Induction by endothelin-1 of epithelium-dependent relaxation of guinea-pig trachea *in vitro*: role for nitric oxide

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1 The purpose of the present experiments was to study the underlying mechanisms responsible for the relaxant action of endothelin-1 (ET-1) in the guinea-pig trachea *in vitro*.

2 In tracheal strips precontracted (60–70% of the maximum) with carbachol, ET-1 (1–100 nM) evoked slowly developing concentration-dependent relaxations. Preincubation of the tissues with the thromboxane A₂/prostaglandin H₂ receptor antagonist, BM 13505 (5 µM) significantly potentiated the relaxant response to ET-1.

3 Removal of the epithelium changed the response of precontracted tracheal preparations to ET-1 from a relaxation to a sustained contraction.

4 ET-1-induced relaxations were abolished by methylene blue (10 µM) and were almost completely attenuated by oxyhaemoglobin (5 µM) and N^G-monomethyl-L-arginine (L-NMMA, 100 µM), an inhibitor of nitric oxide synthesis, but were not altered by indomethacin (10 µM).

5 In tracheal strips under passive tension, ET-1 (1–100 nM) elicited dose-dependent contractions. The sensitivity of tissues to ET-1 was significantly enhanced by removal of the epithelium (apparent EC₅₀ values were 28.1 ± 4.1 and 12.5 ± 0.8 nM in intact and rubbed trachea, respectively, *n* = 7, *P* < 0.01).

6 Preincubation of intact tracheal strips with methylene blue, oxyhaemoglobin or L-NMMA did not mimic the effect of epithelium removal on ET-1-induced contractions.

7 There was a concentration-dependent increase in thromboxane A₂ but not in PGE₂ and prostacyclin release from intact tracheal strips following stimulation with ET-1 (5–100 nM).

8 These results show that ET-1 exerts a dual action on guinea-pig isolated trachea: it evokes contractions at low resting tone, whereas it induces relaxations at higher resting tone. The relaxant action of ET-1 may be mediated by nitric oxide released from epithelial cells and resultant activation of smooth muscle guanylate cyclase.

Keywords: Endothelin-1; epithelium-derived relaxing factor; nitric oxide; guanylate cyclase; isolated trachea (guinea-pig)

Introduction

Recent evidence suggests an important role for the airway epithelium in modulating the responsiveness of the underlying smooth muscle. Hyperreactivity of the airways is associated with damage or loss of the airway epithelium in bronchial asthma (Laitinen *et al.*, 1985). Removal of the epithelial layer from isolated airways of several mammalian species has been shown to enhance the contractile response to various bronchoconstrictor agents, including histamine, acetylcholine, 5-hydroxytryptamine and leukotrienes C₄ and D₄ (Barnes *et al.*, 1985; Holroyde, 1986; Goldie *et al.*, 1986; Hay *et al.*, 1987). These findings led to the concepts that intact epithelium may either act as a protective barrier between constrictors and airway smooth muscle (Holroyde, 1986; Undem *et al.*, 1988) or it may modulate the airway tone through release of relaxant substances, which may include prostanoids (Raeburn, 1990) and epithelium-derived relaxing factor(s) (Vanhoutte, 1988).

Endothelin-1 (ET-1), a 21-amino-acid peptide has been identified in the supernatant of cultured endothelial cells (Yanagisawa *et al.*, 1988) and tracheal epithelial cells (Black *et al.*, 1989). Specific binding of ET-1 to airway smooth muscle cells (Turner *et al.*, 1989b; Rozengurt *et al.*, 1990) as well as to epithelial cells has also been detected by immunohistochemistry (Rozengurt *et al.*, 1990). Thus, being produced and released locally, ET-1 is well situated to modulate airway tone either through acting on epithelial and/or

smooth muscle cells. Indeed, numerous studies have shown that ET-1 exerts an airway constrictor action both *in vitro* and *in vivo*. On a molar basis, it is one of the most potent bronchoconstrictor agents yet described (Uchida *et al.*, 1988; Maggi *et al.*, 1989; Battistini *et al.*, 1990). ET-1 has also been reported to induce relaxation in precontracted rabbit tracheal segments (Grunstein *et al.*, 1991) and in tracheal strips isolated from guinea-pigs following an anaphylactic reaction (Saotome *et al.*, 1991). Furthermore, transient relaxation preceding the contractile response to ET-1 has been reported in some *in vitro* preparations of guinea-pig trachea (Turner *et al.*, 1989a), and in guinea-pig airway smooth muscle *in situ* (White *et al.*, 1991). These findings indicate that ET-1 is capable of releasing a relaxant factor from the trachea under certain circumstances. Bronchodilator prostaglandins (Grunstein *et al.*, 1991), lipoxygenase products derived from non-epithelial tissues (Saotome *et al.*, 1991) as well as an epithelium-derived relaxing factor of unknown nature (Grunstein *et al.*, 1991) have been implicated in mediating the relaxant action of ET-1. Recently, nitric oxide (NO) has been suggested as a mediator of the non-adrenergic, non-cholinergic relaxant response of guinea-pig trachea (Li & Rand, 1991; Kannan & Johnson, 1992). Furthermore, inhalation of NO gas induces rapid and potent bronchodilatation in the methacholine bronchoconstricted guinea-pig (Dupuy *et al.*, 1992). These findings led us to hypothesize that NO might mediate the relaxant action of ET-1 in guinea-pig airways.

The present experiments were designed to characterize further the relaxant action of ET-1 in the guinea-pig trachea *in vitro* and to identify the underlying mechanisms. The present data provide evidence that ET-1 elicits a concentration-dependent relaxation of isolated precontracted trachea,

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which may be mediated by NO released from epithelial cells and resultant activation of smooth muscle guanylate cyclase.

Methods

Tissue preparation

Male Dunkin-Hartley guinea-pigs (420–540 g) were killed by cervical dislocation followed by exsanguination. The trachea was then rapidly removed and placed in oxygenated (95% O₂ and 5% CO₂) Krebs-Henseleit solution (composition in mM: NaCl 118, KCl 4.7, CaCl₂ 2.5, KH₂PO₄ 1.18, NaHCO₃ 25, MgSO₄·7H₂O 1.18 and glucose 11.1) at 37°C, pH 7.0. Following removal of adherent fat and connective tissue the trachea was cut in half. One half was left intact, while in some experiments the other half was stripped of epithelium by gently rubbing the lumen with a cotton-tipped applicator. Proximal and distal halves were randomly selected for removal of the epithelium. Then tracheal segments were cut in spirals (approximately 2.8 mm width by 30 mm long). Tissues were mounted separately in 5 ml organ baths and were connected to isometric force-displacement transducers (Grass model FTO3C) under an initial tension of 1.0 g. Tissues were equilibrated for 60 min in oxygenated Krebs-Henseleit solution. Organ baths were flushed with fresh solution every 15 min. Changes in tension were recorded continuously on a Grass 7D polygraph. In all tissues contractile responses to a single concentration of histamine (4.5 µM) was established before examination of the effects of ET-1 and complete recovery to resting tension was allowed following addition of histamine.

Experimental protocols

Endothelin-1-induced relaxation To study the relaxant effect of ET-1, submaximal tone (usually 60–70% of the maximum) was first induced with carbachol (0.1 µM). Previously we have found that ET-1-induced contraction of guinea-pig isolated airways was, in part, mediated by thromboxane A₂ (Filep *et al.*, 1990; 1991). Therefore, the relaxation experiments were performed either in the absence or in the presence of BM 13505 (5 µM), a specific thromboxane A₂/prostaglandin H₂ receptor antagonist (Patscheke *et al.*, 1987). At the concentration employed, BM 13505 neither affects prostanoid formation (Patscheke *et al.*, 1987; Filep *et al.*, 1991) nor interferes with the biological responses to other bronchoactive agents, including platelet-activating factor, 5-hydroxytryptamine and histamine (Patscheke *et al.*, 1987; Battistini *et al.*, 1990). Cumulative concentrations of ET-1 (1–100 nM) were added to intact and rubbed tracheal strips, the next concentration being added when the preceding one had reached a steady-state effect. In another set of experiments, tracheal strips with intact epithelium were preincubated with methylene blue (10 µM), an inhibitor of soluble guanylate cyclase (Martin *et al.*, 1985), oxyhaemoglobin (5 µM), which binds NO (Hille *et al.*, 1977), N^G-monomethyl-L-arginine (L-NMMA, 100 µM), an inhibitor of NO synthesis (Rees *et al.*, 1989; 1990) or their vehicle for 10 min in the presence of BM 13505 (5 µM) before addition of the first concentration of ET-1. Some tracheal strips were preincubated with indomethacin (10 µM) for 20 min before addition of ET-1. In additional experiments, the reversibility of the action of L-NMMA was studied by use of L-arginine (1 mM) and D-arginine (1 mM).

Endothelin-1-induced contraction Concentration-response curves for ET-1 (1–100 nM) were obtained by its cumulative addition to intact and rubbed tracheal strips. Each ET-1 concentration was left in contact with the preparations until the response reached a steady-state level.

In another set of experiments, tracheal strips with or without intact epithelium were preincubated with methylene blue

(10 µM), oxyhaemoglobin (5 µM), L-NMMA (100 µM) or their vehicle at 37°C for 10 min before cumulative addition of ET-1 to give final concentrations of 1–100 nM. Since methylene blue by itself slightly increased the tone in tracheal strips (3–8% of the contraction evoked by 4.5 µM histamine), tone was reduced, after treatment with this agent, to levels of untreated strips by adjusting the mechanical stretching force.

Only one concentration-response curve for ET-1 was obtained from each preparation. To minimize variation between tissues, ET-1-induced responses are expressed as the percentage of the contraction evoked by 4.5 µM of histamine.

Endothelin-1-induced prostanoid release In another set of experiments the effects of ET-1 on prostaglandin release were also studied. Tracheal strips with intact epithelium were prepared and incubated under the same experimental conditions as described above. Following a control incubation period of 4 min, three consecutive concentrations of ET-1 (5, 10 and 100 nM) were added to the organ baths. In additional experiments the effects of ET-1 were studied in tracheal strips preincubated with indomethacin (10 µM) for 20 min before addition of ET-1 (100 nM). Following an incubation period of 4 min, an aliquot of the incubation medium was removed and stored at –20°C until analysis of immunoreactive prostaglandin E₂ (PGE₂), 6-keto-PGF_{1α}, the stable metabolite of prostacyclin and thromboxane B₂ (TXB₂), the stable metabolite of TXA₂. Each tissue was weighed at the end of the experiment. Prostanoid release is expressed as pg released per 4 min per 100 mg tissue wet weight.

Measurements of prostanoids

Concentrations of immunoreactive PGE₂, 6-keto-PGF_{1α} and TXB₂ in the incubation medium were measured directly by enzyme immunoassays (Cayman Labs, Ann Arbor, MI, U.S.A.). The detection limit of the PGE₂, 6-keto-PGF_{1α} and TXB₂ assays were 1.5, 0.3 and 0.5 pg/tube, respectively. Intraassay coefficients of variation were 4.1% (*n* = 6), 5.8% (*n* = 5) and 4.2% (*n* = 5), respectively. The PGE₂ antiserum cross reacted with TXB₂ 4.0%, PGF_{2α} 2.5% and 6-keto-PGF_{1α} 1.4%. The 6-keto-PGF_{1α} antiserum cross-reacted with PGF_{2α} 12%, 6,15-diketo-PGF_{1α} 8% and TXB₂ 0.2%. The TXB₂ antiserum had 11% cross-reactivity with 2,3-dinor-TXB₂, whereas it had less than 0.7% cross reactivity with other prostanoids.

Histology

After completion of concentration-response studies, tissues were removed from the organ baths and were cut into approximately 3 × 4 mm pieces. Pieces were taken randomly and prepared for light and scanning electron microscopy. For light microscopical examinations, samples were fixed in Bouin fixative, then 5 µm thick sections were prepared and stained with haematoxylin and eosin. For scanning electron-microscopical examinations, samples were prepared according to the method of Postek *et al.* (1980) using critical point drying. Tissues were examined in a Cambridge stereoscan 120 electron-microscope.

Drugs

Endothelin-1 (Peninsula Labs, Belmont, CA, U.S.A.) was dissolved in distilled water and stored in aliquots at –20°C under N₂ for up to 3 weeks. BM 13505 (4-[2-(4-chlorobenzene sulphonyl-amino)ethyl] benzene acetic acid, Boehringer-Mannheim GmbH, Mannheim, Germany) and indomethacin (Merck, Sharp & Dohme, Canada) were dissolved in ethanol and were diluted further with Krebs-Henseleit solution as appropriate. The final concentration of ethanol in the organ bath never exceeded 0.1%. Methylene blue (Fischer Scientific, Fair Lawn, NJ, U.S.A.), L-arginine, D-arginine (free base), carbachol (carbamylocholine chloride, Sigma Chemical

Co., St. Louis, MO, U.S.A.) and N^G -monomethyl-L-arginine (Research Biochemicals Inc., Natick, MA, U.S.A.) were dissolved in Krebs-Henseleit solution. Oxyhaemoglobin was prepared from commercial bovine haemoglobin (Sigma) as described previously (Miller & Vanhoutte, 1989). The purity and concentration of oxyhaemoglobin in the final solution was determined spectrophotometrically. The solution was frozen in aliquots and stored at -20°C up to 10 days.

Statistical analysis

Values are reported as means $\pm$ s.e.mean. Statistical evaluation of the data was performed by (1) two-way analysis of variance using ranks (Friedman test) for repeated measures with a subsequent Wilcoxon-Wilcox test (Wilcoxon & Wilcox, 1964) to identify differences between measurements taken during the control period and measurements taken following various concentrations of ET-1; (2) by Dunn multiple contrast hypothesis test (Dunn, 1964) when different treatments were compared to the same control or (3) by Wilcoxon signed rank test and by Mann-Whitney U test for paired and unpaired observations, respectively. A $P < 0.05$ level was considered significant for all tests.

Results

Endothelin-1-induced epithelium-dependent relaxation

Addition of cumulative concentrations of ET-1 (1–100 nM) to intact tracheal strips precontracted with carbachol resulted in a dose-dependent relaxation both in the absence and presence of BM 13505 (5 μM) (Figures 1, 2). However, the relaxant action of ET-1 was about two fold higher in the presence of BM 13505 (Figure 1). The threshold concentration of ET-1 to evoke relaxation was about 5 nM, whereas statistically significant relaxations were observed at concentrations higher than 10 nM (Figure 1). The relaxation developed following a lag time of 1–2.5 min and reached a plateau within 4–8 min (Figure 2). At higher concentrations (50–100 nM), ET-1-induced relaxations were preceded by small, transitory contractions (Figure 2). After induction of contraction in

rubbed tracheal strips with carbachol, the relaxations elicited by 5 and 10 nM ET-1 were nearly abolished, whereas the relaxations evoked by higher concentrations of ET-1 (50–100 nM) were changed to sustained contractions (Figure 1).

Figure 2 Effects of methylene blue, oxyhaemoglobin and N^G -monomethyl-L-arginine on endothelin-1 (ET-1)-induced relaxation of guinea-pig isolated trachea. Tracheal strips were preincubated with methylene blue (MB, 10 μM) (●), oxyhaemoglobin (Hb, 5 μM) (■), N^G -monomethyl-L-arginine (L-NMMA, 100 μM) (▲) or vehicle (○) for 10 min, then contractions were evoked by carbachol (CCh, 0.1 μM) in the presence of BM 13505 (5 μM) before addition of cumulative concentrations of ET-1 (1–100 nM). (a) Representative tracings; (b) Values are means with s.e.mean. Control $n = 12$, MB $n = 7$, Hb and L-NMMA $n = 5$. * $P < 0.05$; ** $P < 0.01$ compared to control by Dunn multiple contrast test.

Figure 1 Endothelin-1 (ET-1)-induced epithelium-dependent relaxations of the guinea-pig trachea *in vitro*. Tissues were precontracted with 0.1 μM carbachol, then cumulative concentrations of ET-1 were added in the absence (–BM) and presence (+BM) of the specific thromboxane A_2 /prostaglandin H_2 receptor antagonist, BM 13505 (5 μM). Epithelium +, intact epithelium; epithelium –, epithelium was removed. Values are means with s.e.mean of 8–10 experiments. * $P < 0.05$; ** $P < 0.01$ (compared to control by Wilcoxon-Wilcox test).

Effects of methylene blue, oxyhaemoglobin, L-NMMA and indomethacin on ET-1-induced relaxation

Incubation of intact precontracted tracheal strips for 10 min with methylene blue (10 μ M) abolished the relaxation induced by ET-1 (Figure 2). When intact tracheal strips were preincubated with oxyhaemoglobin (5 μ M) for 10 min before inducing contractions with carbachol, ET-1-induced relaxation was markedly attenuated (Figure 2). Oxyhaemoglobin treatment did not affect significantly the lag time of ET-1 (Figure 2). Prolongation of the incubation time did not result in an increase in the inhibitory potency of oxyhaemoglobin (data not shown). Preincubation of the tissues with L-NMMA (100 μ M) for 10 min resulted in similar inhibition of ET-1-induced relaxations as those observed with oxyhaemoglobin (Figure 2). A higher concentration of L-NMMA (1 mM) did not produce additional inhibitory effects. As shown in Table 1, the inhibitory action of L-NMMA was reversed by L-arginine (1 mM), but not by D-arginine (1 mM). The cyclo-oxygenase blocker, indomethacin (10 μ M) failed to alter significantly ET-1-induced relaxations (Table 1).

Effect of epithelium removal on ET-1-induced contraction

In the intact guinea-pig trachea, ET-1 (1–100 nM) evoked tonic, concentration-dependent contractions. The contractions developed slowly, reaching a steady-state level within 3–5 min. In these experiments, we did not observe relaxation following addition of ET-1 to the organ bath. Removal of the epithelium markedly enhanced the responsiveness of guinea-pig trachea to ET-1 as reflected by a 2.3 fold decrease in the apparent EC_{50} values (28.1 ± 4.1 nM in the intact trachea, $n = 7$ versus 12.5 ± 0.8 nM in the trachea without epithelium, $n = 7$, $P < 0.01$) and an increase in the contractile response evoked by 100 nM ET-1 (131 ± 42 and 254 ± 53 mg in the intact and denuded trachea, respectively, $n = 7$, $P < 0.01$).

Effects of methylene blue, oxyhaemoglobin and L-NMMA on ET-1-induced contraction

Addition of methylene blue (10 μ M) to the organ bath resulted in a slowly developing, slight increase in tone (3–8% of the contraction evoked by 4.5 μ M histamine). Consequently, care was taken to match tone with that of control preparations. The contractile response of intact tracheal strips to ET-1 tended to be somewhat higher in the presence of methylene blue than in control tissues. However, these changes did not reach statistical significance (Figure 3). Neither oxyhaemoglobin (5 μ M) nor L-NMMA (100 μ M) affected significantly ET-1-induced contractions (Figure 3). Similarly, methylene blue, oxyhaemoglobin and L-NMMA failed to alter significantly the contractile responses of denuded tracheal strips to ET-1 (data not shown).

Figure 3 Effects of methylene blue, oxyhaemoglobin and N^G -monomethyl-L-arginine on endothelin-1 (ET-1)-induced contraction in guinea-pig tracheal strips *in vitro*. Tissues were preincubated with methylene blue (MB, 10 μ M), oxyhaemoglobin (Hb, 5 μ M), N^G -monomethyl-L-arginine (L-NMMA, 100 μ M) or vehicle (control, C) for 10 min at 37°C before addition of cumulative concentrations of ET-1. Maximum contractions observed after each concentration of ET-1 are expressed as % of the contraction evoked by 4.5 μ M histamine. Values are means with s.e.mean. Control $n = 9$, MB $n = 5$, Hb $n = 9$, L-NMMA $n = 4$.

ET-1-induced prostanoid release

In another set of experiments the effects of ET-1 on PGE_2 , prostacyclin and thromboxane A_2 production by intact tracheal strips were studied. Analysis of the incubation medium showed that the basal release of PGE_2 , 6-keto- $PGF_{1\alpha}$ and TXB_2 were 40–70, 120–200 and 80–140 pg per 4 min per 100 mg tissue wet weight, respectively. Cumulative addition of 5, 10 and 100 nM of ET-1 did not induce significant changes in PGE_2 and 6-keto- $PGF_{1\alpha}$ levels in the medium (Table 2). On the other hand, 10 and 100 nM ET-1 caused about 62 and 190% increases in thromboxane release, respectively (Table 2). Preincubation of the tissues with indomethacin (10 μ M) reduced basal prostanoid production by more than 75% and prevented ET-1 (100 nM)-induced TXA_2 release (Table 2).

Histology

Light microscopical examinations revealed that rubbing the trachea removed the epithelium without producing obvious damage to the underlying submucosa and smooth muscle layers. Examination of randomly selected tissue pieces by scanning electron microscopy revealed that more than 96% of epithelial cells were removed by rubbing the tracheae,

Table 1 Effect of various agents on relaxant responses to endothelin-1 in guinea-pig tracheal strips with intact epithelium

	n	Endothelin-1 (50 nM)		
		Control	+ Agent	P
L-NMMA, 100 μ M	5	40 $\pm$ 4	17 $\pm$ 3	<0.05
L-NMMA, 100 μ M + L-arginine, 1 mM	4	44 $\pm$ 4	40 $\pm$ 2	>0.05
L-NMMA, 100 μ M + D-arginine, 1 mM	3	47 $\pm$ 5	18 $\pm$ 3	<0.05
Indomethacin, 10 μ M	5	45 $\pm$ 4	40 $\pm$ 5	>0.05

Data are expressed as means ($\pm$ s.e.mean) % reductions in the level of carbachol (0.1 μ M)-induced tone. The experiments were performed in the presence of BM 13505 (5 μ M). n = the number of paired observations. P values were obtained by the Wilcoxon signed ranked test. L-NMMA, N^G -monomethyl-L-arginine.

Table 2 Effects of endothelin-1 (ET-1) and indomethacin on prostanoid release from the guinea-pig trachea *in vitro*

	TXB ₂ (pg released 4 min ⁻¹	6-keto PGF _{1α} 100 mg ⁻¹	PGE ₂ tissue wet wt)
Vehicle	99 ± 10	157 ± 17	56 ± 8
ET-1, 5 nM	108 ± 9	139 ± 19	54 ± 10
ET-1, 10 nM	160 ± 15*	182 ± 25	69 ± 12
ET-1, 100 nM	289 ± 32**	193 ± 15	77 ± 20
<i>P</i>	0.001	NS	NS
Indomethacin, 10 μM	13 ± 1	23 ± 5	12 ± 1
Indomethacin, 10 μM plus ET-1, 100 nM	14 ± 1 NS*	25 ± 3 NS*	14 ± 2 NS*

Epithelium-intact tracheal strips were challenged consecutively with saline (vehicle for ET-1) and increasing concentrations of endothelin-1 (ET-1). Following an incubation period of 4 min, an aliquot was removed for determination of prostanoid concentrations. Values are means ± s.e.mean of duplicate measurements for 5 separate experiments. *P* values are the probability levels of two-way analysis of variance using ranks (Friedman test). **P* < 0.05; ***P* < 0.001 values were obtained by Wilcoxon-Wilcox test for multiple comparisons of vehicle versus various concentrations of ET-1. NS, not significant.

*Compared to indomethacin, 10 μM (Mann-Whitney U test).

whereas amongst control tissues 80–90% of the epithelium remained intact following the experiments. Removal of the epithelium potentiated by 2.4 fold the contractions elicited by 0.1–1.0 μM histamine, whereas it did not affect the maximum contractile response to 4.5 μM histamine (235 ± 11 and 225 ± 14 mg contraction in intact and rubbed tracheal strips, respectively, *n* = 6, *P* > 0.05). These findings confirm that the technique used to remove epithelial cells had not damaged the underlying smooth muscle.

Discussion

The present experiments provide further evidence that ET-1 can elicit a dual action in the guinea-pig isolated trachea depending on the basal tone of smooth muscle. ET-1 causes relaxations when basal tone is high and evokes constrictions at lower basal tone. Both the relaxant and constrictor actions of ET-1 depend upon the presence of an intact epithelium, as removal of the epithelial cells abolishes the relaxant effect and potentiates the constrictor action of ET-1.

Our data show that ET-1 produces dose-dependent relaxation of epithelium-intact guinea-pig tracheal strips precontracted with carbachol to 60–80% of the maximum. The relaxations appeared to be slower in onset than those reported for other bronchoconstrictors (Vanhoutte, 1988; Bramley *et al.*, 1990). Threshold concentrations of ET-1 to induce relaxation in precontracted tissues were similar to those required to evoke contractions (Maggi *et al.*, 1989; Filep *et al.*, 1990, 1991). The relaxant effect of ET-1 was augmented by more than twofold in the presence of BM 13505, a thromboxane A₂/prostaglandin H₂ receptor antagonist. We have reported previously that ET-1 induces release of TXA₂ from the guinea-pig trachea *in vitro*, which may, in part, mediate its airway constrictor action in the guinea-pig (Filep *et al.*, 1990, 1991). Since TXA₂ is a very potent constrictor in the guinea-pig airways (Svensson *et al.*, 1977), one may expect that TXA₂ receptor blockade would lead to potentiation of ET-1-induced relaxations.

The present study confirms that ET-1 is a potent constrictor of guinea-pig isolated trachea when the resting tone is low (Uchida *et al.*, 1988; Maggi *et al.*, 1989; Hay, 1990; Filep *et al.*, 1990, 1991; Henry *et al.*, 1990). However, ET-1 was approximately 50 fold less potent in this series of experiments (apparent EC₅₀ = 28.1 nM) than was previously reported by Uchida *et al.* (1988) (EC₅₀ = 0.53 nM), but it was more potent than in the studies of Hay (1990) and Henry *et al.* (1990) (estimated EC₅₀ = 50–60 nM). The reasons for this discrepancy are not clear but might be attributed to differences in tissue preparations (tracheal rings in the earlier experiments versus tracheal strips in the present experiments), differences in the integrity of the airway epithelium, in addition to different strains of guinea-pig used. In these, as well as in the

present experimental conditions, the response to ET-1 was monophasic, i.e. the contractions were not preceded by a transient relaxation as has been observed by using an *in situ* guinea-pig tracheal preparation (White *et al.*, 1991) and tracheal strips (Turner *et al.*, 1989a). However, these latter and the present experimental conditions are not easily comparable. In the *in situ* preparation, resting tension was relatively high compared with active tension (White *et al.*, 1991), whereas in the living system, active tension is relatively low compared with resting tension. Thus, it is possible that the *in situ* preparation used by White *et al.* (1991) may resemble precontracted tracheal strips *in vitro*. On the other hand, tissue damage is reduced and hypoxia is avoided in the *in situ* preparation as arterial circulation of the airways is preserved. These may result in enhanced sensitivity of the tracheal preparations to bronchoactive agents.

Removal of the epithelium from the guinea-pig trachea has pronounced effects on the responses to ET-1. Thus, removal of the epithelium changed the response to ET-1 from a relaxation to a sustained contraction in tissues precontracted with carbachol and markedly enhanced the contractile response to ET-1 under resting conditions (i.e. in the absence of carbachol). In order to study whether or not the epithelium influences the reactivity of the underlying smooth muscle through release of NO, the responses to ET-1 were compared in the absence and presence of chemical agents that are known to interfere with the synthesis or actions of NO.

The relaxant action of ET-1 in precontracted tracheal strips was markedly attenuated by L-NMMA and oxyhaemoglobin, whereas methylene blue mimicked the effect of epithelium removal (i.e. slight contractions rather than relaxations were observed following addition of 50–100 nM ET-1 to the organ bath). L-NMMA has been shown to inhibit formation of endothelium-derived NO from endogenous L-arginine (Rees *et al.*, 1989, 1990), resulting in diminished activation of smooth muscle guanylate cyclase and decreased levels of guanosine 3':5'-cyclic monophosphate (cyclic GMP) in the smooth muscle (Gold *et al.*, 1990). Oxyhaemoglobin generally abolishes NO-induced vascular relaxations because of its high binding affinity for NO (Ignarro *et al.*, 1984). Methylene blue inhibits cytosolic guanylate cyclase (Martin *et al.*, 1985) and accumulation of cyclic GMP by NO (Ignarro *et al.*, 1984; Ignarro, 1989; Gold *et al.*, 1990). Furthermore, methylene blue has been shown to produce free radicals (McCord & Fridovich, 1970) that can destroy relaxing factor(s) (Rubanyi *et al.*, 1986; Gryglewski *et al.*, 1986). The effects of L-NMMA, methylene blue and oxyhaemoglobin in the guinea-pig trachea closely resemble their actions on endothelium-derived relaxing factor/NO in vascular tissues (Palmer *et al.*, 1987; Ignarro, 1989). The inhibitory action of L-NMMA was readily reversible by L-arginine but not by D-arginine, indicating specific inhibition of NO synthase by L-NMMA. It seems unlikely that the inhibitory action of methylene blue or

oxyhaemoglobin was due to a non-specific interaction with ET-1, as neither methylene blue nor oxyhaemoglobin affected significantly ET-1-induced contractions in epithelium-intact and denuded tracheal strips. Since L-NMMA, methylene blue and oxyhaemoglobin are structurally unrelated compounds, it is also unlikely that their inhibitory effect can be attributed to a common non-specific action.

In contrast to the present findings, methylene blue (10–50 μ M) either failed to inhibit relaxation responses to histamine and muscarinic agonists (Güc *et al.*, 1988; Fernandes *et al.*, 1989; Hay *et al.*, 1992) or at a concentration of 50 μ M, it potentiated the maximal relaxant effects of histamine and methacholine in co-axial assemblies (Fernandes & Goldie, 1990). This potentiating action of methylene blue cannot be accounted for by inhibition of soluble guanylate cyclase, but rather it may have been non-specific and might be the consequence of alteration of basal smooth muscle tone. The increased basal tone can lead to an apparent increase in maximal relaxant response to the agonists (Raeburn, 1990). Since methylene blue appears to exert actions other than inhibition of soluble guanylate cyclase, its sole use may not provide a sufficient means to characterize epithelium-derived relaxing factor(s). Furthermore, according to our best knowledge, no information is available on the effects of oxyhaemoglobin and L-NMMA on epithelium-dependent relaxations in co-axial bioassays. Based on the differential effect of methylene blue on tracheal strips and co-axial assemblies, it is possible that the guinea-pig trachea may secrete different relaxant factors (Vanhoutte, 1988) that may be selective for some agonists. Detection of these relaxant factors is further complicated by the type of the assays used. For example, carbachol-induced relaxant factor release can only be detected by a co-axial bioassay system (Fernandes & Goldie, 1990), whereas platelet-activating factor-induced relaxations were observed by using tracheal strips (Brunelleschi *et al.*, 1987) but not by a co-axial bioassay (Fernandes & Goldie, 1990). In addition, it is unclear at present whether or not the epithelium-dependent relaxant responses observed in co-axial bioassay are attributable to hypoxia (Gunn & Piper, 1989; Hay *et al.*, 1992).

Taken together, the present findings suggest that ET-1-induced relaxations can be attributed, in part, to NO release from guinea-pig tracheal strips precontracted with carbachol. The recent observations that NO gas and the relatively stable NO adducts, S-nitrosothiols are potent bronchodilators in the guinea-pig both *in vivo* (Dupey *et al.*, 1992) and *in vitro* (Jansen *et al.*, 1992) give further support to this notion. Since epithelium removal abolished the relaxant effect of ET-1, one possibility is that the synthesis of NO occurs in the epithelial and not in the smooth muscle cells. Alternatively, ET-1 may induce the release of a substance from the epithelium which then releases NO from another source(s). Whether ET-1 could directly stimulate NO release from epithelial cells remains to be investigated. These observations provide strong evidence against the possibility that the epithelium can be considered as a diffusion and/or metabolic barrier, preventing ET-1 from reaching tracheal smooth muscle in precontracted tracheal strips. One should keep in mind that unlike methylene blue, neither L-NMMA nor oxyhaemoglobin inhibited completely the relaxant action of ET-1. Higher concentrations of L-NMMA (1 mM) or oxyhaemoglobin (20 μ M) did

not produce significantly more antagonism. The reason for this is not known, but may be attributed to the release of other relaxing factor(s). Several metabolites of arachidonic acid, in particular PGE₂ and prostacyclin, are bronchodilators (for recent review see Raeburn, 1990). However, it seems unlikely that prostanoids mediate ET-1-induced relaxations since ET-1 did not induce significant PGE₂ and prostacyclin release from guinea-pig tracheal strips and indomethacin failed to inhibit the relaxations elicited by ET-1. Previous studies using co-axial bioassays have also demonstrated that histamine and methacholine-induced relaxations were not affected by cyclo-oxygenase inhibition (Ilhan & Sahin, 1986; Güc *et al.*, 1988; Fernandes *et al.*, 1989; Fernandes & Goldie, 1990). Furthermore, the findings that inhibition of cyclo-oxygenase does not prevent the potentiating effect of epithelium removal on bronchoconstrictor responses (Flavahan *et al.*, 1985; Farmer *et al.*, 1987; Undem *et al.*, 1988) give further support to this notion.

Based on the above conclusion, it might be expected that inhibition of NO synthesis and/or action of NO could potentiate the contractile response to ET-1. However, preincubation of tracheal strips with L-NMMA, methylene blue or oxyhaemoglobin under resting conditions (i.e. in the absence of carbachol) did not potentiate the contractile action of ET-1. On the other hand, removal of the epithelium potentiates ET-1-induced contractions (Maggi *et al.*, 1989; Hay, 1989; 1990, and the present study). One interpretation of these observations is that the NO formation by ET-1 is tone-dependent in the guinea-pig trachea as has been reported for blood vessels (Vargas *et al.*, 1990; Mehta *et al.*, 1992). Thus, like intense vasoconstriction, constriction of airway smooth muscle might lead to increased NO production in response to ET-1. A tone-dependent change in sensitivity of the trachea to NO cannot be ruled out at present. The possibility that epithelium removal led to decreased formation of bronchorelaxant prostanoids seems unlikely as under the same experimental conditions, indomethacin significantly attenuated the contractile action of ET-1 (Filep *et al.*, 1991). Another explanation might be that under resting conditions the epithelium may function primarily as a protective (diffusion and/or metabolic) barrier, reducing the contact between agonists and airway smooth muscle. Indeed, inhibition of neutral endopeptidase has been reported to enhance the contractile response to ET-1 in guinea-pig isolated airways (Maggi *et al.*, 1989; Hay, 1989). Furthermore, ET-1 could have reached smooth muscle cells from the luminal surface in the absence of epithelium.

In summary, the present results indicate that ET-1 can elicit contractions and epithelium-dependent relaxations of the guinea-pig isolated trachea depending on the tone of the tissue. The epithelium may function as a protective barrier under resting conditions. The relaxant action of ET-1 in precontracted tracheal strips is likely to be mediated by NO released from epithelial cells and subsequent activation of guanylate cyclase in smooth muscle cells.

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