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# Necessity of dual blockade of endothelin $ET_A$ and $ET_B$ receptor subtypes for antagonism of endothelin-1-induced contraction in human bronchi

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**1** Endothelin (ET)-1 has been postulated to be involved in the development of obstructive airway diseases in man. In the present study, we attempted to characterize ET receptor subtypes mediating ET-1-induced contraction in human isolated bronchi. The ET receptor antagonists used in the present study were BQ-123 ($ET_A$ receptor-selective), BQ-788 ($ET_B$ receptor-selective) and BQ-928 ($ET_A/ET_B$ dual). Sarafotoxin S6c (S6c) was also used as an $ET_B$ receptor-selective agonist.

**2** In human bronchi, ET-1 and S6c ($10^{-12}$ M to $10^{-7}$ M) produced concentration-dependent contraction with almost equal potency ($pD_2$: $8.88 \pm 0.16$ for ET-1 and $9.42 \pm 0.15$ for S6c). The contraction induced by S6c was competitively antagonized by BQ-788 alone (1 and 10 $\mu$M) with a $pK_B$ value of $7.49 \pm 0.21$, suggesting that the stimulation of $ET_B$ receptors causes a contraction of human bronchi. However, contrary to expectation, the concentration-response curves for ET-1 were not affected by BQ-788. The ET-1- and S6c-induced contractions were not affected by BQ-123 (10 $\mu$M). Thus, ET-1-induced contraction of human bronchi is not antagonized by BQ-123 alone or by BQ-788 alone.

**3** Combined treatment with 10 $\mu$M BQ-123 and 10 $\mu$M BQ-788 significantly antagonized the contraction induced by ET-1 with a dose-ratio of 11. BQ-928 also significantly antagonized ET-1-induced contraction with a $pK_B$ value of $6.32 \pm 0.24$.

**4** The specific binding of [$^{125}$I]-ET-1 to human bronchial membrane preparations was inhibited by BQ-123 (100 pM to 1 $\mu$M) by approximately 40%. Combination treatment with BQ-788 (100 pM to 1 $\mu$M) completely inhibited the BQ-123-resistant component of [$^{125}$I]-ET-1 specific binding.

**5** In conclusion, the present study demonstrates that BQ-788 alone cannot inhibit ET-1-induced contractions in human bronchi, although human bronchial $ET_B$ receptors are BQ-788-sensitive. Furthermore, it was shown that blockade of both receptor subtypes antagonizes ET-1-induced contraction, and that both receptor subtypes co-exist in human bronchial smooth muscles. These findings suggest that $ET_A$ receptors as well as $ET_B$ receptors are involved in ET-1-induced contraction in human bronchi. If ET-1 is involved in human airway diseases, dual blockade of $ET_A$ and $ET_B$ receptors may be necessary to treat the diseases.

**Keywords:** Bronchi (human); endothelin-1; sarafotoxin S6c; $ET_A$ receptors; $ET_B$ receptors; BQ-123; BQ-788; BQ-928

## Introduction

Endothelin-1 (ET-1) is one of the most potent bronchoconstrictor peptides in human isolated airway smooth muscles (Advenier *et al.*, 1990; Henry *et al.*, 1990; Hay *et al.*, 1993). ET-1 is also a potent mitogen of airway smooth muscles (Noveral *et al.*, 1992). The immunoreactive ET level is raised in bronchial washing fluid during status asthmaticus (Nomura *et al.*, 1989). Endothelin expression in airway epithelium is more evident in asthmatic patients than in healthy subjects (Springall *et al.*, 1991; Carpi *et al.*, 1993). Thus, ET-1 appears to play a pathophysiological role in human airway diseases (Barnes, 1994).

At least two distinct ET receptor subtypes, $ET_A$ and $ET_B$ receptors, exist in mammalian tissues (Arai *et al.*, 1990; Sakurai *et al.*, 1990). The concept of ET receptor subtypes is dependent on selectivity for ET isopeptides (Masaki, 1991). $ET_A$ receptors have a higher affinity for ET-1 or ET-2 than for ET-3. $ET_B$ receptors have nonselective high affinity for ET isopeptides. Sarafotoxin S6c (S6c), a peptide structurally related to ET, is an agonist that has the highest selectivity for $ET_B$ receptors over $ET_A$ receptors discovered so far (Williams *et al.*, 1991). In contrast, BQ-123 (Table 1) is an antagonist that has high selectivity for $ET_A$ receptors over $ET_B$ receptors (Ihara *et al.*, 1991). S6c and BQ-123 are often used for pharmacological analysis of ET receptor subtypes. We recently isolated BQ-788, an $ET_B$ selective antagonist (Ishikawa *et al.*, 1994) and BQ-928, an $ET_A/ET_B$ dual antagonist (Yamakawa *et al.*, 1994) (Table 1).

It has been reported that ET-1 causes potent contraction of human isolated bronchi. Because of increased expression of ET-1 in several pulmonary diseases, an ET receptor antagonist might have therapeutic properties in patients with diseases such as bronchial asthma. However, it is not known which ET receptor subtypes are involved in the ET-1-induced contraction of human bronchi. Therefore, it is clinically important to discover which types of ET receptor antagonist ($ET_A$, $ET_B$, $ET_A/ET_B$ dual) effectively antagonize ET-1-induced contraction of human bronchi. In the present study, BQ-123 (an $ET_A$ antagonist), BQ-788 (an $ET_B$ antagonist) and BQ-928 (an $ET_A/ET_B$ dual antagonist) were used to investigate endothelin receptors mediating contraction of human bronchi.

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**Table 1** Characterization of antagonists used in the present study (Yamakawa *et al.*, 1994)

| *Antagonists* | *Structures* | $IC_{50}$ (nM) $ET_A$* | $ET_B$** |
|---|---|---|---|
| BQ-123 | cyclo(-D-Val-Leu-D-Trp-D-Asp-Pro) | 22 | 18000 |
| BQ-788 | Dmpc-γMelLeu-D-Trp(1-COOMe)-D-Nle-OH | 140 | 0.82 |
| BQ-928 | Dmpc-Cprg-D-Trp(2-Br)-D-Nle-OH | 3.8 | 0.81 |

*Inhibition of [$^{125}$I]-ET-1 binding to $ET_A$ receptors on porcine aortic smooth muscle membranes.
**Inhibition of [$^{125}$I]-ET-1 binding to $ET_B$ receptors on porcine cerebellum membranes.
Dmpc: cis-2,6-dimethylpiperidinocarbomyl group. Cprg: cyclopropylglycine.

## Methods

### *Tissue isolation*

Human lung tissue was obtained from 11 patients (4 males and 7 females) aged 27–84 years (60.6±4.7 years; mean± s.e.mean) who were undergoing pulmonary resection for lung cancer at the University of Tsukuba Hospital. None of the patients had signs of obstructive airway diseases (bronchial asthma, emphysema, etc.), vascular disease, metabolic disease, or connective tissue disorders. All of the patients underwent general anaesthesia with inhaled isoflurane and nitrous oxide-oxygen. Each patient was properly informed of the nature of the study, and consent was obtained. The bronchi used in the present study had a macroscopically normal appearance.

### *Contraction studies*

Bronchi with an outer diameter of 2–3 mm were freed from the surrounding connective tissues, lung parenchyma and blood vessels. Each bronchus was cut into rings 4 mm long without damage to the epithelium. The rings were then placed in 5-ml organ baths containing modified Krebs-Henseleit solution (composition in mM: NaCl 115.0, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$-$6H_2O$ 1.2, $KH_2PO_4$ 1.2, $NaHCO_3$ 25.0 and glucose 5.6) maintained at 37°C, continuously aerated with 95% $O_2$ and 5% $CO_2$, and connected to isometric transducers (model TB-651T, Nihon-Kohden, Tokyo, Japan) with sutures. Mechanical responses were recorded isometrically by a multi-channel polygraph (model RMP-6018, Nihon-Kohden); the initial tension was 2.0 g for human bronchi. The tissue was equilibrated for at least 60 min and then contracted by adding 50 mM KCl twice. The response to ET-1 and S6c was expressed as a percentage of the second contraction (% KCl). After the preparations had been washed with Krebs solution and the tone of the preparations was returned to resting tension, concentration-contraction curves for ET-1 and S6c were obtained by cumulative addition of these agonists to the organ bath. The endothelin antagonists were added 20 min before the addition of the agonists. Several protease inhibitors (thiorphan, bestatin and amastatin, 1 μM) were added 40 min before the addition of the agonists to protect the peptides from degradation.

### *Binding experiments*

Bronchi were freed from the surrounding connective tissues, lung parenchyma and blood vessels, and the epithelium was removed by gentle rubbing of the intimal surface with wet filter paper. The tissues were homogenized in 10 volumes of 10 mM 3-[N-morpholino]propane sulphonic acid (MOPS), pH 7.4, containing 20% sucrose with a Polytron (setting 7 for 30 s × 2) at 4°C. The homogenates were centrifuged at 10,000 *g* for 15 min. The supernatants were then centrifuged at 90,000 *g* for 40 min. The pellets were homogenized in 5 mM HEPES/Tris, pH 7.4. The resulting homogenates were incubated at 25°C with 10 pM [$^{125}$I]-ET-1 in the presence or absence of BQ-compounds in 50 mM Tris-HCl buffer, pH 7.4, containing 0.1 mM phenylmethylsulphonyl fluoride, 1 μM pepstatin, 2 μM leupeptin, 1 mM 1,10-phenanthroline, 1 mM EDTA, 10 μM $CaCl_2$, 10 μM $MgCl_2$ and 0.1% BSA. After 4 h of incubation, 2 ml of cold 5 mM HEPES/Tris, pH 7.4, containing 0.3% BSA (Buffer A) was added to the mixture, which was then rapidly filtered through Whatman GF/C glass fibre filters. After the filters were washed with Buffer A, the radioactivity on each filter was determined by a gamma counter. Nonspecific binding was defined by adding 200 nM ET-1 to the assay mixture.

### *Data analysis*

$pD_2$ values for the agonists were calculated after linear regression analyses of the data. $pK_B$ values for the antagonists were calculated using the following formula: $pK_B$ = −log [antagonist]/dose ratio − 1. All data are given as mean± s.e.mean. Statistical comparisons were made by one-way analysis of variance followed by the Neuman-Keuls test.

### *Drugs*

ET-1 and S6c were purchased from Peptide Institute (Osaka, Japan). [$^{125}$I]-ET-1 (2000Ci mmol$^{-1}$) was obtained from Amersham Japan (Tokyo). BQ-123, BQ-788 and BQ-928 were synthesized in our laboratory.

## Results

### *Contraction studies*

ET-1 and S6c potently contracted human bronchi with $pD_2$ values of 8.88±0.16 ($n$=5) and 9.42±0.15 ($n$=6), respectively. The maximum contractions elicited by ET-1 and S6c were 122.7±11.0% and 149.5±21.6%, respectively, of that produced by 50 mM KCl (Figure 1). The concentration-response curve for S6c was shifted to the right by BQ-788 alone (1 and 10 μM) in a competitive manner (Figure 1). Therefore, it was thought that stimulation of $ET_B$ receptors causes contraction

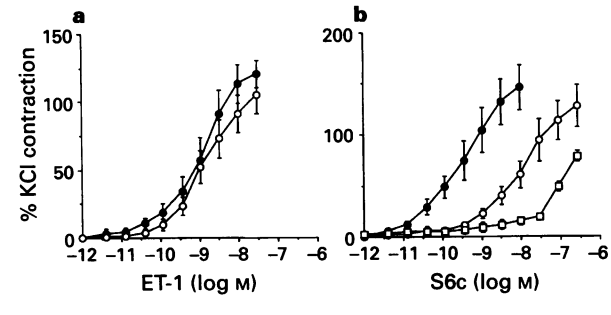


**Figure 1** (a) Effect of BQ-788 (10 μM, ○) on contraction induced by ET-1 in human bronchi; $n$=5. (b) Effect of BQ-788 (1 μM, ○; 10 μM, □) on contraction induced by S6c in human bronchi; $n$=6. (●) control. Data are expressed as mean±s.e.mean.

of human bronchi. The $pK_B$ value of 1 μM BQ-788 against the S6c-induced contraction was 7.49±0.21. However, contrary to expectation, the concentration-response curves for ET-1 were not affected by 10 μM BQ-788 ($pD_2$: 8.77±0.23 in the presence of BQ-788, Figure 1). Neither the ET-1- nor the S6c-induced contraction of human bronchi was affected by 10 μM BQ-123 ($pD_2$: 8.95±0.28 for ET-1, 9.52±0.19 for S6c in the presence of BQ-123) (Figure 2).

We also investigated the effect of simultaneous blockade of both receptor subtypes on the ET-1-induced contraction. Interestingly, combined treatment with 10 μM BQ-123 and 10 μM BQ-788 produced an 11 fold shift of the concentration-response curve for ET-1 without affecting the maximum contraction ($pD_2$: 8.88±0.16 for control, 7.82±0.17 for combined treatment, $P<0.05$) (Figure 3). Thus, BQ-123 and BQ-788 antagonized the contraction in a synergistic manner. Furthermore, 10 μM BQ-928 competitively antagonized the ET-1-induced contraction with a $pK_B$ value of 6.32±0.24 ($pD_2$: 8.88±0.16 for control, 7.73±0.17 for BQ-928, $P<0.05$) (Figure 3).

### *Binding studies*

In human bronchial membrane preparations, BQ-123 (1 μM) inhibited [$^{125}$I]-ET-1 binding to the membranes by approximately 40%, and concomitant treatment with BQ-788 (1 μM) completely inhibited the BQ-123-resistant component of [$^{125}$I]-ET-1 binding to the membranes (Figure 4).

## Discussion

In the present study, ET-1-induced contraction of human bronchi was not antagonized by BQ-123 (an $ET_A$ antagonist) alone or by BQ-788 (an $ET_B$ antagonist) alone but was effectively antagonized by combined treatment with BQ-123 and BQ-788. This result is in accordance with the present finding that BQ-928 (an $ET_A/ET_B$ dual antagonist) effectively antagonizes ET-1-induced contraction of human bronchi. Since the $ET_B$ receptor agonist, S6c produced a contraction that was antagonized by BQ-788 alone, it was thought that stimulation of $ET_B$ receptors causes the contraction of human bronchi. However, as the ET-1-induced contraction of human bronchi was not affected by BQ-788 alone but was antagonized by combined treatment with BQ-123 and BQ-788, it appears that the stimulation of $ET_A$ receptors is also involved in ET-1-induced contraction.

BQ-788 inhibited [$^{125}$I]-ET-1 binding to $ET_B$ receptors on human Girardi heart cells with an $IC_{50}$ value of 1.2 nM (Ishikawa *et al.*, 1994). In the present study, BQ-788 inhibited BQ-123-insensitive components of [$^{125}$I]-ET-1 specific binding to bronchial membrane preparations with an $IC_{50}$ value of 3.0 nM (Figure 4). Thus, BQ-788 has a high affinity for $ET_B$ receptors on human bronchial smooth muscle. Indeed, BQ-788 effectively antagonized the contraction induced by S6c in human bronchi in a competitive manner (Figure 1). Contrary to expectation, BQ-788 alone did not antagonize the contraction induced by ET-1 (Figure 1); however, the blockade of both receptor subtypes by combined treatment with BQ-788 and BQ-123 or by BQ-928 significantly antagonized ET-1-induced contraction in human bronchi (Figure 4). Furthermore, a binding assay revealed that there were BQ-123-sensitive $ET_A$ receptors (approx. 40%) as well as BQ-788-sensitive $ET_B$ receptors (approx. 60%) in human bronchial membrane preparations, although several other cell types in addition to smooth muscle may be contained in these preparations and contribute to the binding profile. These results strongly suggest that $ET_A$ receptors as well as $ET_B$ receptors are simultaneously involved in human bronchoconstriction. The involvement of both $ET_A$ and $ET_B$ receptors in the airway has also been demonstrated in other animal species including the rat (Henry, 1993), mouse (Henry & Goldie, 1994) and rabbit (Yoneyama *et al.*, 1995).

The mechanism of the synergistic inhibition by BQ-123 and BQ-788 of ET-1-induced contraction in human bronchi is not clear. Recently, we reported similar synergistic inhibition in rabbit pulmonary arteries, in which $ET_A$ and $ET_B$ receptors also co-exist (Fukuroda *et al.*, 1994b). One possible explanation of the synergistic inhibition by $ET_A$ and $ET_B$ receptor blockade in tissues where both receptors coexist is that a cross-talk mechanism exists in the signal transduction systems be-

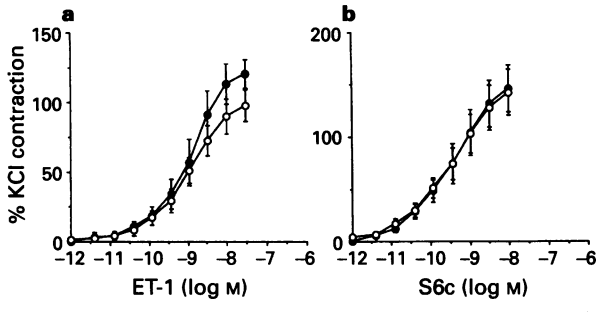


**Figure 2** Effect of BQ-123 (10 μM, ○) on contraction induced by ET-1 (a) or S6c (b) in human bronchi; (●) control. Data are expressed as mean±s.e.mean. $n=5-6$.

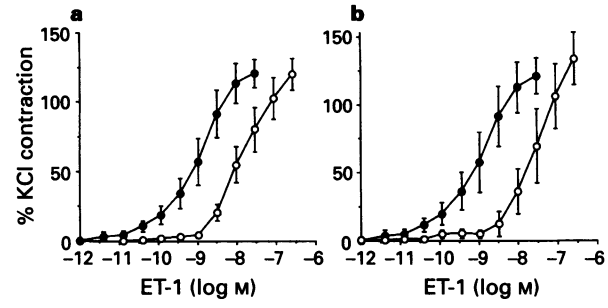


**Figure 3** (a) Effect of combined treatment with BQ-123 and BQ-788 (10 μM each, ○) on contraction induced by ET-1 in human bronchi. (b) Effect of BQ-928 (10 μM, ○) on contraction induced by ET-1 in human bronchi; (●) control. Data are expressed as mean±s.e.mean. $n=5-6$.

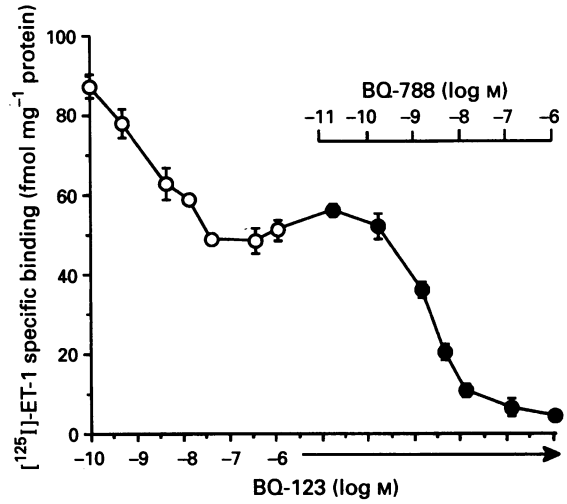


**Figure 4** Effect of BQ-123 (0.1 nM to 1 μM, ○) and 1 μM BQ-123 plus BQ-788 (10 pM to 1 μM, ●) on [$^{125}$I]-ET-1 specific binding to human bronchial membrane preparations. Data are expressed as the mean±s.e.mean of three observations.

tween $ET_A$ and $ET_B$ receptors. In human Girardi heart cells co-expressing $ET_A$ and $ET_B$ receptors in the ratio of 4:6, the displacement profiles of BQ-788 in membrane preparations were very different from those in the whole cells. BQ-788 displaced [$^{125}$I]-ET-1 binding to the membranes in a multiphasic manner with an $IC_{50}$ of 24 nM and that to the whole cells in a monophasic manner with a low affinity ($IC_{50}$ = 890 nM), which was comparable to the displacement profiles previously reported for $ET_A$ receptors, suggesting that the binding of ET-1 to $ET_A$ may convert $ET_B$ receptor to a BQ-788-insensitive form through $ET_A$-mediated intracellular signalling (S. Ozaki, personal communication). Similar mechanisms also may occur in human bronchi and rabbit pulmonary arteries. Alternatively, the activation of only $ET_A$ or only $ET_B$ receptors may be sufficient to produce full signals in these tissues. Indeed, the activation of only $ET_B$ receptors by S6c caused full contraction in human bronchi (Figure 1). However, since no $ET_A$-selective agonist currently exists, whether the activation of only $ET_A$ receptors in human bronchi can cause full contraction awaits further study. When both receptors are stimulated by ET-1, a cross-talk mechanism that limits increases in the intracellular signal by the level stimulated by either receptor may occur. The blockade of either $ET_A$ or $ET_B$ receptors may free the receptors from the inhibitory cross-talk mechanism, compensating for the lack of the other signal.

Some of our results were in accordance with those of Hay *et al.* (1993): ET-1 and S6c produced contractions with equal potency in human bronchi. Since Hay *et al.* (1993) also found that ET-1-induced contraction of human bronchi was not antagonized by an $ET_A$-selective antagonist and they did not study the effects of an $ET_B$-selective antagonist on contraction, they concluded that ET-1-induced contraction of human bronchi is mediated through $ET_B$ receptors. Although our results correspond with theirs, our conclusions are very different. By using the $ET_B$-selective antagonist BQ-788 combined with the $ET_A$-selective antagonist BQ-123, we have shown that $ET_A$ receptors as well as $ET_B$ receptors are involved in the ET-1-induced contraction of human bronchi.

In the present study, combined treatment with 10 $\mu$M BQ-123 and 10 $\mu$M BQ-788 shifted the concentration-response curve for ET-1 by 11 fold in human bronchi. However, the extent of the shift was less than expected because 10 $\mu$M BQ-123 or BQ-788 can antagonize the responses mediated by $ET_A$ or $ET_B$ receptors, respectively, with a dose-ratio of more than 100 fold (Ihara *et al.*, 1992 for BQ-123; present study, Ishikawa *et al.*, 1994 for BQ-788). The antagonistic effect of BQ-928 ($pK_B$: 6.3) in human bronchi was also weaker than expected. BQ-928 inhibited ET-1 binding to $ET_A$ and $ET_B$ receptors with $IC_{50}$ values of 3.8 nM and 0.81 nM respectively, in the binding assay (Table 1) and antagonized $ET_A$-mediated responses in various blood vessels with $pA_2$ values of 7.0–7.9 and $ET_B$-mediated responses in rabbit pulmonary arteries with a $pK_B$ value of 9.2 (unpublished data). Similar decreased potency of $ET_A/ET_B$ dual antagonism was also observed with other $ET_A/ET_B$ dual antagonists against the contraction induced by ET-1 in guinea-pig airways (Battistini *et al.*, 1994), rabbit pulmonary arteries and rat stomachs (Warner *et al.*, 1993). Although the reason for such weak activity of $ET_A/ET_B$ dual antagonism against ET-1 in human bronchi remains unclear, speculative explanations are (1) that there may be other ET receptor subtypes than BQ-123-sensitive $ET_A$ receptors and BQ-788-sensitive $ET_B$ receptors and (2) that an interaction between $ET_A$ and $ET_B$ receptors may occur because the phenomenon is observed in the case of $ET_A/ET_B$ composite-type responses (Clozel & Gray, 1995). The former explanation seems unlikely because [$^{125}$I]-ET-1 binding to human bronchial membrane preparations was completely inhibited by the combination of BQ-123 and BQ-788. The mechanism of the decreased potency of $ET_A/ET_B$ dual antagonists in human bronchi awaits further study.

There is evidence for increased expression of ET-1 in several pulmonary diseases including asthma, fibrosing alveolitis and pulmonary hypertension, suggesting that ET-1 plays a pathophysiological role in their development (Barnes, 1994). It was reported recently that $ET_A$ and $ET_B$ receptors coexist in asthmatic peripheral human lungs at the same density as in non-asthmatic lungs (Knott *et al.*, 1995). Although it is unclear which receptor subtypes participate in the pathogenesis of human airway diseases, an $ET_A/ET_B$ dual antagonist rather than a receptor subtype-selective antagonist may exert protective effects against asthma. Furthermore, since ET-1-induced contraction of human pulmonary arteries is mediated mainly through $ET_A$ receptors (Hay *et al.*, 1993; Fukuroda *et al.*, 1994a), an $ET_A$-selective antagonist such as BQ-123 may selectively affect pulmonary circulation rather than the respiratory system in human lungs charged with ET-1.

In conclusion, in human isolated bronchi the ET-1-induced contraction was not antagonized by BQ-123 alone or by BQ-788 alone but was antagonized by combined treatment with BQ-123 and BQ-788 or by BQ-928, an $ET_A/ET_B$ dual blocker. BQ-123-sensitive $ET_A$ receptors and BQ-788-sensitive $ET_B$ receptors coexist on human bronchi. These results suggested that $ET_A$ receptors as well as $ET_B$ receptors are simultaneously involved in human bronchoconstriction. Although the mechanism of such synergistic inhibition by blockade of $ET_A$ and $ET_B$ receptors remains unknown, there may be a cross-talk mechanism(s) between both receptors. If ET-1 is involved in human airway diseases, dual blockade of $ET_A$ and $ET_B$ receptors may be necessary to treat the diseases.

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