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Localisation and expression of β-adrenoceptor subtype mRNAs in human lung

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Abstract

The cellular localisation and distribution of mRNAs encoding β-adrenoceptor subtypes in human lung were studied by in situ hybridisation and Northern blot analysis. The 851-bp SmaI/PvuII fragment of human β_1-adrenoceptor cDNA, the 439-bp SmaI fragment of human β_2-adrenoceptor cDNA and the 975-bp SmaI fragment of human β_3-adrenoceptor cDNA bound to single mRNA species of approximately 3.2 kb, 2.2 kb and 2.3 kb in size, respectively. Human lung and heart and rabbit lung expressed both β_1- and β_2-adrenoceptor mRNAs with no detectable level of β_3-adrenoceptor mRNA, while rabbit perirenal adipose tissue expressed β_1-, β_2- and β_3-adrenoceptor mRNAs. Cultured human airway epithelial cells and airway smooth muscle cells expressed only β_2-adrenoceptor mRNA. In situ hybridisation in human lung, using ^{35}S-labelled antisense RNA probes revealed a high level of expression of β_1- and β_2-adrenoceptor mRNAs in the pulmonary blood vessels, high level of expression of β_2-adrenoceptor mRNA in the alveolar walls with less expression of β_1-adrenoceptor mRNA. There was a moderate expression of β_2-adrenoceptor but not β_1-adrenoceptor mRNA in airway epithelium and smooth muscle of peripheral airways and no detectable β_3-adrenoceptor mRNA in any lung structures.

Keywords: β-Adrenoceptor; mRNA; Hybridisation, in situ; Northern blot analysis

1. Introduction

β-Adrenoceptors belong to the superfamily of G-protein-coupled receptors. Three subtypes of β-adrenoceptor (β_1–β_3) are known to exist on the basis of their pharmacological properties, physiological effects and molecular cloning. cDNA and genomic clones for different receptor subtypes from several species have been cloned and their sequences deduced (Dixon et al., 1986; Chung et al., 1987; Frielle et al., 1987; Emorine et al., 1989). The cellular distribution of β-adrenoceptor subtype mRNAs in human lung is still unknown. Using selective β_1- and β_2-adrenoceptor antagonists, receptor binding studies have demonstrated a high density of β-adrenoceptor in lung, with β_2-adrenoceptor making up 70% and β_1-adrenoceptor the remaining 30% in human lung (Engel, 1981; Carstairs et al., 1985), whereas in rabbit lung, β_1-adrenoceptor make up 70% and β_2-adrenoceptor 30% (Dickinson and Nahorski, 1983). Autoradiographic mapping has localised β_2-adrenoceptors to airway epithelium and airway smooth muscle, and both β_1- and β_2-adrenoceptors to submucosal glands, pulmonary blood vessels and alveolar walls (Carstairs et al., 1985). Due to the lack of selective β_3-adrenoceptor antagonists, no accurate characterisation of this subtype has been available. However, it has been shown that β_3-adrenoceptors are present functionally in canine bronchi in vitro (Tamoki et al., 1993), but there is no functional evidence for these receptors in human airway smooth muscle (Martin et al., 1994). Using reverse transcription-polymerase chain reaction (RT-PCR), the expression of β_1- and β_2-adrenoceptor mRNAs but not β_3-adrenoceptor mRNA was detected in human lung (Krief et al., 1993; Thomas and Liggett, 1993). Our previous studies have shown that there is a correspondence between the cellular localisation of β_2-adrenoceptor mRNA and β_2-adrenoceptor in human lung (Hamid et al., 1991).

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In the present study, we used Northern blot analysis to demonstrate the presence of β-adrenoceptor subtype mRNAs in human lung and heart, in comparison to rabbit lung and perirenal adipose tissue, as well as in primary cultured human airway epithelial and smooth muscle cells. In situ hybridisation was also used to determine the cellular localisation of mRNAs for the β-adrenoceptor subtypes in human lung.

2. Materials and methods

2.1. Tissue preparations

Fresh human lungs were obtained from normal organ donors who had died in accidents ($n = 6$, 2 male, age 17–43 years). Macroscopically normal areas of peripheral lung tissues containing mainly bronchioles and small pulmonary blood vessels were rapidly frozen in liquid nitrogen and stored at −70°C for RNA extraction. In addition, small pieces of peripheral lung were inflated with tissue-embedding medium (Tissue-Tek OCT compound) diluted 1 : 4 with phosphate-buffered saline (PBS) before freezing. Serial sections (10 μm thick) were cut on a Bright cryostat at −30°C, thawed-mounted onto gelatin-coated glass slides and stored at −70°C until use. Human ventricle was obtained from a single patient undergoing heart transplantation as a control.

Peripheral lung and perirenal adipose tissue were also obtained from New Zealand White rabbits (2–3 kg weight) after lethal administration of an intravenous dose of pentobarbitone (50–60 mg/kg). After removal, the tissues were quickly frozen in liquid nitrogen for RNA extraction.

2.2. Tissue culture

Primary cultures of human airway epithelial cells were established as previously described (Kwon et al., 1994). Briefly, the cartilaginous parts of tracheal or main bronchial segments obtained from either heart or heart/lung transplantation donors were excised, trimmed, washed and incubated with 0.1% protease for 16–20 h at 4°C. The dissociated cells were plated at a density of $1–3 \times 10^4$ cells/cm^2 onto 6-well plastic tissue culture plates coated with collagen gel (Vitrogen100). The cells were grown to confluence (8–10 days) in serum-free, hormone supplemented Ham's F12 medium containing penicillin (100 U/ml), streptomycin (100 μg/ml), amphotericin B (2 μg/ml), L-glutamine (2 mM), insulin (5 μg/ml), transferrin (5 μg/ml), epidermal growth factor (25 ng/ml), endothelial cell growth supplement (15 μg/ml), hydrocortisone (1 μM), cholera toxin (10 ng/ml) and retinoic acid (0.1 μM), and then maintained in fresh Ham's F12 medium containing only L-glutamine and an antibiotic for at least 24 h before RNA extraction.

Human airway smooth muscle cells in culture were established as previously described (Hirst et al., 1992). The human bronchial smooth muscle was obtained during lung resections from patients of either sex undergoing surgery for carcinoma of the bronchus. After removal of the epithelium, portions of smooth muscle not invaded by the carcinoma were dissected free of adherent connective and parenchymal tissue and then dispersed by collagenase and elastase, prior to seeding onto 35-mm dishes at a density of $2–5 \times 10^5$ viable cells/ml in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum supplemented with sodium pyruvate (1 mM), L-glutamine (2 mM), non-essential amino acids (1 ×), gentamicin (50 μg/ml), and amphotericin B (1.5 μg/ml). At confluence, the cells were then subcultured into 75-cm^2 flasks. The cells were grown to confluence before RNA extraction at passages 7–9.

2.3. Molecular probes

A 851-bp SmaI/PvuII fragment of human β_1-adrenoceptor cDNA, a 439-bp SmaI fragment of human β_2-adrenoceptor cDNA and a 975-bp SmaI fragment of human β_3-adrenoceptor cDNA corresponding to the coding region were cut out from the full length human β_1-, β_2- and β_3-adrenoceptor cDNAs in pBluescript SK(−) vectors respectively (gifts from Dr C.D. Strader, Merck Research Laboratories, Rahway, USA). Random primer labelling was carried out with the cDNA fragments using [α-^{32}P]dCTP (3000 Ci/mmol; Amersham, Amersham, UK).

A 341-bp SmaI/PstI fragment of human β_1-adrenoceptor cDNA, a 328-bp PstI/KpnI fragment of human β_2-adrenoceptor cDNA and a 303-bp AccI/SmaI fragment of human β_3-adrenoceptor cDNA corresponding to the coding region were also cut out and subcloned into the multiple cloning site of pGEM-3Z vectors respectively. There is virtually no sequence homology between the three receptors in this region. cRNA probes were produced from the antisense and sense strands of the fragments after linearizing with either EcoRI or HindIII as templates using [^{35}S]UTP (1000 Ci/mmol; Amersham, Amersham, UK) in the presence of SP6 or T7 RNA polymerase.

2.4. RNA isolation and Northern blot hybridisation

Total RNA was isolated by the single-step acid guanidinium thiocyanate-phenol-chloroform extraction method previously described (Chomczynski and Sacchi, 1987). Poly(A)$^+$RNA was then extracted from 5 mg human or rabbit lung total RNA using PolyATtract mRNA Isolation System I (Promega, Southampton, UK). For Northern blot analyses, equal amounts of total RNAs (20 μg/lane) or poly(A)$^+$RNA were subjected to electrophoresis on 1.2% agarose/formaldehyde gels containing 20 mM morpholinosulphonic acid (MOPS), 5 mM sodium acetate and 1 mM EDTA (pH 7.0), and blotted onto Hybond-N membranes (Amersham). Hybond-N membranes containing

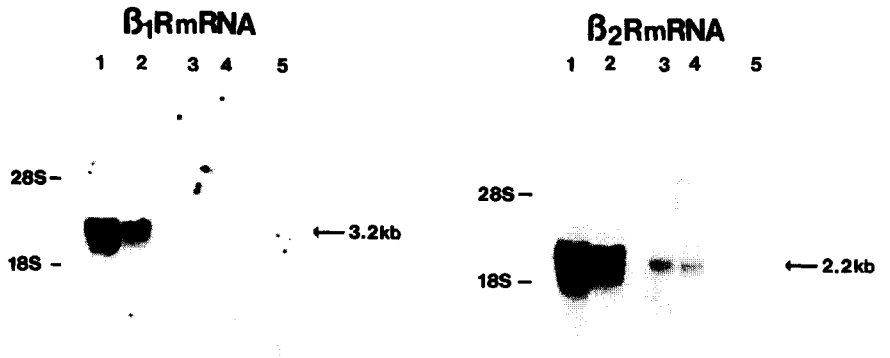

Fig. 1. Northern blot analysis of poly(A)$^+$RNA isolated from 5 mg human lung total RNA (lanes 1 and 2), and total RNA (20 μg/lane) from human lung (lanes 3 and 4), and human ventricle (lane 5) hybridised to human β_1- or β_2-adrenoceptor cDNA probes. The size of mRNA was estimated from 18S and 28S rRNA markers.

RNAs were fixed using UV Stratalinker-1800 (Stratagene, Cambridge, UK). Filters were then prehybridised at 42°C for at least 4 h in a solution containing 50% formamide, 50 mM Tris-HCl (pH 7.5), 5 × Denhardt's solution, 5 × standard saline citrate (SSC), 0.1% sodium dodecyl sulphate (SDS), 5 mM EDTA and 250 μg/ml sonicated denatured salmon sperm DNA. One of the ^{32}P-labelled human β_1-, β_2- or β_3-adrenoceptor cDNA probes was then added to the prehybridisation solution at a concentration of 10^6 cpm/ml. After hybridisation overnight at 42°C, the filters were washed once with 2 × SSC/0.01% SDS at room temperature, once for 30 min with 2 × SSC/0.01% SDS at 42°C, once for 30 min with 0.5 × SSC/0.01% SDS at 50°C, and finally for 30 min with 0.1 × SSC/0.01% SDS at 55°C. The filters were then subjected to autoradiography using Kodak X-OMAT S film with an intensifying screen at −70°C for up to 1 week. After autoradiography, the filters were stripped for subsequent reprobing with other β-adrenoceptor subtype cDNA probes.

2.5. In situ hybridisation

On the day of experimentation, sections were fixed with 4% paraformaldehyde/PBS for 1 h at 4°C, rinsed with PBS, permeabilized with 0.3% Triton X-100/PBS for 15 min and then treated with proteinase K (10 μg/ml) for 7.5 min at room temperature. The tissue was then acetylated by reaction with an 0.1 M triethanolamine/0.25% acetic anhydride, pH 8.0, mixture for 10 min at room temperature. After acetylation, the tissue was dehydrated through increasing concentrations (70, 95 and 100%) of ethanol. The sections were allowed to air-dry for 2 h before hybridisation. In certain experiments, sections were incubated with 100 μg/ml ribonuclease A (RNase A) for 1 h at 37°C, followed by three rinses with 4 × SSC. Hybridisation solution contained 50% formamide, 300 mM NaCl, 20 mM Tris-HCl (pH 7.5), 5 mM EDTA, 10 mM NaH_2PO_4 (pH 8.0), 10% dextran sulphate, 1 × Denhardt's solution, and 500 μg/ml yeast tRNA and the appropriate ^{35}S-labelled antisense and sense β_1-, β_2- or β_3-adrenoceptor riboprobes (50000 cpm/μl). The hybridisation solution (10–20 μl) was applied directly to each section. Hybridisation was allowed to proceed in a humidified chamber for 18–20 h at 50°C. After this time the slides were washed once with 4 × SSC/10 mM dithiothreitol for 5 min at room temperature and twice with 4 × SSC/10 mM dithiothreitol for 30 min at 37°C. Unhybridised single-stranded cRNA probe was then removed by incubating with a

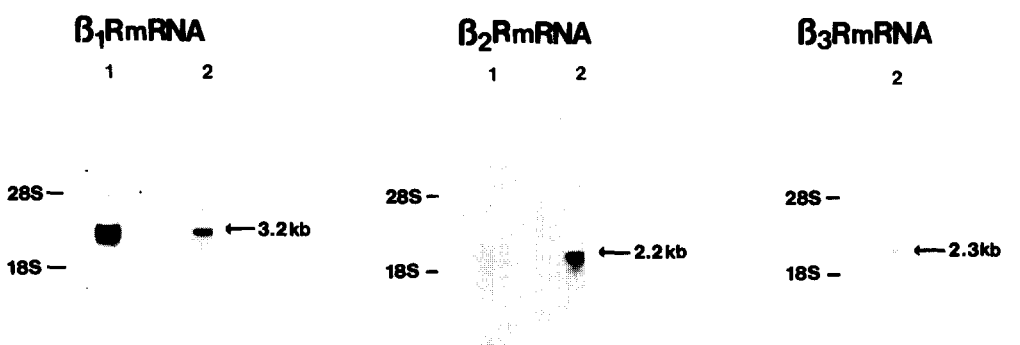

Fig. 2. Northern blot analysis of poly(A)$^+$RNA isolated from 5 mg rabbit lung total RNA (lane 1), and total RNA (20 μg/lane) from rabbit perirenal adipose tissue (lane 2) hybridised to human β_1-, β_2- or β_3-adrenoceptor cDNA probes. The size of mRNA was estimated from 18S and 28S rRNA markers.

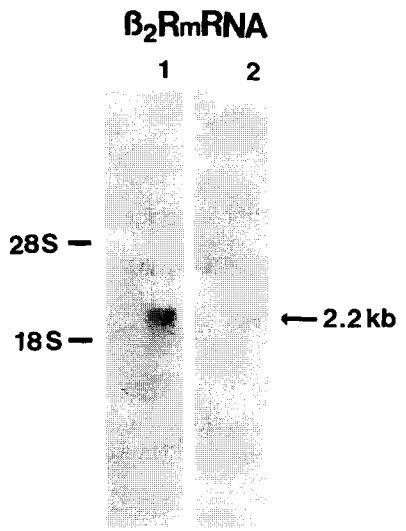

Fig. 3. Northern blot analysis of total RNA (20 μg/lane) from cultured human airway epithelial cells (lane 1), and cultured human airway smooth muscle cells (lane 2) hybridised to human β_2-adrenoceptor cDNA probe. The size of mRNA was estimated from 18S and 28S rRNA markers.

solution containing 50 μg/ml RNase A and 2 × SSC for 30 min at 37°C. The slides were then washed once with 2 × SSC for 30 min at 42°C, once with 0.5 × SSC for 30 min at 50°C, and once with 0.1 × SSC for 30 min at 55°C, and then dehydrated through increasing concentrations (70, 95 and 100%) of ethanol containing 0.3 M ammonium acetate, air-dried, and dipped in Ilford K-5 emulsion diluted 1 : 1 with water and exposed at 4°C for 10–14 days in the presence of dessicant. The slides were developed in Kodak D-19 developer, fixed with Unifix (Kodak), lightly counterstained with haematoxylin, dehydrated, cleared, and mounted with DPX mountant. The slides were visualised using a Zeiss microscope under brightfield and darkfield illumination. Semi-quantification was performed by counting silver grains using an eyepiece graticule. For each patient, at least two sections were hybridised from which two to four fields were counted depending on the pattern of alignment of the grid covering the cellular structures of interest. Results were graded from − (no grains) to + + + (highest grains).

3. Results

3.1. Northern blot analysis

Northern blot analysis of total RNA and poly(A)$^+$RNA from human lung parenchyma, and total RNA from human ventricle, using cDNA probes for human β_1-, β_2- and

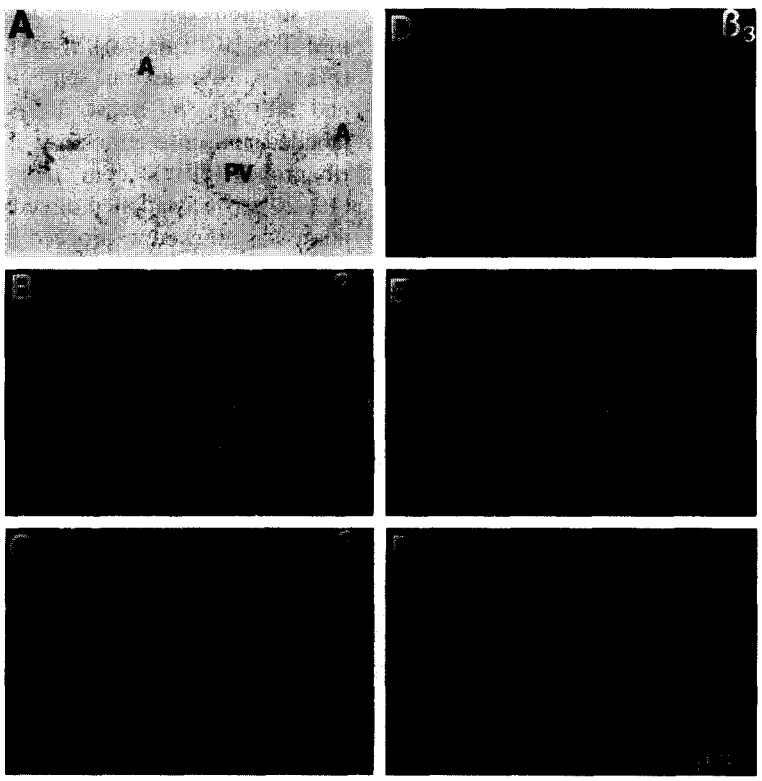

Fig. 4. Distribution of β-adrenoceptor subtype mRNAs in human lung parenchyma. Panel A is brightfield photomicrograph counterstained with haematoxylin. Panels B–F are darkfield photomicrographs of adjacent sections showing the distribution of autoradiographic grains after in situ hybridisation with ^{35}S-labelled antisense β_1-, β_2- or β_3-adrenoceptor riboprobes (B, C and D) or sense β_1- or β_2-adrenoceptor riboprobes (E and F). Labelling of β_1- and β_2-adrenoceptor mRNAs was present over pulmonary blood vessel (PV), and alveolar walls (A). There was no labelling of β_3-adrenoceptor mRNA over any lung structures. Scale bar = 100 μm.

Table 1
Comparison of the cellular expression of β-adrenoceptor subtype mRNAs in human lung tissues

	β_1-Adrenoceptor	β_2-Adrenoceptor	β_3-Adrenoceptor
Alveolar walls	+	+ +	−
Airway epithelium	−	+ +	−
Airway smooth muscle	−	+	−
Submucosal glands	+	+	−
Pulmonary blood vessels	+ + +	+ +	−

− = no labelling; + = sparse labelling; + + = moderate labelling; + + + = dense labelling.

β_3-adrenoceptor, confirmed the expression of β_1- and β_2-adrenoceptor mRNAs in human lung and ventricle, with single transcripts of approximately 3.2 kb and 2.2 kb, respectively (Fig. 1). Similarly, the expression of β_1- and β_2-adrenoceptor mRNAs in rabbit lung was detected as single transcripts of the same size, using human β_1- and β_2-adrenoceptor cDNA probes (Fig. 2). In contrast, rabbit perirenal adipose tissue expressed all of the β-adrenoceptor subtype (β_1–β_3) mRNAs with single transcripts of approximately 3.2 kb, 2.2 kb and 2.3 kb, respectively (Fig. 2). Cultured human airway epithelial cells and airway smooth muscle cells expressed only β_2-adrenoceptor mRNA with single transcript of 2.2 kb (Fig. 3).

3.2. *In situ hybridisation*

A hybridisation signal was revealed by discrete silver grains overlying the tissue sections to antisense cRNA but not to corresponding sense cRNA probes. A high grain density of both β_1- and β_2-adrenoceptor mRNAs was detected over the medial layer of the pulmonary blood vessels, however, the β_1-adrenoceptor mRNA signal intensified towards the intimal surface (Fig. 4B,C). The alveolar walls were also labelled with high grain density with the β_2-adrenoceptor probe, and to a lesser extent with β_1-adrenoceptor probe (Fig. 4B,C). Moderate grain density of β_2-adrenoceptor mRNA was also detected in peripheral airways (not shown). However, there was no obvious, specific hybridisation signal for β_3-adrenoceptor mRNA in any lung structures (Fig. 4D). Specificity of the hybridisation signal is supported by the fact that hybridisation with a sense probe (Fig. 4E,F), or prior treatment with RNase A (not shown) produced no hybridisation signal. The cellular localisation of in situ hybridisation signal in human lung tissues ($n = 4$) is summarised in Table 1.

4. Discussion

Molecular cloning studies have confirmed pharmacological studies in demonstrating the existence of three β-adrenoceptor subtypes. Previous receptor binding and autoradiographic mapping studies have demonstrated the coexistence of β_1- and β_2-adrenoceptors in human lung (Engel, 1981; Carstairs et al., 1985). In the present study, we demonstrated the coexistence of β_1- and β_2-adrenoceptor mRNAs by Northern blot analysis in human lung and ventricle as single transcripts of approximately 3.2 kb and 2.2 kb, respectively. There was no β_3-adrenoceptor mRNA to be detected in human lung and ventricle, in agreement with the recent findings by sensitive and specific RNase protection assays (Berkowitz et al., 1995). Using the more sensitive technique of RT-PCR, β_1- and β_2-adrenoceptor mRNAs but not β_3-adrenoceptor mRNA are expressed in human lung (Krief et al., 1993; Thomas and Liggett, 1993).

Because of the difficulty in obtaining human adipose tissue, a direct comparison on human tissues was not possible. To overcome this problem, we studied β-adrenoceptor subtype mRNA expression in rabbit tissues, including lung and perirenal adipose tissue. We found that rabbit perirenal adipose tissue expressed β_1- and β_2-adrenoceptor mRNAs as well as β_3-adrenoceptor mRNA, consistent with the coexistence of three β-adrenoceptor subtypes in white fat cells of various mammalian species including rabbit (Langin et al., 1991). In addition, Muzzin et al. (1991) have also reported the presence of β_1-, β_2- and β_3-adrenoceptor mRNAs in rat white adipose tissue by Northern blot analysis. There was a correspondence between the expression of β-adrenoceptor subtype mRNAs and their receptor proteins by Northern blot analysis and radioligand binding studies. Human lung, which contains a high proportion of β_2-adrenoceptor, expressed a higher basal level of β_2-adrenoceptor mRNA. By contrast, rabbit lung and human ventricle which have a high proportion of β_1-adrenoceptor, expressed a high level of basal β_1-adrenoceptor mRNA. Both β_1-adrenoceptor and β_2-adrenoceptor mRNAs but not β_3-adrenoceptor mRNA were expressed in rabbit lung.

Using in situ hybridisation, we have shown that a relatively high expression of β_1- and β_2-adrenoceptor mRNAs can be detected in the pulmonary blood vessels over the medial layer as well as alveolar walls, consistent with receptor autoradiography (Carstairs et al., 1985), and known functional effects such as relaxation of pulmonary vascular smooth muscle (Goldie et al., 1982), and surfactant release from type II pneumocytes (Dobbs and Mason, 1979). Both β_1- and β_2-adrenoceptor mRNAs were detected in submucosal glands, in agreement with previous autoradiographic studies (Carstairs et al., 1985). Given the specificity of in situ hybridisation, we conclude that β_3-adrenoceptor mRNA is not expressed to any significant degree in any lung structures, in agreement with our Northern blot analysis and previous studies (Krief et al., 1993; Thomas and Liggett, 1993). β_3-Adrenoceptors have been demonstrated functionally in canine bronchi in vitro (Tamoki et al., 1993), but there is no functional evidence for these receptors in human airway smooth muscle (Martin et al., 1994). The expression of only β_2-adrenoceptor

mRNA in cultured human airway smooth muscle cells by Northern blot analysis and in airway smooth muscle by in situ hybridisation is consistent with the functional studies that relaxation of both central and peripheral human airways is mediated solely via β_2-adrenoceptors (Goldie et al., 1984; Nials et al., 1993). Similarly only β_2-adrenoceptor mRNA was detected in cultured human airway epithelial cells and highly expressed in airway epithelium. This is consistent with previous autoradiographic mapping studies demonstrating only β_2-adrenoceptors in epithelial cells (Carstairs et al., 1985) and with functional studies showing effects of β_2-adrenoceptor agonists (Knowles et al., 1984; Devalia et al., 1992).

Although detection of β_3-adrenoceptor mRNA is important, the extent to which this message is expressed as protein is also crucial. There is no β_3-selective ligand available for estimation of the receptor number and autoradiographic mapping at present. The radiolabelled antagonists available display a markedly lower affinity for β_3-adrenoceptor, and the high concentrations of radioligand that are required to detect β_3-adrenoceptor binding result in high nonspecific binding and therefore prevent accurate characterisation of the binding parameters (Langin et al., 1991). Therefore no detection of β_3-adrenoceptor mRNA in any cellular structures of the human lung reflects the striking interspecies differences in the distribution of β_3-adrenoceptor. It is also possible that β_3-adrenoceptor may be expressed in lung under certain circumstances, such as in disease state. The role of β_3-adrenoceptor cannot be elucidated without the development of specific and selective antagonists. In summary, we report the cellular distribution of β-adrenoceptor subtype mRNAs in human lung by in situ hybridisation, which closely mimicked that of binding sites identified by receptor autoradiographic mapping (Carstairs et al., 1985).

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