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The tissue distribution of the human β_3-adrenoceptor studied using a monoclonal antibody: Direct evidence of the β_3-adrenoceptor in human adipose tissue, atrium and skeletal muscle

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OBJECTIVE: To develop a monoclonal antibody that recognises an epitope of the native β_3-adrenoceptor expressed on the extracellular surface of human cells and tissues.
DESIGN: A high affinity monoclonal antibody, Mab72c, was raised against the human β_3-adrenoceptor expressed on a transfected mammalian cell line.
RESULTS: In CHO (Chinese hamster ovary) cells transfected with β_3-adrenoceptor cDNA, antibody labelling was found to be proportional to receptor density measured by the binding of the radiolabelled β-adrenoceptor antagonist, [^{125}I]-iodocyanopindolol. The use of Mab 72c has demonstrated the expression of the β_3-adrenoceptor in a variety of human tissues, including gall bladder, prostate and colon, where a mRNA signal had been detected previously. This study also provides the first direct demonstration of the expression of β_3-adrenoceptors in human skeletal muscle, atrium and adipose tissue.
CONCLUSION: The development of this antibody represents an important addition to the armentarium of reagents that are available to study the localisation of β_3-adrenoceptors in human tissues.

Keywords: β_3-adrenoceptors; antibody; human; monoclonal antibody; adipose tissue; atrium; skeletal muscle

Introduction

β-adrenoceptors were originally classified into two subtypes, β_1 and β_2, based on the relative potencies of the agonists isoproterenol, adrenaline and noradrenaline.[1] This classification was verified by the isolation of cDNAs encoding the β_1- and β_2-adrenoceptors.[2,3] The human β_3-adrenoceptor was later cloned by Emorine *et al*.[4] The pharmacology of the cloned β_3-adrenoceptor agreed with the pharmacological data previously obtained in rodent adipose tissue, gut, and skeletal muscle in that the β-adrenoceptors in these tissues were insensitive to classical β-adrenoceptor antagonists such as propranolol.[5–8] Aryloxypropanolamine β_1/β_2 adrenoceptor antagonists, exemplified by CGP12177, evoke a lipolytic response in rat adipose tissue through agonism at β_3-adrenoceptors.[9] Similarly, selective β_3-adrenoceptor agonists, such as BRL-37344, showed similar or greater potency than isoproterenol in stimulating lipolysis, but were much less potent than isoproterenol in stimulating responses mediated by β_1- or β_2-adrenoceptors.[10]

The assessment of the pharmacological role of β_3-adrenoceptors in human tissues has proved more controversial. For example, lipolysis in human white adipocytes induced by isoproterenol is sensitive to propranolol.[11] Also, CGP12177-induced lipolytic responses have been demonstrated by some[12–14] but not others.[9,15] It is now evident from comparisons of cloned human and rat β_3-adrenoceptors that there are significant species differences in pharmacology.[16]

Attempts to detect β_3-mRNA in human tissues using reverse-transcription PCR have also given conflicting results. Krief *et al*[17] detected β_3-adrenoceptor mRNA in several tissues, including gall bladder, adipose tissue and colon, while Thomas and Liggett[18] failed to detect a β_3-adrenoceptor signal. Recently, RNAase protection assays, that do not rely on amplification techniques, were used to identify β_3-adrenoceptor mRNA in a variety of human tissues, including

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gall bladder, stomach, small intestine, adipose tissue and prostate gland.[19] In addition, β_3-adrenoceptor mRNA has been reported in human brain,[20] although this was not detected by others.[19] These conflicting results may, in part, be due to the relatively low levels of β_3-adrenoceptor gene expression in human tissue. For example, Northern blotting analyses[21,22] indicate that the β_3-adrenoceptor mRNA level in human white adipose tissue is some 50-fold lower than that in rodent white fat.

Without a specific pharmacological or molecular probe for the β_3-adrenoceptor, it has proved difficult to assess the functional significance of this adrenoceptor subtype in man. Data describing the immunohistochemical localisation of the β_3-adrenoceptor has been limited to a single study using an anti-peptide polyclonal antibody,[23] which detected the receptor only in human gall bladder, a tissue that exhibits a relatively high level of β_3-adrenoceptor mRNA.[17,19] The relative lack of sensitivity of this approach may be due to the difficulty of producing a suitable high affinity antibody using a short polypeptide immunogen.

In this paper we describe the development of a high affinity monoclonal antibody, raised against the human β_3-adrenoceptor expressed on a transfected mammalian cell line, and the immunohistochemical detection of the β_3-adrenoceptor in several human tissues.

Methods

Cell lines

The human β_3-adrenoceptor gene was obtained under licence from Dr S B Liggett (Duke University Medical Center, Durham, NC), subcloned and transfected into CHO (Chinese Hamster Ovary) dhfr$^-$ cells as previously described;[24] four subclones expressing different levels of the human β_3-adrenoceptor were studied, designated C38, C15, D57 and D43 expressing β_3-adrenoceptors at 130, 400, 1300 and 3000 fmol/mg respectively. CHO cells expressing human β_1- (7000 fmol/mg) and β_2- (2300 fmol/mg) adrenoceptors were obtained under licence from A D Strosberg (Universite Paris VII, Institut Cochin de Genetique Moleculaire, Paris) and those expressing the rat β_3-adrenoceptor (770 fmol/mg) from J C Venter (NIH, Bethesda).[25] Cells were cultured in α-MEM (Modified Eagle Medium) growth medium (Life Technologies, Paisley) containing 10% v/v dialysed foetal calf serum. Medium for the CHO β_3 D57 and D43 clones was supplemented with 100 nM methotrexate and 2 mM glutamine. Receptor density values were determined by saturation binding of [^{125}I]-iodocyanopindolol.[24]

Immunisation and hybridoma production

CBH/Cbi rats (National Institute for Medical Research, UK, Mill Hill) were immunised on four occasions with 2×10^7 CHO β_3.D43 cells, at 21–28 d intervals intraperitoneally. Three days after the final immunisation, mesenteric lymph node cells were removed and frozen. After storage in liquid nitrogen for 7 d these cells were thawed and fused with rat myeloma Y3-Ag 1.2.3 cells.[26] Hybridomas were cultured in a 1 : 1 mixture of DMEM (Dulbecco's Modified Eagle Medium) and Ham's F12 (Life Technologies, Paisley) supplemented with 10% v/v FBS (foetal bovine serum) (Hyclone Europe Ltd, Cramlington), cloned by the limiting dilution method and monoclonal antibody purified by Protein G affinity chromatography (Bioprocessing Ltd., UK). Isotype was determined using a commercial kit (Serotec Ltd., Oxford).

Hybridoma screening assay

CHO β_3.D43 or parental CHO non-transfected cells were grown to confluency and then washed with 5% FBS/DMEM. Hybridoma samples were added to plates seeded with parental CHO cells for 30 min at room temperature to pre-adsorb anti-CHO binding and then the pre-adsorbed supernatant samples were transferred to fresh CHO β_3.D43 or parental cell-coated plates and incubated for 30 min at room temperature. The wells were washed and ^{125}I-sheep anti-rat IgG Fab (prepared in-house, 2×10^5 CPM per well in 50 µl of 5% FBS/DMEM) was added and the plates incubated for a further 30 min at room temperature. The plates were washed again and the cells lysed by addition of 1% sarcosyl in 0.5 M NaOH (200 µl/well). After 10 min at room temperature the contents of each well were counted (Innotron Hydragamma 16) and the results expressed as the ratio of counts bound to the CHO β_3.D43 cell line compared with the binding to parental non-transfected CHO cells.

Receptor solubilisation, deglycosylation and Western blotting

CHO cell pellets, prepared from 1×10^8 cells, were resuspended in 2 ml ice-cold TE buffer (10 mM Tris, 1 mM EDTA.Na_2, pH 7.4) in the presence of protease inhibitors (1 µg/ml aprotinin, 200 µM AEBSF (4-(2-aminoethyl) benzene sulphonyl fluoride), 10 µM Leupeptin, 1 µM Pepstatin A). Cells were then homogenised in a Potter homogeniser tube (620 rpm$\times$10 strokes) and the cell membrane fraction sedimented by centrifugation (88,000 g$\times$20 min at 4°C). The pellet was resuspended in 1 ml TEC (TE containing 0.3% w/v sodium deoxycholate) or TEDC (TE containing 0.3% w/v sodium deoxycholate and 1% w/v digitonin) buffer and then left to stand on ice for 45 min with occasional mixing. Solubilised β-adrenoceptor was collected as the supernatant following centrifugation as before. Aliquots were stored at −40°C. For removal of N-linked complex and high mannose type carbohydrate, solubilised receptor in TEC buffer was mixed with an equal volume of a mixture of Endoglycosidase F and Peptide-N-Glyco-

sidase F (Oxford Glycosystems, Oxford, 40 deglycosylation units/ml) and incubated for 30 h at 37°C. A control reaction consisting of one volume solubilised receptor mixed with one volume buffer (enzyme omitted) was run in parallel.

Detergent-solubilised CHO cell membrane fractions prepared as described above were separated by SDS-PAGE on 10–15% polyacrylamide gradient gels using the Pharmacia Phastsystem. Following electrophoretic separation, the proteins were then transferred onto PVDF membrane using the PhastSystem semi-dry western blotting module (1 mA/cm^2 for 15 min, in 48 mM Tris, pH 9.2, 39 mM Glycine, 20% v/v methanol). The membranes were then blocked by overnight incubation in 0.45% v/v fish gelatin (Biocell), 0.01 M NaH_2PO_4, pH 7.4, 0.15 M NaCl, 0.02% w/v polyvinylpyrrolidone-40, 0.02% w/v Ficoll 400. Primary antibody (hybridoma supernatant or purified monoclonal antibody) was diluted in reagent buffer (0.5% w/v BSA, 0.25% w/v bovine γ-globulin, 50 mM Tris, pH 7.4, 0.15 M NaCl, 1 mM $MgCl_2$) and incubated for 1 h at room temperature with shaking. Bound antibody was detected by incubation with goat anti-rat IgG-biotin (Vector, 1/2000) followed by streptavidin-biotinylated HRP complex (Amersham, 1/2000) in reagent buffer, followed by enhanced chemiluminescence (Amersham). The membranes were washed 3–5 times between each incubation step in 10 mM Tris, pH 7.4, 0.15 M NaCl, 0.05% v/v Tween-20.

Flow cytometry

Assessment of Mab binding specificity and kinetics All cells were harvested from an actively growing suspension culture immediately prior to each analysis. Purified monoclonal antibody was incubated with aliquots of cell suspensions containing $3-5\times10^6$ cells for 30 min at room temperature on an end-over-end mixer. Cells were sedimented (300 g×5 min) and resuspended in 2 ml buffer (0.05 M Na_2HPO_4, 0.15 M NaCl, 5 mM EDTA, 0.5% w/v BSA (bovine serum albumin), pH 7.4). The washing step was repeated and the cells were resuspended in 2 ml of buffer containing goat anti-rat IgG-fluorescein (Vector Laboratories, Ltd, Peterborough, 1/500 dilution) followed by a further incubation for 30 min at room temperature. After two final washing steps with buffer the bound fluorescence was measured in a Facstar+ flow cytometer (Becton Dickinson, Oxford). Fluorescence data was collected using a four decade logarithmic amplifier. To calculate the mean fluorescence of each sample the data was gated to exclude dead cells and the fluorescence values converted to equivalent linear values prior to calculation of the mean fluorescence.

To determine the kinetics of antibody binding directly labelled (via fluorescein isothiocyanate) purified monoclonal antibody (designated Mab 72C) and its Fab fragment was used. A modified input system was constructed that allowed unlabelled cells to be analysed. At time 0, an aliquot of the directly labelled probe was injected into the cell suspension without disturbing the flow characteristics. Data was collected at discrete time intervals for several minutes. The mean fluorescence of the cells was then calculated and plotted as a function of the mid time of the data file. In all cases the fluorescence reached a plateau value after 4 min.

Quantitative studies For measurement of receptor density and assessment of the time course of antibody binding, cells were incubated with a Fab fragment of Mab 72c that had been conjugated to fluorescein via an –SH group. This allowed accurate quantitation of the Fab-fluorescein concentration, since the maximal fluorescein : protein will equal 1.0 (only 1 –SH per mole of Fab available). Under saturating binding conditions (10 μg Fab-fluorescein/ml cell suspension containing 5×10^6 cells incubated for 30 min at room temperature) it was possible to correct for the effect of unconjugated Fab (27% of total) on the fluorescein binding response. To correct for non-specific binding, the same conditions were used with CHO β_1 cells and the fluorescence signal subtracted from that measured for CHO β_3 binding.

Immunohistochemistry

For light microscopy, aliquots of 5×10^6 cells from clones D43, D57, C15, C38, CHO-β_1 and CHO-β_2 were sedimented (300 g×5 min) and resuspended in 10% v/v neutral buffered formalin for at least 1 h. In order to facilitate ease of handling, fixed cells were resuspended in 3% w/v agarose (Type IX, Sigma, Poole) in 1.5 ml Eppendorf tubes maintained at 37°C, re-sedimented and allowed to cool to 4°C. The solidified cell pellets were then removed and cut into pieces for processing to wax and 5 μm sections. Human tissue samples were provided by the Department of Histopathology, St George's Hospital, London (with local Ethical Committee approval). All samples were fixed in 10% v/v neutral buffered formalin and processed in a similar manner as the CHO cell agarose pellets.

Light microscopy labelling studies were carried out using Mab 72c at 0.2 μg/ml and detection with anti-rat IgG Elite ABC (Avidin biotin peroxidase complex) kit (Vector Laboratories, Ltd, Peterborough) and DAB (Diaminobenzidine). For indirect immunogold labelling, Mab 72c (2.36 μg/ml) was detected with a goat anti-rat 1 nm gold conjugate (British Biocell International, Cardiff) 1/100 in PBS (phosphate buffered saline) intensified with silver (British Biocell International, Cardiff) and examined using epi-polarized light. For electron microscopy, cells from clones D43, C15, C38 and CHO-β_1 were fixed in 2% paraformaldehyde, 0.05% glutaraldehyde in PBS for 1 h, pelleted in agarose and dehydrated using a progressive

lowering temperature method,[27] in a Reichert AFS freeze-substitution unit (Leica, Milton Keynes). Cells were then embedded in Lowicryl HM20 resin followed by UV polymerization at $-45^{\circ}C$ for 48 h. Sections (50 nm) were cut and indirectly labelled using Mab 72c (0.78–2.36 μg/ml in Tris buffered saline, overnight at 4°C) followed by a 1 h secondary incubation with goat anti-rat 10 nm gold antibody (1/20 in PBS) and uranyl acetate/lead citrate staining. The same negative controls used for flow cytometry were employed for light and Electron microscopy studies.

Time resolved fluorescence detection of β_3-adrenoceptor labelling in human white adipose tissue

Adipose tissue was obtained from female patients undergoing surgery for the treatment of breast cancer at St George's Hospital (University of London, London) after approval of the hospitals ethics committee. Adipocytes were prepared using a modification of the method of Rodbell[28] employing collagenase digestion (272 U/ml collagenase in Krebs buffer) for 30–45 min.

A membrane-bottomed plate (Millipore Ltd, Watford) was pre-wetted with 100 μl/well 10 mM HEPES (N-2-Hydroxyethylpiperazine-2-ethanesulfonic acid), pH 7.4. The fresh adipocyte suspension (100 μl/well) was then added and washed with ice-cold 10 mM HEPES, pH 7.4; non-specific binding sites were 'blocked' by addition of 250 μl/well 1% w/v BSA in Tris buffered saline and incubated for 45 min at room temperature. Wells were then washed twice and 100 μl biotinylated Mab (72c or anti-IL5 5D3), diluted in Europium assay buffer (Wallac, Milton Keynes) containing 100 μg/ml rat IgG was added to each well and incubated for 30 min at room temperature. Wells were washed (four times) and 100 μl streptavidin-europium (200 ng/ml, Wallac) in europium assay buffer was added and incubated for 15 min at room temperature followed by 6 washes prior to the addition of 100 μl enhancer per well. After shaking for 2 min, 90 μl per well was transferred into Delfia plates, and the time-resolved fluorescence signal was measured using the Delfia system as described.[29]

Results

Monoclonal antibody development

Cell fusions were completed using splenocytes from two rats immunised with CHO β_3.D43 cells. 17 hybridomas were generated that gave CHO β_3.D43/non-transfected CHO binding ratios in the range 4.0–13.7. Primary clones were isolated from six of these hybridomas and those giving binding ratios greater than 5.0 at 1/100 dilution of hybridoma supernatant were further examined by SDS-PAGE/Western blotting. One hybridoma, 72c, was taken through a second round of cloning and then scaled up to a 1 l (litre) suspension culture for Mab production. Purified rat IgG2ak was isolated by Protein G affinity chromatography.

Evaluation of Mab72c using cloned β-adrenoceptors

Western blotting of human β_3-adrenoceptors from clone D43 using Mab72c revealed a diffuse band in the 60–70 kDa region. This band was absent from the lanes containing human β_1- or β_2-adrenoceptors. An equivalent amount of cell membranes expressing comparable β-adrenoceptor densities were used (Figure 1a). The magnitude of the β_3 signal was reduced with decreasing receptor density (D43 > C15 > C38, Figure 1b). A band-shift occurred following treatment with deglycosylation enzymes to a region of apparent molecular weight 40 kDa which agrees with the value predicted (43 kDa) from the amino acid sequence of the β_3-adrenoceptor (Figure 1c). The band detected at approximately 100 kDa may represent aggregated receptor. The β_3-specific band was also detected following immunoprecipitation with Mab 72c-Sepharose. This band was not detected for receptor solubilised from CHO cells expressing the rat β_3 adrenoceptor (data not shown).

Flow cytometry

Antibody binding was assessed using a panel of CHO cells expressing a wide range of β-adrenoceptor densities. The binding of a fluoresceinated Fab fragment of Mab 72c to the highest expressing CHO β_3 clone, D43, indicated that half-maximal binding was attained at 0.16 μg/ml, representing a 7-fold molar excess over receptor, with a time to half-saturation of 196 s. Under saturating conditions of the Fab conjugate, the β_3-adrenoceptor density of the D43 clone was estimated at 182,000 receptors per cell, consistent with [^{125}I]-iodocyanopindolol binding (3000 ± 400 fmol ligand/mg protein equating to a mean of $180{,}000 \pm 24{,}000$ receptors per cell, $n = 4$).

Using indirect labelling (Mab 72c at 1 μg/ml followed by anti-rat IgG-fluorescein) a very substantial increase in fluorescence was obtained for the highest-expressing β_3 clone (D43), such that the mean signal was some 20-fold over background (Table 1). This signal reduced with decreasing human β_3-adrenoceptor density, to the extent that the signal obtained for the lowest expressing clone, C38 (130 fmol/mg), was similar to that measured for the D43 clone in the absence of Mab 72c or with an unrelated primary Mab 5D3. Fluorescence for β_1- (7000 fmol/mg) and β_2-adrenoceptor (2300 fmol/mg) clones expressing receptor levels comparable with the β_3 D43 (3000 fmol/mg) clone was also at this background level. A clone expressing an intermediate density of the rat β_3-adrenoceptor (770 fmol/mg) did not yield a signal over background. The high β_3 to β_1 or β_2 differential was maintained after fixation of the cells with 10% v/v formaldehyde (results not shown).

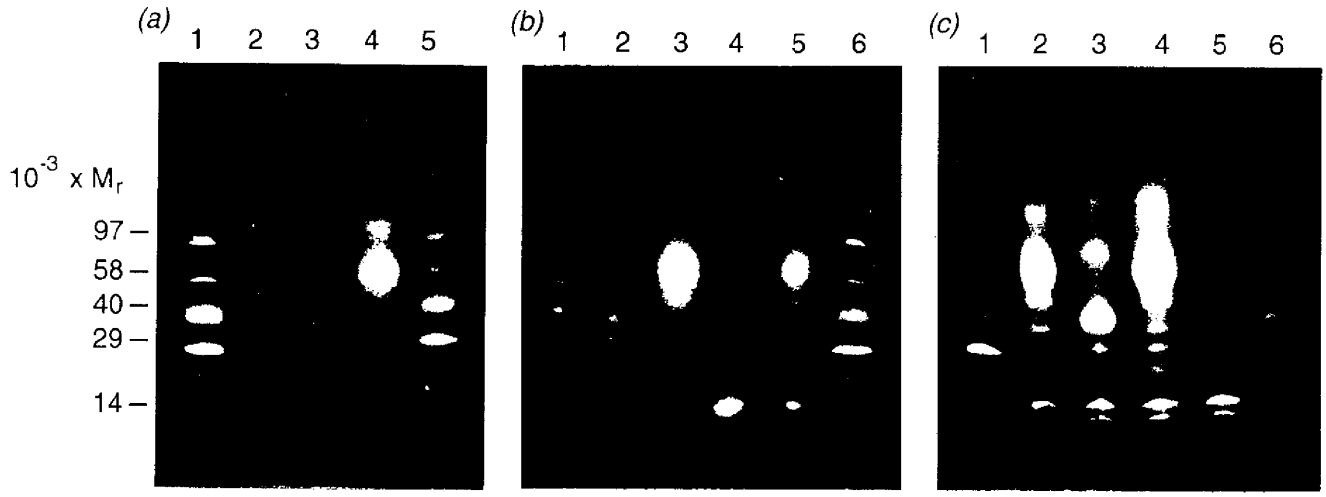

Figure 1 Immunoblotting of human β-adrenoceptors using Mab 72c. Membrane preparations from stably transfected CHO cells expressing β-adrenoceptors were solubilised in the presence of deoxycholate and digitonin and then separated. The proteins were transferred electrophoretically onto PVDF membrane and probed with Mab 72c (1 μg/ml) followed by anti-rat IgG-biotin and anti-biotin-HRP. Bands were detected by enhanced chemiluminescence. (a) CHO cell lines expressing comparable levels of β-adrenoceptor: biotinlylated molecular weight markers (lanes 1 and 5); CHO β_1 (lane 2); CHO β_2 (lane 3): CHO β_3.D43 (lane 4). (b) Influence of β_3-adrenocetpor density on signal detection: CHO β_1 (lane 1); CHO β_2 (lane 2); CHO β_3.D43 (lane 3); CHO β_3.C38 (lane 4); CHO β_3.C15 (lane 5); biotinylated molecular weight standards (lane 6). (c) Effect of receptor deglycosylation: biotinylated molecular weight standards (lane 1); untreated CHO β_3.D43 (lane 2); CHO β_3.D43 post-deglycosylation with EndoF/PNGaseF, 30 h at 37°C (lane 3); CHO β_3.D43 incubation with buffer only, 30 h at 37°C (lane 4); CHO β_1 (lane 5); CHO β_2 (lane 6).

Table 1 Binding of Mab 72c to stably-transfected CHO cells expressing β-adrenoceptors assessed by flow cytometry

Cell type	*Receptor density (fmol/mg)*	*Primary antibody*	*Mean fluorescence*
CHO β_1	7100 ± 260	1 μg/ml 72c	9.1
CHO β_2	2300 ± 120	1 μg/ml 72c	10.2
CHO β_3 (C38)	130	1 μg/ml 72c	16.3
CHO β_3 (C15)	400 ± 80	1 μg/ml 72c	46.7
CHO β_3 (D57)	1300 ± 13	1 μg/ml 72c	115.4
CHO β_3 (D43)	3000 ± 400	1 μg/ml 72c	219.2
CHO rat β_3	770 ± 60	1 μg/ml 72c	4.2
CHO β_3 (D43)	3000 ± 400	1 μg/ml 5D3	9.0
CHO β_3 (D43)	3000 ± 400	no primary ab	9.4

CHO cells were incubated sequentially with Mab 72c followed by anti-rat IgG-fluorescein and then analysed in the flow cytometer. The negative controls in the lower panel were incubated with unrelated (rat anti-human IL5) primary antibody or with the secondary antibody only. Receptor density values (fmoles receptor/mg membrane protein) were determined by saturation binding of [^{125}I]-iodocyanopindolol and represent the mean of 3 or more determinations.

Immunohistochemistry

Light microscopy—CHO cells Light microscopy post-fixation labelling analysis of the CHO cell clones confirmed results obtained by flow cytometry using the panel of β_3-adrenoceptor clones. The D43 and D57 clones showed the highest staining levels of the population with progressively fewer cells staining the C15 and C38 clones. The β_1 and β_2 clones appeared unlabelled as did the controls for non-specific labelling, where the primary antibody was omitted or a rat anti-human IL-5 antibody Mab5D3 was used (Figure 2).

Transmission electron microscopy—CHO cells Post-fixation labelling was found to be of a generally low level with isolated clusters of gold labelling occurring. Labelling was localized on the cell membrane (Figure 2e) and was often seen associated with cytoplasmic processes (Figure 2f).

Light microscopy—human tissues Positive labelling was detected in a small sub-population of smooth muscle cells of the lamina propria of the gall bladder in one out of three tissue samples (Figure 3a). A similar pattern was also consistently observed in colon (three samples, including one of the longitudinal muscles of the taenia coli), prostate, right atrium (in three out of four samples) and gastrocnemius muscle (in two out of three samples; Figure 3b–f). No labelling was detected in lung, left ventricle, appendix, uterus or thyroid.

Examination of eleven samples of adipose tissue from breast, peri-renal and axillary sites proved inconclusive due to problems of interpreting labelling of the thin walled adipocytes.

Time resolved fluorescence—adipocyte membranes To overcome the problem of lack of surface area in the cell wall for labelling by Mab72c in the light microscopy studies, adipocyte membranes were prepared, making a greater surface area available for antibody quantification. In these studies a statistically significant specific labelling of adipocyte membrane preparations was detected at antibody concentrations similar to those used for light microscopy (Figure 4).

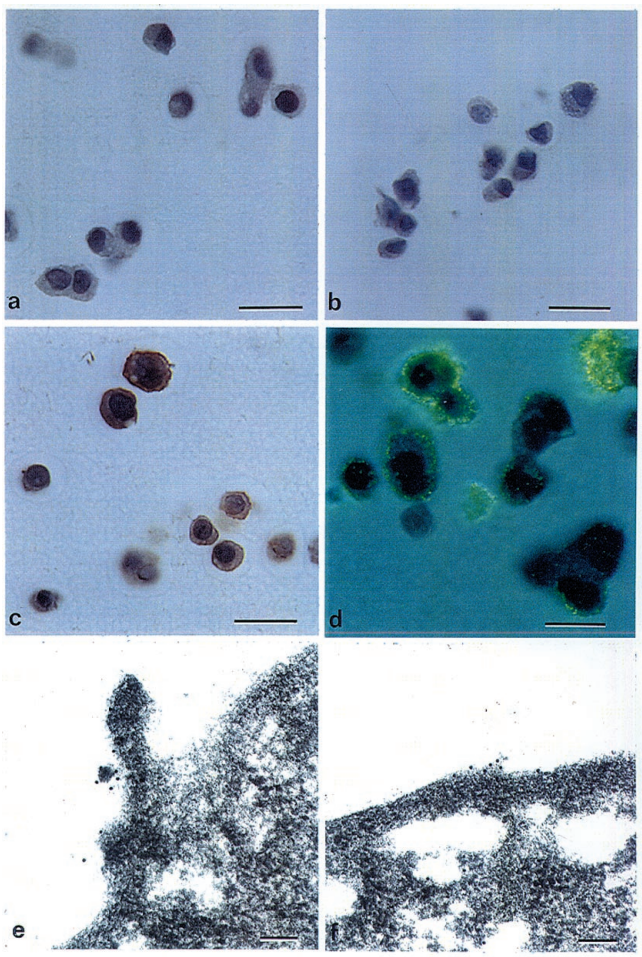

Figure 2 Immunohistochemical localization of β_3-adrenoceptors using Mab 72c in β-adrenoceptor transfected CHO cells by ABC/DAB peroxidase, as described in methods. (a) β_1- and (b) β_2-transfected CHO cells, showing no labelling; (c) β_3-D43 showing overall positive staining with darker staining round the periphery of some cells; (d) immunogold silver-enhanced staining of β_3-D43 viewed by epipolarizing microscopy, showing clearly the peripheral staining of the cells; (e) & (f) β_3-D43: 10 nm immunogold labelling of cell surface detected by transmission electron microscopy, confirming the plasma membrane localisation of the receptor.

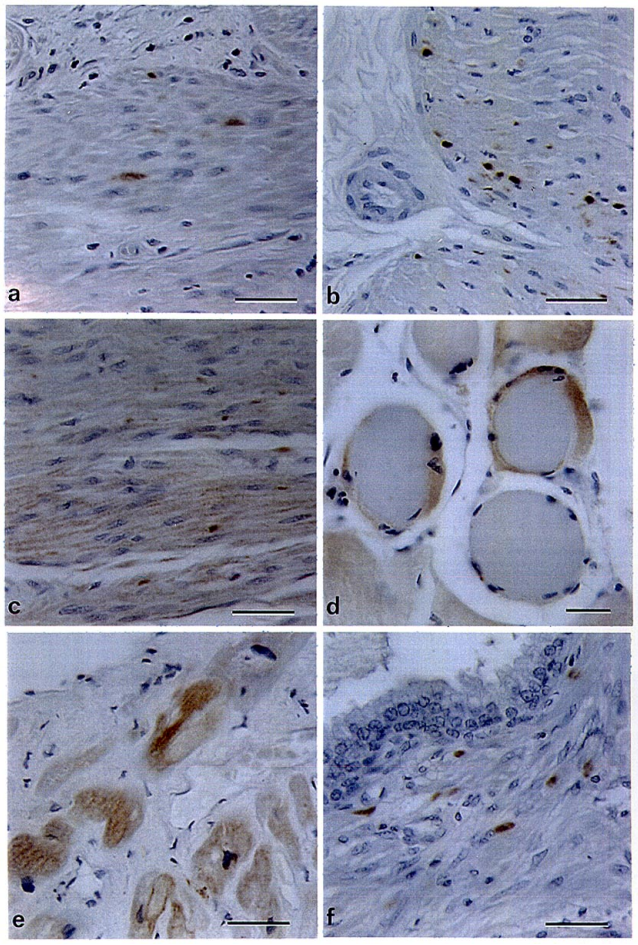

Figure 3 Immunohistochemical localization of β_3-adrenoceptors in human tissues using ABC/DAB peroxidase. (a) Gallbladder, (b) taenia coli, (c) colon, (d) skeletal muscle (gastrocnemius), (e) right atrium, (f) prostate.

Assessment of peptide reactivity

No binding of Mab 72c to either a series of hexameric peptides spanning amino acids 1–28 of the β_3 adrenoceptor (SPOTs kit, Cambridge Research Biochemicals) or the 17–28 peptide in an ELISA was detected (data not shown).

Discussion

In rodents, β_3-adrenoceptors play a significant role in the control of body weight and glucose homeostasis.[10] A similar role has been hypothesised in man such that selective activation of this receptor would lead to therapeutic benefit in obese patients and in Type 2 diabetes.[30] This possibility is supported by the finding in a number of studies of an increased tendency to weight gain and accelerated onset of non-insulin dependent diabetes mellitus in individuals carrying an allelic variant (Trp 64Arg) of the β_3-adrenoceptor.[31] However, contrasting results have also been obtained.[32]

Despite substantial effort, it has proved difficult to unequivocally demonstrate expression of mRNA for this receptor sub-type in the relevant human tissues.[17–22] Furthermore, the only EST hybridising to the human β_3-adrenoceptor so far identified (Gen Bank: HSBAREIX 70811, A1276134) was obtained from a library derived from a pool of three organs: foetal heart, melanocyte and uterus (personal communication, Klaus Duecker and Chris Southan, SB Pharmaceuticals).

The major evidence that the β_3-adrenoceptor is expressed in human tissues comes from functional studies using the β_1/β_2 adrenoceptor antagonist CGP 12177, which has partial agonist activity at the human β_3-adrenoceptor expressed in CHO cells.[33] In human tissues responses to CGP 12177 are not blocked by standard β_1- or β_2-adrenoceptor antagonists. Thus both human circular and taenia coli preparations are relaxed by CGP 12177.[34–36] Similarly in human white

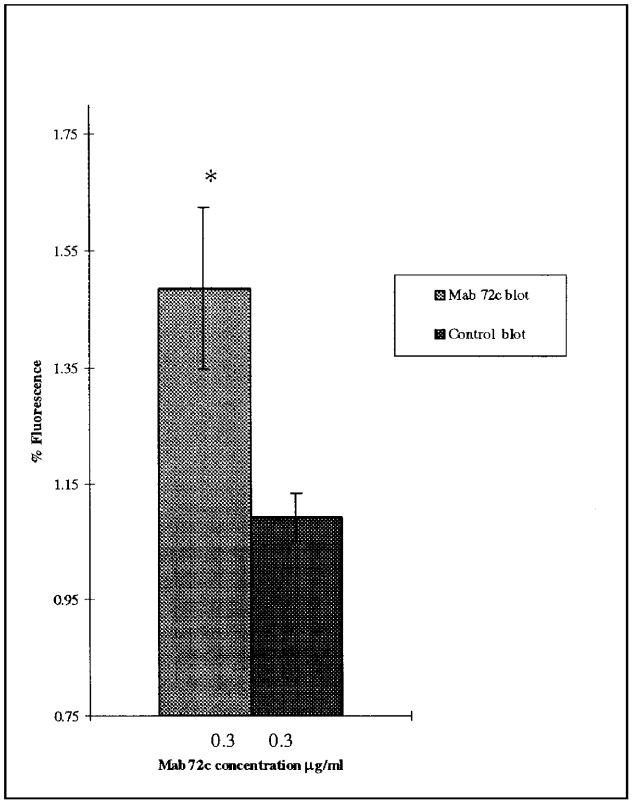

Figure 4 Time-resolved fluorescence labelling of β_3-adrenoceptors in human adipose tissue compared with background non-specific labelling with unrelated (rat Mab 5D5 anti-human IL5) primary antibody. Data shown are the mean ± S.E. (*$P < 0.05$) of three breast preparations taken from separate patients.

adipocytes, CGP 12177 has been demonstrated to have a lipolytic action in most[12–14] but not all studies,[9–5] that is poorly blocked by the standard β_1- and β_2-adrenoceptor antagonists. Interpretation of studies on CGP 12177 is complicated by its mixed actions. In addition to its β_1- or β_2-antagonism, it is claimed to be an activator of the putative β_4-adrenoceptor. Thus, CGP 12177 has an inotropic response on human right atrial appendage that is not mediated by the β_3-adrenoceptor.[37,38] All of these studies have emphasised the need to be able to detect the β_3-adrenoceptor protein in human tissues.

After initial attempts to develop β_3-adrenoceptor specific antibodies against synthetic peptide immunogens had been unsuccessful, we chose to develop monoclonal antibodies from rats immunised with whole CHO cells expressing the human β_3-adrenoceptor at high density. Both the reactive band identified with one antibody, Mab 72c, in the 60–70 kDa region on Western blots of solubilised human β_3-adrenoceptor and the band-shift to approximately 43 kDa following deglycosylation, were consistent with observations made for other β-adrenoceptor subtypes.[39–41] The diffuse nature of the Mab 72c-reactive band most probably reflects heterogeneity of the N-linked complex and high-mannose type carbohydrate chains located close to the N-terminus of the receptor. The titration of this signal with the receptor density in a panel of CHO β_3-adrenoceptor cell lines provided good evidence for specificity.

The identity of the epitope recognised by Mab 72c has not been established. However, as cells examined in the flow cytometer were non-permeabilised, Mab 72c must recognise an epitope on an extracellular domain of the human β_3-adrenoceptor. Stability of recognition to formaldehyde fixation or to reduction of solubilised receptor in the presence of SDS suggested a linear rather than a conformational epitope. Given the lack of reactivity with the rat β_3-adrenoceptor, it seemed most likely that the epitope was located in the first extracellular domain (amino acids 1–35) where there is greatest divergence of the sequence of one of the human extracellular domains from that of the rat. However, we failed to detect Mab 72c binding to a series of hexameric peptides spanning residues 1–28, or to a synthetic peptide comprising residues 17–28. Furthermore, the Mab reacted strongly with both the glycosylated and deglycosylated forms of the solubilised receptor on SDS-PAGE/Western blotting, indicating that the complex carbohydrate associated with the first extracellular domain did not influence recognition of the receptor by Mab 72c. The absence of an effect of the Mab on the binding of [^{125}I]-iodocyanopindolol to CHO β_3-adrenoceptor cell membranes, or on isoproterenol-induced adenylyl cyclase activity or human white adipose tissue lipolysis (data not shown), suggests that the epitope is remote from the ligand binding site and does not influence activation-response coupling.

Fluorescence labelling analysed by flow cytometry was similar for both fresh and fixed cells, proving that the β_3-adrenoceptor withstands formalin fixation. Hence fixed, rather than fresh, human tissue could be considered for immunolocalisation studies. Microscopy showed clear evidence of surface labelling of the CHO cells, particularly in immunogold labelled preparations (light and electron microscopy), which appeared similar to that described for β_2-adrenoceptors in 293 cells,[42] with the presence of clusters of β_3-adrenoceptors located at the cell surface. These receptors were detected also by immunohistochemistry in gall bladder, colon and prostate, confirming previous mRNA studies.[17,19] In these sites, receptors were localised on a sub-population of cells, a fact not detected by whole tissue mRNA analysis. Possibly, this reflects clustering of β_3-adrenoceptors in sympathetically innervated cells. Furthermore, it is noteworthy that the staining pattern obtained in gall bladder was quite different from that reported in a previous study[23] using a polyclonal anti-peptide antibody. In the latter report, staining was associated primarily with arteriolar vascular smooth muscle cells, with negligible staining in the lamina propria. Unfortunately, these authors did not report data from other human tissues for comparison. The detection of Mab 72c binding in longitudinal smooth muscle of the colon agrees with the localisation of atypical (non-β_1-

or β_2-adrenoceptor-related) [^{125}I]-iodocyanopindolol binding in human colon musculature,[43] and supports a role for the β_3-adrenoceptor in mediating relaxation responses.[34–36]

Initial studies on human adipose tissue using immunohistochemistry were inconclusive. To detect a β_3-adrenoceptor signal and therefore confirm the ability of Mab72c to detect β_3-adrenoceptors in adipose tissue, adipocyte membranes were separated and concentrated. This effectively increased the surface area of detection and thereby overcame the inherent inefficiency of the whole tissue studies. Using this approach and time-resolved fluorescence, a significant β_3-adrenoceptor signal was obtained using similar antibody concentrations to those used in the tissue distribution studies. The fact that only a low signal was detected may reflect a low receptor density, as levels of β_3-adrenoceptor mRNA appear lower in subcutaneous than in visceral fat depots,[17] or possibly a difference in expression between subpopulations of adipocytes.

Our observation that Mab 72c produced no labelling in the lung gave added confidence to the β_3 selectivity (over β_2) for the human tissue studies. This was particularly important in substantiating our detection of β_3 adrenoceptors in right atrium and skeletal muscle. In the case of right atrium, Berkowitz *et al*[19] speculated that the weak and variable mRNA signal seen in their samples may have originated in attached fat. The same conclusion was reached by Evans *et al*[44] for heart and skeletal muscle. The present study demonstrates that the location is, in fact, in the myocardial cells and represents the first immunohistochemical localization of β_3-adrenoceptors in the human heart. However, at least some selective agonists at the human β_3-adrenoceptor appear unable to enhance the contractility of human right atrial appendage[45] and it seems probable that much of the effect of CGP 12177 is mediated by the putative β_4-adrenoceptor. Since this latter receptor has not been cloned yet, we cannot rule out the possibility that Mab 72c detects the β_4-adrenoceptor also.

In contrast to human atrium we were unable to detect β_3-adrenoceptors in human ventricle, which was surprising in light of the pharmacological evidence described by Gauthier *et al*,[46] especially as these workers described an order of potency by selective β_3-adrenoceptor agonists in which CGP 12177 was least efficacious, indicating that the effects were unlikely to be mediated via the putative β_4-adrenoceptors.[38]

In addition to the β_3-adrenoceptor signal in the atrium a significant signal was obtained in skeletal muscle using Mab 72c. This finding contrasts with the more tenuous evidence for β_3-adrenoceptor mRNA expression in human skeletal muscle.[19,44] Experiments in rodents have indicated the presence of an atypical β-adrenoceptor in skeletal muscle, although this receptor does not have identical characteristics to the β_3-adrenoceptor.[47,48] The present studies suggest that either the β_3-adrenoceptor or an atypical β-adrenoceptor that cross-reacts with Mab 72c is present in human skeletal muscle.

In summary, Mab 72c is an important addition to the armentarium of reagents that are available to unravel the complexities of β_3-adrenoceptor function in man.

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