Expression of β3-adrenoceptors with low lipolytic action in human subcutaneous white adipocytes

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Abstract β_3-Adrenoceptors are involved in the control of catecholamine-induced lipolysis in rodent adipose tissues. The expression and function of human β_3-adrenoceptors were investigated in subcutaneous white adipocytes of young healthy women. In these cells, β_3-adrenoceptor mRNAs represent 20% of total amount of β-adrenoceptor transcripts and less than half of β_1-adrenoceptor transcripts. Among β_3-agonists known to stimulate β_3-adrenoceptor-mediated lipolysis in rodent fat cells, only CGP12177 was able to mediate such activity in human fat cells. In in vitro lipolysis experiments and in situ microdialysis studies, CGP12177 had a 4- to 5-times lower lipolytic efficacy than isoprenaline, a nonselective β-adrenoceptor agonist. CGP12177-induced lipolysis was antagonized in vitro by bupranolol, a β-adrenergic antagonist potent on rodent β_3-adrenoceptors but not by nadolol, a β_1- and β_2-adrenoceptor antagonist. The in vitro blockade of isoprenaline-stimulated lipolysis by nadolol showed that the agonist acted solely via β_1- and β_2-adrenoceptors. Isoprenaline and CGP12177 were able to increase the nutritive blood flow suggesting an interaction of these molecules with receptors present in adipose tissue vessels. In conclusion, β_3-adrenoceptors are expressed in human subcutaneous white adipocytes but do not significantly contribute to isoprenaline-induced lipolysis.—Tavernier, G., P. Barbe, J. Galitzky, M. Berlan, D. Caput, M. Lafontan, and D. Langin. Expression of β3-adrenoceptors with low lipolytic action in human subcutaneous white adipocytes. *J. Lipid Res.* 1996. 37: 87–97.

Supplementary key words β_3-adrenoceptors • white adipocytes • catecholamines • lipolysis • β_3-agonists

Adipose tissue triacylglycerol is quantitatively the most important source of stored energy in mammals. The release of free fatty acids (FFA) via adipocyte lipolysis for supply to energy-requiring tissues is under acute neural and hormonal control. Catecholamines (adrenaline and noradrenaline) activate hormone-sensitive lipase, the rate-limiting enzyme of lipolysis, through cAMP-dependent phosphorylation. For a long time, catecholamine-induced lipolysis was thought to be mediated via β_1-adrenoceptors and to a lesser extent via β_2-adrenoceptors (1, 2). Studies on laboratory animals based on the use of new synthetic agonists show that part of catecholamine effect is due to an atypical β-adrenoceptor (3–5). The cloning of the β_3-adrenoceptor gene was a major step in the understanding of catecholamine action in rodent adipocytes (6–10). The pharmacological profile of this receptor in transfected host cells is very close to the profile observed in rat and hamster fat cells (11). β_3-Adrenoceptors are expressed in rodent brown and white adipose tissues and in the gastrointestinal tract. This distribution restricted to a limited number of tissues has led to the development of β_3-agonists for their potential role for the treatment of obesity, diabetes, and intestinal hypermotility disorders (12).

Studies of the atypical β_3-adrenergic response of white adipocytes from various mammals reveal important differences between species (5, 13, 14). Catecholamine-induced lipolysis is predominantly mediated by the β_3-adrenoceptors in rodents while human, other primate, and guinea pig adipocytes are poorly responsive to selective β_3-agonists. In human fat cells, the presence of biologically active β_3-adrenoceptors has been controversial. Lipolysis experiments performed on isolated fat cells or tissues reveal little or no β_3-adrenoceptor-mediated lipolysis in most studies (5, 15–17) but the lipolytic effect of CGP12177, a partial β_3-agonist in rodents (5), was reported in human omental fat cells (18). An increased sensitivity to CGP12177 was recently reported

Abbreviations: BSA, bovine serum albumin; CHO, Chinese hamster ovary; ED_{50}, half-maximal effective drug concentration; FFA, free fatty acids; KRB, Krebs-Ringer bicarbonate buffer; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; SSC, sodium chloride citrate buffer.

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in visceral adipocytes from patients with upper-body obesity (19). The contribution of β_3-adrenoceptors to catecholamine-induced lipolysis remains to be established. The absence of a radioligand suitable for the identification and quantitation of β_3-adrenoceptors makes mRNA determination the only way to evaluate β_3-adrenoceptor level of expression. Conflicting results were also obtained concerning the presence of β_3-adrenoceptor mRNA in human adipose tissues (20–22). β_3-Adrenoceptor mRNA is detected, using PCR, in tissues expressing uncoupling protein mRNA, a transcript specific to brown adipocytes, and there is convincing evidence that brown adipocytes express β_3-adrenoceptors (10, 22). No quantitative analysis of β_3-adrenoceptor mRNA levels in human white adipocytes and no comparison to the other β-adrenoceptor subtypes have been performed.

In the present work, we have investigated β_3-adrenoceptor function and expression in white subcutaneous adipocytes of young healthy women. The rationale behind this choice was the possibility to combine the determination of β_3-adrenoceptor mRNA levels as well as lipolysis studies in vitro on isolated fat cells and in situ using the microdialysis technique. Mammary adipocytes were studied in order to perform mRNA studies and lipolysis experiments on samples from non-obese patients. In adults, subcutaneous adipose tissues, at the difference of internal fat stores, are essentially devoid of brown adipocytes which have been shown to express β_3-adrenoceptors (22). β_3-Adrenoceptor mRNA levels

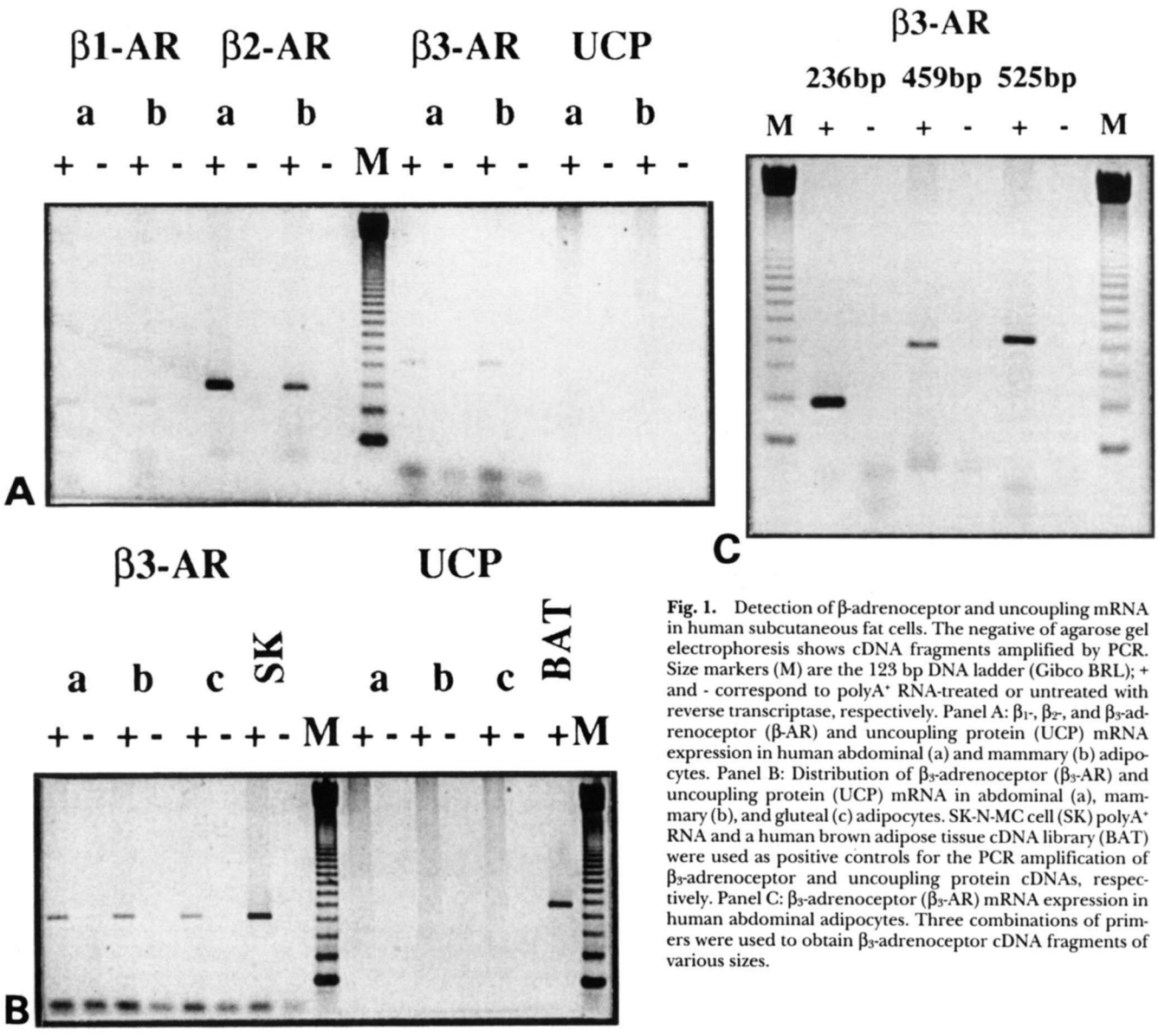


Fig. 1. Detection of β-adrenoceptor and uncoupling mRNA in human subcutaneous fat cells. The negative of agarose gel electrophoresis shows cDNA fragments amplified by PCR. Size markers (M) are the 123 bp DNA ladder (Gibco BRL); + and - correspond to polyA$^+$ RNA-treated or untreated with reverse transcriptase, respectively. Panel A: β_1-, β_2-, and β_3-adrenoceptor (β-AR) and uncoupling protein (UCP) mRNA expression in human abdominal (a) and mammary (b) adipocytes. Panel B: Distribution of β_3-adrenoceptor (β_3-AR) and uncoupling protein (UCP) mRNA in abdominal (a), mammary (b), and gluteal (c) adipocytes. SK-N-MC cell (SK) polyA$^+$ RNA and a human brown adipose tissue cDNA library (BAT) were used as positive controls for the PCR amplification of β_3-adrenoceptor and uncoupling protein cDNAs, respectively. Panel C: β_3-adrenoceptor (β_3-AR) mRNA expression in human abdominal adipocytes. Three combinations of primers were used to obtain β_3-adrenoceptor cDNA fragments of various sizes.

were determined using a quantitative and sensitive dot-blotting technique and compared to β_1-adrenoceptor mRNA levels. The function of β_3-adrenoceptors was assessed in vitro and in vivo. The lipolysis induced by β_3-agonists, suggested as antiobesity drugs, was measured on isolated fat cells and compared to the effect of the nonselective β-agonist, isoprenaline. The microdialysis technique was used to investigate in situ the involvement of the β_3-adrenoceptor subtype in lipolysis.

MATERIAL AND METHODS

Subjects

Group 1 comprised 24 young, healthy, drug-free non-obese women undergoing plastic surgery. Age and body mass index were 32 ± 2 year (mean ± SE, range 18–51) and 22 ± 1 kg/m^2 (range 19–26), respectively. Mammary adipose tissue samples were taken at the beginning of the operation performed in the morning. Mammary fat cells were used for lipolysis and mRNA studies. Additional fat samples for mRNA studies were obtained from abdominal and gluteal fat depots from women undergoing plastic surgery. These subjects were not selected on the basis of age or body mass index. Group 2 subjects undergoing microdialysis experiments comprised six young healthy non-obese women (age 23 ± 1 year, range 20–28, and body mass index 21 ± 1 kg/m^2, range 19–25). The study was approved by the Ethical Committee of Toulouse University Hospitals.

Animals

Male Wistar rats (260–300 g, 6–10 weeks old) that had not undergone any treatment were from Iffa-Credo (L'Arbresle, France). They were housed at 20–22°C with a 16:8 h light–dark cycle and fed ad libitum with free access to water. The animals were killed after an overnight fast. Animal studies were in agreement with Institut National de la Santé et de la Recherche Médicale guidelines for animal care.

Adipocyte isolation

Adipose tissues were carefully dissected out and adipocytes were isolated using the method described by Rodbell (23) with minor modifications in a KRB buffer, containing 1 mg/ml collagenase, 3.5% BSA, and 6 mM glucose adjusted to pH 7.4 just before use (KRB-BSA buffer). Isoprenaline potency was measured on the same cell preparation using different batches of collagenase. Two batches of type I collagenase and four batches of type III collagenase were tested. The type III collagenase batch used in the present study was selected based upon the highest potency in human fat cells (not shown). Digestion and cell isolation procedures were carried out at 37°C. Isolated adipocytes were washed three times and the packed cells were used for lipolysis experiments or total RNA preparations.

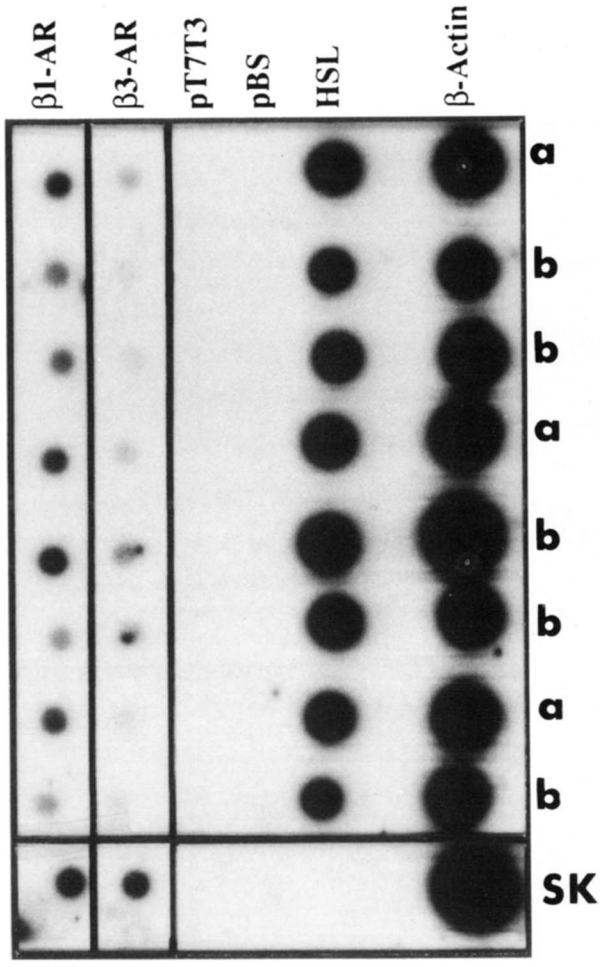


Fig. 2. Comparisons of β_1-adrenoceptor (β_1-AR), β_3-adrenoceptor (β_3-AR), hormone-sensitive lipase (HSL), and β-actin (β-Actin) mRNA levels in human abdominal (a) and mammary (b) adipocytes. This autoradiography shows a typical experiment. pT7T3 and pBS (pBluescript) correspond to the plasmids without inserts (see Methods). β_1- and β_3-adrenoceptor mRNA levels were also measured in SK-N-MC cells (SK). Quantitation of the dots was performed using InstantImager electronic autoradiography system (Packard).

RNA preparation

Total RNA was prepared from isolated fat cells using a single-step guanidinium thiocyanate-phenol-chloroform extraction (24), with some modifications. Isolated adipocytes were washed twice with PBS to remove BSA from the medium, then frozen at -80°C after vigorous shaking in denaturing solution. After thawing, the mixture was decanted and the lipid layer was removed. The rest of the RNA extraction was performed as described (24). RNA samples were stored in ethanol at -80°C. Integrity of total RNA was systematically checked by agarose gel electrophoresis (not shown). RNA concen-

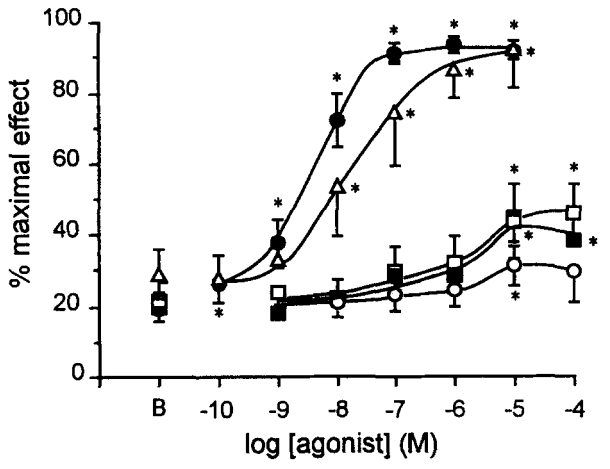


Fig. 3. Dose–response curves of β-adrenergic agonists in human mammary subcutaneous adipocytes. Lipolysis induced by β_3-adrenoceptor agonists, BRL37344 (□), CL316243 (○), CGP12177 (■), was compared to the lipolysis induced by isoprenaline, a nonselective β-adrenergic agonist (●) and noradrenaline in the presence of 10^{-5} M of the α_2-adrenergic antagonist RX821002 (△). Values are mean ± SE of 11 experiments except for noradrenaline (6 experiments). Basal lipolysis and maximal lipolysis rates induced by isoprenaline were 0.14 ± 0.03 and 0.62 ± 0.12 μmol glycerol/90 min per 100 mg lipid, respectively. Paired *t*-test was used to compare the effect of each concentration of drug with basal lipolysis; $*P < 0.05$.

trations were determined by measuring OD at 260 nm. PolyA$^+$ RNA was prepared from 100 μg total RNA using Dynabeads mRNA purification kit (Dynal, Compiègne, France). Briefly, total RNA was heated at 65°C for 5 min and then hybridized to dynabeads-oligo(dT)$_{25}$ for 10 min at room temperature. The beads were washed twice, eluted with 40 μl 2 mM EDTA, pH 8.0, and used immediately for PCR or dot-blotting analyses.

PCR analysis

One hundred ng of polyA$^+$ RNA was treated with or without 200 U Superscript reverse transcriptase (GIBCO BRL, Epargny, France) in 20 μl reverse transcriptase buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$) containing 5 mM DTT, 0.5 mM each dNTP, 100 ng oligo(dT)$_{12-18}$ and 2 U/μl RNase Inhibitor (Pharmacia, St Quentin en Yvelines, France). cDNA was heated 5 min at 92°C and subjected to 30 cycles of amplification (92°C 1 min, 55°C 1.5 min, 72°C 1.5 min) followed by 7 min of extension at 72°C in a temperature cycler (Biometra, Göttingen, Germany) in 100 μl of PCR buffer containing 2.5 U of *Thermus aquaticus* polymerase (Promega, Madison, WI), 2.5 mM $MgCl_2$, 50 μM each dNTP, 125 nM each sense and antisense oligonucleotide primers, and 10% (vol/vol) dimethylsulfoxide. Formamide [5% (vol/vol)] was added for β_1-adrenoceptor cDNA amplification and 2.5% (vol/vol) formamide was added for β_2-adrenoceptor and β_3-adrenoceptor cDNA amplification. Uncoupling protein cDNA amplification was performed with 2.5% (vol/vol) formamide without dimethylsulfoxide. The preliminary denaturation step was 92°C for 3 min followed by 30 cycles (92°C 1 min, 50°C 1.5 min, 72°C 1.5 min) and 5 min of extension at 72°C. PCR products (10-μl aliquots) were visualized by electrophoresis through 2% agarose ethidium bromide-stained gels.

Sequences of the sense and antisense oligonucleotide primers, respectively, were: 5′ - TCGTGTGCACCGT GTGGGCC-3′ and 5′-AGGAAACGGCGCTCGCAG CTGTCG - 3′ for β_1 - adrenoceptor, 5′ - GGATC CAAGCTTCCCATCCTGCTCCACCT-3′ and 5′- GGA TCCAAGCTTGCCTGCTGACCAAGAATAAGGCC-3′, for β_2-adrenoceptor, 5′-TAGGTATAAAG GTGTCC TGG-3′ and 5′-CACTTTTGTACTGTCCTGGTGG - 3′ for uncoupling protein. The expected lengths of the fragments calculated from cDNA sequences were 265, 353, and 590 bp for β_1 -, β_2 -adrenoceptors and uncoupling protein, respectively. For β_3-adrenoceptor, three combinations of primers were used: 5′-GCAT GCTCCGTGGCCTCACGAGAA-3′and 5′-CTGGCTCA TGATGGGCGC-3′, 5′-CGCTGACTGGCCACTGGC CGTTGGG-3′ and 5′-CTGGCTCATGATGGG CGC-3′, 5′-GCCTCCAACATGCCCTA-3′ and 5′-GGCTGCGG CAGTAGATG-3′ that should give fragments of 525, 236, and 459 bp.

Dot-blotting analysis

For labeled cDNA synthesis, 500 ng polyA$^+$ RNA was treated with 200 U Superscript reverse transcriptase in 40 μl buffer containing 100 ng oligo(dT), 50 μCi [α^{32}P]dCTP, 10 mM DTT, 20 μM each dNTP and 4 U RNase inhibitor. The incubation proceeded at 37°C for 1 h and 50°C for 10 min. The reaction was stopped by addition of 3 μl 0.5 M EDTA, pH 8.0, and heating at 65°C for 5 min. The labeled probe was separated from free nucleotides with P10 gel fine columns (Bio-Rad, Ivry sur Seine, France). An aliquot was precipitated with trichloracetic acid to determine labeled cDNA concentrations. The quality of the synthesis was appreciated by agarose gel electrophoresis of 5000 cpm of labeled cDNAs. Typical specific activity was 100,000 cpm/ng cDNA.

Two hundred ng of human cDNAs in pT7T3 (Pharmacia, St Quentin en Yvelines, France) and pBluescript (Stratagene, Paris, France) vectors and vectors alone were blotted on positively charged nylon membrane (Hybond N$^+$ from Amersham, Les Ulis, France). DNA dot blotting and alkali fixation protocols were done as described by the manufacturer. The membrane was immediately prehybridized in 6 × SSC, 5 × Denhardt's, 50% formamide, 0.2% SDS, and 100 μg/ml salmon sperm DNA at 42°C for at least 5 h. Hybridization proceeded in the same buffer with 5 ng/ml radioactive probe at 42°C for 40 h. Filters were washed 15 min at room temperature in 2 × SSC and 0.1% SDS and twice

45 min at 60°C in 0.1 × SSC and 0.1% SDS. Signals were quantitated using InstantImager electronic autoradiography system (Packard, Meriden, CT). To test the quality of the membranes, increasing quantities of the various vectors (20 pg to 200 ng) were blotted and hybridized to a primer corresponding to the ampicillin resistance gene (5′-GTCATGCCATCCGTAAGATG-3′). The signals increased linearly with the quantities of DNA and were similar for the various vectors (not shown).

Lipolysis measurements

Isolated adipocytes (2000–5000 cells/ml) were incubated in plastic vials in 100 µl KRB-BSA buffer with gentle shaking in a water bath under an air phase at 37°C. After 90 min, the incubation tubes were placed in an ice bath and 20 µl of the infranatant was removed for the determination of glycerol using a highly sensitive radiometric technique (25). Pharmacological agents were added just before the beginning of the incubation in 5-µl portions in vehicle to obtain the indicated final concentration. The intrinsic activity of each agonist was calculated by dividing its maximal lipolytic effect by the maximal lipolytic effect of the full agonist isoprenaline. Half-maximal effective drug concentration (ED_{50}) values were obtained by computer-fitting of dose–response curves of various β-agonists and expressed as pD_2 values (-log M for ED_{50}). Dose–response curves for agonists were constructed from experiments made in the absence and in the presence of 4 (human) to 6 (rat) antagonist concentrations. Schild plots were constructed and their slopes were determined by linear regression analysis. When the slope did not differ from 1, pA_2 (-log M) values were calculated.

Microdialysis technique

The subjects were studied at 8 AM in the supine position after an overnight fast. Adipose tissue microdialysis was performed using 20 × 0.5 mm probes with a 20,000 Da molecular weight cut-off (Carnegie Medicine, Stockholm, Sweden). One probe was inserted percutaneously after light intradermal anesthesia (100 µl of 1% lidocaine, Roger-Bellon, France) into the abdominal subcutaneous adipose tissue at a distance of 100 mm to the right or the left of the umbilicus. The probe was connected to a multichannel microinjection pump (Harvard Apparatus, South Natick, MA) and continuously perfused with a sterile Ringer's solution (154 mM NaCl, 4 mM KCl, 2.5 mM $CaCl_2$) supplemented with ethanol (1.7 g/l) at 0.8 µl/min. No collection of the outgoing dialysate was executed during the first 30 min after implantation. Then, the in vivo recovery rate at 2.5 µl/min was evaluated for each probe using the measurement of dialysate glycerol concentrations at various perfusion rates (26). Briefly, the probes were perfused at four successive rates (0.8 µl/min, 1.5 µl/min, 3.5 µl/min, and 2.5 µl/min), and the glycerol concentrations were determined in the dialysate for each perfusion rate at the steady state. These concentrations were plotted (after log-transformation) against the perfusion rate values. Regression analysis was used to calculate the glycerol concentration at the "zero flow", corresponding to the interstitial glycerol concentration. The ratio of dialysate glycerol concentration at 2.5 µl/min to interstitial glycerol concentration expressed the in vivo recovery rate at 2.5 µl/min of the probe in the experimental conditions.

After this calibration period, the perfusion flow rate was maintained at 2.5 µl/min in order to evaluate the effects of the pharmacological agents. Ten-min fractions

TABLE 1. Pharmacological properties of β-adrenoceptor agonists in human subcutaneous and rat white fat cells

Agonist	Human		Rat	
	pD_2	IA	pD_2	IA
Isoprenaline	8.3±0.2	1	8.4±0.1	1
Noradrenaline + RX821002	7.4±0.4	0.99±0.02	7.3±0.1	1.00±0.02
CGP12177	5.9±0.3	0.27±0.06	6.5±0.1	0.80±0.04
CL316243	5.7±0.3	0.16±0.04	8.5±0.1	0.95±0.04
BRL37344	5.8±0.3	0.32±0.06	8.5±0.1	0.95±0.06

Values are mean ± SE of 11 (6 for noradrenaline + 10^{-5} M RX821002) and 5 experiments on human and rat isolated adipocytes, respectively. IA, intrinsic activity relative to isoprenaline. pD_2 values are -log (M for ED_{50}). ED_{50} values were calculated from agonist dose–response curves. Isoprenaline and noradrenaline concentrations ranged from 10^{-10}M to 10^{-5} M. BRL37344, CL316243, and CGP12177 concentrations ranged from 10^{-9} M to 10^{-4} M. Basal lipolysis rates were 0.14 ± 0.03 and 0.35 ± 0.07 µmol glycerol/90 min per 100 mg lipid in human and rat, respectively. Maximal lipolysis rates induced by isoprenaline were 0.62 ± 0.12 and 3.53 ± 0.35 µmol glycerol/90 min per 100 mg lipid in human and rat, respectively.

TABLE 2. Blockade of β_3-adrenoceptor agonist-induced lipolysis by β-adrenoceptor antagonists in human subcutaneous adipocytes

	Lipoytic Effect
Basal lipolysis	22.5±1.1
BRL37344	37.8±1.8[a]
BRL37344+nadolol	22.7±1.1[b]
CL316243	29.1±3.0[a]
CL316243+nadolol	22.9±1.7[b]
CGP12177	30.3±0.4[a]
CGP12177+nadolol	27.2±3.0
CGP12177+bupranolol	24.6±1.9[b]

Values are mean ± SE of 5 experiments. β_3-Adrenoceptor agonists (BRL37344, CL316243, and CGP12177) were used at 10^{-5} M. Nadolol, a β_1- and β_2-adrenoceptor antagonist, and bupranolol, a β_1-, β_2-, and β_3-adrenoceptor antagonist, were used at 10^{-4} M. Comparisons were performed using two-way ANOVA followed by the Newman-Keuls multiple comparison procedure. Maximal lipolysis induced by isoprenaline and basal lipolysis were 0.71 ± 0.12 and 0.16 ± 0.01 μmol glycerol/90 min per 100 mg lipid.

[a] $P < 0.05$: agonist-induced lipolysis vs. basal lipolysis.

[b] $P < 0.05$: agonist plus antagonist-induced lipolysis vs. agonist-induced lipolysis.

of the outgoing dialysate were collected. Glycerol (as lipolysis index) was assayed in each collected fraction. In the same way, ethanol level was measured in each fraction to assess the changes occurring in the nutritive blood flow of the adipose tissue, using the ethanol outflow/inflow ratio, as previously described (27, 28). Four fractions were collected to evaluate basal glycerol and blood flow levels before adding the various β-adrenergic agonists in the perfusate. In the first experiment, the effects of two successive concentrations of isoprenaline (0.1 μM and 1 μM) were studied. In the second experiment, two successive doses of CGP12177 (10 μM and 100 μM) were used. Each dose was applied for 40 min.

Drugs and chemicals

BRL37344, CL316243, CGP12177, RX821002, and SR58611 were generous gifts from SmithKline-Beecham (Epsom, UK), Lederle-American Cyanamide (Pearl River, NY), Ciba-Geigy (Basel, Switzerland), Reckitt and Colman (Kingston-upon-Hull, UK), and Sanofi (Milano, Italy), respectively. Other drugs came from the following sources: lidocaine (Roger Bellon, Paris, France); bupranolol (Sanol, Monheim, Germany); nadolol (Squibb, Paris, France); isoprenaline hydrochloride (Winthrop, Clichy, France); (-)noradrenaline, fraction V BSA and type I *Clostridium histiolyticum* collagenase (Sigma Chemical Co., St. Louis, MO); adenosine deaminase, type III *Clostridium histiolyticum* collagenase, NAD, and enzymes for glycerol assays (Boehringer Mannheim, Mannheim, Germany). [γ^{32}P]ATP and [α^{32}P]dCTP (3000 Ci/mmol) were from ICN (Bordeaux, France). All other chemicals and organic solvents were of reagent grade.

Statistical analysis

Values are mean ± SE. Student's *t*-test, Wilcoxon's test, and ANOVA were used for comparisons.

RESULTS

Measurement of β_3-adrenoceptor mRNA

β_3-Adrenoceptor transcripts were first detected in human subcutaneous fat cells using a PCR assay. Uncoupling protein mRNA was also investigated to evaluate the presence of brown adipocytes. Products of expected sizes corresponding to β_1-, β_2-, and β_3-adrenoceptor mRNAs were detected in subcutaneous fat cells (**Fig. 1A**). β_3-Adrenoceptor transcripts were present in white adipocytes from gluteal, mammary, and abdominal fat depots (Fig. 1B). Identity of the PCR-amplified transcripts was confirmed using three combinations of primers that yielded fragments of expected sizes (Fig. 1C). No products corresponding to uncoupling protein were seen on ethidium bromide-stained gels. Southern blotting experiments using a human uncoupling protein cDNA probe (29) revealed weak signals in some mammary adipocyte preparations (not shown).

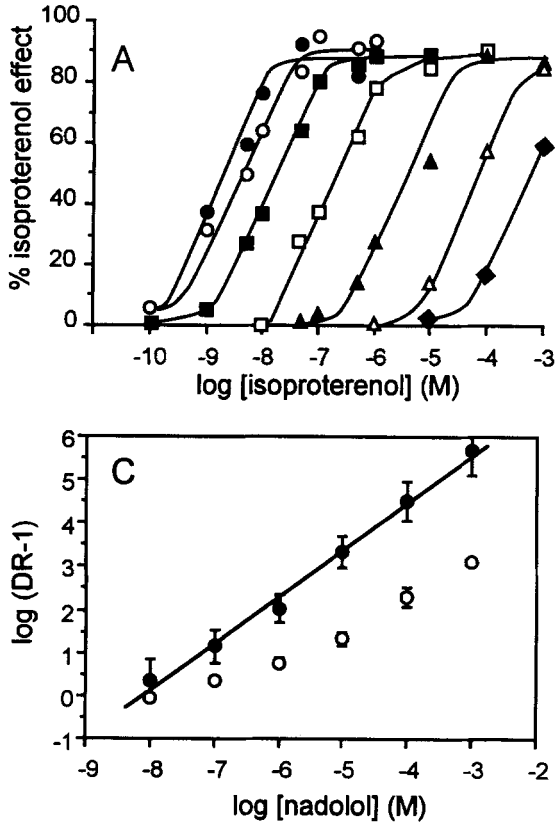
We first attempted to quantitate β_1- and β_3-adrenoceptor mRNA levels using a PCR-based technique spiking the cellular RNA with a multi-specific internal probe (30). Unfortunately, upon comparing relative β_1- and β_3-adrenoceptor mRNA quantities in SK-N-MC cells, a human neuroblastoma cell line expressing the two receptors (31), by Northern blotting analysis and quantitative PCR, it was clear that the PCR-based method underestimated β_1-adrenoceptor mRNA level by a factor of 15 (unpublished data). A dot-blotting technique was therefore developed for the quantitation of rare transcripts (**Fig. 2**). A β_1-/β_3-adrenoceptor mRNA ratio of 1.1–1.2 was measured in SK-N-MC cells that corresponded to the value obtained from Northern blotting analysis (not shown). The coefficient of variation of β_3-adrenoceptor mRNA level in seven independent experiments performed on SK-N-MC cells was 11%. The dot-blotting technique was sensitive enough to detect transcripts that represent only 0.5 ± 0.1% of β-actin mRNAs (n = 22) and 2.6 ± 0.7% of mRNAs encoding hormone-sensitive lipase, the rate-limiting enzyme of adipose tissue lipolysis (n = 15). To appreciate the rela-

tive importance of β_3-adrenoceptor transcripts in human subcutaneous fat cells, we determined β_3- and β_1-adrenoceptor mRNA levels in parallel. The ratio of β_3-/β_1-adrenoceptor mRNA levels was 0.4 ± 0.1 in mammary adipocytes from young women (n = 12), 0.3 ± 0.1 in abdominal adipocytes (n = 6), and 0.4 ± 0.1 in 22 samples including 4 gluteal adipocyte preparations. β_3-Adrenoceptor transcripts were much less abundant in human white adipocytes than in rat white adipocytes (human β_3-/rat β_3-adrenoceptor mRNA ratio of 0.07 ± 0.01, n = 7).

Lipolysis experiments on isolated adipocytes

The lipolytic effects of β-agonists were measured in mammary fat cells from 11 young women (**Fig. 3**). Isoprenaline, a nonspecific β-agonist, was studied in parallel with four β_3-agonists. The intrinsic activities and pD_2 values in human and rat adipocytes are shown on **Table 1.** BRL37344, CL316243, and CGP12177 showed low intrinsic activities and similarly low pD_2 values in humans whereas BRL37344 and CL316243 were full agonists with high potencies in rats. CGP12177 pD_2 values were in the same range in both species. Human fat cells were 300-times more sensitive to isoprenaline than to the three β_3-agonists. SR58611 did not induce lipolysis in human fat cells at concentrations up to 10^{-3} M (not shown).

The subtype selectivity of BRL37344, CL316243, and CGP12177 was measured using nadolol, a nonselective β_1- and β_2-adrenoceptor antagonist with low β_3-adrenoceptor antagonistic property (unpublished data) (6, 32). BRL37344 and CL316243 effects were completely blocked by nadolol showing that these agonists interact at high concentrations with β_1- and/or β_2-adrenoceptors (**Table 2**). Nadolol was without effect on CGP12177-induced lipolysis which is in agreement with the β_1- and β_2-adrenoceptor blocking property of CGP12177 (33). Bupranolol, a β-adrenoceptor antagonist potent on rodent β_3-adrenoceptors (5), partially antagonized the CGP12177 effect. To determine whether the interaction of catecholamines with β_3-adrenoceptors could contribute to their lipolytic effect, the antagonist potency of nadolol on isoprenaline-induced lipolysis was evaluated by calculating pA_2 values in rat fat cells and human adipocytes responsive to CGP12177 (i.e., with intrinsic activities above 0.2) (**Fig. 4**). The calculated pA_2 value (8.0 ± 0.2, n = 6) and the linearity of the Schild plot (slope of 1.08, not different from 1) in human adipocytes were consistent with an interaction of isoprenaline solely with β_1- and β_2-adrenoceptors. The Schild plot was curvilinear in rat adipocytes. At low concentrations of nadolol (10^{-8} to 10^{-6} M), the slope of 0.62 showed the interaction



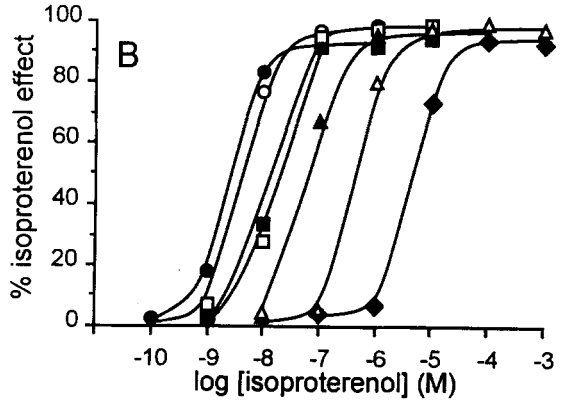


Fig. 4. Determination of the subtype specificity of isoprenaline-stimulated lipolysis in subcutaneous mammary adipocytes. Dose–response curves of isoprenaline, a nonselective β-adrenergic agonist, were constructed in the absence (●) or presence of 10^{-8} M (○), 10^{-7} M (■), 10^{-6} M (□), 10^{-5} M (▲), 10^{-4} M (△), 10^{-3} M (◆) of nadolol, a β_1- and β_2-adrenoceptor antagonist. Panels A and B represent experiments performed in human and rat fat cells, respectively. Schild plots (-log(dose ratio(DR)-1) vs. -log(antagonist)) for human (●) and rat (○) fat cells are shown on panel C. Values are mean of 6 experiments in both species. SE were below 10%.

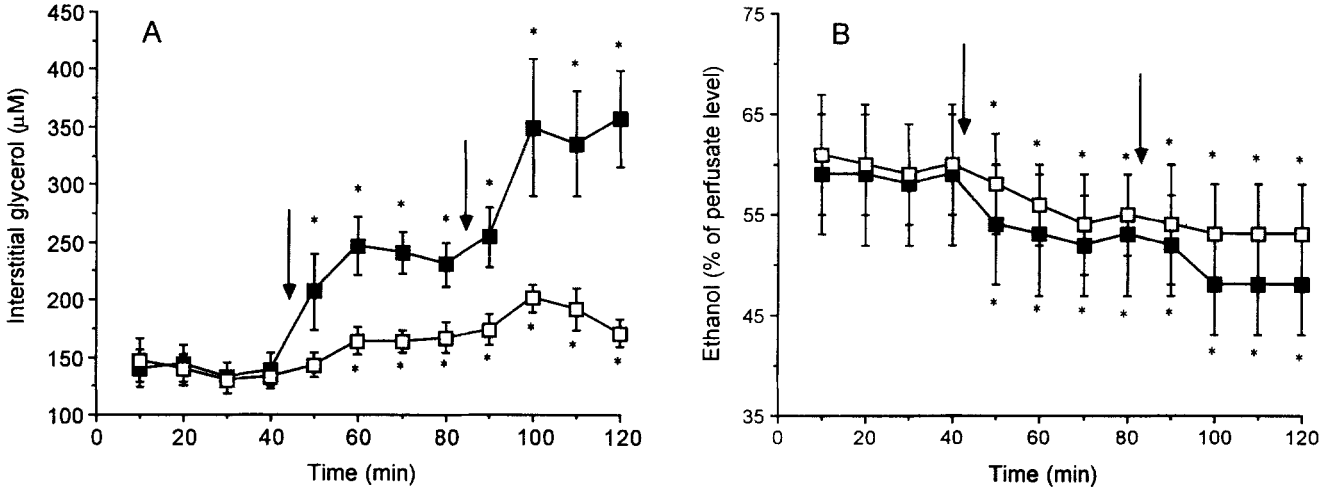


Fig. 5. Effects of isoprenaline, a nonselective β-adrenergic agonist (■), and CGP12177, a β_3-adrenergic agonist (□), on the extracellular glycerol concentration (panel A) and the ethanol ratio (panel B) in human subcutaneous adipose tissue. After a calibration period, the dialysate was collected at 10-min intervals. Four fractions were collected to evaluate basal glycerol and blood flow levels before adding the agonist. Isoprenaline, 0.1 μM then 1 μM, was perfused for 40 min. In a second experiment, CGP12177, 10 μM then 100 μM, was perfused for 40 min. The start of perfusion with the different concentrations of agonist is indicated by the arrow. Ethanol = ethanol in the outgoing dialysate divided by ethanol in the ingoing dialysate × 100. Values are mean ± SE (n = 6). Wilcoxon's test was used to compare each fraction to the last fraction before perfusion of drugs (40-min fraction); $P < 0.05$.

of the antagonist with a heterogenous population of receptors, i.e., β_1-/β_2-, and β_3-adrenoceptors. At higher concentrations of nadolol (10^{-5} to 10^{-3} M), the slope was not different from unity (0.99) and the pseudo pA_2 value of 6.2 ± 0.1 showed the interaction of nadolol with the predominant population of β_3-adrenoceptors. This latter value was in agreement with the antagonistic potency of nadolol on BRL37344-induced lipolysis in rat fat cells (unpublished data).

In situ determination of lipolysis

The in situ effects of increasing doses of isoprenaline and CGP12177 on interstitial glycerol levels and ethanol ratio on the abdominal subcutaneous adipose tissue of young healthy women are depicted in **Fig. 5.** The in vivo recovery rates were not different in the two probes (34 ± 3% vs. 35 ± 3%). ANOVA for interstitial glycerol concentration was not significant when comparing the first four fractions of the two curves ($F = 0.01$, $P = 0.93$). When 0.1 μM and 1 μM of isoprenaline were added to the perfusate, there was a strong dose-dependent increase of the interstitial glycerol concentration (182 ± 10% and 268 ± 35%, respectively) when compared to the last value measured just before the perfusion (basal value). Addition of CGP12177 significantly increased the interstitial glycerol concentration after 10-min perfusion of the lowest dose (10 μM). This effect was sustained but weak (127 ± 12% of the basal value). Increasing the dose to 100 μM led to a maximal effect corresponding to 154 ± 16% of the basal value after 10-min perfusion but did not increase the glycerol outflow in a dose-dependent manner (ANOVA with repeated measures from 60 to 120 min was not significant: $F = 1.66$, $P = 0.16$). Isoprenaline promoted a significant dose-dependent decrease of the ethanol outflow/inflow ratio (Fig. 5B), indicating that an increase in nutritive blood flow (vasodilation) occurred in the adipose tissue. The 10-μM dose of CGP12177 induced a significant vasodilation after 10 min of perfusion; the 100-μM dose did not promote any additional decrease of the ethanol outflow/inflow ratio. The difference between the curves of CGP12177 and isoprenaline was not significant (ANOVA: $F = 0.14$, $P = 0.72$).

DISCUSSION

In this study, we analyzed the expression and function of β_3-adrenoceptors and the importance of these receptors in lipolysis in human subcutaneous white adipocytes. We provide for the first time a quantitative comparison between β_1- and β_3-adrenoceptor mRNA levels in combination with analyses of the importance of β_3-adrenoceptors in lipolysis in vitro and in vivo.

The characterization of an "atypical" β-adrenergic response in rodent white and brown adipocytes led to the synthesis of new β-agonists that potently stimulate lipolysis and thermogenesis while having little effect on β_1- and β_2-adrenergic responses (34). Regarding the existence and the role of β_3-adrenoceptors in human

white adipose tissues, fundamental questions remain to be answered (35). What is the level of expression of β_3-adrenoceptors in human white adipocytes? Are selective β_3-adrenoceptor agonists defined on transfected cells and rodent fat cells good candidates as antiobesity drugs in man? Do β_3-adrenoceptors contribute to catecholamine-induced lipolysis?

β_3-Adrenoceptor mRNA was expressed in mammary, abdominal, and gluteal white adipocytes (Fig. 1B). In abdominal and gluteal adipocytes, β_2-adrenoceptor mRNAs represent 60% of β_1-adrenoceptor mRNAs (36) and the β_1- to β_3-adrenoceptor mRNA ratio was found to be 2.5 (present study). Therefore, β_3-adrenoceptor transcripts represent less than 20% and more than 90% of the overall β-adrenoceptor transcripts in human and rodent white adipocytes, respectively (37, 38). Consequently, the level of expression of the β_3-adrenoceptor is much lower in human subcutaneous white adipocytes than in rodent white fat cells and, probably, than in human brown adipocytes (22).

Full β_3-agonists on rat adipocytes, i.e., BRL37344, CL316243, and SR58611 (3–5, 39, 40), showed low lipolytic potencies and efficacies in human adipocytes. BRL37344 and CL316243 act via β_1- and β_2-adrenoceptors at high concentrations (Table 2) (41). Accordingly, in vivo administration of BRL35135, the precursor of BRL37344, produces a rise in serum FFA and glycerol concentrations which appears to be exclusively mediated via β_2-adrenoceptors (42). Species-specific pharmacological differences can only partly explain the discrepancy in the lipolytic effects of β_3-agonists between human and rodent fat cells. In transfected Chinese hamster ovary (CHO) cells, BRL37344 and CL316243 potencies are 30- and 90-fold lower for the human than for the rodent β_3-adrenoceptors, respectively. However, SR58611, which is equipotent in CHO cells expressing human and murine β_3-adrenoceptors (43), was not lipolytic in human fat cells. The present data clearly indicate that transfected CHO cells and rodent adipocytes are not predictive of the lipolytic efficacy and potency of β_3-agonists on human fat cells.

CGP12177 was the only compound to show a lipolytic activity that was not due to interactions with β_1- and β_2-adrenoceptors (Table 2). The increase of extracellular glycerol concentration elicited in situ by CGP12177 and isoprenaline was comparable to the lipolytic effect of these molecules on isolated fat cells (Table 1, Fig. 5A). During the completion of the present work, an elevation of glycerol outflow in the microdialysis probe after CGP12177 infusion was reported (44). At this point, it must be stressed that the pharmacology of β_3-adrenoceptors on human fat cells is only defined by the capacity of CGP12177 to induce lipolysis. CGP12177 is a hydrophilic molecule that does not penetrate the cells and is therefore very likely to interact with cell surface proteins (33). We have previously shown in hamster fat cells that CGP12177 operates via the same receptor as BRL37344 and noradrenaline (5). Moreover, bupranolol, a β_3-antagonist in rodent fat cells (5), was able to partially antagonize CGP12177-induced lipolysis in human adipocytes (Table 2). Therefore, if β_3-adrenoceptors are present on human adipocyte plasma membrane, CGP12177 is expected to bind this receptor.

Another point addressed by this study was the importance of β_3-adrenoceptors in catecholamine-induced lipolysis in human fat cells. The antagonistic action of nadolol on isoprenaline-induced lipolysis indicated an interaction of the agonist and antagonist with an homogeneous population of receptors (Fig. 4) that can be defined as β_1- and β_2-adrenoceptors because, under our experimental conditions, nadolol and isoprenaline do not discriminate between the two subtypes (45). Therefore, in human subcutaneous adipocytes, β_3-adrenoceptors do not play a significant role in isoprenaline-induced lipolysis. This conclusion is in agreement with the low efficacy of β_3-agonists in vitro and in situ and the exclusively β_1- and β_2-adrenoceptor-mediated effects of isoprenaline in vivo on serum FFA and glycerol levels (46, 47).

As changes in local blood flow may be of importance for substrate mobilization from adipose tissue and may modify interstitial glycerol levels, a parallel determination of glycerol concentrations and blood flow was necessary to evaluate the effect of perfused molecules (27, 28). The ethanol outflow/inflow ratio decreased after isoprenaline and CGP12177 perfusions (Fig. 5). This vasodilating effect was most likely due to the interaction of the drugs with β-adrenoceptors located on adipose tissue vessels as shown in dog adipose tissue (48). A vasodilating effect of CGP12177 was not observed by Enocksson et al. (44). The discrepancy may be due to technical differences, e.g., the length of the microdialysis probe and infusion rates. Vascular β_3-adrenoceptors in adipose tissue may constitute a new and, as yet, unexplored target for β_3-agonists and the importance of the local vascular effects of catecholamines in human adipose tissue needs further studies.

In summary, β_3-adrenoceptor mRNA is expressed in human subcutaneous adipocytes. Full β_3-agonists characterized on rodent fat cells do not mediate specific β_3-adrenergic lipolysis in human adipocytes. CGP12177 is the only compound eliciting a response that is not attributable to β_1- and β_2-adrenoceptors. In vivo administration of β_3-agonists to obese and nonobese individuals gave strikingly different results in humans and animals (12). Further development of β_3-agonists for use as potential therapeutic drugs should include a characterization on human adipocytes.

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REFERENCES

1. Lafontan, M., and M. Berlan. 1993. Fat cell adrenergic receptors and the control of white and brown fat cell function. *J. Lipid Res.* **34:** 1057–1091.
2. Lands, A. M., A. M. Arnold, J. P. McAuliff, and F. P. Luduena. 1967. Differentiation of receptor systems activated by sympathomimetic amines. *Nature.* **214:** 597–598.
3. Arch, J. R. S., A. T. Ainsworth, M. A. Cawthorne, V. Piercy, M. V. Sennitt, V. E. Thody, C. Wilson, and S. Wilson. 1984. Atypical β-adrenoceptor on brown adipocytes as target for anti-obesity drugs. *Nature.* **309:** 163–165.
4. Hollenga, C., and J. Zaagsma. 1989. Direct evidence for the atypical nature of functional β-adrenoceptors in rat adipocytes. *Br. J. Pharmacol.* **98:** 1420–1424.
5. Langin, D., M. Portillo, J-S. Saulnier-Blache, and M. Lafontan. 1991. Coexistence of three β-adrenergic receptor subtypes in white fat cells of various mammalian species. *Eur. J. Pharmacol.* **199:** 291–301.
6. Emorine, L. J., S. Marullo, M. M. Briend-Sutren, G. Patey, K. Tate, C. Delavier-Klutchko, and A. D. Strosberg. 1989. Molecular characterization of the human β_3-adrenergic receptor. *Science.* **245:** 1118–1121.
7. Muzzin, P., J-P. Revelli, F. Kuhne, J. D. Gocayne, W. R. McCombie, J. C. Venter, J-P. Giacobino, and C. Fraser. 1991. An adipose tissue specific β-adrenergic receptor. Molecular cloning and down-regulation in obesity. *J. Biol. Chem.* **266:** 24053–24058.
8. Granneman, J. G., K. N. Lahners, and A. Chaudry. 1991. Molecular cloning and expression of the rat β_3-adrenergic receptor. *Mol. Pharmacol.* **40:** 895–899.
9. Nahmias, C., N. Blin, J-M. Elalouf, M. G. Mattei, A. D. Strosberg, and L. J. Emorine. 1991. Molecular characterization of the mouse β_3-adrenergic receptor: relationship with the atypical receptor of adipocytes. *EMBO J.* **10:** 3721–3727.
10. Lelias, J. M., M. Kaghad, M. Rodriguez, P. Chalon, J. Bonnin, I. Dupre, B. Delpech, M. Bensaid, G. L. Fur, P. Ferrara, and D. Caput. 1993. Molecular cloning of a human β_3-adrenergic receptor cDNA. *FEBS Lett.* **324:** 127–130.
11. Zaagsma, J., and S. R. Nahorski. 1990. Is the adipocyte β-adrenoceptor a prototype for the recently cloned atypical "β_3-adrenoceptor"? *Trends Pharmacol. Sci.* **11:** 3–7.
12. Howe, R. 1993. β_3-Adrenergic agonists. *Drugs Future.* **18:** 529–549.
13. Carpéné, C., I. Castan, P. Collon, J. Galitzky, J. Moratinos, and M. Lafontan. 1994. Adrenergic lipolysis in guinea pig is not a β_3-adrenergic response: comparison with human adipocytes. *Am. J. Physiol.* **266:** R905–R913.
14. Bousquet-Mélou, A., J. Galitzky, C. Carpéné, M. Lafontan, and M. Berlan. 1994. β-Adrenergic control of lipolysis in primate white fat cells: a comparative study with nonprimate mammals. *Am. J. Physiol.* **267:** R115–R123.
15. Hollenga, C., M. Haas, J. T. Deinum, and J. Zaagsma. 1990. Discrepancies in lipolytic activities induced by β-adrenoceptor agonists in human and rat adipocytes. *Horm. Metab. Res.* **22:** 17–21.
16. Rosenbaum, M., C. C. Malbon, J. Hirsch, and R. L. Leibel. 1993. Lack of β_3-adrenergic effect on lipolysis in human subcutaneous adipose tissue. *J. Clin. Endocrinol. & Metab.* **77:** 352–355.
17. Van Liefde, I., A. Van Ermen, and G. Vauquelin. 1994. No functional atypical β-adrenergic receptors in human omental adipocytes. *Life Sci.* **54:** PL209–214.
18. Lönnqvist, F., S. Krief, A. D. Strosberg, B. Nyberg, L. J. Emorine, and P. Arner. 1993. Evidence for a functional β_3-adrenoceptor in man. *Br. J. Pharmacol.* **110:** 929–936.
19. Lönnqvist, F., A. Thörne, K. Nilsell, J. Hoffstedt, and P. Arner. 1995. A pathogenic role of visceral fat β_3-adrenoceptors in obesity. *J. Clin. Invest.* **95:** 1109–1116.
20. Thomas, R. F., and S. B. Liggett. 1993. Lack of β_3-adrenergic receptor mRNA expression in adipose and other metabolic tissues in the adult human. *Mol. Pharmacol.* **43:** 343–348.
21. Granneman, J. G., K. N. Lahners, and D. D. Rao. 1992. Rodent and human β_3-adrenergic receptor genes contain an intron within the protein-coding block. *Mol. Pharmacol.* **42:** 964–970.
22. Krief, S., F. Lönnqvist, S. Raimbault, B. Baude, A. V. Spronsen, P. Arner, A. D. Strosberg, D. Ricquier, and L. J. Emorine. 1993. Tissue-distribution of β_3-adrenergic receptor mRNA in man. *J. Clin. Invest.* **91:** 344–349.
23. Rodbell, M. 1964. Metabolism of isolated fat cells. I. Effects of hormones on glucose metabolism and lipolysis. *J. Biol. Chem.* **29:** 375–380.
24. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162:** 156–159.
25. Bradley, D. C., and H. R. Kaslow. 1989. Radiometric assays for glycerol, glucose, and glycogen. *Anal. Biochem.* **180:** 11–16.
26. Stahle, L., P. Arner, and U. Ungerstedt. 1991. Drug distribution studies with microdialysis. III extracellular concentration of caffeine in adipose tissue in man. *Life Sci.* **49:** 1853–1858.
27. Hickner, R. C., H. Rosdahl, I. Borg, U. Ungerstedt, L. Jorfeldt, and J. Henriksson. 1991. Ethanol may be used with the microdialysis technique to monitor blood flow changes in skeletal muscle: dialysate glucose concentration is blood flow-dependent. *Acta Physiol. Scand.* **143:** 355–356.
28. Galitzky, J., M. Lafontan, J. Nordenström, and P. Arner. 1993. Role of vascular α_2-adrenoceptors in regulating lipid mobilization from human adipose tissue. *J. Clin. Invest.* **91:** 1997–2003.
29. Cassard, A. M., F. Bouillaud, M. G. Mattei, E. Hentz, S. Raimbault, M. Thomas, and D. Ricquier. 1990. Human uncoupling protein gene structure, comparison with rat gene and assignment to the long arm of chromosome 4. *J. Cell. Biochem.* **43:** 255–264.

30. Legoux, P., C. Minty, B. Delpech, A. J. Minty, and D. Shire. 1992. Simultaneous quantitation of cytokine mRNAs in interleukin-1β-stimulated U373 human astrocytoma cells by a polymerisation chain reaction method involving co-amplification with an internal multi-specific control. *Eur. Cytokine Netw.* **3:** 553–563.
31. Esbenshade, T. A., C. Han, T. L. Theroux, J. G. Granneman, and K. P. Minneman. 1992. Coexisting β_1- and atypical β-adrenergic receptors cause redundant increase in cyclic AMP in human neuroblastoma cells. *Mol. Pharmacol.* **42:** 753–759.
32. Galitzky, J., M. Reverte, C. Carpéné, M. Lafontan, and M. Berlan. 1993. β_3-Adrenoceptors in dog adipose tissue: studies on their involvement in the lipomobilizing effect of catecholamines. *J. Pharmacol. Exp. Ther.* **266:** 358–366.
33. Lacasa, D., P. Mauriège, M. Lafontan, M. Berlan, and Y. Giudicelli. 1986. A reliable assay for β-adrenoceptors in intact isolated human fat cells with a hydrophilic radioligand, [^{3}H]CGP12177. *J. Lipid Res.* **27:** 368–376.
34. Arch, J. R. S., and A. J. Kaumann. 1993. β_3- and atypical β-adrenoceptors. *Med. Res. Rev.* **13:** 663–729.
35. Lowell, B. B., and J. S. Flier. 1995. The potential significance of β_3-adrenergic receptors. *J. Clin. Invest.* **95:** 923.
36. Arner, P., L. Hellström, H. Wahrenberg, and M. Brönnegard. 1990. β-Adrenoceptor expression in human fat cells from different regions. *J. Clin. Invest.* **86:** 1595–1600.
37. Granneman, J. G., and K. N. Lahners. 1992. Differential adrenergic regulation of β_1- and β_3-adrenoceptor messenger ribonucleic acids in adipose tissues. *Endocrinology.* **130:** 109–114.
38. Collins, S., K. W. Daniel, E. M. Rohlfs, V. Ramkumar, I. L. Taylor, and T. W. Gettys. 1994. Impaired expression and functional activity of the β_3- and β_1-adrenergic receptors in adipose tissue of congenitally obese (C57BL/6J ob/ob) mice. *Mol. Endocrinol.* **8:** 518–527.
39. Himms-Hagen, J., J. Cui, E. Danforth, D. J. Taatjes, S. S. Lang, B. L. Waters, and T. H. Claus. 1994. Effect of CL-316,243, a thermogenic β_3-agonist, on energy balance in brown and white adipose tissue in rats. *Am. J. Physiol.* **266:** R1371–R1382.
40. Nisoli, E., C. Tonello, and M. O. Carruba. 1994. SR 58611A: a novel thermogenic β-adrenoceptor agonist. *Eur. J. Pharmacol.* **259:** 181–186.
41. Dolan, J. A., H. A. Muenkel, M. G. Burns, S. M. Pellegrino, C. M. Fraser, F. Pietri, A. D. Strosberg, E. E. Largis, M. D. Dutia, J. D. Bloom, A. S. Bass, T. K. Tanikella, A. Cobuzzi, F. M. Lai, and T. H. Claus. 1994. β_3-Adrenoceptor selectivity of the dioxolane dicarboxylate phenethanolamines. *J. Pharmacol. Exp. Ther.* **269:** 1000–1006.
42. Wheeldon, N. M., D. G. McDevitt, L. C. McFarlane, and B. J. Lipworth. 1994. β-Adrenoceptor subtypes mediating the metabolic effects of BRL 35135 in man. *Clin. Sci.* **86:** 331–337.
43. Blin, N., C. Nahmias, M. F. Drumare, and A. D. Strosberg. 1994. Mediation of most atypical effects by species homologues of the β_3-adrenoceptor. *Br. J. Pharmacol.* **112:** 911–919.
44. Enocksson, S., M. Shimizu, F. Lönnqvist, J. Nordenström, and P. Arner. 1995. Demonstration of an in vivo functional β_3-adrenoceptor in man. *J. Clin. Invest.* **95:** 2239–2245.
45. Kenakin, T. 1993. Competitive antagonism. *In* Pharmacologic Analysis of Drug-Receptor Interaction. T. Kenakin, editor. Raven Press, New York. 278–322.
46. Wheeldon, N. M., D. G. McDevitt, and B. J. Lipworth. 1993. Do β_3-adrenoceptors mediate metabolic responses to isoprenaline? *Q. J. Med.* **86:** 595–600.
47. Van Baak, M. A., E. Thijssen, E. E. Blaak, and W. H. Saris. 1995. β-Adrenoceptor subtypes mediating the stimulation of energy expenditure and lipolysis by isoprenaline. *Int. J. Obesity.* **19:** S2. 41 (Abs.).
48. Shen, Y-T., H. Zhang, and S. F. Vatner. 1994. Peripheral vascular effects of β_3-adrenergic receptor stimulation in conscious dogs. *J. Pharmacol. Exp. Ther.* **268:** 466–473.