Journal of Cell Science 110, 801-807 (1997)
Printed in Great Britain © The Company of Biologists Limited 1997
JCS3511

Human immortalized brown adipocytes express functional β_3-adrenoceptor coupled to lipolysis

Vladimir Zilberfarb[1], France Piétri-Rouxel[1], Ralf Jockers[1], Stéphane Krief[1], Claude Delouis[2], Tarik Issad[1] and A. Donny Strosberg[1,*]

[1]Institut Cochin de Génétique Moléculaire – Laboratoire d'Immuno-Pharmacologie Moléculaire, CNRS UPR 0415 et Université de Paris – 22, rue Méchain, 75014 Paris, France
[2]URA-INRA de Génétique Moléculaire, Ecole Nationale Vétérinaire d'Alfort, 7 av. du Général de Gaulle, 94704, Maisons-Alfort, Cedex, France

*Author for correspondence

SUMMARY

Human brown pre-adipocytes were immortalized by microinjection of the genes encoding simian virus 40 T and t antigens under the control of the human vimentin promotor. The transfected pre-adipocytes were cultured for several months with no loss of their morphological characteristics. These cells accumulate lipids and differentiate into adipocytes when treated with insulin, triiodothyronine and dexamethazone. The mRNA of various adipocyte markers was detected by reverse transcriptase-polymerase chain reaction analysis, including hormone-sensitive lipase, lipoprotein lipase, adipsin, glucose transporters 1 and 4, the uncoupling protein (specific of brown adipocytes), and leptin, the product of the *ob* gene. Pharmacological analyses indicated that the β_3-adrenoceptor is the predominant β-adrenoceptor subtype in PAZ6 cells and that this receptor subtype is functionally coupled to adenylate cyclase and lipolysis. The immortalization of human adipocytes will permit pharmacological analysis of the human β_3-adrenoceptor function in adipose cells and will allow detailed studies of human adipocyte differentiation.

Key words: Human brown adipocyte, Immortalization, Beta-adrenergic receptor

INTRODUCTION

Obesity, which results from an imbalance between caloric intake and energy expenditure, is highly correlated with insulin resistance and diabetes in experimental animals and humans. Since adipose tissue is the major site of energy storage and mobilization, considerable efforts have been made to study adipocyte physiology and metabolism in intact animals, primary fat cells and in murine adipocyte-like cell lines, such as 3T3 L1, 3T3-F442A, Ob 1771 and RBM-Ad (Green and Kehinde, 1974; Negrel et al., 1978; Marko et al., 1995), but no human preadipocyte cell line has, until now, been maintained in culture long enough to study differentiation under a variety of conditions. This report describes the successful immortalization of human brown preadipocytes and shows that insulin treatment converts these cells into brown adipocytes which store fat in multilocular droplets and express a number of markers often found in or specific to adipocytes, including adipsin, the glucose transporters Glut1[1] and Glut4, leptin, lipoprotein lipase (LPL), hormone-sensitive lipase (HSL), uncoupling protein (UCP) and β_3-adrenoceptor.

Studies on catecholamine-induced lipolysis in these cells suggest that the β_3-adrenoceptor (β_3-AR) (Emorine et al., 1989, 1994; Strosberg and Piétri-Rouxel, 1996) is essential for regulating fat metabolism in these human adipocytes. While it is now widely accepted that rodent β_3-AR acts in this way, there is still debate about the human receptor (Arch and Wilson, 1996), despite evidence from studies on lipolysis in human mammary (Tavernier et al., 1996) and omental fat (Lönnqvist et al., 1993), as well as the discovery of an association between a single amino acid substitution in the human β_3-adrenoceptor and the early onset of non-insulin-dependent diabetes mellitus in Pima Indians (Walston et al., 1995), greater frequency of obesity in Japanese patients (Kadowaki et al., 1995), and a correlation with greater dynamic capacity to put on weight in French (Clément et al., 1995) and Finnish obese patients (Widen et al., 1995). The present study shows the immortalization of human adipocytes which are an appropriate tool for defining the precise role played by the β_3-adrenoceptor in human physiology, and specifically in the regulation of lipid metabolism.

MATERIALS AND METHODS

Isolation and immortalization of human adipocytes

Human vascular stromal cells from infant brown adipose tissue were obtained by collagenase digestion as described by Hauner et al. (1989). Tissue was digested in Krebs-Ringer buffer containing 2 mg/ml collagenase (collagenase A, Boehringer) and 20 mg/BSA at 37°C for 40 minutes in a shaking waterbath at 100 strokes/minutes. The digest was filtered through a nylon screen (pore size 190 μm) and the tissue

remnants were again digested for 30 minutes and filtered. Cells were collected by centrifugation at 200 ***g*** for 10 minutes, washed once with Krebs-Ringer containing BSA and once with DMEM/Ham's F12 medium (1:1, v:/v). Red blood cells were removed by treatment with Gey's buffer. The vascular stroma cells were seeded into 35 mm culture dishes at a density of 20,000/cm^2 in 2 ml DMEM/Ham's F12 (1:1, v/v) supplemented with 100 μg/ml penicillin, 0.1 mg/ml streptomycin, 7.5% donor calf serum (Gibco) and 2.5% newborn calf serum (Boehringer). The cells were allowed to attach by incubation for 16 hours at 37°C in an atmosphere of 90% air/10% CO_2. The attached cells were washed with PBS and refed with DMEM/Ham's F12 medium (1:1, v/v) supplemented with 33 μM biotin, 17 μM pantothenate, 15 mM Hepes, 0.2 nM triiodothyronine, 100 U/ml penicillin, 0.1 mg/ml streptomycin (ITT medium), 7.5% donor calf serum and 2.5% newborn calf serum. The medium was changed every 2 days. Cells were transfected by microinjecting the genes coding for SV 40 large and small t antigens under the control of the vimentin promotor (Schwartz et al., 1991). The vector was kindly provided by Dr D. Paulin. Transfected preadipocytes were amplified and kept frozen in liquid nitrogen. These cells (referred to as PAZ6 cells) could be thawed, placed in culture dishes, grown and passaged weekly. The cells used for differentiation studies were seeded at 10,000 cells/cm^2 and cultivated for 3 days in medium containing 6% fetal calf serum until they become confluent. These cells were placed in ITT medium supplemented with 0.1 μM dexamethasone, 850 nM insulin, 1 nM triiodothyronine and 1 μM pioglitazone. The medium also contained 3-isobutyl-1 methyl-xanthine (IBMX; 0.25 mM) for the initial 4 days of differentiation. Cells were maintained under these culture conditions for 15 to 21 days, washed with PBS and placed in medium without insulin, dexamethasone and serum but supplemented with 10 μg/ml transferin for 24 hours before experiments.

Crude membrane preparation

The cell monolayers were washed with PBS, detached, harvested and centrifuged at 700 ***g*** for 10 minutes and incubated in 1 mM EDTA, 10 mM Tris-HCl, pH 7.5, 0.5 mM PMSF, 10 μg/ml benzamidine, 50 μg/ml leupeptin and 0.25 mg/ml concanavalin A for 60 minutes at 4°C. The cells were homogenized with a potter and centrifuged at 700 ***g*** for 10 minutes. The pellet was washed twice and the supernatants were collected and centrifuged at 50,000 ***g*** for 30 minutes at 4°C. The resulting membrane pellet was resuspended in 25 mM Tris-HCl, 1 mM EDTA, 10% glycerol and protease inhibitors. Protein concentration was determined by the method of Bradford using a Bio-Rad protein assay system with bovine serum albumin as standard.

Radioligand binding assay

Cell membranes (20-50 μg of proteins) were incubated in the presence of 500 pM [^{125}I]CYP with or without 50 μM bupranolol (to define non-specific binding) or with 100 nM (±)-2-(3-t-carbamoyl-4-hydroxyphenoxy)-ethylamino)-3-(4-(1-methyl-4-trifluoromethyl-2-imidazolyl)-phenoxy)-2-propanol methane sulfonate (CGP20712A) and/or 50 nM erythro-(±)-1-(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol (ICI118551) for 90 minutes at 25°C in a final volume of 250 μl (25 mM Tris-HCl, pH 7.4, 2 mM EDTA, 12.5 mM $MgCl_2$, supplemented with 0.50 mg/ml BSA and 1 μM desipramine). The reaction was terminated by rapid filtration through Whatman GF/C glass fiber filters that had been soaked for 30 minutes in 0.3% polyethyleneimine in PBS (to reduce non-specific binding).

Saturation experiments were done by incubating membrane samples (20-50 μg) for 30 minutes at 37°C with [^{125}I]CYP 1-4,000 pM) with or without 100 μM bupranolol for non-specific binding determination. The final volume of binding buffer was 250 μl, the reaction was terminated as described previously. Saturation experiments were analysed with the program EBDA/LIGAND (Biosoft, Elsevier, Cambridge, England) using Scatchard plot representation.

Assay of intracellular cyclic AMP

Cells were washed once in PBS and incubated in PBS containing 0.5 mM IBMX and 0.5 mM ascorbic acid, with or without 10 μM isoprenaline or (±)-4-(3-t-butylamino-2 hydroxypropoxy) benzimidazol-2-one (CGP12177A) for 15 minutes at 37°C. The incubation buffer was discarded, and the cells were lysed in 1 M NaOH for at least 20 minutes at 37°C. The lysate was neutralized with 1 M acetic acid and centrifuged in a microfuge at maximum speed for 5 minutes. The cyclic AMP in the supernatant was measured using an ^{3}H-cyclic AMP assay system (Amersham).

RNA analysis

Total RNA was extracted from PAZ6 cells (Cathala et al., 1983) and digested for 20 minutes at 37°C with 0.3 U RNase-free DNAse I (RQ1 DNase, Promega) per μg nucleic acid in 40 mM Tris-HCl, pH 7.9, 10 mM NaCl, 6 mM $MgCl_2$ plus 2 U/μl placenta RNase inhibitor (RNasin, Promega). RNA was then extracted with phenol/chloroform and precipitated with ethanol.

RT-PCR experiments

cDNA was synthesized at 37°C for 30 minutes with 100 U Moloney murine leukemia virus reverse transcriptase (Gibco BRL) using 200 ng DNased RNA in 10 μl reverse transcriptase buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$ and 10 mM DTT) containing 0.4 mM each dNTP, 60 μM random hexanucleotides (Pharmacia, France), and 2 U/μl placenta RNase inhibitor. The enzyme was then inactivated by heating the mixture at 94°C for 4 minutes. A control without reverse transcriptase was done to verify that residual genomic DNA was not amplified. Samples were then supplemented with 5 μl 10× PCR buffer (1× PCR buffer is: 50 mM KCl, 10 mM Tris-HCl, pH 9.0, at 25°C, 0.1% Triton X-100, and 1.5 mM $MgCl_2$), 0.5 μl 25 mM each dNTP, 10 μl 50% dimethylsulfoxide (for β_1-, β_2-, β_3-, α_{2A}-ARs, ob and adipsin), 0.5 μl 25 mM each sense and antisense oligonucleotides, 1.5 U *Thermus aquaticus* DNA polymerase (Promega) and water to 50 μl. cDNAs were denatured for 2 minutes at 94°C and amplified with 29 cycles: 94°C for 15 seconds; 60°C for 30 seconds (55°C for 30 seconds for ob and UCP); 72°C for 30 seconds, followed by 4 minutes of final extension at 72°C in a temperature cycler (GeneAmp PCR System 9600, Perkin-Elmer Cetus). The sequences of the sense and antisense oligonucleotides were: 5′-TCGTGTG-CACCGTGTGGGCC-3′ and 5′-AGGAAACGGCGCTCGCAGCT-GTCG-3′ and 265 base pairs for β_1-AR; 5′-GCCTGCTGACCAA-GAATAAGGCC-3′ and 5′-CCCATCCTGCTCCACCT-3′ and 329 base pairs for β_2-AR; 5′-ATGGCTCCGTGGCCTCAC-3′ and 5′-CCCAACGGCCAGTGGCCAGTCAGCG -3′and 317 base pairs for β3-AR (Krief et al., 1993). 5′-TTAGGAAGCAAGATTTTAGC-3′ and 5′-AAGTCGCAAGAAGGAAGGTA-3′ and 498 base pairs for UCP. 5′-GAGACCTTCAACACCCC-3′ and 5′-GTGGTGGT-GAAGCTGTAG-3′ and 236 base pairs for β-actin. 5′-CGAGCGAGCCAGGTGAAGCC-3′ and 5′-GCCAGCGGAAACCT-CACACG-3′ and 403 base pairs for α2-AR. 5′-TGCTGGCTGTGGGAGGA-3′ and 5′-GAGGATGCCGACGAC-GAT-3′ and 470 base pairs for GLUT1; 5′-TCCTGCTGCCCTTCT-GTC-3′ and 5′-GGCCTACCCCTGCTGTCT-3′ and 309 bp for GLUT4. 5′-CGGCTGGGGCATAGTCA-3′ and 5′-GCACGCCCC-CGCACACC-3′ and 200 base pairs for adipsin. 5′-GGGGCT-GAGTTTGAGCG-3′ and 5′-GCTCCTCACTGTCCTGTCC-3′ and 286 base pairs for hormone-sensitive lipase; 5′-CCTGCTCGTGCT-GACTCTG-3′ and 5′-GGGCTCCAAGGCTGTATC-3′ and 473 base pairs for lipoprotein lipase. 5′-GCTGTGCCCACTCAAAAAGT-3′ and 5′-ACTGCCAGTGTCTGGTCCAT-3′ and 182 base pairs for ob. All oligonucleotide sequences were determined using Oligo 4.0 (National Biosciences, Plymouth, MN 5547, USA) primer analysis software.

Northern blot experiments

Total RNA (35 μg) was denaturated by heating at 95°C for 3 minutes, size fractionated by electrophoresis on 1% agarose gels and transferred to a nylon membrane (Hybond-N, Amersham). The membrane

was hybridized with a human cDNA UCP probe (kindly provided by Dr Daniel Ricquier, CNRS, Centre de Recherche sur l'Endocrinologie Moléculaire et le Développement, Meudon-Bellevue, France). This probe was labeled using a rediprime labelling system kit (Amersham, Bucks, UK). The membranes were incubated overnight at 42°C and autoradiographed using Hyperfilm MP (Amersham) at –80°C with intensifiying screens.

Lipolysis experiments

Lipolysis was assessed by measuring the glycerol released from adherent cells in 6-well plates. Mature adipocyte monolayers were washed with PBS and pre-incubated overnight in DMEM/Ham's F12 (1:1, v/v) containing 1% fatty acid free BSA. The adipocytes were then incubated in Krebs-Ringer phosphate buffer, pH 7.4, supplemented with 2% fatty acid free BSA, 1 g/liter glucose and 50 µg/ml $Na_2S_2O_5$, with or without lipolytic agents for 2 hours at 37°C. Samples of incubation medium (400 µl) were removed and the glycerol content measured by monitoring the reduction of NAD to NADH at 340 nm after addition of 10 µg/ml *Enterobacter aerogenes* glycerol dehydrogenase (Boehringer Mannheim). The reaction buffer was 125 mM $K_2CO_3/NaHCO_3$, pH 10, 3.5 mM NAD, 330 mM $(NH_4)_2SO_4$.

Statistical analysis

The data are expressed as means ± s.e.m. for at least two to three experiments performed in duplicate. Significance between differences were assessed using Student's *t*-test.

RESULTS

Immortalization of human brown preadipocytes

Human vascular stromal cells from infant brown adipose tissue were isolated and transfected with a vector containing the genes coding for SV40 T and t antigens under the control of the vimentin promoter. The immortalized brown preadipocytes (PAZ6 preadipocytes) were passaged in culture for several months without losing their morphological characteristics, or their molecular markers. These markers included β_1 and β_2-AR (Fig. 1a), hormone sensitive lipase, adipsin and the glucose transporters Glut 1 and Glut 4 (Fig. 1b). These immortalized brown preadipocytes (Fig. 2a) could be converted to adipocytes at any time by treatment with insulin and dexamethasone, following which they accumulated multilocular fat (Fig. 2b). The rate of conversion was accelerated by pioglitazone (not shown). PCR analysis indicated that these differentiated cells express β_1-, β_2-, β_3-AR, α_{2A}-AR, lipoprotein lipase (Fig. 3a-b), the product of the ob gene, leptin (Fig. 4) and UCP (Fig. 5a). A signal corresponding to UCP mRNA was detected in preadipocytes and in differentiated adipocytes. This signal was more readily detectable in differentiated adipocytes that had been incubated with 10^{-5} M norepinephrine for 4 hours (Fig. 5a, lane 3). UCP mRNA was also demonstrated by northern blot hybridization of total RNA (Fig. 5b).

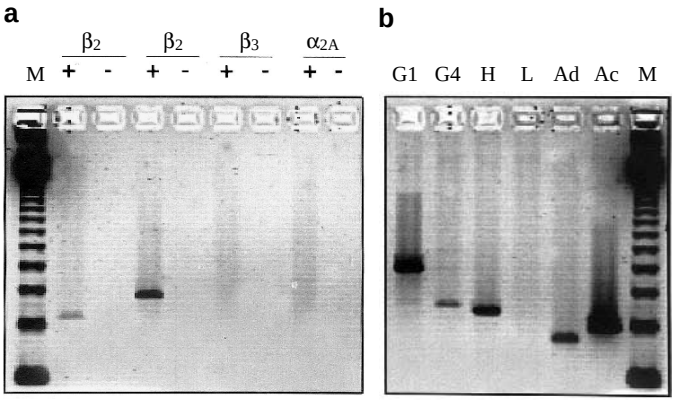

Fig. 1. Molecular markers in PAZ6 preadipocytes. (a) RT-PCR analysis of β_1-, β_2-, β_3-, α_{2A}-adrenoceptor mRNAs in PAZ6 preadipocytes; + and – symbols indicate the presence or absence of reverse transcriptase during reverse transcription. (b) G1, Glut 1; G4, Glut 4; H, HSL; L, LPL; Ad, adipsin and Ac, β-actin. No signal was detected in control experiments performed without reverse transcriptase (not shown). Size markers (M) were the 123 base pairs DNA ladder (Gibco BRL). Each lane contained 200 ng total RNA. cDNAs were amplified for 29 cycles of PCR.

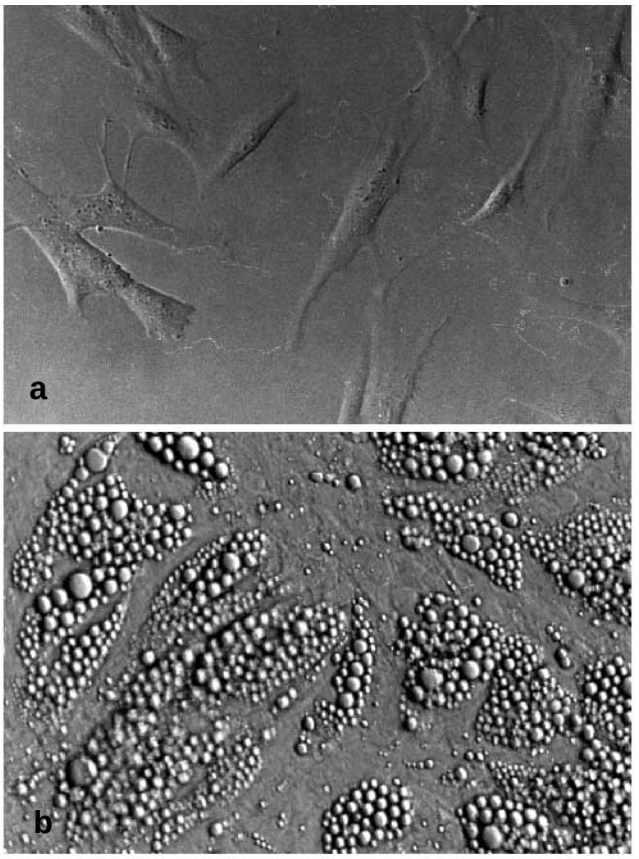

Fig. 2. Morphological changes associated with the differentiation of PAZ6 cells. The human brown preadipocytes (a) were differentiated to mature adipocytes (b) by incubation in ITT medium supplemented with 1 nM T3, 850 nM insulin, 100 nM dexamethasone and 1 µM pioglitazone for 3 weeks.

Determination of ICYP-binding sites in human brown adipocytes

RT-PCR experiments (Fig. 3a) revealed mRNA coding for the three β-adrenoceptor subtypes in differentiated adipocytes (PAZ-6 adipocytes). [^{125}I]Iodocyanopindolol (ICYP)-binding experiments were performed with subtype-specific ligands to determine whether these receptors were functional in PAZ-6 adipocytes. The capacity of β_1-AR selective (CGP20712A 100 nM) and β_2-AR selective (ICI 118551 50 nM) ligands to compete with 500 pM ICYP were tested (a saturating concen-

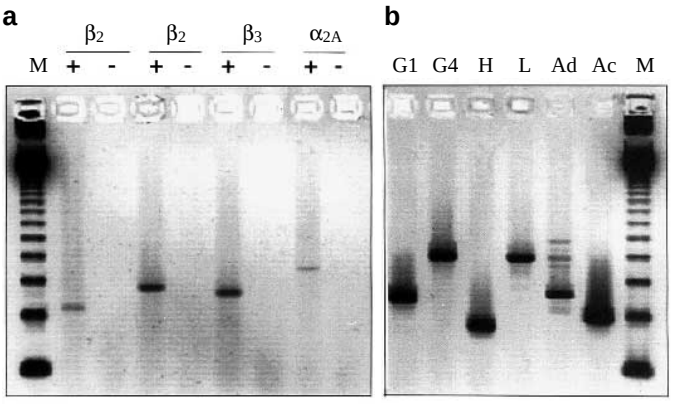

Fig. 3. Molecular markers in PAZ6 adipocytes. PAZ6 cells were differentiated as described in the legend to Fig. 2. (a) RT-PCR analysis of β_1-, β_2-, β_3-, α_{2A}-adrenoceptor mRNA in PAZ6 adipocytes; + and – symbols indicate the presence or absence of reverse transcriptase during reverse transcription. (b) H, HSL; L, LPL; Ad, adipsin; G1, Glut1; G4, Glut 4 and Ac, β-actin. No signal was detected in control experiments performed without reverse transcriptase (not shown). Size markers (M) are the 123 base pairs DNA ladder (Gibco BRL). Each lane contained 200 ng of total RNA. cDNAs were amplified for 29 cycles of PCR.

tration for β_1- and β_2-AR and a sub-saturating concentration for β_3-AR) (Blin et al., 1993).

Specific ICYP binding was decreased by 51±15% by the β_2-AR-selective antagonist ICI 118551. The β_1-AR-selective antagonist CGP 20712A decreased ICYP binding by 24±11%. Both antagonists used at the same time diminished the binding of ICYP by 74±9%. This indicates the presence of β_1-AR and β_2-AR plus other binding sites which could correspond to the β_3-AR subtype. However, binding is likely to be mainly by β_1-AR and β_2-AR receptor subtypes, under these experimental conditions (saturating concentration for β_1- and β_2-ARs and sub-saturating concentrations for β_3-ARs), leading to an underestimation of the proportion of β_3-adrenergic binding sites. ICYP binding sites were therefore assayed by saturation binding experiments.

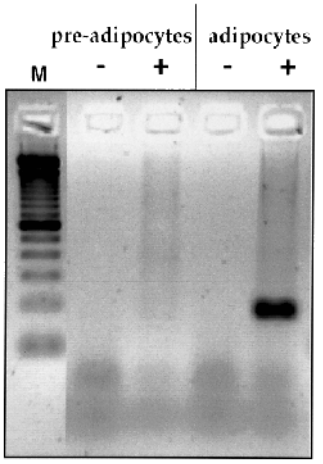

Fig. 4. Expression of the *ob* gene in preadipocytes and in differentiated adipocytes. PAZ6 cells were differentiated as described in the legend to Fig. 2. *ob* gene expression was analysed by RT-PCR. Each lane contained 200 ng total RNA. Size of the amplification product: 182 base pairs. cDNAs were amplified for 29 cycles; + and – symbols indicate the presence or absence of reverse transcriptase during reverse transcription. Size markers (M) are the 100 base pair DNA ladder (Gibco BRL).

Saturation binding analysis

The β-adrenocepter subtypes present on the differentiated PAZ6 cells were further characterized and quantified by saturation binding experiments on membrane preparations. The specific radioligand binding curve best fitted a model for a single class of low affinity binding sites (B_{max} = 596±263 fmoles/mg proteins; K_d = 0.52±0.23 nM). This K_d value is in good agreement with the values reported for human (Tate et al., 1991), mouse (Nahmias et al., 1991), rat (Granneman et al., 1991) and bovine (Piétri-Rouxel et al., 1995) β_3-AR. Thus, although there appear to be β_1 and β_2 binding sites on these cells according to radioligand competition experiments using selective antagonists, these sites are nearly undetectable in saturation binding experiments. The β_3-adrenoceptor therefore appears to be the most abundant subtype in these cells.

Accumulation of cAMP in the PAZ-6 cells

Stimulation of differentiated PAZ-6 cells with isoproterenol (10 μM) increased intracellular cAMP 10-fold over basal values (from 134±11 pmoles cAMP/mg protein to 1,349±149 pmoles cAMP/mg protein (n=4; P<0.001). The β1/β2-AR antagonist CGP 12177A (10 μM), which is a partial β3-AR agonist, increased cAMP production 2.3-fold (from 134±11 pmoles cAMP/mg proteins in control to 309±55 pmoles cAMP/mg proteins (n=4; P<0.02), suggesting the presence of a significant number of adenylyl cyclase-coupled β3-AR.

Lipolysis in PAZ-6 cells

Lipolysis in PAZ-6 cells was measured by determining the glycerol released by mature adipocytes treated with lipolytic agents. Forskolin produced a modest (30%), but significant, increase in lipolysis (Fig. 6). The physiological catecholamines epinephrine and norepinephrine had similar effects, as did isoproterenol, a general β-adrenergic agonist. The selective β_3-AR agonist CGP 12177A, also fully stimulated lipolysis, suggesting that β3-AR in PAZ-6 cells is functionally coupled to lipolysis.

DISCUSSION

β_3-adrenoceptors in human brown adipocytes

The cloning of the genes encoding human (Emorine et al., 1989), mouse (Nahmias et al., 1991) and rat (Granneman et al., 1991; Muzzin et al., 1991) β_3-AR species homologues led to extensive pharmacological characterization of the β_3-AR proteins in non-adipocyte cells (Blin et al., 1993). The significant differences found between pharmacology of the human and rodent β_3-AR have led some authors to suggest that they could be different subtypes. The cloned rodent β_3-AR and the previously described 'atypical' β-AR have been shown to constitute species that are homologues by comparative analyses of adipocyte-like 3T3-F442A cells (Fève et al., 1991), Chinese hamster ovary cells transfected with the human β_3-AR gene (Blin et al., 1994) and animal tissues (Granneman et al., 1991;

Muzzin et al., 1991; Granneman and Lahners, 1992). Doubts initially raised about the existence of a functional β_3-AR in human fat tissues (Zaagsma and Nahorski, 1990) were supported by certain negative data (Langin et al., 1991; Rosenbaum et al., 1993), obtained mostly with subcutaneous and omental white fat tissues (Van Liefde et al., 1994). The resulting controversy was not completely resolved by the description of β_3-AR specific mRNA in brown and white fat tissues (Krief et al., 1993; Berkowitz, et al., 1995). Several investigators suggested that the role of the β_3-AR in lipolysis and thermogenesis was less important in man than in the rodent.

Lönnqvist et al. (1993) used a more sensitive microdialysis technique than that used previously and confirmed that a β_3-AR agonist could indeed modulate lipolysis in human omental fat. More recently, Tavernier et al. (1996) obtained a similar result using mammary subcutaneous fat. According to Enocksson et al. (1995), β_2-AR is the most important receptor for the mobilization of lipids from human abdominal subcutaneous adipose tissue because it stimulates both lipolysis and blood flow, while β_3-AR acts on lipolysis but not on blood flow, as measured by ethanol escape. The sensitivity of β_3-AR, in white adipose tissue of obese patients (Lönnqvist et al., 1995), is enhanced about 50-fold, and the coupling efficiency is increased from 37 to 56%. Clément et al. (1995) showed that the single Trp 64 Arg substitution in the β_3-AR of patients with morbid obesity (Walston et al., 1995) is correlated with an increased dynamic capacity for these subjects to gain weight. This suggests that the β_3-AR plays a major role in the pathophysiology of obesity.

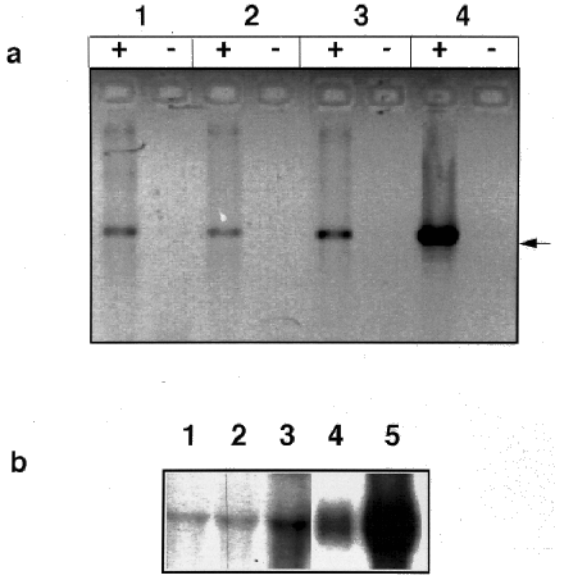

Fig. 5. Expression of the *UCP* gene in preadipocytes and differentiated adipocytes. PAZ6 cells were differentiated as described in the legend to Fig. 2. (a) RT-PCR analysis of the *UCP* gene expression: lane 1, PAZ6 preadipocytes; lane 2; PAZ6 differentiated adipocytes; lane 3; PAZ6 differentiated adipocytes incubated with 10^{-5} M norepinephrine for 4 hours; lane 4, positive control: human brown adipose tissue from a patient with pheochromocytoma; + and – symbols indicate the presence or absence of reverse transcriptase. Each lane contained 200 ng total RNA. Size of the amplification product: 498 base pairs. cDNAs were amplified for 33 cycles. (b) Northern blot analysis of UCP mRNA; 35 μg total mRNA were loaded in each lane, except in lane 4, where only 2 μg total mRNA were loaded. Lane 1, PAZ6 preadipocytes; lane 2, PAZ6 differentiated adipocytes; lane 3, PAZ6 differentiated adipocytes incubated with 10^{-5} M norepinephrine for 4 hours; lanes 4 and 5 positive control: human brown adipose tissue from a patient with pheochromocytoma. The size of the transcript is 1.9 kb. Densitometric scanning of the 18 S ribosomal RNA band stained with ethidium bromide indicated that equivalent amounts of RNA were indeed loaded in lanes 1, 2, 3 and 5 (not shown).

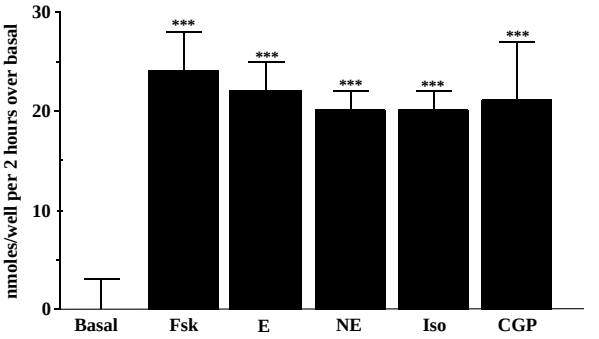

Fig. 6. Lipolysis in PAZ6 adipocytes. Lipolysis in PAZ6 cells as measured by glycerol released by incubation with 100 μM of various lipolytic agents. Basal glycerol release was 70±2 nmol/well per 2 hours. Fsk, forskolin; E: (–)-epinephrine; NE: (–)-norepinephrine; Iso: (–)-isoproterenol. ***P<0.001 when compared to the basal lipolysis.

Although β3-AR mRNA is present in human brown adipose tissue (Krief et al., 1993), pharmacological analysis of β3-AR in human brown adipocytes has not been performed yet, because it is difficult to routinely obtain such cells. Our RT-PCR results show that β_3-AR is expressed in PAZ-6 cells at the mRNA level using moderate (29 cycles) amplification and at the receptor protein level by saturation binding analysis which suggests that β_3-AR may be the predominant β-adrenoceptor subtype in human brown adipocytes.

The β_3-adrenoceptor is functionally coupled to lipolysis in human adipocytes

β_3-AR was shown to be coupled to adenylyl cyclase and lipolysis in PAZ-6 cells using the β_1/β_2-antagonist, CGP 12177A, which is a partial β_3-agonist. Although CGP 12177A had much less effect on the cAMP concentration than did isoproterenol, the efficacy to stimulate lipolysis was similar for the two agonists. This suggests that maximal lipolysis can be obtained with sub-maximal activation of adenylyl cyclase in these cells. Similar results have been obtained by others using freshly isolated rat adipocytes (Murphy et al., 1993).

Effect of pioglitazone on the differentiation of PAZ-6 preadipocytes into adipocytes

Insulin and dexamethasone cause PAZ6 preadipocytes to differentiate from fibroblast-like cells to mature adipocytes capable of accumulating fat globules and carrying out lipolysis under well regulated conditions. The rate of PAZ6 cell differentiation is considerably enhanced by pioglitazone. Thiazolidinediones have been shown to enhance insulin and dexamethasone-induced differentiation of murine 3T3-L1 fibroblasts into adipocytes (Kletzien et al., 1992), probably by acting as high affinity ligands for the peroxisome proliferator-activated receptor γ (PPARγ), a nuclear receptor that plays a

crucial role in adipogenesis (Tontonoz et al., 1994; Lehmann et al., 1995).

Leptin in brown adipocytes

A remarkable feature of the PAZ-6 cells is that they produce leptin once they have been converted into adipocytes. Leptin decreases appetite and increases energy expenditure when injected into ob/ob mice in which the corresponding gene is mutated, resulting in decreased obesity (Pelleymounter et al., 1995).

Leptin is also present in rat interscapular brown adipose tissue, and its amounts are markedly elevated in Zucker fatty rats, suggesting the presence of an obesity-linked regulation of the *ob* gene expression in brown adipose tissue as well as in white adipose tissue (Ogawa et al., 1995). The finding that human PAZ6 adipocytes also contain leptin thus provides a convenient system for studying the regulation of the human *ob* gene.

Conclusion

The establishment of human immortalized preadipocytes with cognate markers represents a first step towards obtaining clonal cell lines which should make possible precise pharmacological analyses of β_3-AR function, including ligand binding, adenylyl cyclase activation and lipolysis. This should yield information directly applicable to the development of therapeutically useful drugs. These cells are also suitable for studies on the various stages of adipocyte differentiation, i.e. the appearance of new markers and the possible disappearance of others, and should allow analysis of the influence of various conditions (insulin, glucocorticoids, T3, β_3-AR agonists and NPY) on adipocyte differentiation.

This work was mainly supported by the Centre National de la Recherche Scientifique and the Ministry for Science, Education and Research. We also thank the Ligue Nationale contre le Cancer, the Fondation pour la Recherche Médicale, the Association pour la Recherche contre le Cancer, MIEC CHRX-CT 94-0490 contract for Human Capital and Mobility, and ENBEST CHRX-CT 94-0689, for financial support. We thank Prof. Helardot (Hôpital St-Vincent-de-Paul, Paris) for providing the human brown adipose tissue, Prof. D. Paulin for advice on immortalization of cells, L. Guillaud for microinjection of the preadipocytes, Prof. B. Cannon (Stockholm) for help and advice in performing lipolysis experiments and Dr D. Ricquier (CNRS, Paris) for advice for UCP characterization.

REFERENCES

Arch, J. R. S. and Wilson, S. (1996). Prospects for beta(3)-adrenoceptor agonists in the treatment of obesity and diabetes. *Int. J. Obes.* **20**, 191-199.

Berkowitz, D. E., Nardone, N. A., Smiley, R. M., Price, D. T., Kreutter, D. K., Fremeau, R. T. and Schwinn, D. A. (1995). Distribution of beta 3-adrenoceptor mRNA in human tissues. *Eur. J. Pharmacol.* **289**, 223-228.

Blin, N., Camoin, L., Maigret, B. and Strosberg, A. D. (1993). Structural and conformational features determining selective signal transduction in the beta 3-adrenergic receptor. *Mol. Pharmacol.* **44**, 1094-1104.

Blin, N., Nahmias, C., Drumare, M. F. and Strosberg, A. D. (1994). Mediation of most atypical effects by species homologues of the β3-adrenoceptor. *Br. J. Pharmacol.* **112**, 911-919.

Cathala, G., Savouret, J. F., Mendez, B., West, B. L., Karin, M., Martial, J. A. and Baxter, J. D. (1983). A method for isolation of intact, translationally active ribonucleic acid. *DNA* **2**, 329-335.

Clément, K., Vaisse, C., Manning, B. S., Basdevant, A., Guy, G. B., Ruiz, J., Silver, K. D., Shuldiner, A. R., Froguel, P. and Strosberg, A. D. (1995). Genetic variation in the beta 3-adrenergic receptor and an increased capacity to gain weight in patients with morbid obesity. *New Eng. J. Med.* **333**, 352-354.

Emorine, L. J., Marullo, S., Briend-Sutren, M.-M., Patey, G., Tate, K., Delavier-Klutchko, C. and Strosberg, A. D. (1989). Molecular characterization of the human β3-adrenergic receptor. *Science* **245**, 1118-1121.

Emorine, L., Blin, N. and Strosberg, A. D. (1994). The human beta(3)-adrenoceptor – the search for a physiological function. *Trends Pharmacol. Sci.* **15**, 3-7.

Enocksson, S., Shimizu, M., Lönnqvist, F., Nordenstrom, J. and Arner, P. (1995). Demonstration of an in vivo functional beta 3-adrenoceptor in man. *J. Clin. Invest.* **95**, 2239-2245.

Fève, B., Emorine, L. J., Lasnier, F., Blin, N., Baude, B., Nahmias, C., Strosberg, A. D. and Pairault, J. (1991). Atypical β-adrenergic receptor in 3T3-F442A adipocytes. Pharmacological and molecular relationship with the human β3-adrenergic receptor. *J. Biol. Chem.* **266**, 20329-20336.

Granneman, J. G., Lahners, K. N. and Chaudhry, A. (1991). Molecular cloning and expression of the rat β3-adrenergic receptor. *Mol. Pharmacol.* **40**, 895-899.

Granneman, J. G. and Lahners, K. N. (1992). Differential adrenergic regulation of β1- and β3-adrenoreceptor messenger ribonucleic acids in adipose tissues. *Endocrinology* **130**, 109-114.

Green, H. and Kehinde, O. (1974). Sublines of mouse 3T3 cells that accumulate lipid. *Cell* **1**, 113-116.

Hauner, H., Entenmann, G., Wabitsch, M., Gaillard, D., Ailhaud, G., Negrel, R. and Pfeiffer, E. F. (1989). Promoting effect of glucocorticoids on the differentiation of human adipocyte precursor cells cultured in a chemically defined medium. *J. Clin. Invest.* **84**, 1663-1670.

Kadowaki, H., Yasuda, K., Iwamoto, K., Otabe, S., Shimokawa, K., Silver, K., Walston, J., Yoshinaga, H., Kosaka, K. and Yamada, N. (1995). A mutation in the beta 3-adrenergic receptor gene is associated with obesity and hyperinsulinemia in Japanese subjects. *Biochem. Biophys. Res. Commun.* **215**, 555-560.

Kletzien, R. F., Clarke, S. D. and Ulrich, R. G. (1992). Enhancement of adipocyte differentiation by an insulin-sensitizing agent. *Mol. Pharmacol.* **41**, 393-398.

Krief, S., Lönnqvist, F., Raimbault, S., Baude, B., Van Spronsen, A., Arner, P., Strosberg, A. D., Ricquier, D. and Emorine, L. J. (1993). Tissue distribution of β3-adrenergic receptors mRNA in man. *J. Clin. Invest.* **91**, 344-349.

Langin, D., Portillo, M. P., Saulnier-Blache, J.-S. and Lafontan, M. (1991). Coexistence of three β-adrenoceptor subtype in white fat cells of various mammalian species. *Eur. J. Pharmacol.* **199**, 291-301.

Lehmann, J. M., Moore, L. B., Smith, O. T., Wilkison, W. O., Willson, T. M. and Kliewer, S. A. (1995). An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor gamma (PPAR gamma). *J. Biol. Chem.* **270**, 12953-12956.

Lönnqvist, F., Krief, S., Strosberg, A. D., Nyberg, B., Emorine and L. J. Arner, P. (1993). Evidence for a functional β3-adrenoceptor in man. *Br. J. Pharmacol.* **110**, 929-936.

Lönnqvist, F., Thome, A., Nilsell, K., Hoffstedt, J. and Arner, P. (1995). A pathogenic role of visceral fat beta 3-adrenoceptors in obesity. *J. Clin. Invest.* **95**, 1109-1116.

Marko, O., Cascieri, M. A., Ayad, N., Strader, C. D. and Candelore, M. R. (1995). Isolation of a preadipocyte cell line from rat bone marrow and differentiation to adipocytes. *Endocrinology* **136**, 4582-4588.

Murphy, G. J., Kirkham, D. M., Cawthorne, M. A. and Young, P. (1993). Correlation of beta 3-adrenoceptor-induced activation of cyclic AMP-dependent protein kinase with activation of lipolysis in rat white adipocytes. *Biochem. Pharmacol.* **46**, 575-581.

Muzzin, P., Revelli, J.-P., Kuhne, F., Gocayne, J. D., McCombie, W. R., Venter, J. C., Giacobino, J.-P. and Fraser, C. M. (1991). An adipose tissue-specific β-adrenergic receptor. Molecular cloning and down-regulation in obesity. *J. Biol. Chem.* **266**, 24053-24058.

Nahmias, C., Blin, N., Elalouf, J.-M., Mattei, M. G., Strosberg, A. D. and Emorine, L. J. (1991). Molecular characterization of the mouse β3-adrenergic receptor: relationship with the atypical receptor of adipocytes. *EMBO J.* **10**, 3721-3727.

Negrel, R., Grimaldi, P. and Ailhaud, G. (1978). Establishment of preadipocyte clonal line from epididymal fat pad of ob/ob mouse that responds to insulin and to lipolytic hormones. *Proc. Nat. Acad. Sci. USA* **75**, 6054-6058.

Ogawa, Y., Masuzaki, H., Isse, N., Okazaki, T., Mori, K., Shigemoto, M.,

Satoh, N., Tamura, N., Hosoda, K. and Yoshimasa, Y. (1995). Molecular cloning of rat obese cDNA and augmented gene expression in genetically obese Zucker fatty (fa/fa) rats. *J. Clin. Invest.* **96**, 1647-1652.

Pelleymounter, M. A., Cullen, M. J., Baker, M. B., Hecht, R., Winters, D., Boone, T. and Collins, F. (1995). Effects of the obese gene product on body weight regulation in ob/ob mice. *Science* **269**, 540-543.

Piétri-Rouxel, F., Lenzen, G., Kapoor, A., Drumare, M. F., Archimbault, P., Strosberg, A. D. and Manning, B. S. (1995). Molecular cloning and pharmacological characterization of the bovine beta 3-adrenergic receptor. *Eur. J. Biochem.* **230**, 350-358.

Rosenbaum, M., Malbon, C. C., Hirsch, J. and Leibel, R. L. (1993). Lack of beta 3-adrenergic effect on lipolysis in human subcutaneous adipose tissue. *J. Clin. Endocrinol. Metab.* **77**, 352-355.

Schwartz, B., Vicart, P., Delouis, C. and Paulin, D. (1991). Mammalian cell lines can be efficiently established in vitro upon expression of the SV40 large T antigen driven by a promoter sequence derived from the human vimentin gene. *Biol. Cell* **73**, 7-14.

Strosberg, A. D. and Piétri-Rouxel, F. (1996). Function and regulation of the β3-adrenoceptor. *Trends Pharmacol. Sci.* **17**, 373-381.

Tate, K. M., Briend-Sutren, M.-M., Emorine, L. J., Delavier-Klutchko, C., Marullo, S. and Strosberg, A. D. (1991). Expression of three human β-adrenergic-receptor subtypes in transfected Chinese hamster ovary cells. *Eur. J. Biochem.* **196**, 357-361.

Tavernier, G., Barbe, P., Galitzky, J., Berlan, M., Caput, D., Lafontan and M. Langin, D. (1996). Expression of β3-adrenoceptors with low lipolytic action in human subcutaneous white adipocytes. *J. Lipid. Res.* **37**, 1-11.

Tontonoz, P., Hu, E. and Spiegelman, B. M. (1994). Stimulation of adipogenesis in fibroblasts by PPAR gamma 2, a lipid-activated transcription factor. *Cell* **79**, 1147-1156.

Van Liefde, I., Van Ermen, A. and Vauquelin, G. (1994). No functional atypical beta-adrenergic receptors in human omental adipocytes. *Life Sci.* **54**, 209-214.

Walston, J., Silver, K., Bogardus, C., Knowler, W. C., Celi, F. S., Austin, S., Manning, B., Strosberg, A. D., Stern, M. P. and Raben, N. (1995). Time of onset of non-insulin-dependent diabetes mellitus and genetic variation in the beta 3-adrenergic-receptor gene. *New Eng. J. Med.* **333**, 343-347.

Widen, E., Lehto, M., Kanninen, T., Walston, J., Shuldiner, A. R. and Groop, L. C. (1995). Association of a polymorphism in the beta 3-adrenergic-receptor gene with features of the insulin resistance syndrome in Finns. *New Eng. J. Med.* **333**, 348-351.

Zaagsma, J. and Nahorski, S. R. (1990). Is the adipocyte β-adrenoceptor a prototype for the recently cloned atypical 'β3-adrenoceptor'. *Trends Pharmacol. Sci.* **11**, 3-7.

(Received 2 December 1996 – Accepted 31 January 1997)