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DIFFERENT DISTRIBUTION OF ENDOTHELIN RECEPTOR SUBTYPES IN PULMONARY TISSUES REVEALED BY THE NOVEL SELECTIVE LIGANDS BQ-123 AND [Ala^{1,3,11,15}]ET-1

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SUMMARY: We have demonstrated the different distribution of two distinct endothelin (ET) receptor subtypes in porcine pulmonary tissues using a radioligand binding assay. The clear differentiation of the subtypes was made possible by the discovery of two compounds, BQ-123 and [Ala^{1,3,11,15}]ET-1 (4AlaET-1), that are highly selective for ET_A and ET_B receptors, respectively. In the bronchus and lung parenchyma, BQ-123 inhibited 65% and 30% of [^{125}I]ET-1 binding on the sensitive sites, while 4AlaET-1 displaced 25% and 60%, respectively. The combination of the two compounds completely inhibited ET-1 binding in both tissues. An autoradiographic study of [^{125}I]ET-1 binding using BQ-123 and 4AlaET-1 also supported the different localization of two ET receptor subtypes in pulmonary tissues. In particular, the blood vessels and bronchi are rich in ET_A, but the lung parenchyma is rich in ET_B.

Endothelin (ET)-1 is one of the most potent vasoconstrictive peptides recently discovered from the cultured medium of vascular endothelial cells (1). Subsequent studies have revealed the existence of the family peptides, ET-1, ET-2 and ET-3, and have demonstrated their biological effects on blood vessels, the heart, the kidney and the central nervous system (2).

ET-1 also elicits various biological responses in pulmonary tissues (3). In particular, ET-1 is a potent constrictor of the trachea, bronchus and lung parenchyma (4,5,6) and induces the release of thromboxaneA_2 from the airway tissues (5). It has been proved that ET-1 induces a marked increase in pulmonary resistance and a marked decrease in lung compliance *in vivo* (7,8). An autoradiographic study of the lung revealed that ET receptors are widespread at a high density in pulmonary tissues, namely bronchi, blood vessels and alveoli (9). Therefore, these above-mentioned pulmonary responses may be induced by the binding of ET-1 to the ET receptors that are distributed in the airway. Furthermore, immunoreactive ET levels in the bronchial lavage fluid and airway epithelium of asthmatic patients are higher than those of normal subjects (10,11), suggesting that ET-1 plays a pathophysiological role in pulmonary diseases such as asthma.

Two distinct ET receptors, 'ET-1-selective' ET_A and 'ET isopeptides-nonselective' ET_B, were recently cloned from the cDNA library in bovine and rat lungs, respectively (12,13).

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However, the distribution of the ET receptor subtypes in pulmonary tissues has not yet been clarified due to a lack of selective ligands.

In the present study, we will describe the different distribution of two distinct ET receptors in porcine pulmonary tissues as demonstrated by two novel compounds that are a potent ET_A-selective antagonist cyclo(-D-Trp-D-Asp-Pro-D-Val-Leu-) (BQ-123) (14) derived from the modification of a natural lead of the ET_A antagonist BE-18257B (15) and an ET_B-selective agonist [$Ala^{1,3,11,15}$]ET-1 (4AlaET-1), which is a linear analog of ET-1 (16).

MATERIALS AND METHODS

Materials: ET-1 and ET-3 were purchased from Peptide Institute Inc. (Osaka, Japan). BQ-123 and 4AlaET-1 were prepared in our laboratories. [^{125}I]ET-1 and Hyperfilm-[^{3}H] were purchased from Amersham Japan Inc. (Tokyo). O.C.T. compound and Kodak D-19 were purchased from Miles Inc. (Elkart, USA) and Eastman Kodak Co. (New York, USA), respectively.

Binding experiments: Porcine tissues were obtained from a slaughterhouse. The large bronchi, lung parenchyma and cerebellum were dissected and the membranes of these tissues were prepared as previously described (15). The membranes were incubated at 25°C with 12pM [^{125}I]ET-1 in the presence or absence of test compounds in 50mM Tris/HCl buffer, pH 7.4, containing 0.1mM phenylmethylsulfonyl fluoride, 1μM pepstatin, 2μM leupeptin, 1mM 1,10-phenanthroline, 1mM EDTA, 10μM $CaCl_2$, 10μM $MgCl_2$ and 0.1% BSA. After 4 hours of incubation, ice-cold 5mM Hepes/Tris, pH 7.4, containing 0.3% BSA (Buffer A) was added to the mixture, followed by rapid filtration through GF/C glass fiber filters (Whatman, England). After the filters were washed with buffer A, the radioactivity remaining on the filter was determined by a gamma counter (PACKARD: COBRA 5002). Nonspecific binding was defined by adding 0.2μM ET-1 to the assay mixture. Cultured porcine aortic smooth muscle cells (VSMCs) were obtained according to a previously described method (17) with minor modifications. The confluent VSMCs in the 3rd to 14th passages were used in these experiments. Binding experiments on VSMCs were performed as previously described (15). Briefly, the confluent VSMCs were incubated at 37°C with 10pM [^{125}I]ET-1 in the presence or absence of test compounds for 2 hours in Hank's balanced salt solution containing 0.1% glucose and 0.3% BSA, and the cell-bound radioactivity was determined.

Autoradiography: Fresh porcine lung was embedded in O.C.T. compound and frozen on dry ice. Sections were cut by cryotome to a thickness of 20 microns and were thaw-mounted onto poly-L-lysine-coated slides. The [^{125}I]ET-1 binding study was performed according to a previously described method (18). After incubation at room temperature for 10 minutes in 50mM Tris/HCl buffer, pH 7.4, containing 0.1M NaCl, 5mM $MgCl_2$, 40μg/ml bacitracin and 1% BSA (Buffer B), the serial tissue sections were incubated for 2 hours with 50pM [^{125}I]ET-1 in the presence or absence of either 1μM BQ-123 or 0.1μM 4AlaET-1 alone and combined. The sections were then washed twice in buffer B at 4°C for 10 minutes. Nonspecific binding was assessed in serial sections in the presence of 0.5μM ET-1. Labelled sections were placed in radiographic cassettes and apposed to Hyperfilm-[^{3}H] at 4°C for 7 days. The films were developed in a Kodak D-19 developer at 19°C for 5 minutes.

RESULTS AND DISCUSSION

A high density of [^{125}I]ET-1 binding sites was present in porcine bronchus and lung parenchyma membranes. The inhibition profiles of [^{125}I]ET-1 binding by ET-1 in these tissue membranes were monophasic, and the Hill coefficient (n_H) values (19) were not different from unity. Furthermore, analysis of the inhibitory profiles by the LIGAND PROGRAM (20) showed that both tissues have a single class of high-affinity binding sites for ET-1 (bronchus: K_D 307±23 pM, B_{max} 1.47±0.15 pmol/mg protein; lung parenchyma: K_D 258±14 pM, B_{max} 1.08±0.15 pmol/mg protein, mean±S.E.M. (n=4)). In contrast, the n_H values for the ET-3 displacement of [^{125}I]ET-1 binding to both tissues were significantly lower than unity (0.5-

Table 1. IC_{50} values and Hill coefficients for ET-1, ET-3, 4AlaET-1 and BQ-123 in displacing [^{125}I]ET-1 binding in porcine tissue membranes

Tissues		IC_{50} (nM) ET-1	ET-3	4AlaET-1	BQ-123
	ET_A+ET_B#	0.10 (0.92)§	4.00 (0.60)	0.52* (2-phase)	6.38* (2-phase)
Bronchus	ET_A¶	0.15 (1.02)	24.3 (0.94)	--------	7.96 (0.96)
	ET_B£	0.15 (1.14)	0.11 (0.91)	0.61 (0.93)	--------
	ET_A+ET_B#	0.10 (1.06)	0.31 (0.50)	0.57* (2-phase)	6.50* (2-phase)
Lung	ET_A¶	0.18 (0.92)	23.9 (0.97)	---------	7.67 (0.89)
	ET_B£	0.15 (0.94)	0.11 (1.07)	0.61 (0.91)	---------
VSMC	ET_A#	0.10	70	2200	7.3
Cerebellum	ET_B#	0.11	0.09	0.33	18000

The values in this table represent mean values determined in at least three different preparations.
Neither BQ-123 nor 4AlaET-1 were present in the incubation buffer.
¶ Determined in the presence of 0.1μM 4AlaET-1.
£ Determined in the presence of 1μM BQ-123.
* Half maximum inhibition concentration for the high-affinity site.
§ Hill coefficient (n_H).

0.6) (Table 1). Such low n_H values for ET-3 have been previously described in several studies and suggested that there are multiple, different affinity sites for ET-3 in several tissues including lung and trachea (21,22). However, there was no direct evidence that the lower n_H values were due to multiple receptor subtypes. In this study, ET_A-selective BQ-123 and ET_B-selective 4AlaET-1 were used to elucidate the existence of ET receptor subtypes in porcine pulmonary tissues.

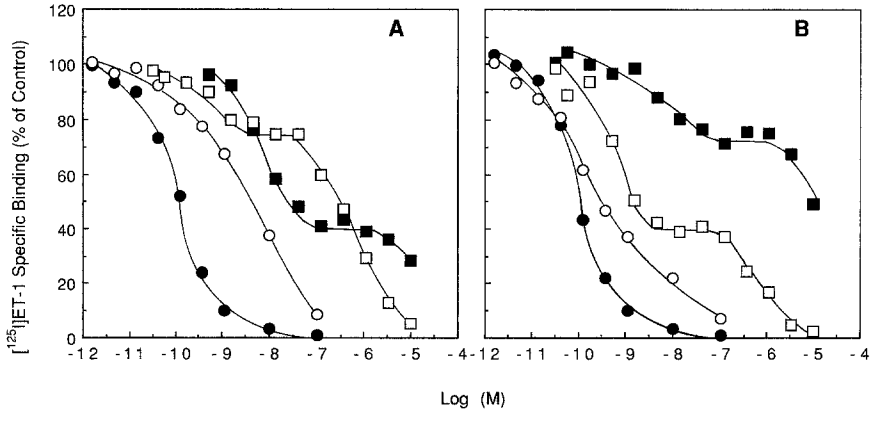

Fig.1. Displacement of [^{125}I]ET-1 binding to porcine bronchus (A) and lung parenchyma(B) membranes by ET-1 (●), ET-3(○), BQ-123 (■) and 4AlaET-1 (□). Data points represent the mean of four preparations.

As shown in Table 1, BQ-123 was 2,500 times more selective for ET_A receptors on vascular smooth muscle cells (VSMC) than for ET_B receptors in cerebellum membranes, while 4AlaET-1 was 6,700 times more selective for ET_B compared with ET_A receptors. These two compounds displaced specific [^{125}I]ET-1 binding to bronchus and lung parenchyma in a biphasic manner, indicating the presence of the high and low affinity sites. BQ-123 inhibited approximately 65% (bronchus) and 30% (lung parenchyma) of [^{125}I]ET-1 binding with IC_{50} values of 6.4-6.5 nM on the sensitive sites, whereas 4AlaET-1 inhibited approximately 25% and 60 % with IC_{50} values of 0.52-0.57 nM on the sensitive sites, respectively (Fig.1).

In the displacement studies using sufficient concentrations of BQ-123 (1μM) or 4AlaET-1 (0.1μM) to completely occupy the high-affinity sites, 4AlaET-1 and BQ-123 monophasically inhibited the specific binding of [^{125}I]ET-1 (Fig.2) with IC_{50} values of 0.61 and 8.0 nM in bronchus and 0.61 and 7.7 nM in lung parenchyma, respectively. The above-mentioned values correspond to the half maximum inhibition concentration for the high-affinity sites in the biphasic inhibition curves (Table 1). These data revealed that the high-affinity sites for BQ-123 and 4AlaET-1 were distinct and corresponded to the low-affinity sites for 4AlaET-1 and BQ-123, respectively. Furthermore, in the presence of 0.1μM of 4AlaET-1, the lower n_H values for ET-3 shifted toward unity and the IC_{50} values increased to 24 nM, which are close to that of VSMC having only ET_A receptors (Table 1). In the presence of 1μM of BQ-123, the displacement curve for ET-3 was similar to that of ET-1, as shown in cerebellum membrane

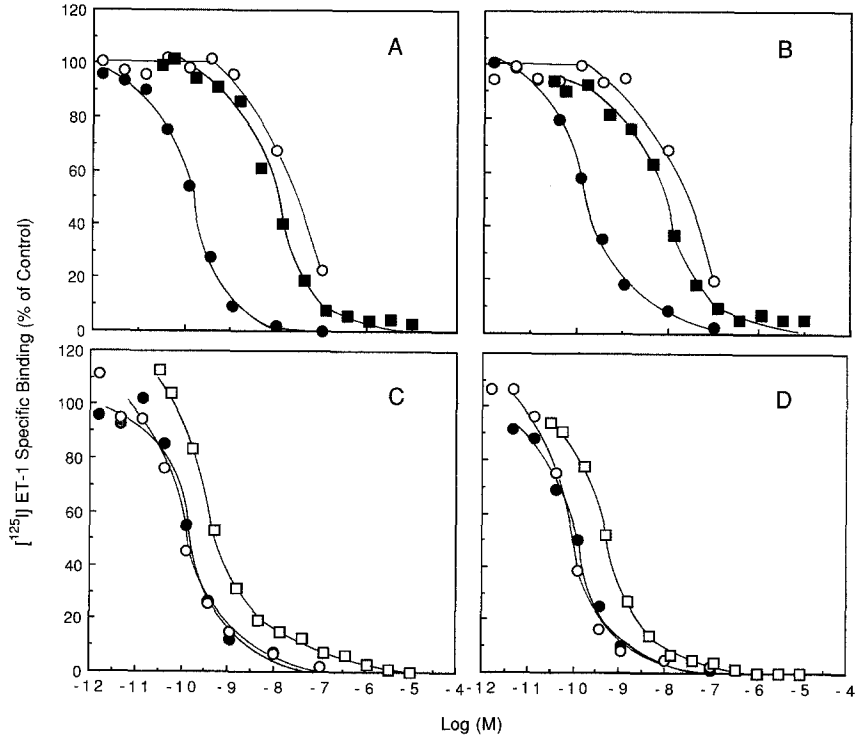

Fig.2. Displacement of [^{125}I]ET-1 binding to porcine bronchus and lung parenchyma membranes by ET-1 (●), ET-3(○), BQ-123 (■) and 4AlaET-1 (□) in the presence of 0.1μM 4AlaET-1 (A:bronchus, B: lung) or 1μM BQ-123 (C: bronchus, D: lung). Data points represent the mean of four preparations.

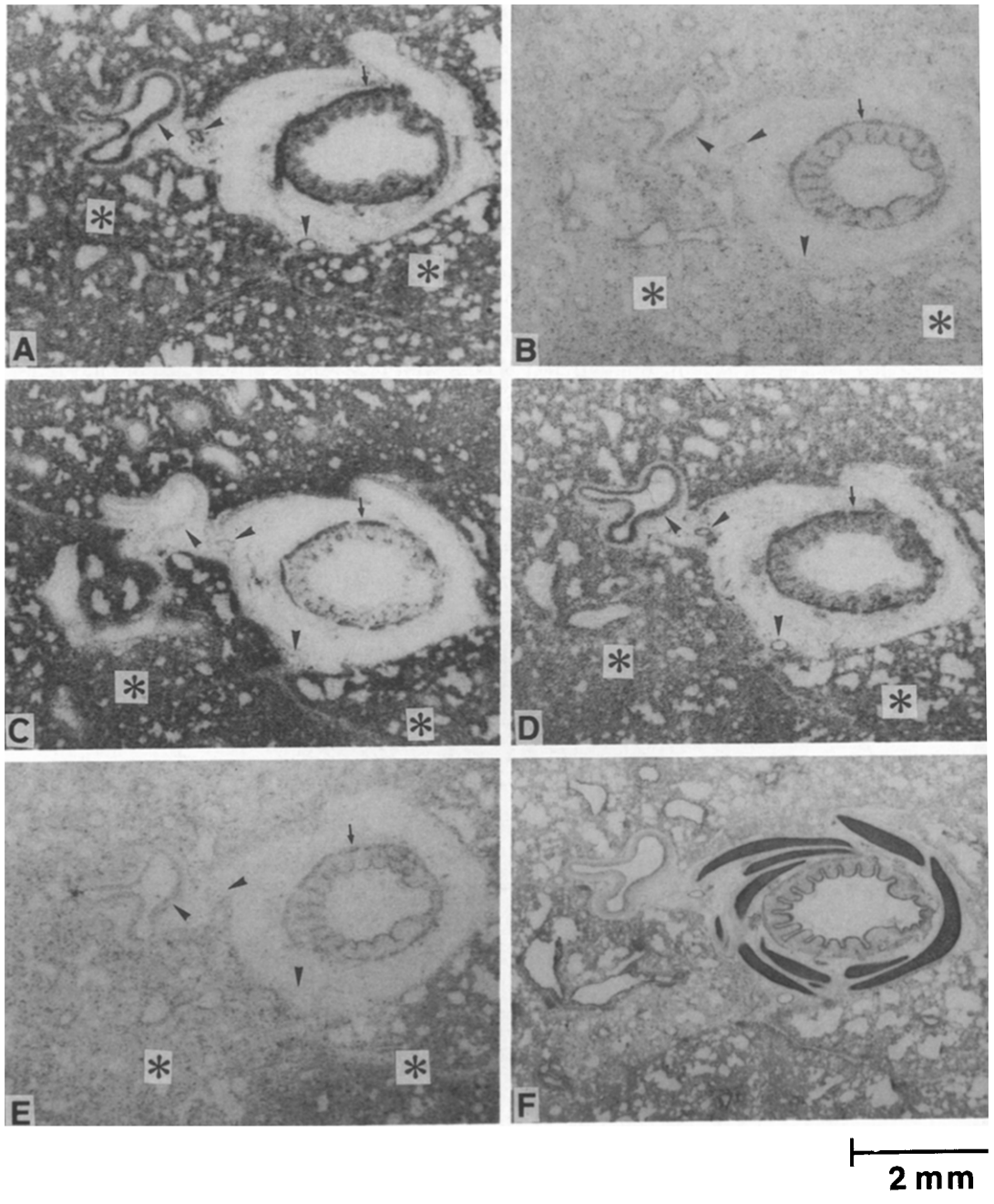

Fig.3. Autoradiograms of the distribution of [^{125}I]ET-1 binding sites in porcine lung incubated with [^{125}I]ET-1 alone (A), in the presence of 0.5μM of unlabelled ET-1 (B), in the presence of 1μM BQ-123 (C), in the presence of 0.1μM 4AlaET-1 (D) and in the presence of BQ-123 and 4AlaET-1 (E). Toluidine blue staining in the serial section (F). Binding sites for [^{125}I]ET-1 are demonstrated on the vasculature (arrowhead), bronchus (arrow) and alveoli (asterisk).

which has only ET_B receptors. These data indicate that ET-3 has insufficient selectivity for ET_B receptors to clearly distinguish the two receptor subtypes and that this insufficient selectivity lowers the n_H values for ET-3 displacement of ET-1 binding.

Autoradiographic examination of [^{125}I]ET-1 binding to porcine pulmonary tissues was undertaken to reveal the different localization of the ET receptor subtypes. As shown in Fig.3A, high- density [^{125}I]ET-1 was widespread in the pulmonary tissues. Binding sites for [^{125}I]ET-1 were demonstrated on the vasculature (arrowhead), bronchi (arrow) and alveoli (asterisk) (Fig.3A). The binding was almost abolished in the presence of 0.5μM of unlabelled ET-1 (Fig.3B). In the presence of 1μM BQ-123 to inhibit [^{125}I]ET-1 binding to ET_A receptors, binding density on the vasculature and bronchi was markedly diminished, whereas that of alveoli was only slightly reduced (Fig.3C). In contrast, 0.1μM of 4AlaET-1 slightly reduced ET-1 binding on the vasculature and bronchi while markedly decreased ET-1 binding on alveoli (Fig.3D). When both compounds were applied in combination, ET-1 labeling was almost completely inhibited in all areas to the level of ET-1 nonspecific binding observed in Fig.3B (Fig.3E). These results indicate that ET receptors in porcine pulmonary tissues are composed of ET_A and ET_B receptors, and that the localization of ET receptor subtypes is different in various parts of the pulmonary tissue. In particular, ET_A receptors are abundant in the bronchi and vasculature while ET_B receptors are abundant in the lung parenchyma, which is mostly composed of alveoli. Thus, the autoradiographic data also support the different distribution of the two ET receptor subtypes observed in the binding assay in the bronchus and lung parenchyma membranes.

In conclusion, using two highly selective ligands, BQ-123 and 4AlaET-1, we clearly demonstrated that two ET receptor subtypes, ET_A and ET_B, exist with different distribution in pulmonary tissues. ET_A receptors are preferentially localized in the bronchi and vasculature, while ET_B receptors are commonly found in lung parenchyma. The distinct localization of ET receptor subtypes in pulmonary tissues suggests that ET-1 has a complex pathophysiological role as a pulmonary regulating substance.

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