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Differential modulation of endothelin ligand-induced contraction in isolated tracheae from endothelin B (ET_B) receptor knockout mice

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1 The role of endothelin B (ET_B) receptors in mediating ET ligand-induced contractions in mouse trachea was examined in ET_B receptor knockout animals.

2 Autoradiographic binding studies, using [^{125}I]-ET-1, confirmed the presence of ET_A receptors in tracheal and bronchial airway smooth muscle from wild-type (+/+) and homozygous recessive (−/−) ET_B receptor knockout mice. In contrast, ET_B receptors were not detected in airway tissues from (−/−) mice.

3 In tracheae from (+/+) mice, the rank order of potencies of the ET ligands was sarafotoxin (Stx) S6c > ET-1 > ET-3; Stx S6c had a lower efficacy than ET-1 or ET-3. In tissues from (−/−) mice there was no response to Stx S6c (up to 0.1 μM), whereas the maximum responses and potencies of ET-1 and ET-3 were similar to those in (+/+) tracheae. ET-3 concentration-response curve was biphasic in (+/+) tissues (*via* ET_A and ET_B receptor activation), and monophasic in (−/−) preparations (*via* stimulation of only ET_A receptors).

4 In (+/+) preparations SB 234551 (1 nM), an ET_A receptor-selective antagonist, inhibited the secondary phase, but not the first phase, of the ET-3 concentration-response curve, whereas A192621 (100 nM), an ET_B receptor-selective antagonist, had the opposite effect. In (−/−) tissues SB 234551 (1 nM), but not A192621 (100 nM), produced a rightward shift in ET-3 concentration-response curves.

5 The results confirm the significant influence of both ET_A and ET_B receptors in mediating ET-1-induced contractions in mouse trachea. Furthermore, the data do not support the hypothesis of atypical ET_B receptors. In this preparation ET-3 is not an ET_B receptor-selective ligand, producing contractions *via* activation of both ET_A and ET_B receptors.

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Abbreviations: ET_A, endothelin A receptor; ET_B, endothelin B receptor; ET-1, endothelin-1; ET-2, endothelin-2; ET-3, endothelin-3; PCR, polymerase chain reaction; Stx S6c, sarafotoxin S6c

Introduction

The endothelin isopeptides (ET-1, ET-2, and ET-3) are a family of distinct gene products, the effects of which are mediated by G-protein coupled, seven-transmembrane spanning receptors. In mammalian systems, two major subtypes (ET_A and ET_B) have been characterized pharmacologically and by molecular biological techniques (Hosoda *et al*., 1992; Masaki *et al*., 1992; 1994; Arai *et al*., 1993; Rubanyi & Polokoff, 1994). These receptors mediate a multiplicity of biological actions of ET ligands (reviewed in Masaki *et al*., 1992; Rubanyi & Polokoff, 1994). The most widely studied isoform, ET-1, was the first member of the family identified (Yanagisawa *et al*., 1988). Although recognized initially for its potent vasoconstrictor activity, ET-1 has been shown subsequently to induce various effects in the pulmonary system, notably airway smooth muscle contraction, and has been implicated as a mediator in several lung diseases, including asthma (Hay *et al*., 1993a; Goldie & Henry, 1999; Michael & Markewitz, 1996).

With the exception of ovine airway smooth muscle, in which only ET_A receptors are expressed (Goldie *et al*., 1994), airway smooth muscle from other animal species, including mouse (Carr *et al*., 1996, rat (Henry, 1993), guinea-pig and pig (Goldie *et al*., 1996a,b), and from the human (Goldie *et al*., 1995; Fukuroda *et al*., 1996; Hay *et al*., 1993a,b; 1996; Yoneyama *et al*., 1995), contain both receptor subtypes which contribute to ET-1-induced contraction. Furthermore, both receptor subtypes must be blocked before significant inhibition of ET-1-evoked contraction is obtained, indicating marked functional reserve (Henry, 1993; Goldie *et al*., 1996a, b; Fukuroda *et al*., 1996). However, based upon pharmacological data using ET agonists and antagonists in various tissues, including the lung, it has been speculated that there may exist ET receptor subtypes in addition to the ET_A and ET_B receptor populations (Bax & Saxena, 1994; Warner *et*

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al., 1993; Hay *et al*., 1996; 1998; Yoneyama *et al*., 1995). This hypothesis appears to be based, to a large extent, on the insensitivity of ET ligand-induced contractions to the currently available ET receptor antagonists, and, thus far, has not been supported by molecular biological, structural or operational experiments. This phenomenon is dependent upon the ET ligand used, and thus may not be due to the presence of novel ET receptor subtypes, but reflect differences in the kinetics of ligand/receptor interactions (Devadason & Henry, 1997; Henry & King, 1999). For example, the apparent failure of antagonists, such as BQ-123, to effectively block ET_A receptor-mediated contraction to ET-1 in some models, appears to be related to the rapid dissociation of BQ-123 from the ET_A receptor and its replacement by ET-1, which does not dissociate quickly from the receptor. In contrast, BQ-123 inhibits responses to sarafotoxin (Stx) S6b, since both ligands readily dissociate from ET_A receptors.

An alternative, complementary approach towards pharmacological evaluation and receptor classification involves the use of knockout mice, in which a specific receptor is targeted for deletion. This strategy was employed in the current study, which involved utilization of contraction and quantitative autoradiography experiments in airways from ET_B receptor knockout mice (Hosoda *et al*., 1994) to investigate further the distribution, density and function of ET_B receptors in the murine respiratory tract. In addition, the effects of the ET receptor antagonists, SB 234551, a nonpeptide ET_A receptor-selective antagonist (Ohlstein *et al*., 1998), and A192621, a nonpeptide ET_B receptor-selective antagonist (Douglas, 1997), against ET ligand-induced contractions in tracheae from wild-type and ET_B receptor knockout animals were evaluated. It is noteworthy that Berthiaume and colleagues have demonstrated recently that heterozygous knockout of ET_B receptors, but not ET_A receptors, in the mouse produces hypertension. This hypertensive state is sensitive to BQ-123, the peptide ET_A receptor-selective antagonist, or SB 209670, the nonpeptide, mixed ET_A/ET_B receptor antagonist, but unaffected by BQ-788, the peptide ET_B receptor-selective antagonist (Berthiaume *et al*., 2000).

Methods

Compounds and reagents

Stx S6c, ET-1 and ET-3 were obtained from American Peptide (Sunnyvale, CA, U.S.A.). [^{125}I]-ET-1 (specific activity 2200 Ci mmol^{-1}) was obtained from New England Nuclear (Boston, MA, U.S.A.) A192621 (2R, 3R, 4S)-2-(4-propoxyphenyl)-4-(1,3-benzodioxol-5-yl)-1-(N-[(2,6-diethylphenyl]acetamido)pyrrolidine-3-carboxylate, SB 234551 (E)-alpha-[[1-butyl-5-[2-[(2-carboxyphenyl)methoxy]-4-methoxyphenyl]-1H-pyrazol-4-yl] methylene] -6-methyoxy-1, 3-benzodioxole-5-propanoic and BQ-123 [cyclo(-D-Asp-L-Pro-D-Val-L-Leu-D-Trp-)] were synthesized in the Department of Medicinal Chemistry, SmithKline Beecham Pharmaceuticals (King of Prussia, PA, U.S.A.).

Animals

ET_B receptor knockout mice were obtained from an in-house colony originally derived from heterozygous *129/Sv-Ednrb*^{tmlYwa} breeding pairs purchased from Jackson Laboratory (Bar Harbor, ME, U.S.A.) and described previously (Hosada *et al*., 1994; Puffenberger *et al*., 1994). All animals were housed in a barrier facility and fed water and pellet food *ad libitum*. Wild type mice (+/+) and heterozygote ET_B receptor knockout animals (+/−) were of healthy appearance and normal lifespan and were used in a weight range of 18–20 g. The homozygous recessive (−/−) mice were used at 6–14 g and had a healthy appearance at the time of use; the (−/−) mice, however became ill with megacolon within several days post-weaning. All procedures utilized were approved by the Animal Care and Use Committee and met or exceeded standards of the American Association for the Accreditation of Laboratory Animal Care, the United States Department of Health and Human services and all local and federal animal welfare laws. All of the experiments described were terminal.

Note in the text and figures, tissues from wild type mice (+/+), heterozygote ET_B receptor knockout animals (+/−) and the homozygous recessive (−/−) ET_B receptor knockout mice are referred to often as '(+/+)', '(+/−)' and '(−/−)', respectively.

Genotyping and characterization of ET_B receptor knockout mice

As previously outlined (Griswold *et al*., 1999), individual genotype analysis was performed by PCR using genomic DNA (0.2 μg) isolated from tail snip samples. Briefly, amplification used a 'two step' protocol consisting of primary high stringency amplification (11 cycles; annealing: 64°C, 45 s; extension: 72°C, 45 s; denaturation: 95°C, 35 s) followed by secondary low stringency PCR (21 cycles; annealing: 58°C).

Quantitative autoradiographic studies

Mice were anaesthetized with halothane and killed by cervical dislocation and exsanguination. The lungs and trachea were removed intact and rinsed in ice-cold Krebs bicarbonate solution (KBS) of the following composition (in mM): NaCl 117, KCl 5.36, $NaHCO_3$ 25.0, KH_2PO_4 1.03, $MgSO_4.7H_2$ 0.57, $CaCl_2.2H_2O$ 2.5 and glucose 11.1. The lungs were inflated *via* tracheal instillation with OCT embedding medium diluted 1:4 with 0.9% w v^{-1} NaCl solution. Autoradiographic studies were conducted as previously described (Henry *et al*., 1990a,b).

Briefly, inflated lung and attached tracheal tissue was frozen by immersion in isopentane, quenched with liquid nitrogen. Serial transverse sections (10 μm) of trachea or lung containing upper bronchial tissue were cut at −20°C and thaw-mounted onto gelatin chrom-alum coated glass microscope slides. These sections were pre-incubated for 5 min at 22°C in buffer (50 mM Tris-HCl, 100 mM NaCl, 0.25% bovine serum albumin, pH 7.4) containing the protease inhibitor phenylmethysulphonylfluoride (10 μM) and then for 3 h in buffer containing 0.2 nM [^{125}I]-endothelin-1 alone (total binding) or in the presence of BQ-123 (ET_A receptor-selective ligand; 1 μM) or sarafotoxin S6c (Stx S6c; ET_B receptor-selective ligand; 100 nM). Nonspecific binding was determined in the combined presence of BQ-123 (1 μM) and Stx S6c (100 nM). After 3 h, tissue sections were washed twice for 10 min in buffer, rinsed in distilled water and dried under

a stream of cold dry air. Emulsion-coated coverslips (Kodak NTB-2) were attached to one end of the glass slides with cyanoacrylate adhesive and exposed for 2–5 days at 4°C. These were developed for 3 min in Kodak Dektol diluted 1 : 1 with water, rinsed for 15 s in dilute acetic acid (2%) containing hardener (Ilford) and fixed (Ilford Hypam, 1 : 4) for 2.75 min. Tissue sections were then stained for 30 s with Gill's double strength haematoxylin, dehydrated in ethanol, cleared in xylene and mounted (Depex, BHD) for light microscopy. Autoradiographic grain densities were determined by computer-assisted image analysis (Henry *et al*., 1990a,b) using the MD-20 image analysis system (Flinders Imaging, Adelaide, Australia). Five separate fields were viewed from each tissue section. The emulsion background grain density was measured over non-tissue areas within the lumen of airway tissues from three fields for each slide.

Contraction studies

Male or female wild-type, heterozygous or homozygous recessive ET_B receptor knockout mice were killed and exsanguinated. The trachea from each animal was removed and cleaned of adherent tissue under a dissecting stereomicroscope. Two rings (approximately 2 mm diameter, 4 cartilage rings in length) were cut from the trachea; the epithelium was left intact. Tissues were put into modified Krebs-Henseleit solution which was gassed with 95% O_2: 5% CO_2 and maintained at 37°C; the composition of the modified Krebs-Henseleit solution was (mM): NaCl 113.0, KCl 4.8, $CaCl_2$ 2.5, KH_2PO_4 1.2, $MgSO_4$ 1.2, $NaHCO_3$ 25.0 and dextrose 11.0. Experiments were run in the presence of 1 μM meclofenamic acid to inhibit the synthesis of cyclooxygenase products and to standardize the experimental conditions. Individual tissues were suspended *via* stainless steel hooks and silk suture in 10-ml water-jacketed organ baths containing Krebs-Henseleit solution and connected to Grass FTO3C force-displacement transducers. Mechanical responses were recorded isometrically by MP100WS/Acknowledge data acquisition system (BIOPAC systems, Santa Barbara, CA, U.S.A.; www.biopac.com) run on Macintosh computers. The tissues were equilibrated under a resting tension of 0.2–0.3 g, and washed with modified Krebs-Henseleit solution every 15 min for 1 h. After the equilibration period, each tissue was contracted with 10 μM carbachol until the tension reached a plateau. Tissues were then rinsed every 15 min over 1 h until reaching baseline tone, and then left for at least 30 min before the start of the experiment.

ET-1, ET-3 and Stx S6c concentration-response curves were obtained by cumulative addition of the individual agonist in half-log increments. Each concentration was left in contact with the preparation until the response plateaued before the addition of the subsequent agonist concentration. At the end of the experiment, tissues were exposed to 10 μM carbachol, which served as a reference contraction for data analysis. Paired tissues were exposed to SB 234551 or A192621, alone or in combination, or vehicle for 30 min before ET ligand cumulative concentration-response curves were generated. A concentration of 100 nM of SB 234551 or A192621 was utilized in all of these experiments, except for those examining the influence of SB 234551 against ET-3 concentration-response curves, where a concentration of 1 nM was used; in the latter experiments a higher concentration of SB 234551 (30 nM) essentially abolished responses to concentrations of ET-3 up to 10 μM. A concentration of 100 nM of SB 234551 or A192621 has been shown previously to potently antagonize ET_A or ET_B receptor-mediated responses, respectively, in mouse pulmonary tissues *in vitro* (Griswold *et al*., 1999).

Statistical analysis

All binding and contractile responses are expressed as the mean or the mean ± standard error of the mean. For contraction studies, agonist-induced responses, including the maximum contractile response for each tissue, were expressed as a percentage of the reference contraction to carbachol 10 μM, obtained after the construction of agonist concentration-response curves ('post-carbachol'). Geometric mean EC_{50} values (pD_2s) were calculated from linear regression analyses of data. It is recognized that the ETs and related ligands may not interact with receptors in a classical manner which will result in a reversible competitive interaction between agonist, antagonist and receptor (Marsault *et al*., 1991; Waggoner *et al*., 1992; Hay *et al*., 1996). However, antagonist potencies were calculated assuming a classical competitive interaction. Where appropriate, the effect of antagonists were assessed as shifts in the concentration-response curves and expressed as apparent pK_Bs; $pK_B = -\log$ [antagonist]/X − 1, where X is the ratio of agonist concentration required to elicit 50% of the maximal contraction in the presence of the antagonist compared with that in its absence (Arunlakshana & Schild, 1959). Statistical evaluation was conducted using Student's *t*-test or ANOVA where appropriate, with a probability value, $P < 0.05$, considered statistically significant.

Results

Characterization of knockout mice

Three different genotypes of mice originally derived from the ET_B receptor gene targeted disruption mice were utilized for the experiments described: wild type (+/+), heterozygote (+/−) and homozygous recessive (−/−). As reported previously (Griswold *et al*., 1999), PCR amplification of samples of genomic DNA produced a single reaction product of either 400 bp (wild type ET_B receptor) or a 280 bp (neomycin-insert) which reflected the wild type (+/+) and homozygous (−/−) knock out genotypes, respectively. In contrast, samples which resulted in a 280 bp/400 bp doublet upon PCR amplification corresponded to the heterozygous (+/−) mice. These results were consistent with the phenotypes of the animals, i.e., agouti (+/+) and (+/−) genotypes or piebald (−/−) genotype, coat colour (data not shown).

Quantitative autoradiography

Autoradiographic binding studies were conducted using mouse tracheal and lung tissue containing major bronchi. High levels of specific binding of [^{125}I]-ET-1 (non-selective ET ligand) were detected in tracheal (Figure 1) and bronchial airway smooth muscle (Figure 2), with approximately four times more total specific binding in bronchial than in tracheal

smooth muscle from mice of all three genotypes. Binding levels were clearly highest in tissue from (+/+) mice, with an obvious reduction in binding density in tissue from (+/−) mice and a further decline in tissue from (−/−) mice (Figures 1 and 2c,d). Total specific binding was 23 and 46% lower in tracheal smooth muscle from (+/−) and (−/−) mice, respectively, and 16 and 41% lower, respectively, in bronchial airway smooth muscle. This indicates that the total ET receptor densities in tracheal airway smooth muscle from (+/−) and (−/−) mice relative to (+/+) mice were 100 (+/+): 77 (+/−): 54 (−/−) and in bronchial airway smooth muscle were 100 : 84 : 59.

Both ET_A and ET_B binding sites were detected in airway smooth muscle as evidenced by the reductions in binding observed in the presence of either the ET_A receptor-selective antagonist, BQ-123 (1 μM), or the ET_B receptor-selective ligand, Stx S6c (100 nM) (Table 1; Figures 2e–h and 3). Figure 3 and Table 1 summarize the quantitative analysis of autoradiographic data. In tracheal smooth muscle from (+/+) mice, the ratio of ET_A (BQ-123-sensitive): ET_B (Stx S6c-sensitive) receptors was 34 : 66 (Table 1; Figure 3), and in bronchial smooth muscle, it was 40 : 60 (Table 1). In contrast, in tracheal (Table 1; Figures 1 and 3) and bronchial smooth muscle (Table 1; Figure 2) from (−/−) animals, almost no Stx S6c-sensitive specific binding was detected (Figure 2f), demonstrating the apparent absence of ET_B receptors in these preparations. BQ-123 abolished specific binding in airway smooth muscle from (−/−) (Figures 2h and 3), consistent with the presence of only ET_A receptors in tissue with this genotype. In both tracheal and bronchial airway smooth muscle from (+/−) mice, the ratio of ET_A : ET_B receptors was 50 : 50 (Table 1).

Contraction studies

Functional studies, exploring the contractile responses to the ET ligands, ET-1, Stx S6c and ET-3, in the presence and absence of the ET_A receptor-selective antagonist, SB 234511, and the ET_B receptor-selective antagonist, A192621, were conducted in mouse tracheal preparations. The results are summarized in Tables 2 and 3 and Figures 4–7.

The selectivity of SB 234551 and A192621 for ET_A and ET_B receptors, respectively, was confirmed in mouse pulmonary artery, a tissue in which ET ligands produce contraction exclusively *via* activation of ET_A receptors, e.g., Stx S6c does not elicit contraction. In (+/+), (+/−) or

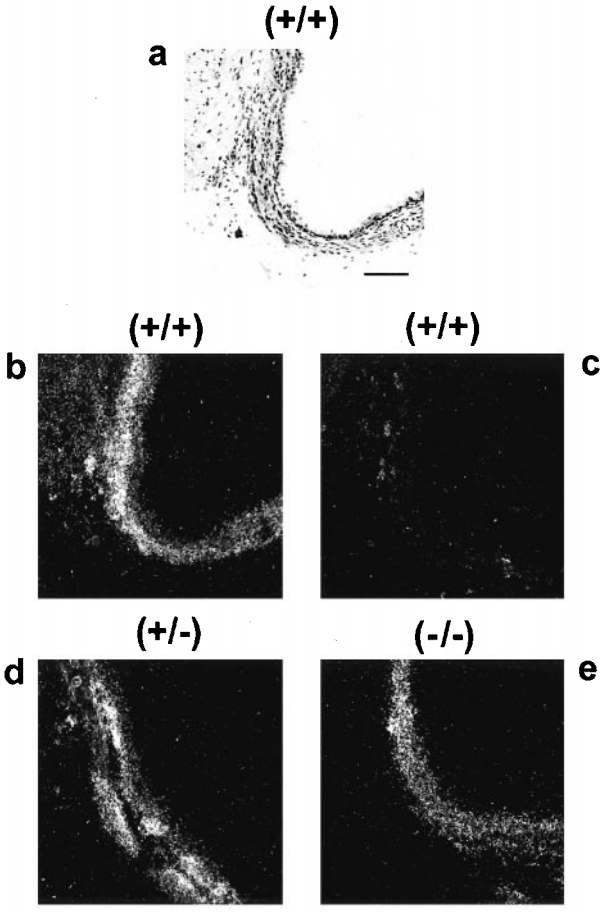

Figure 1 Montage of darkfield (b–e) autoradiograms showing the distribution of total binding of [^{125}I]-ET-1 in murine tracheal smooth muscle from (+/+) (wildtype, panel b; panel a = brightfield image), (+/−) (panel d) and (−/−) (panel e) ET_B receptor knockout mice. Non-specific binding, determined in the combined presence of BQ-123 (1 μM) and Stx S6c (100 nM), is shown in panel c. Bar = 100 μm.

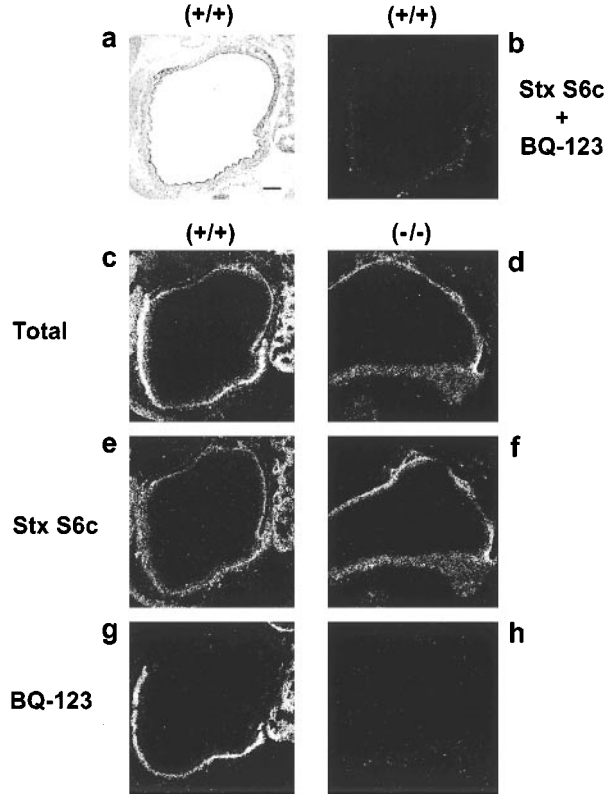

Figure 2 Montage of darkfield (b–h) autoradiograms showing the distribution total binding of [^{125}I]-ET-1 in murine bronchial smooth muscle from (+/+) (wildtype, panel c; panel a = brightfield image) and (−/−) (homozygous ET_B receptor knockout; panel d) mice. Nonspecific binding, determined in the combined presence of the ET_A receptor-selective ligand BQ-123 (1 μM) and the ET_B receptor-selective ligand Stx S6c (100 nM), is shown in panel b. The impact of Stx S6c (100 nM) on binding is shown in panels e (+/+) and f (−/−) and that of BQ-123 (1 μM) is shown in panels g (+/+) and h (−/−). Bar = 100 μm.

(−/−) preparations SB 234551 (1–100 nM) potently inhibited ET-1- or ET-3-induced contractions with pK_Bs ranging from 8.5–8.9 and 9.7–10.3, respectively, whereas A192621 (100 nM) was without effect (data not shown).

Effects of ET ligands alone

A comparison of ET-1, Stx S6c and ET-3 concentration-response curves in tracheae from wild-type (+/+), heterozygous (+/−) and homozygous (−/−) ET_B receptor knockout mice was performed (Figure 4). In (+/+) preparations the rank order of potencies was Stx S6c > ET-1 > ET-3. However, ET-1 and ET-3 elicited similar maximum responses (about 70% of contraction to 10 μM carbachol), whereas Stx S6c induced a lower maximum effect (about 45% of 10 μM carbachol with 0.1 μM Stx S6c; Table 2). It is noteworthy that the ET-3 concentration-response curve was biphasic, whereas those to ET-1 and Stx S6c were monophasic (Figure 4a).

Similar results were obtained with ET-1 and ET-3 in tissues from (+/−) mice (Table 2 and Figure 4b). However, Stx S6c was a significantly less potent and efficacious agonist in (+/−) compared to (+/+) preparations (Table 2 and Figure 4a,b). Furthermore, in tracheae from (−/−) mice Stx S6c was inactive, in concentrations up to 0.1 μM (Table 2; Figure 4c). The maximum responses and potencies of ET-1 and ET-3 in (−/−) preparations were similar to those in (+/+) tissues. In contrast to the findings in (+/+) tissues, in both (+/−) and (−/−) preparations the ET-3 concentration-response curve was monophasic rather than biphasic.

Table 1 Ratio of ET_A:ET_B receptors in tracheal and bronchial smooth muscle from wild type (+/+), heterozygous (+/−) and homozygous ET_B receptor knockout (−/−) mice

Genotype	ET_A:ET_B receptor ratio: Tracheal smooth muscle	Bronchial smooth muscle
Wild type (+/+)	34:66	40:60
Heterozygous ET_B receptor knockout (+/−)	50:50	50:50
Homozygous ET_B receptor knockout (−/−)	93:7	96:4

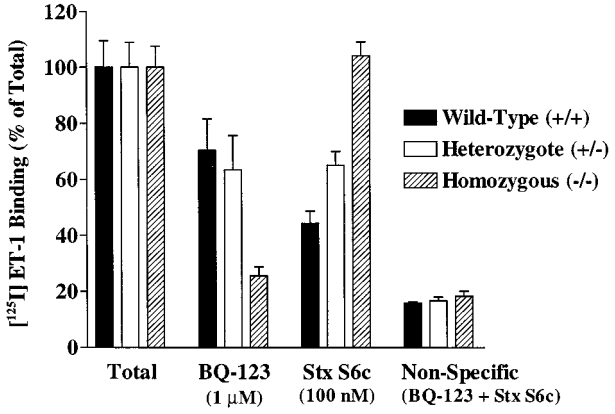

Figure 3 Histogram showing quantitative autoradiographic data for the binding of [^{125}I]-ET-1 (% total binding) in mouse tracheal airway smooth muscle from wild-type (+/+; filled columns), heterozygous ET_B receptor knockout (+/−; unfilled columns) and homozygous ET_B receptor knockout mice (−/−; hatched columns). Data are presented as total binding, and binding detected in the presence of the ET_A receptor-selective ligand BQ-123 (1 μM), or the ET_B receptor-selective ligand Stx S6c (100 nM) or in the combined presence of BQ-123 (1 μM) and Stx S6c (100 nM) (nonspecific binding).

Effects of the antagonists, SB 234551 and A192621

For the sake of simplicity, and to highlight any differences more clearly, the effects of SB 234551 and/or A192621 against responses to ET ligands are presented in graph form only for tracheal preparations from (+/+) and (−/−) mice.

(i) ET-1 In (+/+) mouse trachea SB 234551 (100 nM) or A192621 (100 nM) were without effect on ET-1-induced responses (Figure 5a and b, respectively), whereas the combination produced a marked (16.2 fold) shift to the right of the ET-1 concentration-response curve (Figure 5c). In (−/−) preparations the effects of A192621 alone (Figure 5e) and the combination of A192621 and SB 234551 (Figure 5f) were the same as in (+/+) tissues. However, unlike in (+/+) tissues, SB 234551 alone produced a marked shift to the right in ET-1 concentration-response curves in (−/−) preparations with a $pK_B = 8.3$ (Figure 5d; Table 3).

In (+/−) tissues, the influence of A192621 (no effect at 100 nM) and the combination of A192621 and SB 234551 ($pK_B = 8.0$; $n = 3$; Table 3) on ET-1-induced contractions were similar to those obtained in (+/+) preparations. However, in contrast to the results in (+/+) tissues, SB 234551 (100 nM) produced a significant inhibition of ET-1 concentration-response curves, with a pK_B of 7.7 ($n = 3$; Table 3).

Table 2 Concentration-response curves of Stx S6c, ET-1 and ET-3, in trachea from wild type (+/+), heterozygote (+/−) and homozygote (−/−) ET_B receptor knockout mice

Genotype	Stx S6c		Trachea ET-1		ET-3	
	pD_2	Max	pD_2	Max	pD_2	Max
Wild type (+/+)	8.8 (8)	48.6±2.2	8.0 (12)	71.1±3.9	7.1 (6)	77.8±0.6
Heterozygote (+/−)	8.5 (8)	32.2±3.5	8.2 (9)	78.3±1.2	7.3 (8)	73.7±2.3
Homozygous (−/−)	N.E. (4)	N.E.	8.1 (9)	69.8±1.1	6.6 (12)	67.2±3.9

Results are expressed as pD_2 (−log M) or maximum response (% 10 μM carbachol) and are given as the mean (pD_2) or mean±s.e.mean (maximum response; 'Max'); the n values are given in parentheses. N.E. = No effect.

Table 3 Effects of the ET_A receptor antagonist, SB 234551 (1 or 100 nM), or the ET_B receptor antagonist, A192621 (100 nM), against contractions to Stx S6c, ET-1 or ET-3 in wild-type (+/+), heterozygous (+/−) and homozygous (−/−) ET_B receptor knockout mice

	S6c		*ET-1*		*ET-3*	
Genotype	*SB 234551*	*A192621*	*SB 234551*	*A192621*	*SB 234551*	*A192621*
Wild type (+/+)	N.E. (4)	$pK_B = 9.9$ (4)	N.E. (6)	N.E. (3)	Inhibition* (4)	Inhibition† (3)
			SB 234551 + A192621: $pK_B = 8.2$ (3)			
Heterozygous (+/−)	N.E. (3)	$pK_B = 9.1$ (5)	$pK_B = 7.7$ (3)	N.E. (3)	Inhibition* (3)	$pK_B = 7.6$ (5)
			SB 234551 + A192621: $pK_B = 8.0$ (3)			
Homozygous (−/−)	No contraction		$pK_B = 8.2$ (3)	N.E. (3)	$pK_B = 9.8$ (7)	N.E. (5)
			SB 234551 + A192621: $pK_B = 8.4$ (3)			

Results are expressed as pK_B (−log M) are given as the mean, the *n* values are given in parentheses. N.E. = no effect. *Dual effect: i.e., no effect against 1st phase of ET-3 concentration-response curve and inhibition of the 2nd phase. †Dual effect: i.e., no effect against 2nd phase of ET-3 concentration-response curve and inhibition of the 1st phase.

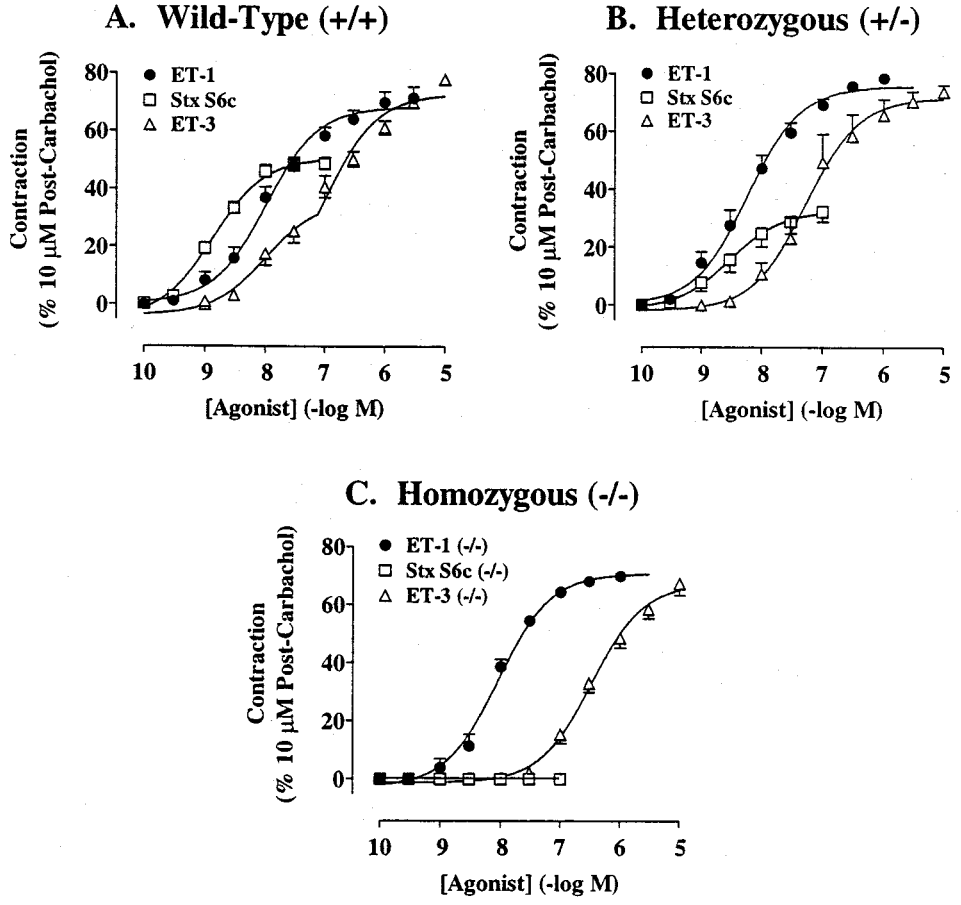

Figure 4 ET-1, Stx S6c or ET-3 concentration-response curves in trachea from (a) wild-type (+/+); (b) heterozygous (+/−) and (c) homozygous (−/−) ET_B receptor knockout mice. The results are presented as a percentage of the contraction to 10 μM carbachol and are the mean ± s.e.mean; (a) $n = 6–12$; (b) $n = 8–9$; (c) $n = 4–12$.

(ii) Stx S6c As indicated above, Stx S6c (up to 0.1 μM) produces no contraction in (−/−) preparations. In (+/+) tissues SB 234551 (100 nM) had no effect on Stx S6c concentration-induced contractions (Figure 6a), whereas A192621 (100 nM) potently inhibited Stx S6c concentration-response curves, with a pK_B of 9.9 ($n = 4$) (Figure 6b; Table 2). Similar results were obtained in (+/−) preparations: A192621 (100 nM) had a pK_B of 9.1, and SB 234511 (100 nM) had no effect on Stx S6c concentration-response curves (Table 3).

(iii) ET-3 The concentration-response curve to ET-3 was biphasic in (+/+) preparations (Figure 7a,b) and monophasic in (−/−) and monophasic in (−/−) tissues (Figure 7c,d). In (+/+) preparation SB 234551 (1 nM) alone had no effect on the first phase of the ET-3 concentration-response curve, but inhibited the second phase (Figure 7a). In (−/−) tissues SB 234551 (1 nM) potently inhibited ET-3-induced contractions, with a pK_B of 9.8. Note, the low concentration of SB 234551 utilized in these experiments; a higher

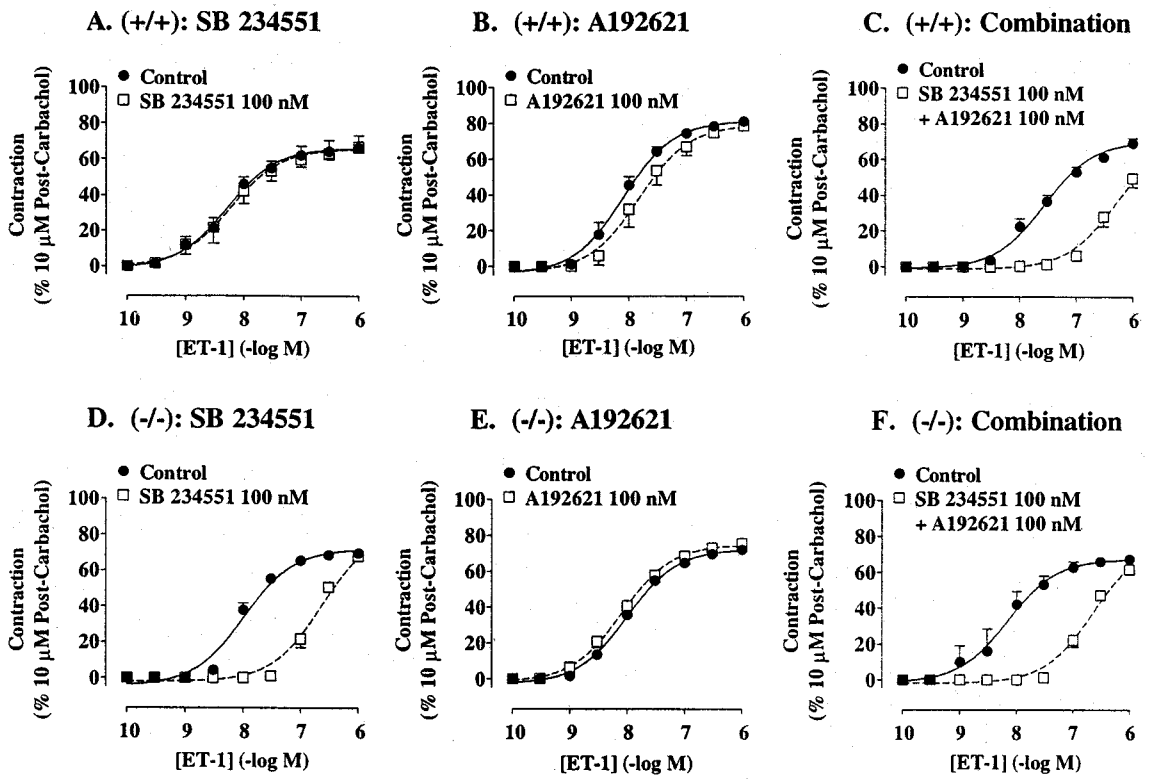

Figure 5 Effects of SB 234551 (ET_A receptor-selective antagonist; 100 nM) or A192621 (ET_B receptor-selective antagonist; 100 nM), alone and in combination, against ET-1 concentration-response curves in trachea from (a–c) wild-type (+/+) or (d–f) homozygous (−/−) ET_B receptor knockout mice. The results are presented as a percentage of the contraction to 10 μM carbachol and are the mean ± s.e.mean; (a) $n=6$, (b) $n=3$, (c) $n=3$, (d) $n=3$, (e) $n=3$, (f) $n=3$.

concentration, 30 nM, essentially abolished contractions to ET-3 in (−/−) tracheal preparations (data not shown).

In (+/+) tissues A192621 (100 nM) inhibited the response to lower concentrations of ET-3 (10, 30 and 100 nM) (Figure 7b), whereas in (−/−) preparations A192621 was without significant effect (Figure 7d).

Discussion

To our knowledge, this study represents the first one analysing ET ligand-induced contractions and ET receptor distribution and density in airways from ET_B receptor knockout mice. The major findings are: (1) quantitative autoradiographic binding studies in tracheal and bronchial airway smooth muscle confirm the presence of ET_A receptors in tissues from wild-type (+/+), heterozygous (+/−) and homozygous recessive (−/−) ET_B receptor knockout mice, and indicate the reduction and absence of ET_B receptors in (+/−) and (−/−) tissues respectively, compared with (+/+) preparations; (2) in tracheal airway ring preparations from (+/+) mice, the rank order of contractile potencies for the ET ligands examined was Stx S6c > ET-1 > ET-3; Stx S6c elicited a lower maximum response than ET-1 or ET-3; (3) in tissues from (−/−) mice, there was no response to Stx S6c (up to 0.1 μM), whereas the maximum responses and potencies of ET-1 and ET-3 were similar to those in (+/+) tracheae; (4) the ET-3 concentration-response curve was biphasic in (+/+) tissues and monophasic in (−/−) preparations, whereas the ET-1 and Stx S6c curves were monophasic in the two groups of preparations; (5) in (+/+) preparations SB 234551 (1 nM), the ET_A receptor antagonist, inhibited the secondary phase, but not the first phase of the ET-3 concentration-response curve, whereas A192621 (100 nM), the ET_B receptor antagonist had the opposite effect and (6) in (−/−) tissues SB 234551 (1 nM), but not A192621 (100 nM), produced a rightward shift in ET-3 concentration-response curves.

The current autoradiographic data, utilizing the non-selective ligand [^{125}I]-ET-1, confirm our earlier findings that both ET_A (35–40%) and ET_B (60–65%) receptors are expressed in airway smooth muscle in the mouse (Carr *et al.*, 1996; Knott *et al.*, 1996), although these previous studies indicated subtype ratios closer to 50 : 50. This may reflect a mouse strain difference (CBA/CaH versus Balb c) between the studies. Interestingly, bronchial airway smooth muscle contained an approximately four times greater density of both receptor subtypes than tracheal airway smooth muscle. Data generated using tracheal and bronchial smooth muscle from (+/+), (+/−) and (−/−) animals support the conclusions from genotyping that (−/−) represents the phenotype that does not express the ET_B receptor subtype. Use of the ET_B receptor-selective agonist, Stx S6c, and the ET_A receptor-selective antagonist, BQ-123, clearly indicated the presence of only BQ-123-sensitive specific [^{125}I]-ET-1 binding in the (−/−) mice, with significantly reduced levels of ET_B receptor binding in (+/−) animals compared to that observed in the (+/+) mice. Results indicate that the total

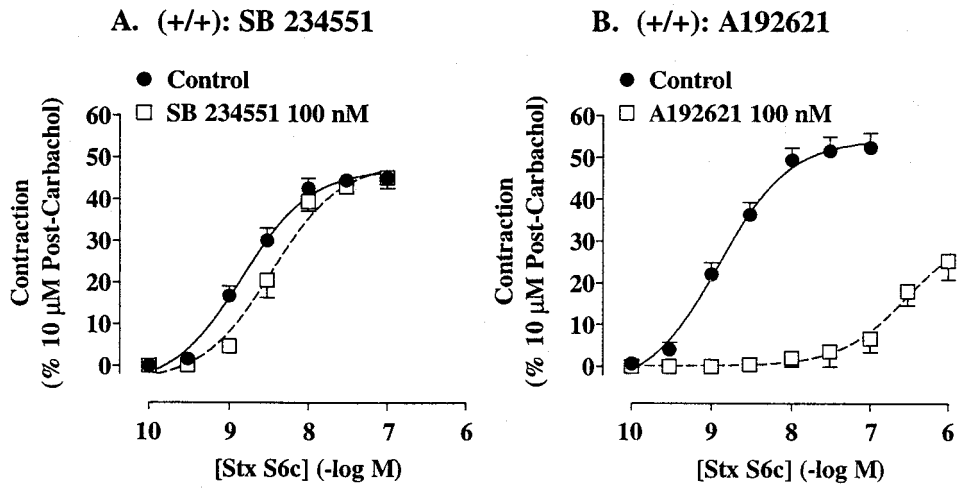

Figure 6 Effects of (a) SB 234551 (ET_A receptor-selective antagonist; 100 nM) or (b) A192621 (ET_B receptor-selective antagonist; 100 nM) against Stx S6c concentration-response curves in trachea from wild-type (+/+) mice. The results are presented as a percentage of the contraction to 10 μM carbachol and are the mean±s.e.mean; (a) $n=4$, (b) $n=4$. Note, no response to Stx S6c (up to 0.1 μM) is observed in homozygous (−/−) ET_B receptor knockout mice.

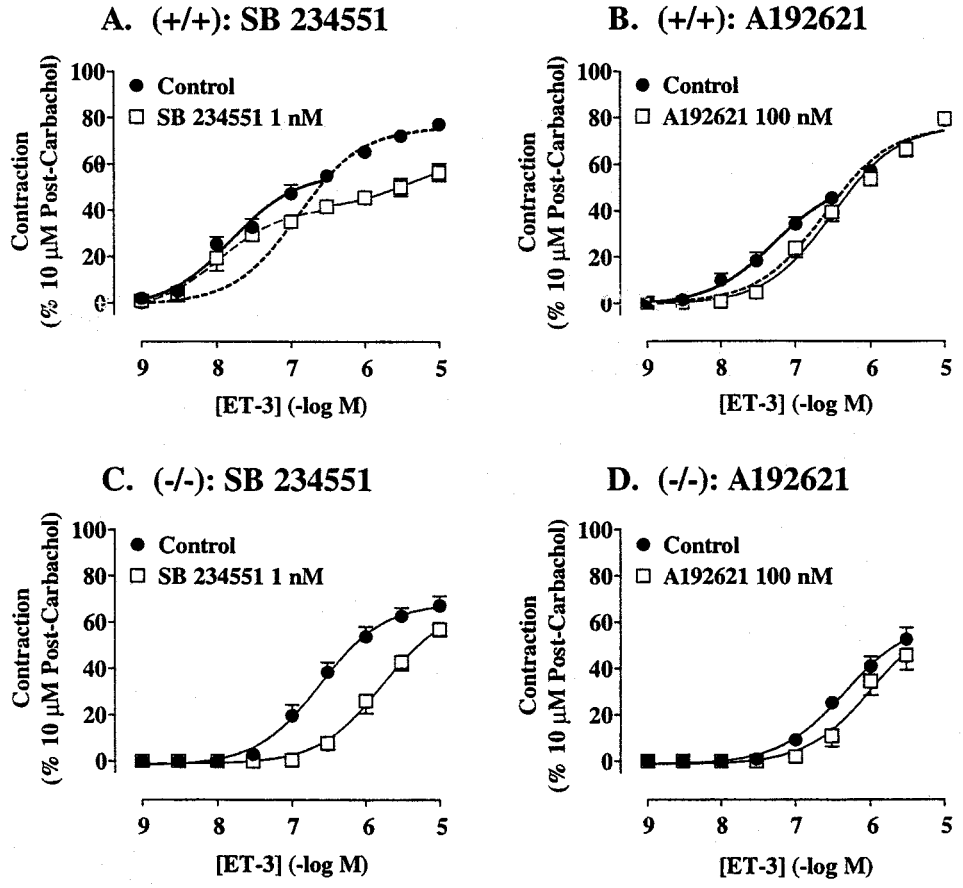

Figure 7 Effects of (a, c) SB 234551 (ET_A receptor-selective antagonist; 1 nM) or (b, d) A192621 (ET_B receptor-selective antagonist; 100 nM) against ET-3 concentration-response curves in trachea from (a, b) wild-type (+/+) or (c, d) homozygous (−/−) ET_B receptor knockout mice. The results are presented as a percentage of the contraction to 10 μM carbachol and are the mean±s.e.mean; (a) $n=4$, (b) $n=3$, (c) $n=7$, (d) $n=5$. Note, there was evidence of a biphasic ET-3 concentration-response curve; the dashed lines represent the theoretical extension of the second component of the control curve.

ET receptor number in (+/−) tracheal and bronchial smooth muscle was 23 and 16% lower than in similar tissue from (+/+) mice and that these values fell to 46 and 41%, respectively in tissue from (−/−) mice.

The findings from contraction studies support those from the binding experiments. For example, concomitant with the reduction and absence of ET_B receptors in (+/−) and (−/−) preparations, respectively, there was an attenuation in the maximum contractile response to the ET_B receptor-selective ligand, Stx S6c, in (+/−) tissues and abolition of Stx S6c-induced contractions in trachea from (−/−) mice.

Substantial differences were apparent in the profiles of ET-1, Stx S6c and ET-3 and/or their sensitivities to antagonists. These qualitative and quantitative discrepancies can be reconciled by consideration of the different selectivity profiles for the three ET ligands for the ET_A and ET_B receptors: Stx S6c is ET_B receptor-selective, ET-1 has equivalent, potent affinities for ET_A and ET_B receptors, ET-3 has higher affinity for ET_B compared to ET_A receptors. For example, contractile responses to Stx S6c were absent in (−/−) mouse tracheal preparations, whereas ET-1 and ET-3 concentration-response curves were similar in (−/−) compared to (+/+) or (+/−) tissues, reflective of their potent ability to produce contractions *via* ET_A receptor activation.

Furthermore, in (+/+) preparations the ET-3 concentration-response curve was biphasic, whereas the curve to ET-1 was monophasic. These profiles reflect the differences in the affinities of ET-3 for ET_A (lower) and ET_B (higher) receptors, while ET-1 has equivalent affinities for both receptor populations (Masaki *et al*., 1992; Rubanyi & Polokoff, 1994). This former proposal is supported by the experiments with the antagonists, in which SB 234551 (ET_A receptor-selective) inhibited the second, but not the first, phase of the ET-3 concentration-response curve whereas A192621 attenuated the first, but not the second phase. In addition, the differences in the profiles of the ET-3 concentration-response curves in tissues from wildtype (biphasic) compared to heterozygous and homozygous (monophasic) animals provides further evidence for an interaction of ET-3 with both ET_A and ET_B receptors in the former but not the latter preparations. Thus, although ET-3 has been regarded to some extent as an ET_B receptor-selective agonist (see reviews by Masaki *et al*., 1992; Bax & Saxena, 1994; Rubanyi & Polokoff, 1994), the current results highlight the caution that should be exercised with this categorization, with agonists with greater selectivity, such as Stx S6c, more appropriate tool ligands to selectively activate ET_B receptors.

ET-1-induced contraction in human bronchus (Goldie *et al*., 1995; Fukuroda *et al*., 1996; Hay *et al*., 1993a,b; 1996; Yoneyama *et al*., 1995), which contain both ET receptor subtypes (Goldie *et al*., 1995; Fukuroda *et al*., 1996), is insensitive to ET_A or ET_B receptor-selective antagonists alone, with significant inhibition of the response occurring only with a combination of both antagonists; this indicates a marked functional reserve (Goldie *et al*., 1996a,b; Fukuroda *et al*., 1996). Similar results were obtained in the current study in trachea from (+/+) mice. However, in mouse trachea the inhibition of ET-1 concentration-response curves was markedly greater than obtained in human bronchus (Goldie *et al*., 1996a,b; Fukuroda *et al*., 1996). Furthermore, the functional potency of the nonpeptide ET_B receptor antagonist, A192621, against Stx S6c-induced contractions of wildtype mouse trachea was significantly (about 2-log units) greater than previously reported for the potent, peptide ET_B receptor antagonist, BQ-788 in human and rabbit bronchus (Fukuroda *et al*., 1996; Hay *et al*., 1996; 1998), despite the similar potencies of the two compounds from binding studies using the cloned human ET_B receptor (unpublished observations). Collectively, these data suggest that there may exist marked species differences in the potencies of the ET receptor antagonists.

There are several reports proposing the existence of additional ET receptor subtypes, in particular for the ET_B receptor category (Bax & Saxena, 1994; Warner *et al*., 1993; Hay *et al*., 1996; 1998; Yoneyama *et al*., 1995). These hypotheses are based on binding and, in particular, contraction experiments exploring the effects of receptor antagonists against responses elicited by various ET agonists. For example, functional experiments have uncovered a limited or lack of effect of ET_B receptor antagonists against ET_B receptor-mediated responses in different tissues, including rabbit and human bronchi (Hay *et al*., 1996; 1998). There are severe limitations in utilizing only pharmacological data for receptor classification, with the need for supportive molecular biological, operational and structural information. A further issue in the ET receptor arena is the differences in the nature of the interactions of different ET ligands with the ET receptors (classical competitive versus pseudoirreversible), which complicate further the interpretation of pharmacological results (Devadason & Henry, 1997; Henry & King, 1999). In addition, there has been a limited availability of non-peptide ET_B receptor antagonists from different structural classes, with most studies utilizing the peptide antagonists, such as BQ-788. The utilization of tissues from knockout mice, in which a specific gene is disrupted, is an important technique to assist in receptor categorizing and investigation of pharmacologically heterogenous responses. A key study for the ET area was reported by Mizuguchi and co-workers (Mizuguchi *et al*., 1997) in which they investigated in ET_B receptor knockout mice the PD142893-sensitive, vasodilator response in aorta and the PD142893-resistant contractile response in gastric fundus, which had been postulated to have been mediated *via* distinct ET_B receptors. It was determined that both responses were absent in tissues from ET_B receptor knockout animals, and are thus mediated *via* a receptor population derived from the same gene (Mizuguchi *et al*., 1997). The results of the current study support this postulate of a homogenous receptor population mediating ET_B receptor-induced responses, at least in the mouse. The evidence in support of this hypothesis includes: (1) the abolition of Stx S6c-induced contractions in (−/−) mouse trachea; (2) the lack of effect of the ET_A receptor antagonist, SB 234551, alone against ET-1-induced responses in (+/+) preparations, but potent inhibition ($pK_B = 8.3$) in (−/−) tissues and (3) the biphasic profile of the ET-3 concentration-response curve in (+/+) tissues and monophasic nature of the curve in (−/−) trachea.

In summary, the current results confirm the significant influence of both ET_A and ET_B receptors in mediating ET ligand-induced contractions in mouse trachea. Furthermore, in this preparation, ET-3 was not a particularly selective ET_B receptor agonist, since it was a potent activator of both ET_A and ET_B receptors. The present data do not support the concept of the presence of atypical ET_B receptors in airway smooth muscle.

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