁻² ($n = 3$). There was no significant inter-species differences in the density of non-specific [¹²⁵I]-ET-1 binding sites in airway smooth muscle (Figure 7). The relationship between the observed density of specific [¹²⁵I]-ET-1 binding sites and the contractile potency of ET-1 in airway smooth muscle is presented for each of the animal species in Figure 8.

Discussion

In the current study, a dense population of specific binding sites for the recently identified vasoactive peptide ET-1 was localized over human bronchial smooth muscle. Additional *in vitro* studies indicated that some of these ET-1 binding sites may be linked to functional responses, since ET-1 caused marked constrictor responses in human isolated bronchial

Figure 5 (a) Dark-field photomicrograph of a 10 μ m frozen section of human isolated bronchus showing the distribution and localization of autoradiographic grains derived from [¹²⁵I]-endothelin-1 ([¹²⁵I]-ET-1) (0.2 nM) binding. (b) Bright-field photomicrograph of the above section. SM = smooth muscle, E = epithelium, SG = submucosal glands. Bar = 100 μ m. (c) Dark-field photomicrograph showing the distribution of non-specific autoradiographic grains in a neighbouring section incubated with [¹²⁵I]-ET-1 (0.2 nM) and unlabelled ET-1 (1 μ M).

smooth muscle. In terms of spasmogenic potency, ET-1 was approximately 3 times more active in human isolated bronchial smooth muscle than either the cholinergic agonist carbachol or histamine (Goldie *et al.*, 1986).

Tracheal preparations from guinea-pig, rats and mice also contained populations of specific binding sites for ET-1 which were highly localized to airway smooth muscle. All airways preparations examined were sensitive to the constrictor actions of ET-1; however, significant inter-species differences in ET-1 potency were observed. The order of airway responsiveness to ET-1 was: mouse trachea > rat trachea > human bronchus > guinea-pig trachea, with mouse trachea being at least 10 and 70 times more sensitive to ET-1 than human and guinea-pig airways respectively. In the current study, we examined whether these significant interspecies differences in

Figure 6 (a) Dark-field photomicrograph of mouse isolated trachea showing the distribution of autoradiographic grains derived from [125 I]-endothelin-1 ([125 I]-ET-1) (0.2 nM) binding. (b) Bright-field photomicrograph of the above section. SM = smooth muscle, C = cartilage, E = epithelium. Bar = 100 μ m. (c) Dark-field photomicrograph showing the distribution of non-specific autoradiographic grains in a neighbouring section incubated with [125 I]-ET-1 (0.2 nM) and unlabelled ET-1 (1 μ M).

airway smooth muscle contractile responsiveness to ET-1 may be related to differences in ET-1 binding characteristics in airway smooth muscle. Clearly, various parameters can be used to describe binding characteristics (K_d , B_{max}) or airway smooth muscle responsiveness (potency, maximal effect) and thus the validity of any link made between ET-1 binding and airway smooth muscle responsiveness may depend specifically on which parameters have been correlated. In addition, any attempt to relate specific binding site density and contractile response depends on the binding sites being receptors that are functionally linked. At present, it is not known whether receptor reserves exist for ET-1 or the extent to which receptor reserves are species-dependent.

In Figure 8 the relationship between the density of specific ET-1 binding sites and the contractile activity of ET-1 is illustrated for each of the animal species examined. The contractile

Figure 7 Concentration-dependence of [125 I]-endothelin-1 ([125 I]-ET-1) binding to smooth muscle of (a) rat trachea, (b) mouse trachea, (c) human bronchus and (d) guinea-pig trachea showing specific (●) and non-specific (○) binding. [125 I]-ET-1 binding density was estimated from quantitative autoradiographic analyses of grain densities (see Methods). Each curve represents the mean responses of preparations from 3 animals; vertical bars show s.e.mean.

Figure 8 Relationship between [125 I]-endothelin-1 ([125 I]-ET-1) binding site density (hatched columns, $n = 3$ animals, Figure 7) and contractile activity (closed columns, $n = 3$ –11 animals, Figure 2) in airway smooth muscle preparations from guinea-pig, human, rat and mouse. ET-1 activity is expressed as $-\log$ (concentration of ET-1 producing 30% C_{max}).

activity of ET-1 has been expressed in terms of the concentration of ET-1 producing 30% of the maximal contractile response to the cholinergic agonist carbachol, because a clear maximal contractile response to ET-1 was not obtained for all species and thus conventional EC_{30} or EC_{50} values could not be derived. However, in view of the clear separation of ET-1 concentration-effect curves between the different animal species (Figure 2a), it is unlikely the rank order of potencies of ET-1 would change significantly if conventional EC_{30} values were able to be derived. For man, rat and guinea-pig the order of airway responsiveness to ET-1 was apparently positively correlated with the order of ET-1 binding site density. For example, guinea-pig tracheal smooth muscle contained the lowest density of ET-1 binding sites and was the least responsive of the species to the constrictor actions of ET-1. Similarly, the rat trachea and human bronchus which contained the highest densities of ET-1 binding sites were proportionately more responsive to the constrictor effects of ET-1. These results suggest that for some species at least, the density of ET-1 binding sites may be an important determinant of ET-1 constrictor potency. However, an exception to

this relationship was observed. The mouse trachea contained a lower density of ET-1 binding sites than either rat trachea or human bronchus despite being the most responsive of the airway preparations to ET-1.

In addition to receptor density, ET-1 potency is likely to be dependent on a number of other factors. We have investigated the possibility that some of these factors may be different in the mouse compared to the other animal species. Firstly, it has been suggested that in some animal species, the potent bronchoconstrictor actions of ET-1 may be at least in part, attributable to the capacity of ET-1 to release potent spasmogens. Eglen and co-workers (1989) have suggested that the high potency of ET-1 observed in guinea-pig trachea ($EC_{50} = 0.5 \text{ nm}$) by Uchida and associates (1988) may be due, at least partially, to the production of the potent bronchoconstrictor agent thromboxane A_2 , via stimulation of cyclo-oxygenase. Consistent with this view, ET-1 has been shown to release thromboxane A_2 from guinea-pig lung (Antunes *et al.*, 1989). Furthermore, the cyclo-oxygenase inhibitor indomethacin has been shown to reduce (Braquet *et al.*, 1989) or abolish (Payne & Whittle, 1988) the *in vivo* bronchoconstriction produced following ET-1 administration. However, the inter-species differences in ET-1 activity described in the current study cannot be attributed to the differential release by ET-1 of thromboxanes, because the 70 fold potency difference observed between guinea-pig and mouse trachea was maintained in the face of cyclo-oxygenase inhibition by indomethacin. Indeed in the current study, indomethacin augmented rather than attenuated constrictor responses to ET-1, suggesting that any thromboxane release played only a minor role in determining the tracheal constrictor potency of ET-1 *in vitro*. Similarly, removal of the tracheal epithelium had no significant effect on the actions of ET-1 in mouse isolated tracheal preparations. These results suggest that the observed constrictor effects of ET-1 occurred via a direct action on the smooth muscle.

There was no evidence from the current study to suggest that compared to other animal species mouse tissues *per se* were hyperreactive to ET-1 or to spasmogens in general. For example, airway smooth muscle preparations from mice were no more sensitive to the constrictor actions of the cholinergic agonist carbachol than were preparations from other animal species. Moreover, the mouse thoracic aorta preparation, irrespective of whether the endothelium was intact or removed, responded poorly to the constrictor actions of ET-1. This was particularly surprising given that ET-1 was a potent vasoconstrictor agent in almost all other isolated vascular preparations so far examined (D'Orleans-Juste *et al.*, 1989;

Eglen *et al.*, 1989). Thus it is clear that the high sensitivity of mouse tracheal smooth muscle to ET-1 is not a general indication of the responsiveness of mouse tissues to ET-1.

An alternative explanation for the apparent high sensitivity of mouse tracheal smooth muscle to ET-1 involves a more efficient coupling between ET-1 binding sites and post-receptor mechanisms. At present, the intracellular processes mediating the constrictor actions of ET-1 have not been fully elucidated. ET-1-induced contractions of airway smooth muscle cells are associated with an increase in intracellular Ca^{2+} levels (Sakata *et al.*, 1989) although the source of these Ca^{2+} ions is not clear. The removal of extracellular Ca^{2+} has been shown in rat trachea (Turner *et al.*, 1989) and canine trachea (Sakata *et al.*, 1989) to attenuate contractile responses to ET-1, indicating an important role for extracellular Ca^{2+} . However, ET-1-induced contractions of guinea-pig trachea (Hay, 1989) were not influenced significantly by an extracellular Ca^{2+} -free environment. A dependence of the ET-1 bronchoconstriction on the opening of dihydropyridine-sensitive Ca^{2+} -channels has been suggested from studies using airways preparations of guinea-pig (Uchida *et al.*, 1988; Hay, 1989; Maggi *et al.*, 1989), rat (Borges *et al.*, 1989) and rabbit (Grunstein *et al.*, 1989), although other investigators have not found any role for these Ca^{2+} -channels in mediating ET-1-induced contractions of rat trachea (Turner *et al.*, 1989). No role for phosphoinositide metabolism in elevating intracellular Ca^{2+} levels has so far been identified for ET-1 in contracting airway smooth muscle (Hay, 1989). In addition to these studies, Sakata and coworkers (1989) have suggested that a portion of the ET-1-induced contraction may occur through a Ca^{2+} -independent mechanism. The nature of the post-receptor mechanisms involved in ET-1-induced responses in airway smooth muscle is clearly complex. Thus any explanation for the apparently greater efficiency of this receptor-effector process in mouse trachea would be premature without further experimentation.

In view of the ability of ET-1 to induce potent sustained increases in tone in human airway smooth muscle, the potential role of this peptide in obstructive airway disease requires detailed examination. For example, ET-1 may be an important mediator of asthma since this disease involves pulmonary hypoxia, a condition conducive to the synthesis and release of ET-1.

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