International Journal of Obesity (2002) 26, 1442–1450
 0307–0565/02 $25.00
www.nature.com/ijo

PAPER

Immunohistochemical identification of the β_3-adrenoceptor in intact human adipocytes and ventricular myocardium: effect of obesity and treatment with ephedrine and caffeine

R De Matteis[1], JRS Arch[2], ML Petroni[3], D Ferrari[3], S Cinti[1]* and MJ Stock[4]

[1]*Institute of Normal Human Morphology-Anatomy, University of Ancona, Ancona, Italy;* [2]*GlaxoSmithKline, New Frontiers Science Park, Harlow, Essex, UK;* [3]*Nutrition Research Laboratory, Istituto Auxologico Italiano, Verbania, Italy; and* [4]*Department of Physiology, St George's Hospital Medical School, London, UK*

OBJECTIVES: To investigate whether the β_3-adrenoceptor could be identified by immunohistochemistry in intact human white and brown adipocytes and other human tissues, and to investigate the influence of obesity and its treatment with ephedrine and caffeine on the expression of the β_3-adrenoceptor in adipocytes.
METHODS: Morbidly obese patients were given a hypoenergetic diet (70% of energy expenditure) and some were also treated with ephedrine and caffeine (20/200 mg, three times daily) for 4 weeks. Adipose tissue and other tissues were taken during surgery. Immunohistochemistry was carried out using a monoclonal antibody raised against the human β_3-adrenoceptor.
RESULTS: Staining was localized to the periphery of cells. All white adipocytes were stained. Those from lean subjects and obese subjects treated with ephedrine and caffeine showed more intense staining than those from untreated obese subjects. Staining was more intense in brown than in white adipocytes in perirenal adipose tissue from phaeochromocytoma patients. Staining was also seen in ventricular myocardium, and in smooth muscle of the prostate, ileum, colon and gall bladder.
DISCUSSION: The tissue and subcellular distribution of staining was consistent with it being due to binding of the antibody to the human β_3-adrenoceptor. The presence of the β_3-adrenoceptor in human white adipocytes is consistent with evidence that it can mediate lipolysis in human white adipocytes. The increased expression of the β_3-adrenoceptor in obese subjects treated with caffeine and ephedrine supports the potential of β_3-adrenoceptor agonists in the treatment of obesity and type 2 diabetes. Its expression in ventricular myocardium is consistent with evidence that the β_3-adrenoceptor mediates a negative inotropic effect in this tissue.
International Journal of Obesity (2002) **26,** 1442 – 1450. doi:10.1038/si.ijo.0802148

Keywords: white adipocyte; brown adipocyte; ventricular myocardium; β_3-adrenoceptor; obesity; immunohistochemistry

Introduction

Mammalian (including human) adipose tissue is composed mainly of white and brown adipocytes. White adipocytes store lipids in unilocular droplets, whereas brown adipocytes store lipids in multilocular droplets. This reflects the different functions of these two cell types. White adipocytes store lipids and release fatty acids in the intervals between meals to provide other tissues with a source of energy other than glucose. Brown adipocytes, on the other hand, oxidize most of the stored intracellular lipid and release the energy as heat. The oxidation of lipids in brown adipocytes can be very rapid because it is uncoupled from the synthesis of ATP by the mitochondrial uncoupling protein-1 (UCP-1).[1,2]

Brown and white adipocytes are localized in specific depots, but often these two cell types are found in the same depot. Unilocular white adipocytes that do not express UCP-1 are frequently seen in brown adipose depots. Conversely, adipocytes with a brown phenotype can appear among the unilocular adipocytes within white adipose depots.[3] In a recent study[4] it was found that, after treatment

***Correspondence: Professor S Cinti, Institute of Normal Human Morphology-Anatomy, University of Ancona, via Tronto 10/a, Ancona, 60020, Italy.**
E-mail: cinti@popcsi.unian.it
†Current address: Clore Laboratory, University of Buckingham, Buckingham MK18 1EG, UK.
‡Mike Stock died on 26 March 2001, a few weeks after drafting this manuscript. This paper is dedicated to his memory.
Received 15 October 2001; revised 28 May 2002; accepted 2 June 2002

of rats with the β_3-adrenoceptor agonist CL-316243 for 7 days, multilocular adipocytes arose directly from conversion of unilocular white adipocytes. Only a few of these 'convertible' brown adipocytes expressed UCP-1, however. Moreover, only a minority showed the complete differentiation of mitochondria characteristic of brown adipocytes, and some showed morphological features typical of white adipocytes during lipolysis.[4]

Lipolysis in white and brown adipocytes is activated by sympathetic stimulation of β-adrenoceptors. In rodents the β_3-adrenoceptor plays the dominant role in these cells. This partly explains why β_3-adrenoceptor agonists are highly effective in causing weight loss and improving insulin sensitivity in animal models of obesity and type 2 diabetes: brown adipose tissue (together with skeletal muscle) is an important site of thermogenesis, whilst white adipose tissue provides much of the fuel for thermogenesis.[5] The conversion of unilocular to multilocular adipocytes by repeated administration of β_3-adrenoceptor agonists is probably part of the mechanism by which the thermogenic effect of β_3-adrenoceptor agonists increases with time.[6]

The role of the β_3-adrenoceptor in humans is more controversial. No β_3-adrenoceptor agonist has so far progressed beyond phase II clinical trials for obesity or type 2 diabetes. It is unclear whether this is because a compound with suitable selectivity for the β_3-adrenoceptor and drug properties (eg oral bioavailability) has not been found, or because the β_3-adrenoceptor has a lesser role in humans than in rodents. There is some functional evidence for β_3-adrenoceptors in human white adipocytes, but such evidence is more difficult to obtain than with rodent white adipocytes. β_3-Adrenoceptor mRNA is expressed in human adipose tissue, but in lower amounts than in rodents.[5] Immunohistochemistry using one antibody has demonstrated the presence of the receptor in gallbladder and in vascular and non-vascular smooth muscle, but not, it seems, in adipose tissue.[7] Another antibody has been used to demonstrate the presence of the receptor in human white adipose membranes using time-resolved fluorescence. It was not possible, however, in that study to identify the receptor in intact adipocytes owing to the difficulty of interpreting labelling of these thin-walled cells.[8]

Nevertheless, using this latter antibody, we are now able to show unequivocally using light microscopy that the β_3-adrenoceptor is present in adipose tissue of lean and obese humans. Furthermore, the receptor was detected on ventricular myocardium, with weak staining detected on smooth muscle cells of the small intestine, gall bladder and prostate.

Patients and methods

Patient selection for white adipose tissue biopsies

Morbidly obese patients were selected from the waiting list for bariatric surgery at the Surgical Department, Molinette Hospital, Turin. In order to be admitted for bariatric surgery, they had to have BMI > 40 kg/m^2, a history of unresponsiveness to previous, medically supervised, weight-reduction therapy, and no major psychiatric illness. Only premenopausal females who were non-smokers or smoking less than five cigarettes per day were selected for the research protocol. Exclusion criteria were: ischaemic heart disease, cardiac insufficiency, hypertension requiring pharmacological treatment, tachyarrhythmias, sick sinus syndrome, AV block, two-bundle ventricular block, cerebrovascular disease, occlusive peripheral artery disease, renal insufficiency and current treatment with drugs that might affect metabolic rate (eg beta-blockers, thyroid hormones).

Patients were randomized to treatment with either caffeine/ephedrine (200/20 mg three times daily) or placebo of identical appearance. Treatment was started 4 weeks before the scheduled date of the operation at an intial dose of a half tablet three times daily for the first week, before proceeding to the full dose of one tablet three times daily. Blood pressure was measured three times a day and an electrocardiogram was recorded weekly, but no cardiovascular side-effects were detected. Three patients (one in the placebo and two in the treatment group) complained of insomnia after the first week and were returned to half dosage for the entire treatment period.

Patients were hospitalized during the whole treatment period, first in a metabolic ward and then for 3–6 days prior to surgery in the Surgical Department in Turin. Treatment was discontinued on the day before the operation. During the treatment period the patients were put on a hypoenergetic diet of total energy content equal to about 70% of energy expenditure as measured by indirect calorimetry, and containing 20% protein, 55% carbohydrate and 25% fat, of which half was monounsaturates, plus 35 g/day of fibre. Weight loss (expressed as body mass index units) over the 4 weeks of treatment before surgery averaged about 3 kg/m^2 in those patients on the ephedrine/caffeine treatment and about 2 kg/m^2 in those on placebo.

Surgery consisted of either gastroplasty or gastric banding via a laparoscopic approach. At the time of laparotomy adipose tissue samples from omental ($n=9$) and subcutaneous ($n=14$) sites were excised and fixed as described below.

The study included subcutaneous adipose tissue from three lean patients (two men and one woman, 17–44 y old) undergoing surgery from clinical purposes.

In summary, we studied three lean, six obese untreated and eight obese patients treated with ephedrine and caffeine.

The study protocol was approved by the Ethical Committee of the Istituto Auxologico Italiano and by the Regional Ethical Committee of Piedmont. All patients were required to sign a written consent form.

Other tissue biopsies

Specimens of prostate ($n=4$), gall bladder ($n=1$), small intestine (ileum; $n=2$), large intestine (colon; $n=2$), skeletal muscle (m. soleus and m. pectoralis; $n=3$), bladder ($n=2$)

and lung ($n=2$) were obtained during surgery for clinical purposes from patients at the Faculty of Medicine Hospital, Ancona, Italy.

Perirenal adipose tissue from phaeochromocytoma patients ($n=4$) and ventricular biopsies ($n=11$) were collected from the Anatomy Pathology Institute, 'Casa Sollievo della Sofferenza' Hospital, S. Giovanni Rotondo, Italy.

Right ventricular myocardial biopsies ($n=9$) were obtained from patients (seven men and two women, 20–50 y old), of whom four had dilatative cardiomyopathy, two had arrhythmogenic cardiomyopathy, one had ischaemic cardiomyopathy, one had restrictive cardiomyopathy and one studied for a suspected arrhythmogenic displeasure that was found to be 'normal' after histological examination. Autoptic left ventricular myocardium ($n=2$) was also studied.

Informed consent was obtained from the patients or their relatives. None of the patients, except those with phaeochromocytoma, had diseases known to interfere with the β-adrenergic system.

Microscopy

Tissues were fixed by immersion in 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4. After washing in this buffer overnight, the samples were dehydrated in a graded series of ethanol solutions and embedded in paraffin blocks ready for light microscopy. For immunohistochemistry 10 μm (white adipose tissue) or 3 μm sections (other tissues) were immunostained by the avidin-biotin peroxidase (ABC) technique.[9]

Dewaxed sections were processed through the following steps: (1) hydrogen peroxide (0.3%) in methanol for 30 min to block endogenous peroxide; (2) normal rabbit serum (1:75) for 20 min to reduce non-specific background staining; (3) monoclonal anti-human β_3-adrenoceptor antibody

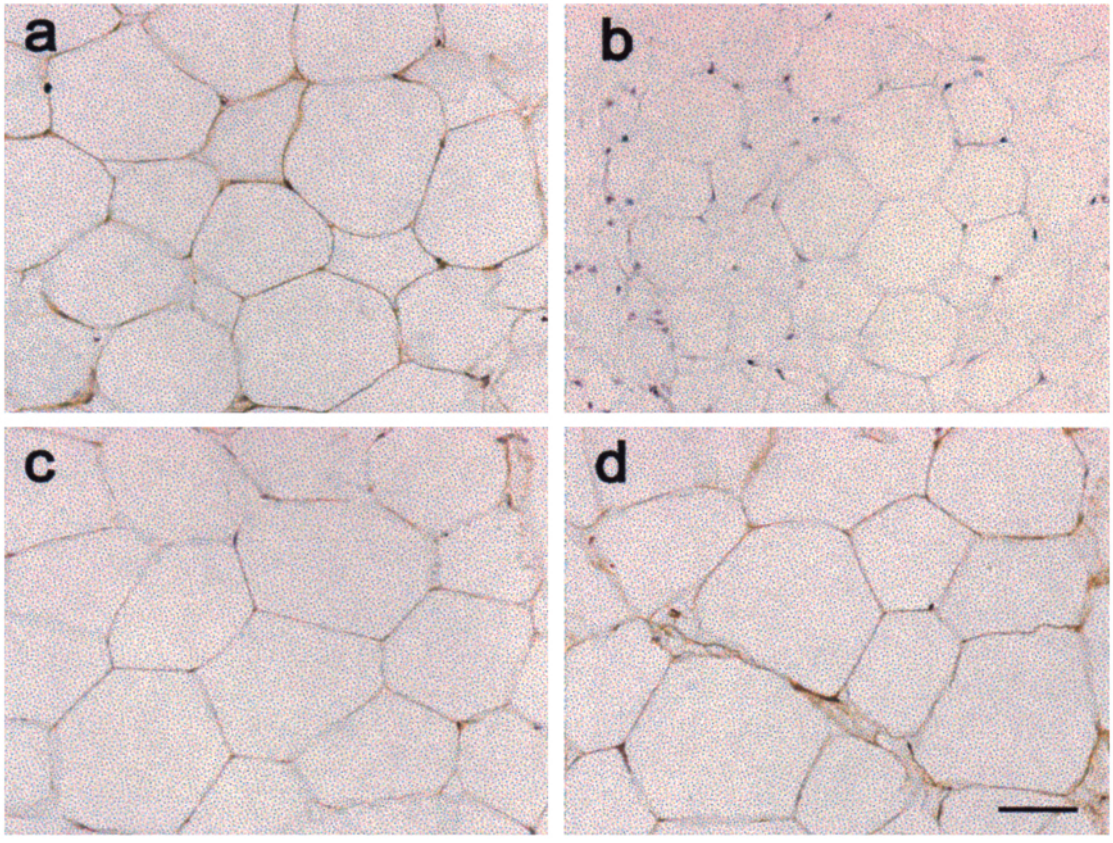

Figure 1 Immunohistochemistry of human adipose tissues. The primary antibody is a monoclonal antibody anti human β_3 adrenoceptor (mab 72c, see Chamberlain *et al*[8] for details) diluted 1:1500. Paraffin embedded tissues, ABC method. All images were taken by a digital camera Nikon-DXM 1200 (Nikon Europe BV, The Netherlands), mounted on a Nikon-Eclipse E800 Microscope (Nikon Europe BV, The Netherlands) at the same enlargment (20×) and using the same light filter and the same intensity of luminosity. (a) Subcutaneous adipose tissue of a lean patient. The thin cytoplasm rim of adipocytes is immunoreactive for the β_3 adrenoceptor (compare with b and c). (b) Subcutaneous adipose tissue. Control test: phosphate buffer substituted for the primary antibody. No staining is visible (compare with a, c and d). (c) Subcutaneous adipose tissue of an obese untreated patient. The thin cytoplasmic rim of adipocytes is weakly positive. (compare with a, b and d). (d) Subcutaneous adipose tissue of an obese patient treated with caffeine and ephedrine. Note the intense staining of the thin cytoplasmic rim (compare with a, b and c). Bar: (a)–(d) = 59 μm.

(Mab 72c[8]) raised in the rat and diluted 1:1500 in PBS overnight at 4°C; (4) biotinylated secondary rabbit anti-rat IgG (1:200) for 30 min (Vector Laboratories, Burlingame, CA, USA); (5) ABC complex for 1 h (vectastain ABC kit, Vector); and (6) 3′,3′-diaminobenzidine hydrochloride cromogen (Sigma, St Louis, MO, USA) in Tris buffer 0.05 M, pH 7.6 for visualization of peroxidase. Sections were counterstained with haematoxylin and mounted in Eukitt (Kindler, Germany). Specificity tests were performed by omitting the primary antiserum in the staining and by using preimmune serum instead of the primary antiserum. The β_3-adrenoceptor antibody was also used at dilutions of 1:1000 and 1:2000, but the figures are from the tests at a dilution of 1:1500.

Hydrated sections of perirenal adipose tissue from phaeochromocytoma patients were processed for UCP1 primary antibody, (generously provided by Dr D Ricquier, Meudon, France; diluted 1:10 000) and labelled with the ABC method as described above using normal rabbit serum and biotinylated rabbit anti-sheep IgG.

Results

All the white adipocytes from subcutaneous and omental depots were β_3-adrenoceptor-immunoreactive (Figure 1). Staining at the peripheral cytoplasmic rim was clearly evident in adipocytes from both lean (Figure 1a) and

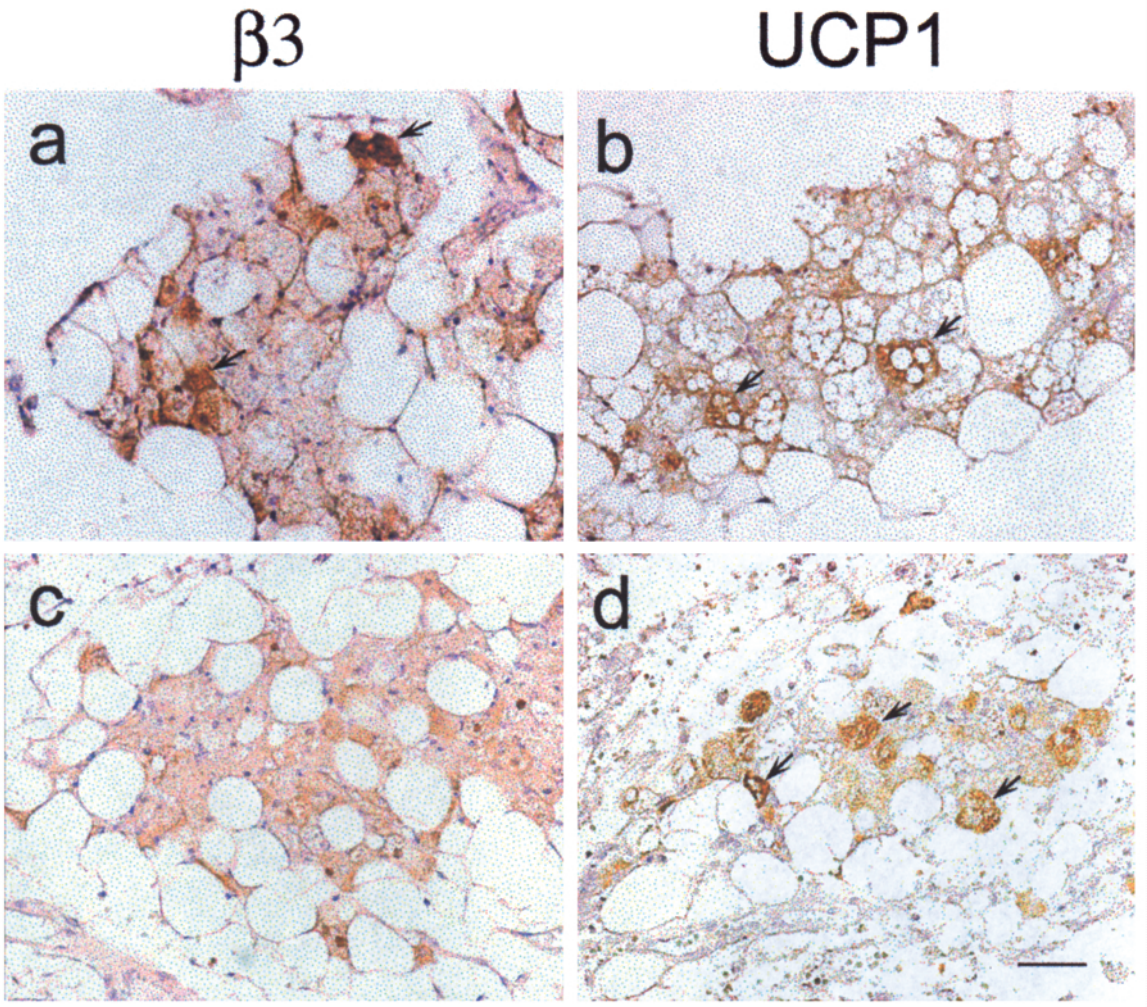

Figure 2 Periadrenal adipose tissues from two phaeochromocytoma patients was stained with anti human β_3 adrenoceptor antibody (a and c) and anti UCP1 antibody (b and d). The primary antibody is a monoclonal antibody anti human β_3 adrenoceptor (mab 72c, see Chamberlain *et al*[8] for details) diluted 1:1500 (a and c) and a polyclonal antibody anti-UCP1, (generously provided by Dr D Ricquier, Meudon, France; diluted 1:10 000; b and d). Paraffin embedded tissues, ABC method. All images were taken by a digital camera Nikon-DXM 1200 (Nikon Europe BV, The Netherlands), mounted on a Nikon-Eclipse E800 Microscope (Nikon Europe BV, The Netherlands) at the same enlargment (20×) and using the same light filter and the same intensity of luminosity. In both unilocular white and multilocular brown adipocytes are visible. Both resulted β_3 adrenoceptor immunoreactive in their cytoplasm (a and c). Some brown adipocytes appear more intensely stained than others (arrows). Brown adipocytes located in the same region of the tissue were also UCP1 stained (b and d). Some of them resulted more intensely stained (arrows). Bar: (a) – (d) = 53 μm.

obese (Figure 1c) subjects, but was more intense in adipocytes from the lean subjects. No staining was seen when the primary antibody was omitted (Figure 1b). Adipocytes from obese patients treated with caffeine and ephedrine (Figure 1d) showed more intense staining than adipocytes from placebo-treated obese subjects (Figure 1c), and comparable staining to that of lean subjects (Figure 1a).

Several clusters of brown adipocytes in between white adipocytes were observed in the perirenal adipose tissue of phaeochromocytoma patients. In these samples both white and especially brown adipocytes were β_3-adrenoceptor-immunoreactive (Figure 2a and c). The intensity of staining of brown adipocytes varied greatly from cell to cell (Figure 2a).The samples analysed for β_3-adrenoceptor were processed with anti-UCP1 antibodies. Some multilocular adipocytes also express UCP1 (Figure 2b and d).

Immunoreactivity was also detected in ventricular myocardial cells. Figure 3a shows strong staining in myocardium from a 'normal' living patient; not all the myocardial cells were stained. Endothelial cells and smooth muscle fibres in the wall of the vessels were negative. The immunoreactivity was weaker in one of the two samples from autopsies (left ventriculum) and in seven of the nine biopsies from living patients (right ventriculum; Figure 3c). The enlargement of Figure 3d shows that the immunoreactivity was often localised to the periphery of the cells.

We found positive staining in several other tissues: prostate, intestine and gall bladder (Figure 4). In the prostate only stromal smooth muscle cells were immunoreactive, but the intensity of the staining was highly variable (Figure 4a). Weaker staining was present in smooth muscle cells of the lamina propria of the gall bladder (Figure 4c) and the ileum and colon (not shown).

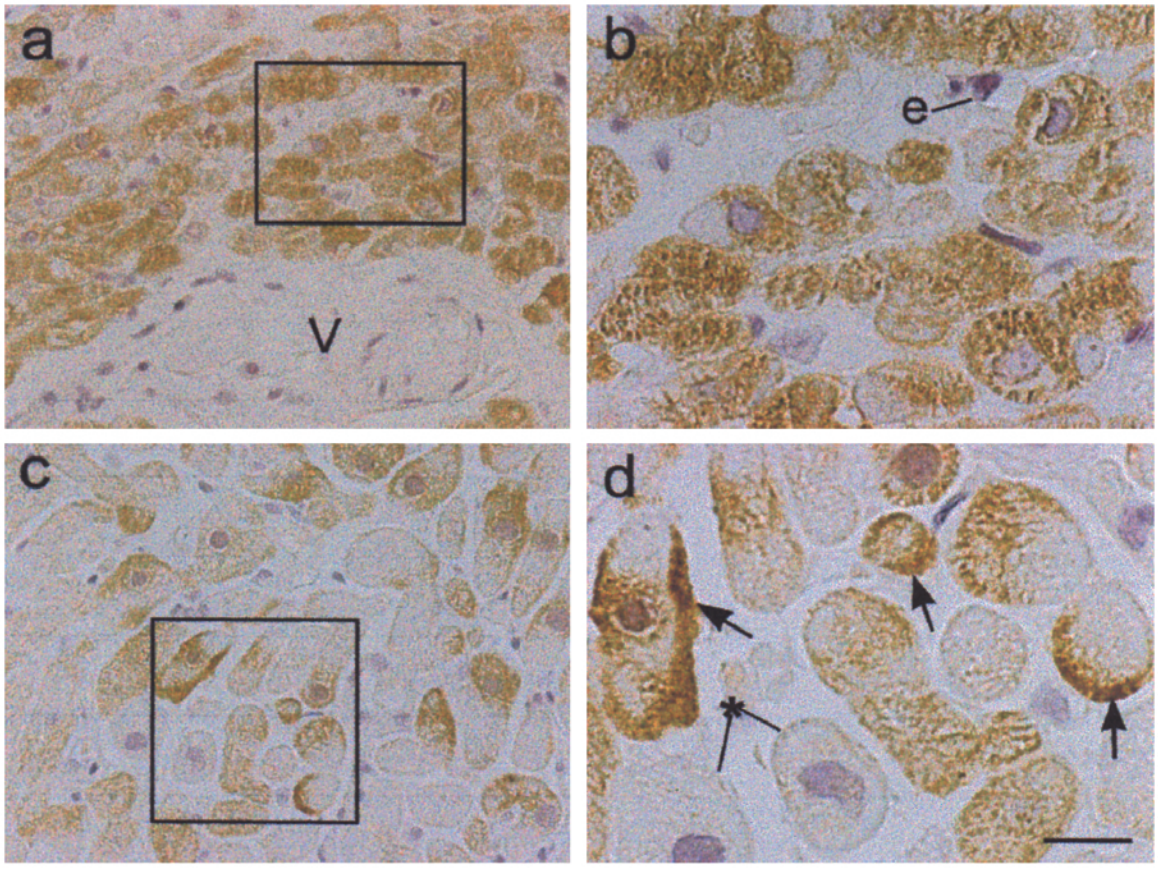

Figure 3 Immunohistochemistry of human ventricular myocardial tissue. The primary antibody is a monoclonal antibody anti human β_3 adrenoceptor (mab 72c, see Chamberlain *et al*[8] for details) diluted 1:1500. Paraffin embedded tissues, ABC method. All images were taken by a digital camera Nikon-DXM 1200 (Nikon Europe BV, The Netherlands), mounted on a Nikon-Eclipse E800 Microscope (Nikon Europe BV, The Netherlands) at the enlargment 40× (a and c) and 100× (b and d) and using the same light filter and the same intensity of luminosity. (a) Right ventricular myocardial tissue from a 'normal' patient (studied for a suspected arrhythmogenic displeasure). An intense labelling is evident in a number of fibers but also negative myocardial cells are visible. Vessels resulted negative (V). (b) Enlargement of the framed area in a. Endotelial cells result negative (e). (c) Right ventricular myocardial tissue from a living patient affected by dilatative cardiomyopathy. Myocardial cells are weakly stained (compare with a). (d) Enlargement of the framed area in (c). High magnification of myocardial cells show that immunoreactivity is localised at the periphery of the cells (arrows). Negative myocardial cells are also visible (*). Bar: (a) and (c) = 34 μm; (b) and (d) = 14 μm.

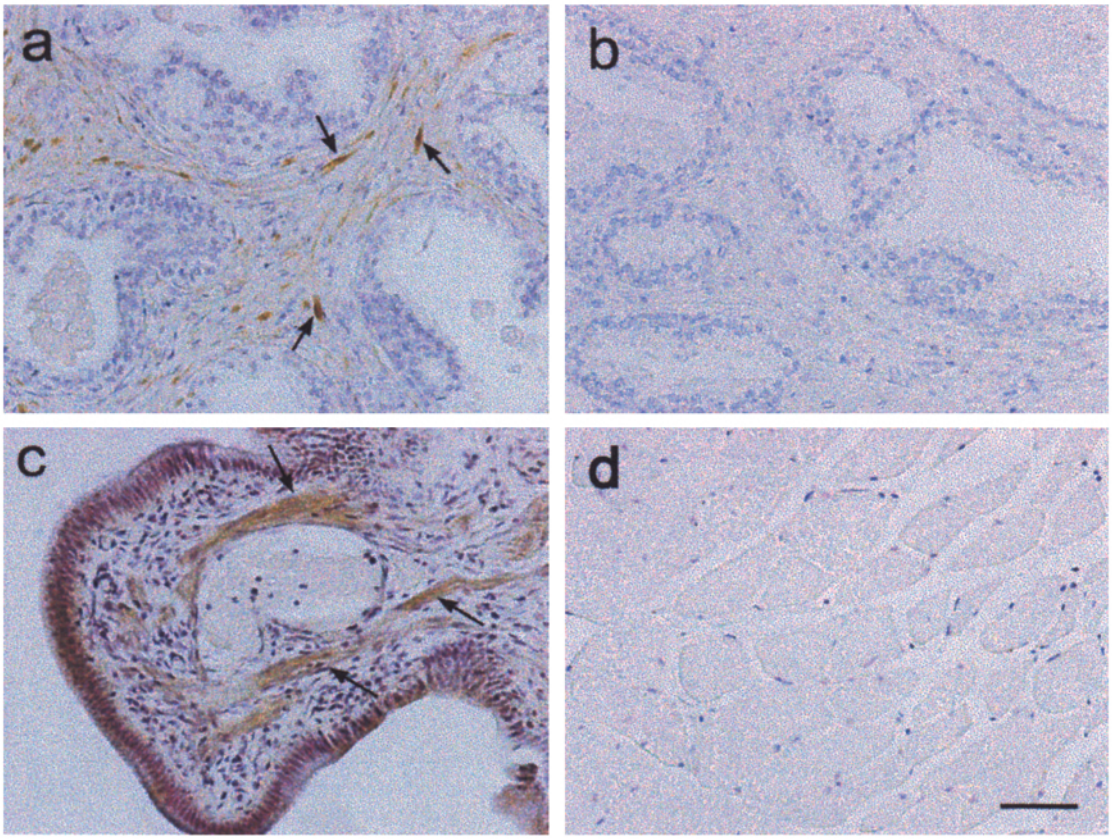

Figure 4 Immunohistochemistry of human tissues. The primary antibody is a monoclonal antibody anti human β_3 adrenoceptor (mab 72c, see Chamberlain *et al*[8] for details) diluted 1:1500. Paraffin embedded tissues, ABC method. All images were taken by a digital camera Nikon-DXM 1200 (Nikon Europe BV, The Netherlands), mounted on a Nikon-Eclipse E800 Microscope (Nikon Europe BV, The Netherlands) at the same enlargment (20×) and using the same light filter and the same intensity of luminosity. (a) Normal area of a prostate. Smooth muscle cells and not the epithelial cells are immunoreactive for the β_3 adrenoceptor. Some muscle cells appear more immunoreactive than others (arrows). (b) Normal area of the prostate of the same patient shown in (a). Control test: preimmune serum instead of the primary antibody. No staining is visible. (c) Normal area of a gall bladder. Smooth muscle cells and not the epithelial cells are weakly immunoreactive for the β_3 adrenoceptor (arrows). (d) Normal skeletal muscle (m. pectoralis). All citotypes result negative. Bar: (a) – (d) = 59 μm.

All cell types in the lung, urinary bladder and skeletal muscle (Figure 4d) of all the patients were unstained, as were the smooth muscle fibres of the wall of the arterioles and veins present in all organs examined (adipose tissue, intestine, bladder, prostate, gall bladder, lung).

Discussion

It is well established that β_3-adrenoceptor mRNA is expressed in rodent adipocytes and that β_3-adrenoceptors play a functional role. The existence and role of β_3-adrenoceptors in human adipocytes is more controversial, however. Most,[10–14] although not all[15,16] workers have detected β_3-adrenoceptor mRNA in human adipose tissue, but expression is some 50-fold lower than in rodent adipose tissue[13,14] and β_3-adrenoceptor transcripts represent less than 20% of total β-adrenoceptor transcripts compared with more than 90% in rodents.[12] Studies in transgenic mice indicate that the promoter for the human β_3-adrenoceptor drives expression mainly in brown adipose tissue, of which there is relatively little in adult humans.[17]

Functional evidence for β_3-adrenoceptors in white adipocytes was largely based upon the lipolytic activity of CGP-12177, which was believed to be a potent $\beta_{1/2}$-adrenoceptor antagonist and a β_3-adrenoceptor agonist at somewhat higher concentrations. CGP-12177 has now, however, been shown also to activate a conformation of the β_1-adrenoceptor (previously called the β_4-adrenoceptor).[18,19] The activation of β_1-adrenoceptors by CGP-12177 is less sensitive than activation by standard agonists to β_1-adrenoceptor antagonists. The pharmacology of the β_1-adrenoceptor when stimulated by CGP-12177 therefore resembles that of the β_3-adrenoceptor. Nevertheless, there are selective β_3-adrenoceptor agonists (SB-226552; SB-251023) which do not activate β_1- (or 'β_4-') adrenoceptors and which stimulate lipolysis in human white adipocytes.[19–21] Thus the low

levels of β_3-adrenoceptor mRNA in human white adipocytes appear to produce sufficient β_3-adrenoceptors to mediate functional responses to highly selective β_3-adrenoceptor agonists.

In the present study we provide direct evidence for the existence of β_3-adrenoceptor protein in white adipocytes from both lean and obese subjects and in brown adipocytes from phaeochromocytoma patients using immunohistochemistry coupled with light microscopy. A previous study demonstrated the selectivity of the antibody that we have used for the human cloned β_3-adrenoceptor over β_1- and β_2-adrenoceptors, and detected the β_3-adrenoceptor in adipocyte membranes by time-resolved fluorescence.[8] The authors were unable, however, to detect the β_3-adrenoceptor in intact adipocytes. Our success in this respect may be due to our experience in fixing and embedding adipose tissue samples.[22] We also demonstrate the presence of the β_3-adrenoceptor in some other human tissues, notably myocardium.

Staining was often localized to the periphery of cells (eg Figure 3d), as would be expected of a 7-transmembrane receptor. Moreover, the tissue distribution of immunoreactivity stongly supports the evidence of the previous paper.[8] that the antibody selectively detects the β_3-adrenoceptor rather than β_1- or β_2-adrenoceptors. First, no immunoreactivity was detected in lung, skeletal muscle or arterioles, which are known to express high numbers of β_2-adrenoceptors. Secondly, staining of cardiac tissues and intestine was far less pronounced than would be expected of an antibody that reacts with β_1-adrenoceptors. Thirdly, staining was especially pronounced in multilocular adipocytes of perirenal tissue from phaeochromocytoma patients, consistent with the higher expression of β_3-adrenoceptor mRNA in brown compared to white adipocytes.[10,16] In contrast, expression of the β_1-adrenoceptor is lower in brown than in white adipocytes, at least during the transformation of bovine perirenal adipose tissue from brown into white fat.[23] Lastly, staining was less in obese than in lean subjects but was restored to the level in lean subjects by treatment with ephedrine and caffeine. Since ephedrine promotes noradrenaline release as well as causing some direct stimulation of adrenoceptors, and with caffeine it enhances sympathetic activity, it is to be expected that it would have a similar effect to phaeochromocytoma on β_3-adrenoceptor expression. Indeed there is evidence that the thermogenic effect of ephedrine in humans is in part mediated by β_3-adrenoceptors[24] and that the β_3-adrenoceptor-mediated component of this effect is enhanced by repeated administration of ephedrine or ephedrine plus caffeine.[25,26] This latter finding also accords with the expectation that the thermogenic response to a β_3-adrenoceptor agonist should increase during chronic therapy, at least in part because it leads to increases in brown adipocyte and therefore β_3-adrenoceptor numbers. Indeed, clinical data from the obese subjects of the present study on body composition, resting energy expenditure and lipid metabolism are overall compatible with a more pronounced thermogenic and lipolytic effect by ephedrine plus caffeine plus energy restriction as compared to energy restriction alone (manuscript in preparation).

The role of the β_3-adrenoceptor in the human heart, like that in human white adipose tissue, is unclear (see Gauthier *et al*[27] for review). Some workers have detected β_3-adrenoceptor mRNA in human atrium and some in ventricle.[10,11,28] There is, however, no evidence that the β_3-adrenoceptor mediates a functional response in human atrium. The work of Kaumann and colleagues demonstrated the presence of a β_4-adrenoceptor-mediated positive inotropic response in human atrium, but the β_4-adrenoceptor is now believed to be a form of the β_1-adrenoceptor.[29] β_3-Adrenoceptor agonists that have no effect at the β_1-adrenoceptor were ineffective in human atrium.[20] In contrast, Gauthier, Balligand and colleagues have described a β_3-adrenoceptor-mediated response in human ventricle, but surprisingly this is an inhibition rather than stimulation of contraction,[27] emphasizing the potential of β_3-adrenoceptor agonists to stimulate metabolic rate without increasing heart rate in humans.

The present study shows that β_3-adrenoceptor immunoreactivity is present in human ventricle. The same antibody has been used previously to detect the β_3-adrenoceptor in left ventricular myocytes,[30] but the first study using this antibody detected the β_3-adrenoceptor in human atrium but not ventricle.[8] The balance of evidence is nevertheless that the β_3-adrenoceptor is present in human ventricle where it mediates a negative inotropic response.

Our finding of β_3-adrenoceptor immunoreactivity in intestine agrees with work using a different antibody[31] and is consistent with evidence that β_3-adrenoceptor mRNA is expressed in human small intestine.[11,14] Functional evidence for β_3-adrenoceptors in the human gastrointestinal tract is restricted to work with colon tissue. The finding of immunoreactivity in gall bladder and prostate is also consistent with previous work.[8,10,11,32–34]

We did not detect β_3-adrenoceptor immunoreactivity in human skeletal muscle. β_3-Adrenoceptor expression in skelatal muscle remains controversial. Skeletal muscle appears to be a site of β_3-adrenoceptor-stimulated thermogenesis,[35] functional studies suggest that the receptor is present in rat soleus muscle,[36] and previous work with the same antibody did detect β_3-adrenoceptor immunoreactivity in a proportion of human myofibrils in two out of three samples of gastrocnemius muscle.[8] These discrepancies may reflect an irregular β_3-adrenoceptor distribution in the sites from which skeletal muscle was obtained. Differences in β-adrenoceptor density among muscle fibre types have been described previously.[37]

In conclusion, we have detected the β_3-adrenoceptor by immunohistochemistry in intact human adipocytes. The receptor was more abundant in multilocular than in unilocular adipocytes. Its abundance was lower in adipocytes from obese than from lean subjects, but therapy with ephedrine and caffeine increased expression in the obese subjects.

These findings support the potential of β_3-adrenoceptor agonists in the treatment of obesity and type 2 diabetes.

Acknowledgements

We wish to thank Dr Mauro Toppino for providing adipose tissue biopsies and Professor Franco Balzola, Dr Sebastiano Avagnina (Molinette Hospital, Turin) and Professor Francesco Cavagnini (Istituto Auxologico Italiano, Milano) for their valuable contribution to the clinical protocol in the obese patients; we thank Dr Michele Bisceglia ('Casa Sollievo della Sofferenza' Hospital, S. Giovanni Rotondo), Professor Aldo Bertani, Professor Aroldo Fianchini, Professor Edoardo Landi, Professor Cristina Marmorale, Professor Giovanni Muzzonigro (University Hospital, Ancona) for providing human tissue biopsies and Dr Claudia Venturini for her valuable help (Ancona University). This work was supported by grants from the Italian Ministry of Health (RF 1996) to Istituto Auxologico Italiano and from the Italian Ministery of University and Scientific Research (MIUR) and the University of Ancona to S Cinti.

References

1 Himms-Hagen J. Brown adipose tissue thermogenesis and obesity. *Prog Lipid Res* 1989; **28**: 67–115.
2 Cannon B, Nedergaard J. In: Blatteis, CM (ed). *Physiology and Pathophysiology of Temperature Regulation*. World Scientific: Memphis, TN; 1998. pp 63–77.
3 Cinti S. *The Adipose Organ*. Editrice Kurtis: Milan; 1999. plate 44.
4 Himms-Hagen J, Melnyk A, Zingaretti MC, Ceresi E, Barbatelli G, Cinti S. Multilocular fat cells in WAT of CL-316243-treated rats derive directly from white adipocytes. *Am J Physiol Cell Physiol* 2000; **279**: C670–C681.
5 Arch JRS. The β_3-adrenergic system and β_3-adrenergic agonists. *Rev Endocr Metab Dis* 2001; **2**: 385–393.
6 Arch JRS, Ainsworth AT, Ellis RDM, Piercy V, Thody VE, Thurlby PL, Wilson C, Wilson S, Young P. Treatment of obesity with thermogenic β-adrenoceptor agonists: studies on BRL 26830A in rodents. *Int J Obes*. 1984; **8**(Suppl): 1–11.
7 Strosberg AD, Gerhardt CC. In: Strosberg AD (ed.). *The β_3-adrenoceptor*. Taylor & Francis: London; 2000. pp 1–19.
8 Chamberlain PD, Jennings KH, Paul F, Cordell J, Berry A, Holmes SD, Park J, Sennitt MV, Stock MJ, Cawthorne MA, Young PW, Murphy GJ. The tissue distribution of the human β_3-adrenoceptor studies using a monoclonal antibody. *Int J Obes Relat Metab Disord* 1999; **23**: 1057–1065.
9 Hsu SM, Raine L, Fanger H. Use of avidin-biotin-peroxidase complex (ABC) in immonoperoxidase technique: a comparison between ABC and unlabelled antibody (PAP procedures). *J Histochem Cytochem* 1981; **29**: 577–580.
10 Krief S, Lonnqvist F, Raimbault S, Baude B, Van Spronson A, Arner P, Strosberg AD, Ricquier D, Emorine LJ. Tissue distribution of β_3-adrenergic receptor mRNA in man. *J Clin Invest* 1993; **91**: 344–349.
11 Berkowitz DE, Nardone NA, Smiley RM, Price DT, Kreutter DK, Fremeau RT, Schwinn DA. Distribution of β_3-adrenoceptor mRNA in human tissues. *Eur J Pharmac* 1995; **289**: 223–228.
12 Tavernier G, Barbe P, Galitzky J, Berlan M, Caput D, Lafontan M, Langin D. Expression of β_3-adrenoceptors with low lipolytic action in human subcutaneous white adipocytes. *J Lipid Res* 1996; **37**: 87–97.
13 Granneman JG, Lahners KN, Chaudhry A, Rao DD. Characterization of the human β_3-adrenergic receptor gene Rodent and human β 3-adrenergic receptor genes contain an intron within the protein-coding block. *Mol Pharmac* 1992; **42**: 964–970.
14 Granneman JG, Lahners KN, Chaudhry A. Characterization of the human β 3-adrenergic receptor gene. *Mol Pharmac* 1993; **44**: 264–270.
15 Thomas RF, Liggett SB. Lack of β_3-adrenergic receptor mRNA expression in adipose and other metabolic tissues in the adult human. *Mol Pharmac* 1993; **43**: 343–348.
16 Deng C, Paoloni-Giacobino A, Kuehne F, Boss O, Revelli J-P, Moinat M, Cawthorne MA, Muzzin P, Giacobino JP. Respective degree of expression of β_1-, β_2- and β_3-adrenoceptors in human brown and white adipose tissues. *Br J Pharmac* 1997; **118**: 929–934.
17 Ito M, Grujic D, Abel ED, Vidalpuig A, Susulic VS, Lawitts J, Harper, ME, Himms-Hagen J, Strosberg AD, Lowell BB. Mice expressing human but not murine β-adrenergic receptors under the control of human gene regulatory elements. *Diabetes* 1998; **47**: 1464–1471.
18 Konkar AA, Zhai Y, Granneman JG. β_1-adrenergic receptors mediate β_3-adrenergic-independent effects of CGP 12177 in brown adipose tissue. *Mol Pharmac* 2000; **57**: 252–258.
19 Konkar AA, Zhu Z, Granneman JG. Aryloxypropanolamine and catecholamine ligand interactions with the β_1-adrenergic receptor: evidence for interaction with distinct conformations of β_1-adrenergic receptors. *J Pharmac Exp Ther* 2000; **294**: 923–932.
20 Sennitt MV, Kaumann AJ, Molenaar P, Beeley LJ, Young PW, Kelly J, Chapman H, Henson SM, Berge JM, Dean DK, Kotecha NR, Morgan HKA, Rami HK, Ward RW, Thompson M, Wilson S, Smith SA, Cawthorne MA, Stock MJ, Arch JRS. The contribution of classical ($\beta_{1/2}$-) and atypical adrenoceptors to the stimulation of human white adipocyte lipolysis and right atrial appendage contraction by novel β_3-adrenoceptor agonists of differing selectivities. *J Pharmac Exp Ther* 1998; **285**: 1084–1095.
21 Arch JRS, Kaumann AJ, Molenaar P, Sennitt MV, Berge JM, Chapman H, Kelly J. Studies on a novel selective β_3-adrenoceptor agonist in human right atrial appendage and human white adipocytes. *Br J Pharmac* 1999; **126**: 100P.
22 Cinti S, Zingaretti MC, Cancello R, Ceresi E, Ferrara P. Morphologic techniques for the study of brown adipose tissue and white adipose tissue. In: Ailhaud G (ed.). *Methods in molecular biology: adipose tissue protocols*. Humana Press: Totowa, NJ; 2001. pp 21–51.
23 Casteilla L, Muzzin P, Revelli J-P, Ricquier D, Giacobino J-P. Expression of β_1- and β_3-adrenergic-receptor messages and adenylate cyclase β-adrenergic response in bovine perirenal adipose tissue during its transformation from brown into white fat. *Biochem J* 1994; **297**: 93–97.
24 Liu Y-L, Toubro S, Astrup A, Stock MJ. Contribution of β_3-adrenoceptor activation to ephedrine-induced thermogenesis in humans. *Int J Obes Relat Metab Diso* 1995; **19**: 678–685.
25 Astrup A, Lundsgaard C, Madsen J, Christensen NJ. Enhanced thermogenic responsiveness during chronic ephedrine treatment in man. *Am J Clin Nutr* 1985; **42**: 83–94.
26 Greenway F, de Jonge L, Tucker E, Rood J, Smith S. Caffeine and ephedrine become more beta-3 selective with time. *Obesi Res* 2000; **8**(Suppl 1): 70S.
27 Gauthier C, Langin D, Balligand J-L. β_3-Adrenoceptors in the cardiovascular system. *Trends Pharmac Sci* 2000; **21**: 426–431.
28 Gauthier C, Tavernier G, Charpentier F, Langin D, Le Marec H. Functional β_3-Adrenoceptor in the human heart. *J Clin Invest* 1996; **98**: 556–562.
29 Kaumann AJ, Engelhardt S, Hein L, Molenaar P, Lohse M. Abolition of (−)-CGP 12177-evoked cardiostimulation in double β_1/β_2-adrenoceptor knockout mice. Obligatory role of β_1-adrenoceptors for putative β 4-adrenoceptor pharmacology. *Naunyn-Schmiedeberg's Arch Pharmac* 2001; **363**: 87–93.
30 Moniotte S, Kobzik L, Feron O, Trochu J-N, Gauthier C, Balligand J-L. Upregulation of β_3-adrenoceptors and altered contractile response to inotropic amines in human failing myocardium. *Circulation* 2001; **103**: 1649–1655.

31 Anthony A, Schepelmann S, Guillaume J-L, Strosberg AD, Dhillon AP, Pounder RE, Wakefield AJ. Localization of the β (beta)$_3$-adrenoceptor in the human gastrointestinal tract: an immunohistochemical study. *Aliment Pharmac Ther* 1998; **12**: 519–525.
32 Igawa Y, Yamazaki Z, Takeda H, Hayakawa K, Akahane M, Ajisawa Y, Yoneyama T, Nishizawa O, Andersson KE. Functional and molecular biological evidence for a possible beta3-adrenoceptor in the human detrusor muscle. *Br J Pharmac* 1999; **126**: 819–825.
33 Takeda M, Obara O, Mizusawa T, Tomita Y, Arai K, Tsutsui T, Hatano A, Takahashi K, Nomura S. Evidence for β_3-adrenoceptor subtypes in relaxation of the human urinary bladder detrusor: analysis by molecular biological and pharmacological methods. *J Pharmac Exp Ther* 1999; **288**: 1367–1373.
34 Fujimura T, Tamura K, Tsutumi T, Yamamoto T, Nakamura K, Koibuchi Y, Kobayashi M, Yamaguchi O. Expression and possible functional role of the β_3-adrenoceptor in human and rat detrusor muscle. *J Urol* 1999; **161**: 680–685.
35 Thurlby PL, Ellis RDM. Differences between the effects of noradrenaline and the β-adrenoceptor agonist BRL 28410 in brown adipose tissue and hind limb of the anaesthetized rat. *Can J Physiol Pharmac* 1986; **64**: 1111–1114.
36 Challis RAJ, Leighton B, Wilson S, Thurlby PL, Arch JRS. An investigation of the β-adrenoceptor that mediates metabolic responses to the novel agonist BRL 28410 in rat soleus muscle. *Biochem Pharmac* 1988; **37**: 947–950.
37 Martin WHI, Korte E, Tolley TK, Saffitz JE. Skeletal muscle β-adrenoceptor distribution and responses to isoproterenol in hyperthyroidism. *Am J Physiol* 1992; **262**: E504–E510.