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The distribution and density of receptor subtypes for endothelin-1 in peripheral lung of the rat, guinea-pig and pig

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1 Quantitative autoradiographic studies were conducted to determine the distributions and densities of endothelin-A (ET_A) and ET_B receptor subtypes in peripheral lung alveolar wall tissue of the rat, guinea-pig and pig, with a view to assessing the potential suitability of these tissues as models for investigations of ET receptor function in human alveolar tissue.

2 High levels of specific [^{125}I]-ET-1 binding were detected in peripheral lung components from all three species tested. In mature porcine alveolar wall tissue, specific binding increased in a time-dependent manner to a plateau, consistent with the previously described pseudo-irreversible binding of this ligand to a finite population of specific binding sites.

3 [^{125}I]-ET-1 was associated specifically with both ET_A and ET_B binding site subtypes in alveolar wall tissue of foetal pig lung as early as 36 days gestation, raising the possibility of a functional role for ET-1 in lung development. In addition, both ET_A and ET_B binding site subtypes were detected in alevolar wall tissue and in peripheral airway smooth muscle of mature lung parenchyma from all three species. However, the binding subtype proportions differed in these tissues. For example, in porcine peripheral bronchial smooth muscle, ET_A sites apparently predominated, whereas ET_B sites constituted the major subtype detected in alveolar wall in this species. These data suggest significant shifts in ET receptor subtype expression at different levels in the respiratory tract.

4 ET binding site subtype proportions in the alveolar wall also differed markedly between species. In rat lung alveoli, ET_A and ET_B sites were detected in similar proportions ($52\pm3\%$ and $43\pm5\%$ respectively). In contrast, in guinea-pig peripheral lung, ET_B binding sites clearly predominated, constituting approximately 80% of total specific binding, with ET_A sites accounting for only 12%. Porcine alveolar wall tissue also contained a mixture of these ET receptor subtypes, with ET_A and ET_B binding comprising $23\pm3\%$ and $65\pm1\%$ respectively of the total population of specific binding sites detected. These latter proportions are similar to values previously obtained in human peripheral lung tissue, suggesting that porcine lung might be a useful model of the human peripheral lung in subsequent studies of the functions of these pulmonary ET receptor subtypes.

Keywords: Peripheral lung alveoli; endothelin; ET_A and ET_B receptors; airway smooth muscle

Introduction

Radioligand binding and autoradiographic studies have demonstrated the presence of both ET_A and ET_B receptors in the airways of the human (Goldie *et al.*, 1995) and of various animal species (Henry, 1993; Goldie *et al.*, 1994a; Henry & Goldie, 1994). Although both receptor subtypes co-exist in human bronchial smooth muscle, contraction is predominantly mediated via the ET_B receptor subtype (Goldie *et al.*, 1995), whereas both ET_A and ET_B receptors mediate contraction in guinea-pig (Tschirhart *et al.*, 1991; Hay *et al.*, 1993), rabbit (Yoneyama *et al.*, 1995), rat (Henry, 1993) and mouse tracheal smooth muscle (Henry & Goldie, 1994). In sharp contrast, endothelin-1 (ET-1)-induced contraction of ovine tracheal smooth muscle involves stimulation of only ET_A sites (Abraham *et al.*, 1993; Goldie *et al.*, 1994a). Importantly, both ET_A and ET_B sites have also been found in abundance in the alveolar walls of human (Knott *et al.*, 1995) and ovine peripheral lung (Goldie *et al.*, 1994a), although as in airway smooth muscle, there are marked species differences in the proportions of these subtypes expressed in alveoli. For example, in human alveoli, ET_B sites predominated, accounting for approximately 70% of specific binding, whereas in sheep lung this figure was only about 40%. The present study assessed the distributions and densities of alveolar receptor subtypes for ET-1 with a view to identifying an animal model which more closely resembled human peripheral lung tissue with respect to ET receptor subtype expression.

Methods

Tissue preparation

Lung parenchymal tissue was obtained from male pigs 20-25 weeks of age, from 8 week old male Sprague Dawley rats and from 6-8 week old male guinea-pigs (SR/C Tricolour). Pigs were anaesthetized with sodium thiopentone (25 mg kg^{-1}, i.v.) and killed with a dose of potassium chloride (100 mg kg^{-1}, i.v.). Foetal pig lung tissue, 36, 47 and 57 days of age was obtained from a local abattoir. At these gestation times, the foetal weights were approximately 10 g, 35 g and 100 g respectively. The total gestation period for the pig is approximately 114 days, at which time the birth weight is approximately 1 kg. Guinea-pigs were killed by cervical dislocation and rats by stunning and exsanguination. Lungs from each species were removed and transferred to ice-cold Krebs bicarbonate solution, the composition of which was (mM); NaCl 117, KCl 5.36, $NaHCO_3$ 25.0, KH_2PO_4 1.03, $MgSO_4.7H_2O$ 0.57, $CaCl_2$ 2.5 and glucose 11.1. Mature lung parenchymal tissue was inflated by bronchial instillation with OCT embedding medium diluted 1:4 with 0.9% w/v NaCl solution and snap frozen in isopentane quenched in liquid nitrogen as previously described (Goldie *et al.*, 1994a). Whole

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foetal lung lobes were placed in small aluminium foil pans and immersed in OCT-saline and snap frozen. Transverse sections (10 μm) of all preparations were cut at −20°C and thaw-mounted onto gelatin/chrome alum-coated glass slides.

Autoradiographic studies

Autoradiographic studies with [^{125}I]-ET-1 in lung parenchymal tissue were conducted essentially as previously described (Goldie *et al.*, 1994a).

Time course of [^{125}I]-ET-1 binding in porcine alveolar wall tissue

The relative proportions of specific ET_A and ET_B binding sites in lung alveolar wall tissue was determined when total specific binding was maximal i.e. at B_{max}. Since [^{125}I]-ET-1 binds irreversibly to its specific sites (Marsault *et al.*, 1991; Waggoner *et al.*, 1992), it was not appropriate to use an equilibrium binding approach to derive B_{max} e.g. from quantitative autoradiographic data describing the concentration-dependence of specific binding. However, B_{max} and the incubation time required to attain B_{max} can be derived from an analysis of the time course of [^{125}I]-ET-1 binding, as previously described in ovine tracheal smooth muscle (Goldie *et al.*, 1994a). This binding is described by the relationship

$$B_t = B_{max}\ (1 - e^{-tk_1L})$$

where B_t is the specific binding at time t, k_1 is association rate constant and L is the ligand concentration (Waggoner *et al.*, 1992). B_{max} is attained at the plateau of the time-course curve.

In these experiments, slide-mounted tissue sections were incubated for 2 × 5 min at 22°C in 170 mM Tris-HCl buffer (pH 7.6) containing 0.25% (w/v) bovine serum albumin and the protease inhibitor phenylmethylsulphonyl fluoride (10 μM). Sections were then incubated for 10–240 min in buffer containing 0.3 nM [^{125}I]-ET-1 in the absence (total binding) or combined presence of 1 μM BQ 123 (ET_A receptor-selective ligand; Ihara *et al.*, 1992), and 100 nM sarafotoxin S6c (ET_B receptor-selective ligand; Williams *et al.*, 1991) (non-specific binding). In some experiments, non-specific binding was also determined in the presence of non-radiolabelled ET-1 (100 nM).

Autoradiographic grain densities over alveolar wall tissue were determined with an automated grain detection and counting system (Henry *et al.*, 1990). Autoradiographic grain densities were measured in a total of 720 fields [(4 fields per tissue section) × (5 tissue sections per slide) × (3 slides per treatment) × (2 treatments per time point) for each of 6 time points]. Specific autoradiographic grain densities were expressed as grains 1000 μm^{-2}.

Assessment of ET_A and ET_B binding site proportions in alveolar wall

Autoradiograms were produced as described above using tissue incubated with 0.3 nM [^{125}I]-ET-1 for 180 min in the absence (total binding) or presence of 1 μM BQ-123 or 100 nM sarafotoxin S6c to determine the extent of ET_A and ET_B site binding respectively. Non-specific binding was assessed in the combined presence of these ligands. For each species, autoradiographic grain densities were measured in a total of 240 tissue fields [(4 fields per section) × (5 tissue sections) × (3 slides per treatment) × (4 treatments)].

Drugs

Drugs used were; [^{125}I]-ET-1 (2000 Ci $mmol^{-1}$), ET-1, sarafotoxin S6c (Auspep, Melbourne, Australia), phenylmethylsulphonyl fluoride (Calbiochem, La Jolla, U.S.A.), BQ-123 (cyclo[D-Trp-D-Asp-L-Pro-D-Val-L-Leu]; gift from Dr D.W.P. Hay of SmithKline Beecham Pharmaceuticals, U.S.A.). Stock solutions of ET-1 (50 μM) and sarafotoxin S6c (50 μM) were prepared in 0.1 M acetic acid and dilutions made in 0.9% NaCl solution (saline). BQ-123 was prepared in 100 mM Na_2CO_3 and diluted in saline as required.

Statistical analyses

Differences between treatment means were assessed by analysis of variance followed by a modified t statistic (Wallenstein *et al.*, 1980) or by Student's unpaired t test as appropriate. P values less than 0.05 were considered to be statistically significant.

Results

Autoradiographic studies

Time course of [^{125}I]-ET-1 binding in porcine alveolar wall tissue Results show that specific binding of [^{125}I]-ET-1 at a concentration of 0.3 nM increased in a time-dependent manner to a plateau that was reached between 120 and 240 min (Figure 1). Thereafter, it was assumed that B_{max} for specific binding to both ET_A and ET_B binding sites was attained in all tissues at the 180 min incubation time point. Accordingly, in all subsequent autoradiographic experiments, [^{125}I]-ET-1 binding was assessed in the absence or presence of ET receptor subtype-selective ligands at this time point.

Distribution and density of ET_A and ET_B binding sites in alveolar wall tissue

Porcine lung High densities of autoradiographic grains derived from [^{125}I]-ET-1 binding were observed in foetal pig lung at 36, 47 (Figure 2) and 57 days gestation. Very low levels of non-specific binding were observed in each case. Although specific grain densities were not quantified in foetal porcine lung, Figure 2 clearly shows high densities of specific [^{125}I]-ET-1 binding

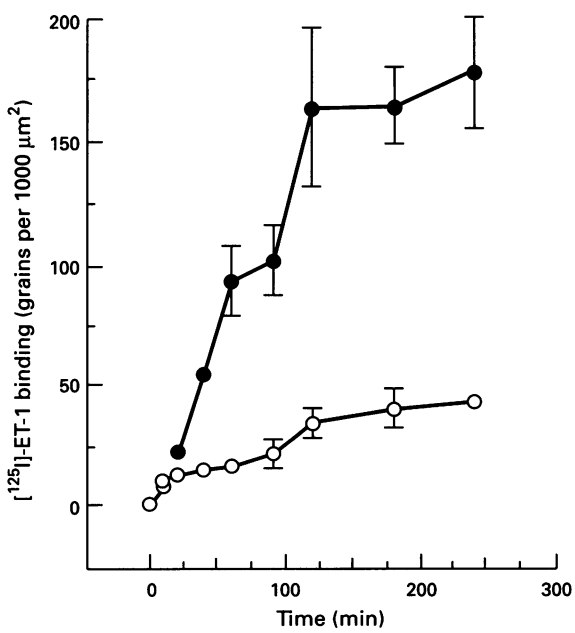

Figure 1 Time-dependence of [^{125}I]-ET-1 (0.30 nM) specific (●) and non-specific (○) binding in 10 μm transverse frozen sections of slide-mounted porcine alveolar wall tissue. Non-specific binding was assessed in the combined presence of the ET_A receptor-selective ligand, BQ-123 (1 μM) and the ET_B receptor-selective ligand, sarafotoxin S6c (100 nM). Data are presented as mean ± s.e.mean of mean of estimates from 3 separate lung samples.

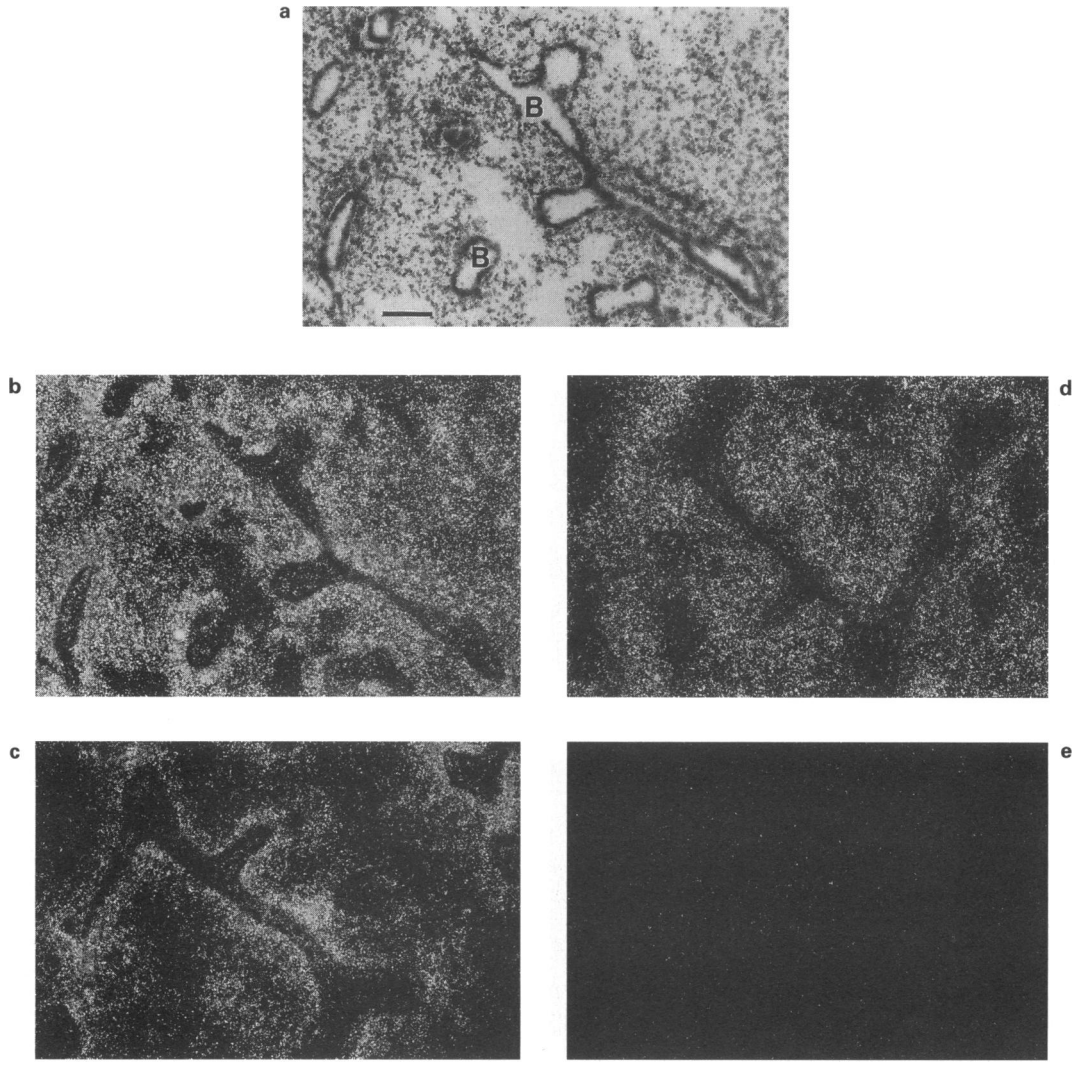

Figure 2 Autoradiographic detection of binding sites for [^{125}I]-endothelin-1 ([^{125}I]-ET-1, 0.30 nM, 180 min) in foetal porcine peripheral lung. (a) Bright-field photomicrograph of a 10 μm transverse frozen section of foetal porcine peripheral lung (47 days gestation). B = bronchus. (b–e) Dark-field photomicrographs showing the distribution of autoradiographic grains derived from [^{125}I]-ET-1. (b) Total [^{125}I]-ET-1 binding in the section shown in the light-field photomicrograph. (c–e) Serial sections showing [^{125}I]-ET-1 binding in the presence of (c) the ET_A receptor-selective ligand BQ-123 (1 μM), (d) the ET_B receptor-selective ligand sarafotoxin S6c (100 nM) and (e) in the combined presence of 1 μM BQ-123 and 100 nM sarafotoxin S6c (i.e. nonspecific binding). Bar = 100 μm.

associated with immature bronchi and with parenchymal cells of developing alveoli. Furthermore, sarafotoxin S6c (100 nM) and BQ-123 (1 μM) caused similar, significant reductions in total specific binding, indicating the presence of similar numbers of ET_A and ET_B binding sites at this very early stage of lung development. Figure 3 shows the distribution of specific ET_A and ET_B binding sites in porcine peripheral lung from a 20 week old animal. High densities of specific [^{125}I]-ET-1 binding were associated with porcine bronchial airways and with alveolar wall tissue. It is clear that both ET_A and ET_B binding sites co-existed in the peripheral lung, with sarafotoxin S6c-sensitive ET_B sites predominating in the alveolar wall. A mixture of BQ-123-sensitive ET_A sites and ET_B binding sites was also evident in peripheral bronchioles. Quantitation of specific binding grain densities over alveolar wall tissue in the absence and presence of BQ-123 or sarafotoxin S6c revealed that ET_A and ET_B binding sites co-existed in the proportion $23 \pm 3\%$ to $65 \pm 1\%$ respectively ($n = 5$).

Guinea-pig lung Specific [^{125}I]-ET-1 binding was also detected in guinea-pig peripheral lung airways and alveoli (Figure 4). Once again, ET_B binding sites predominated in alveolar wall tissue. The proportions of ET_A to ET_B binding sites were $12 \pm 1\%$ and $80 \pm 1\%$ respectively ($n = 5$).

Rat lung As in pig and guinea-pig peripheral lung, specific binding of [^{125}I]-ET-1 was associated with alveoli and with airways containing airway smooth muscle. Figure 5 suggests that ET_A and ET_B binding sites co-existed in these areas in approximately equal proportions. Interestingly, BQ-123 abolished specific binding to epithelial and adjacent submucosal tissue, indicating the predominance of ET_A sites in these areas. Quantitation of data in the alvelolar wall demonstrated that ET_A and ET_B binding sites were present in the proportions $52 \pm 3\%$ to $43 \pm 5\%$ respectively ($n = 5$).

Discussion

This study has clearly demonstrated that specific ET_A and ET_B binding sites existed in the lung parenchymal tissue of the pig, guinea-pig and rat. In pig and guinea-pig lung, the ET_B population clearly predominated, whereas in rat lung these populations were of approximately similar sizes. Specific binding

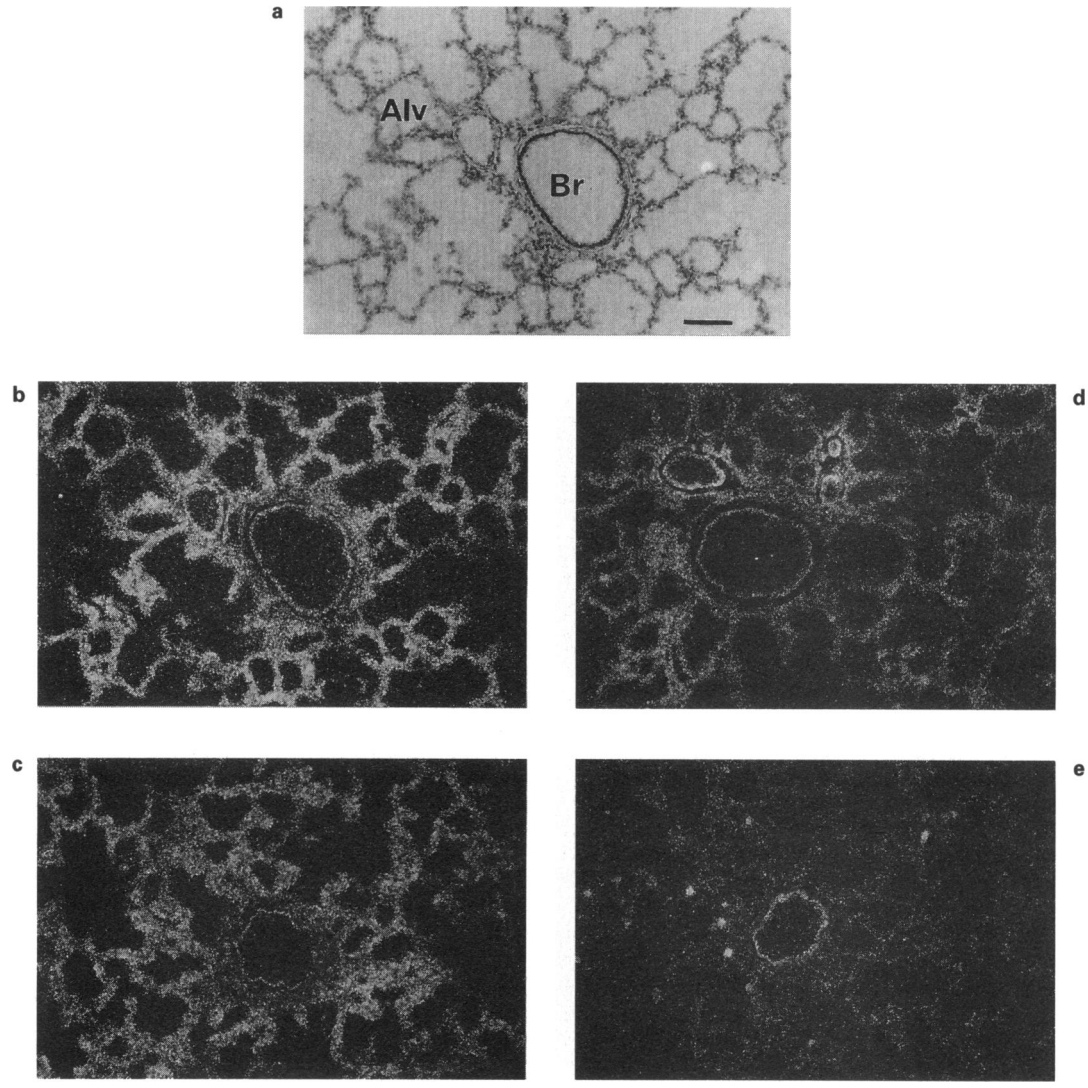

Figure 3 Autoradiographic detection of binding sites for [^{125}I]-endothelin-1 ([^{125}I]-ET-1, 0.30 nM, 180 min) in mature porcine peripheral lung. (a) Bright-field photomicrograph of a 10 μm transverse frozen section of mature porcine peripheral lung (20 weeks post birth). Br = bronchiole, Alv = alveolus. (b–e) Dark-field photomicrographs showing the distribution of autoradiographic grains derived from [^{125}I]-ET-1. (b) Total [^{125}I]-ET-1 binding in the section shown in the light-field photomicrograph. (c–e) Serial sections showing [^{125}I]-ET-1 binding in the presence of (c) the ET_A receptor-selective ligand, BQ-123 (1 μM), (d) the ET_B receptor-selective ligand, sarafotoxin S6c (100 nM) and (e) in the combined presence of 1 μM BQ 123 and 100 nM sarafotoxin S6c (i.e. nonspecific binding). Bar = 100 μm.

sites for [^{125}I]-ET-1 have been detected previously in peripheral lung alveoli and bronchi from sheep (Goldie *et al.*, 1994a), rat and guinea-pig lung (Power *et al.*, 1989). However, no previous studies have been conducted which estimate the relative densities of alveolar ET_A and ET_B subtypes. The present research addressed this issue and provided comparative data for porcine, guinea-pig and rat lung.

Most previous investigations of ET-1-induced responses and of binding site distribution and density have focussed on airway smooth muscle from the trachea and bronchus (Hay *et al.*, 1993; Goldie *et al.*, 1994a; Henry, 1994). These have suggested or shown that the proportions of ET_A and ET_B receptors in these tissues differ between species. Evidence has also been provided that demonstrates a change in the proportion of ET_A and ET_B receptors throughout the respiratory tract within a species. For example, although airway smooth muscle contraction to ET-1 was mediated via both ET_A and ET_B receptors in guinea-pig bronchus and trachea, the efficacy of the ET_B receptor-selective agonist sarafotoxin S6c was significantly greater in the main bronchus than in the upper trachea (Hay *et al.*, 1993). This suggests that the proportions of ET_B receptors increased down the respiratory tract in this species. Evidence has also been provided that demonstrates a change in ET_A and ET_B proportions in moving from human bronchial smooth muscle (87% ET_B; Goldie *et al.*, 1994a, b) to alveolar wall (68% ET_B sites; Knott *et al.*, 1995).

The present studies in pig lung have also provided some preliminary evidence that there are differences in the proportions of ET-1 binding site subtypes at different levels in the respiratory tract. Thus, in peripheral porcine bronchial smooth muscle, ET_A sites were apparently in the majority as previously reported (Nakamichi *et al.*, 1992; Hislop *et al.*, 1995), whereas in the lung parenchyma, the present study shows that ET_B receptors were the major subtype (ET_B 65%; ET_A 23%). This is also consistent with non-quantitative data from the study by Hislop and co-workers (1995). Interestingly, this proportion is similar to the values of 68% and 32% respectively observed in human lung alveolar wall (Knott *et al.*, 1995), suggesting that porcine peripheral lung tissue might serve as an appropriate model of human lung for the study of alveolar wall ET receptors.

ET_A and ET_B sites were also detected in peripheral airway smooth muscle and in alveolar wall tissue from rat and guinea-pig lung, since both BQ-123 and sarafotoxin S6c caused sig-

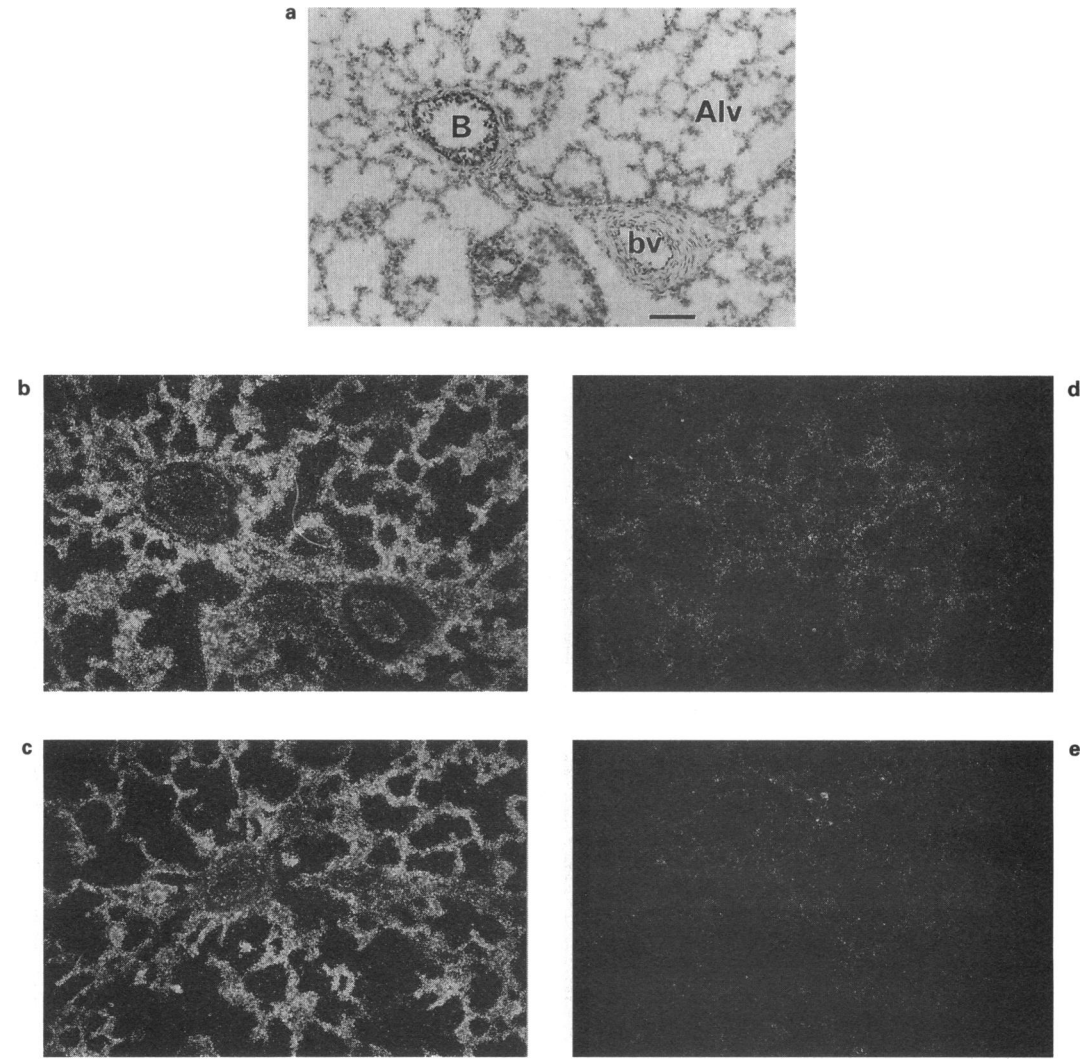

Figure 4 Autoradiographic detection of binding sites for [^{125}I]-endothelin-1 ([^{125}I]-ET-1, 0.30 nM, 180 min) in guinea-pig peripheral lung. (a) Bright-field photomicrograph of a 10 μm transverse frozen section of guinea-pig peripheral lung. B = bronchus, Alv = alveolus, bv-blood vessel. (b–e) Dark-field photomicrographs showing the distribution of autoradiographic grains derived from [^{125}I]-ET-1. (b) Total [^{125}I]-ET-1 binding in the section shown in the light-field photomicrograph. (c–e) Serial sections showing [^{125}I]-ET-1 binding in the presence of (c) the ET_A receptor-selective ligand, BQ-123 (1 μM), (d) the ET_B receptor-selective ligand, sarafotoxin S6c (100 nM) and (e) in the combined presence of 1 μM BQ-123 and 100 nM sarafotoxin S6c (i.e. nonspecific binding). Bar = 100 μm.

nificant but incomplete reductions in specific [^{125}I]-ET-1 binding in these locations. The ratios of alveolar ET_A and ET_B sites were markedly different in these species, with ET_B sites greatly outnumbering ET_A sites in the guinea-pig, whereas these subtypes were detected in approximately equal proportions in the rat.

It was also of interest to determine whether the lung expressed high densities of specific ET binding sites at an early stage of development, since the process of lung maturation has previously been shown to involve changes in the densities of some receptor types. For example, β-adrenoceptors are very sparsely distributed in foetal rabbit alveoli, but are found increasingly with lung maturation (Barnes *et al.*, 1984). The gestation period in the pig is approximately, 114 days (Ullrey *et al.*, 1965). Both ET_A and ET_B binding sites were detected in the peripheral lung of the foetal pig, at least as early as 36 days gestation, at which stage the foetus weighs approximately 10 g and is nearing the end of the first trimester of gestation. The fact that high densities of specific [^{125}I]-ET-1 binding sites were seen throughout the porcine lung at this early stage of growth, raises the possibility of a functional role(s) for ET-1 in the development of the lung. Certainly, ET-1 has been shown to promote airway smooth muscle proliferation (Noveral *et al.*, 1992; Tomlinson *et al.*, 1994). Thus, ET-1 may act as a growth factor in the foetal respiratory tract. A role for endothelin-1 in foetal development has also been suggested by results in newborn mice derived from animals which had been genetically engineered not to express ET_A receptors. In such knockout mice, significant abnormalities occurred in craniofacial development resulting in the production of non-viable offspring (Kurihara *et al.*, 1994). A role for ET-1 in lung development is also consistent with the presence of mRNA for this peptide in human foetal lung (Giaid *et al.*, 1991).

No attempt was made to determine the precise cellular locations of the alveolar ET-1 binding sites detected in the present study. However, specific [^{125}I]-ET-1 binding has been detected in rat alveolar capillary endothelial cells, fibroblasts (Furuya *et al.*, 1991; 1992) and epithelial cells (Markewitz *et al.*, 1995). ET-1 causes contraction of peripheral lung strip preparations (Goldie *et al.*, 1994a). It seems likely that contractile responses of alveolar interstitial myofibroblasts (Kapanci *et al.*, 1974) contributed to the increase in lung strip tone in response to ET-1 and thus also contained receptors for ET-1, since this tissue component in lung strips has been previously reported to be active in response to spasmogens (Bertram *et al.*, 1983). Recent studies in the rat and rabbit have

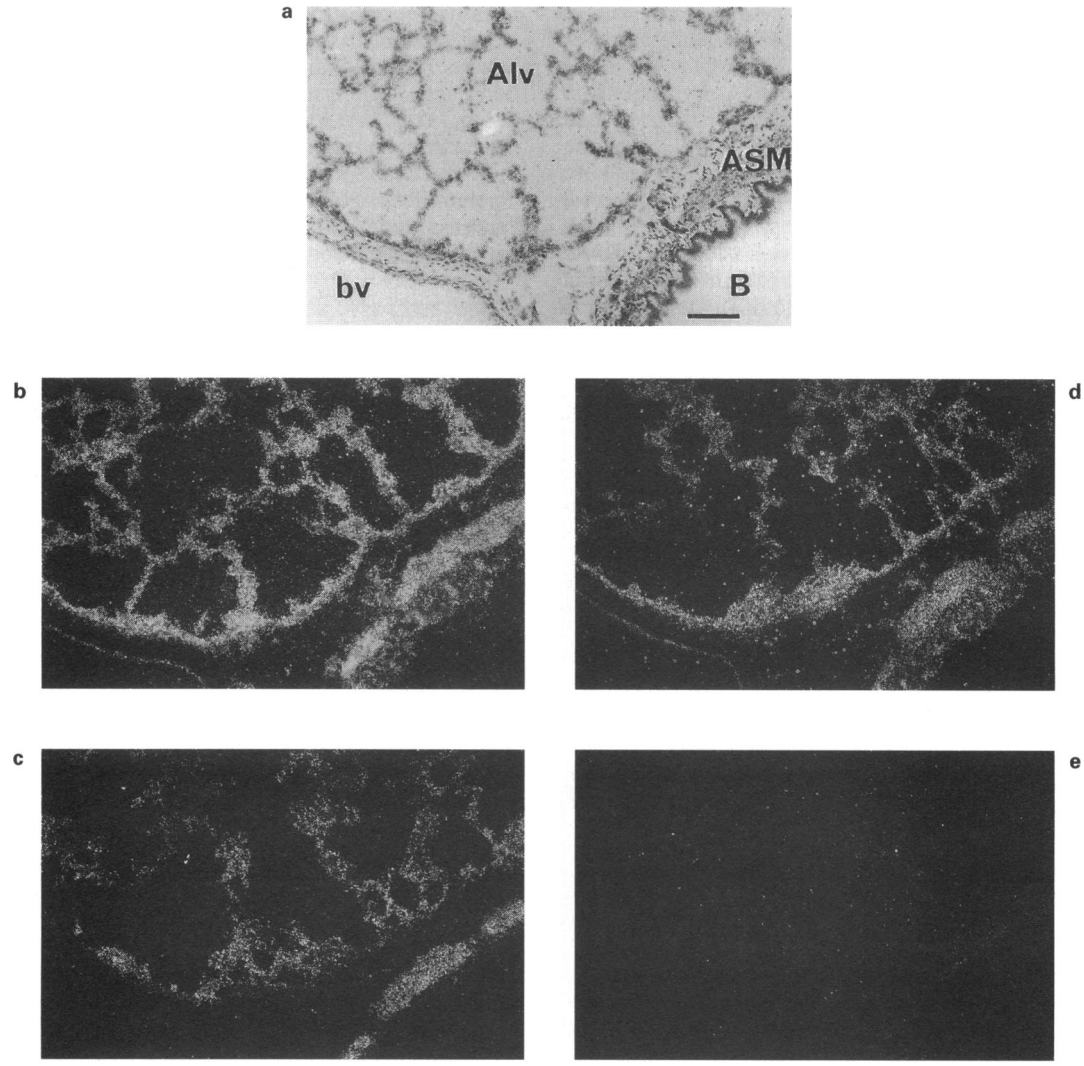

Figure 5 Autoradiographic detection of binding sites for [^{125}I]-endothelin-1 ([^{125}I]-ET-1, 0.30 nM, 180 min) in rat peripheral lung. (a) Bright-field photomicrograph of a 10 μm transverse frozen section of rat peripheral lung. B = bronchus, Alv = alveolus, ASM = airway smooth muscle. (b–e) Dark-field photomicrographs showing the distribution of autoradiographic grains derived from [^{125}I]-ET-1. (b) Total [^{125}I]-ET-1 binding in the section shown in the light-field photomicrograph. (c–e) Serial sections showing [^{125}I]-ET-1 binding in the presence of (c) the ET_A receptor-selective ligand, BQ-123 (1 μM), (d) the ET_B receptor-selective ligand sarafotoxin S6c (100 nM) and (e) in the combined presence of 1 μM BQ-123 and 100 nM sarafotoxin S6c (i.e. nonspecific binding). Bar = 100 μm.

shown that ET_B receptors were associated with alveolar type II pneumocytes, suggesting that ET-1 may play a role in either the synthesis or secretion of surfactant from these cells (Durham *et al.*, 1993), while in a rat cultured alveolar epithelial cell line, ET_A receptors were detected which mediated ET-1-induced increases in intracellular prostaglandin E_2 and adenosine 3′:5′-cyclic monophosphate (cyclic AMP) production (Markewitz *et al.*, 1995). Studies in rat lung have also shown that ET_B sites were involved in the clearance of ET-1 from the circulation (Fukuroda *et al.*, 1994). ET_A and ET_B receptors may have similar functions in human, pig, guinea-pig and rat lung parenchyma.

It is interesting to note that while the sum of the numbers of ET_A and ET_B sites in rat lung alveolar wall tissue accounted for about 100% of the specific [^{125}I]-ET-1 binding, this was apparently not the case in similar tissue from the pig and guinea-pig, where on average, this sum fell about 10% short of the value for total specific [^{125}I]-ET-1 binding. The possibility of a third receptor subtype can be excluded, since no significant difference in the levels of non-specific binding were detected when either ET-1 (100 nM) or a combination of BQ-123 and sarafotoxin S6c were used to assess this component. A similar discrepancy was reported in rat tracheal smooth muscle (Henry, 1993) and is presumably the result of normal variability in estimating the values for ET_A and ET_B binding levels.

In conclusion, the purpose of this study was to determine if porcine peripheral lung suitably represented the peripheral human lung in regard to ET-1 binding site distribution and the proportions of ET_A and ET_B subtypes. This study has shown that, the proportion of ET_A and ET_B subtypes in lung parenchyma of the pig closely reflect those of the human lung. Thus, pig peripheral lung alveoli might serve as a suitable model of human alveolar wall for studies, examining the functional effects of ET-1.

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