Early alterations in the brown adipose tissue adenylate cyclase system of pre-obese Zucker rat *fa/fa* pups: decreased G-proteins and β3-adrenoceptor activities

Christine CHARON,* Stéphane KRIEF,† Francine DIOT-DUPUY,* A. Donny STROSBERG,† Laurent J. EMORINE†‡ and Raymond BAZIN*§

*INSERM U 177, 15 rue de l'Ecole de Médecine, 75270 Paris, cedex 06, France, and †CNRS UPR 0415 and Université Paris VII, Institut Cochin de Génétique Moléculaire, 22 rue Méchain, 75014 Paris, France

This study was undertaken to determine whether receptor and non-receptor components of the adenylate cyclase (AC) cascade were altered in brown adipose tissue (BAT) of 14-day-old pre-obese (*fa/fa*) rats, before endocrine status is strongly modified by *fa* gene expression. Activity of the AC catalytic subunit did not differ between the two genotypes. In *fa/fa* rats compared with control *Fa/fa* rats, there was a 50% decrease in the activity of αG_s (stimulated by NaF or guanosine 5'-[γ-thio]triphosphate) but no change in protein content (Western blotting). αG_i function, assessed by the inhibitory action of low concentrations of guanosine 5'-[βγ-imido]triphosphate upon 10^{-4} M forskolin-stimulated AC activity, was equally low in both genotypes. Analysis of dose–response curves for different β-agonists revealed that (i) both the basal and the maximally stimulated activity of AC were 2-fold lower in *fa/fa* rats than in *Fa/fa* rats; (ii) BRL37344 and CGP12177 (β3 agonists) were less potent in *fa/fa* than in *Fa/fa* rats ($K_{act.}$ multiplied by 2); (iii) noradrenaline and isoprenaline (Iso), at the low-affinity site (β3-AR), were less potent in *fa/fa* than in *Fa/fa* pups ($K_{act.}$ increased by 30 and 20% respectively). At the high-affinity site (mainly β1) these two agonists were more potent in *fa/fa* than in *Fa/fa* rats ($K_{act.}$ decreased by 40 and 80% respectively). In good agreement with the latter result, the β1-adrenergic receptor (β1-AR)-selective antagonist CGP20712A had more effect on the Iso-stimulated AC activity in pre-obese than in lean pups (2-fold decreased in IC_{50}). Binding experiments with [^{3}H]CGP12177 show that in BAT of suckling rats, β3-ARs represent 80% of the total β-ARs. $B_{max.}$ values for the two sites were not affected by the genotype, although the β3-AR mRNA concentration in BAT (quantitative reverse-transcriptase PCR) was 3-fold lower in *fa/fa* rats than in *Fa/fa* pups. In conclusion, these results provide evidence for alterations in β1- and β3-AR signalling in BAT of 14-day-old suckling pre-obese Zucker rats with a decreased activity of αG_s. The impaired AC responsiveness to catecholamines might be a primary contributor to the development of this genetic obesity.

INTRODUCTION

In brown adipose tissue (BAT), thermogenesis is regulated by noradrenaline (NA) acting primarily via β-adrenergic receptors (β-ARs). Binding of NA results in the activation of adenylate cyclase (AC), which requires the interaction of at least three membrane-bound proteins: the β-AR, a signal-transducing G-protein (G_s) and the catalytic subunit C (for review see Levitzki [1]).

Although a significant population of β1-adrenoceptors exists in rat BAT, together with a small number of β2-adrenoceptors (for review see Lafontan and Berlan [2]), considerable evidence has suggested that the β-AR which mediates lipolysis and thermogenesis in this tissue displays pharmacological properties inconsistent with the β1/β2-AR subclassification [3]. In recent years, a third subtype of β-AR, the β3-adrenoceptor (β3-AR) [4], has been characterized in rodent brown and white adipose tissues [5–9] where it is thought to be of paramount importance in transducing noradrenergic messages in BAT.

In genetic models of obesity, such as the *ob/ob* mouse and the *fa/fa* Zucker rat, obesity occurs as a result of a single gene mutation. The mouse *ob* gene has been cloned recently [10] and the rat *fa* gene has been localized on chromosome 5 [11]. However, neither the primary defect nor the product of these genes is known. Decreased AC activity in response to catecholamines has been characterized in adipose tissues of both adult *fa/fa* Zucker rats and *ob/ob* mice [5,12] and has been correlated with a decrease in β-AR expression [7,13]. However, the AC system is also composed of signal-transducing heterotrimeric G-proteins which bind and hydrolyse GTP. One form of G_s and three different forms of G_i (G_{i1}, G_{i2}, G_{i3}), consisting of a distinct GTP-binding and similar β-γ subunits have been identified in fat-cell membranes [14].

Several hormones including insulin, thyroid hormones, glucocorticoids and catecholamines regulate the expression and the activity of both β-adrenoceptors (for review see Lafontan and Berlan [2]) and G-proteins [15–18]. In genetic models of obesity, when the syndrome is clearly expressed, numerous alterations in

Abbreviations used: AC, adenylate cyclase; AR, adrenergic receptor; BAT, brown adipose tissue; $B_{max.}$, receptor density; BRL37344, sodium-4{2-[2-hydroxy-2-(3-chlorophenyl)ethyl-amino]propyl}phenoxyacetate sesquihydrate; [^{3}H]CGP12177, 4-(3-t-butylamino-2-hydroxypropoxy)[5,7-^{3}H]benzimidazole-2-one; CGP20712A, [2-(3-carbamoyl-4-hydroxyphenoxy)-ethylamino]-3-[4-(1-methyl-4-trifluormethyl-2-imidazolyl)-phenoxy]-2-propanolmethanesulphonate; metrizamide, (2-[3-acetamido-5-*N*-methylacetamido-2,4,6-tri-iodobenzamido]-2-deoxy-D-glucose); GTP[S], guanosine 5'-[γ-thio]triphosphate; p[NH]ppG, guanosine 5'-[βγ-imido]triphosphate; Iso, isoprenaline; NA, noradrenaline; RT, reverse-transcriptase.

‡ Present address: Laboratoire de Pharmacologie et Toxicologie Fondamentales, 205 route de Narbonne, 31077 Toulouse cedex, France.

§ To whom correspondence should be addressed.

the endocrine status, including those of the above-mentioned hormones, are detectable [19] and could be responsible for defects in the AC system reported in adult animals by several investigators [5,15].

BAT dysfunction has been suggested to play an important role in the positive energy balance [20] in Zucker *fa/fa* rats. The positive energy balance is, at the onset of obesity, mainly due to defective energy expenditure [21] and a decreased capacity for BAT thermogenesis [22]. Impaired BAT responsiveness to catecholamines is now clearly established in adult rats; however, the AC system has never been investigated in BAT of young pre-obese rats and it is not clear whether this abnormality is a consequence of endocrine disorders or one of the primary defects responsible for obesity development. We previously reported that NA content and T4-to-T3 conversion were decreased in the BAT of suckling *fa/fa* rats compared with their lean *Fa/fa* littermates [23,24]. More recently, administration of thermogenic β-adrenergic agonists to *fa/fa* pups was shown to correct all the known metabolic abnormalities of BAT, and to prevent emergence of their hyperinsulinaemia [24,25]. Treatment of Zucker neonates with NA also prevented an excess of fat deposition in *fa/fa* pups [26], suggesting that impaired sympathetic activity is present early in life in *fa/fa* Zucker rats. Thus, if impaired cellular responsiveness to catecholamines was also present in BAT, it might play a role in the development of this genetic obesity.

The current study was undertaken to obtain further insight into the mechanisms involved in defective β-adrenergic stimulation of BAT (receptor and non-receptor components of the AC system including G-proteins) in pre-obese Zucker pups. In 14-day-old *fa/fa* rats, at a time when BAT dysfunction plays a crucial role in the positive energy balance, we reported a decrease in β3-mRNA levels and impaired basal and agonist-stimulated AC activity which seemed to be due to impaired αG_s activity.

MATERIALS AND METHODS

Materials

BRL37344 was generously provided by Beecham Pharmaceuticals. [^{3}H]CGP12177 (46 Ci/mmol) was from Amersham International. Unlabelled CGP12177 and CGP20712A were gifts from Ciba-Geigy.

Animals and protocols

Zucker rats were bred in our laboratory from pairs originally provided by the Harriet G. Bird Memorial Laboratory (Stowe, MA, U.S.A.). Animals used in this study were obtained from obese (*fa/fa*) males and heterozygous lean (*Fa/fa*) females. Pups and their mothers were housed in a temperature-controlled room (22 ± 1 °C) on a fixed 12:12-h light/dark cycle (lights on from 07:00h to 19:00h). The dams were fed on stock diet (UAR, France) *ad libitum*. At 2 days of age, litter size was standardized at 10 pups.

Genotype identification

The genotype of the pups (*Fa/fa* and *fa/fa*) was identified through the study of the correlation between inguinal adipose weight and body weight [27].

Tissue sampling and processing

At 14 days of age, rats of either sex were killed by decapitation. Interscapular BAT was carefully dissected out and immediately frozen at −70 °C for further isolation of RNA or for preparation of membranes.

Preparation of crude membranes

Membranes were prepared according to Grannemann and MacKenzie [13]. Briefly, one tissue (80–100 mg) was homogenized in 2.5 ml of cold 25 mM Tris/HCl buffer (pH 7.4) containing 250 mM sucrose and 1 mM $MgCl_2$ and then filtered through glass wool. The filtrate was centrifuged at 1100 *g* for 10 min at 4 °C, the supernatant removed and centrifuged at 48000 *g* for 10 min at 4 °C. The resulting pellet was washed twice in fresh buffer. Finally, membranes were resuspended in 25 mM Tris/HCl (pH 7.5) buffer containing 1 mM EDTA.

Preparation of plasma membranes

BAT plasma membranes were prepared according to Giacobino [28]. Three tissues (300–400 mg) were homogenized with a glass–Teflon homogenizer in 2 ml of ice-cold 0.05 mM $CaCl_2$/0.1 mM $NaHCO_3$/0.02 mM $MgSO_4$ buffer (pH 7.5). Homogenate was centrifuged at 1100 *g* for 10 min at 4 °C, the supernatant removed and the pellet resuspended and centrifuged a second time. The two supernatants were pooled and centrifuged at 35000 *g* for 20 min at 4 °C. The resulting pellet was homogenized in fresh buffer, layered on to 5 ml of 18.5% (w/v) metrizamide and centrifuged at 156000 *g* for 90 min at 4 °C. Plasma membranes were found in a layer above the metrizamide, they were collected and centrifuged at 180000 *g* for 45 min at 4 °C. The pellet was resuspended in 10 mM Tris/HCl (pH 7.4) buffer containing 25 mM sucrose and 1 mM EDTA and filtered on a 60-μm-pore-size filter (Nybond HC60). Plasma membranes were frozen at −80 °C, until utilization for Western-blot analysis and binding experiments.

Marker enzyme assays

5′-Nucleotidase was the marker used for plasma membranes and was determined as described by Avruch and Wallach [29]. Contamination by mitochondria (the major subcellular component in brown fat) was assessed by measurement of cytochrome *c* oxidase activity as described by Yonetani and Ray [30]. The proteins were measured according to the method of Bradford [31].

AC assay

AC (EC 4.6.1.1) activity was measured according to the method of Feve [36], for 10 min at 35 °C in a 50 μl standard assay consisting of 0.2 mM [α-^{32}P]ATP (PB 171; Amersham Co.), 1 mM cyclic AMP, 10 mM phosphocreatine, 0.5 unit of creatine phosphokinase, 5 μM GTP, 5 mM $MgCl_2$, 0.2 mM EDTA and 50 mM Tris/HCl (pH 7.5). Membranes (20–30 μg of protein) were preincubated for 15 min at 4 °C with or without additional compounds to allow equilibration. The reaction was initiated by addition of this mixture to the incubation medium and stopped by addition of cold 1 M HCl (200 μl). After 5 min at 95 °C, followed by neutralization with 200 μl of 3.34 M imidazole, the [α-^{32}P]cyclic AMP was separated from AMP by column chromatography on alumina (neutral aluminium oxide 90, active). The [α-^{32}P]cyclic AMP eluted with 3 ml of 50 mM imidazole (pH 7.5) was quantified by liquid scintillation spectroscopy. AC activity was expressed as the amount of cyclic AMP formed/min per mg of protein.

Activity of the AC catalytic subunit was estimated according to Chaudry and Granneman [33], in the presence of 2 μM Mn^{2+},

100 μM guanosine 5'-[β-thio]diphosphate (GDP[S]) and without Mg^{2+} (to inhibit G_s activity) in basal conditions, or after stimulation by 100 μM diterpene forskolin. Other drugs, which exert their actions directly through G_s or G_i, i.e. NaF, non-hydrolysable GTP analogues such as guanosine 5'-[γ-thio]triphosphate (GTP[S]) and guanosine 5'-[$\beta\gamma$-imido]-triphosphate (p[NH]ppG) [5], were used to estimate the activity of the G-proteins. Furthermore, β-adrenergic effectors such as NA, isoprenaline (Iso), BRL37344 (a selective β3-agonist) and CGP12177A (a specific β1/β2-antagonist but β3-agonist) were used to activate AC when they were bound to their specific receptor.

Western-blot analysis of G_s protein

Plasma membranes were electrophoresed on an SDS/12%-polyacrylamide gel, electroblotted and incubated with a 1/1000 dilution of an anti-αG_s protein antibody (NEI-805; NEN Dupont de Nemours, France). The immune complex was detected by autoradiography after incubation with ^{125}I-labelled Protein A (Amersham). Bands corresponding to αG_s proteins were detected at 42 and 45 kDa.

RNA analysis

RNA analysis was performed according to Krief et al. [34]. Total RNA was extracted from BAT by the CsCl (5.7 M) method and treated for 15 min at 37 °C with 0.3 unit of RNase-free DNase I (RQ1 DNase; Promega) per μg of nucleic acid in 40 mM Tris/HCl (pH 7.9)/10 mM NaCl/6 mM $MgCl_2$ in the presence of 2 units/μl of placenta RNase inhibitor (Promega). RNA was then extracted with phenol/chloroform (1:1, v/v) and precipitated. cDNA synthesis was performed with 100 units of Maloney murine leukaemia virus reverse-transcriptase (RT) (Life Technologies Inc.) using 50–100 ng of total RNA in 10 μl of RT buffer [67 mM Tris/HCl (pH 8.4), 6.7 mM $MgCl_2$, 6.7 μM EDTA, 10 mM 2-mercaptoethanol and 16 mM $(NH_4)_2SO_4$] containing 0.4 mM of each dNTP, 10 μM of random hexanucleotides (Pharmacia, France), and 2 units/μl of placenta RNase inhibitor (Promega). Samples were then supplemented with 4 μl of 10 × PCR buffer [1 × PCR buffer: 50 mM KCl/10 mM Tris/HCl (pH 8.3) at 25 °C/1.5 mM $MgCl_2$/0.1 mg/ml gelatin] containing 0.5 μl of 25 mM of each dNTP, 10 μl of 50% DMSO, 0.5 μl of solutions (25 μM) of each sense and antisense oligonucleotide, 1.25 units of *Thermophilus aquaticus* DNA polymerase (Perkin-Elmer), and water to 40 μl. cDNAs were denatured for 2 min at 94 °C and amplified with 29 temperature cycles (94 °C, 15 s; 60 °C, 30 s; 72 °C, 30 s) followed by 3 min of final extension at 72 °C in a temperature cycler (GeneAmp PCR System 9600; Perkin-Elmer). Sequences of the sense and antisense oligonucleotides for β3-AR were as follows: 5'-ATGGCTCCGTG-GCCTCAC-3' and 5'-CCCAACGGCCAGTGGCCAGTCAG-CG-3' respectively. For quantitative purposes, PCR site-directed mutagenesis was performed to create a *Bam*HI restriction site within the sequence of the human β3-AR cloned gene corresponding to those amplified using the above primers. Mutated DNA were cloned into pGEM4Z transcription vectors, and RNA was synthesized using an RNA transcription kit (Promega); 50 ng and 100 ng of RNA were used for lean (*Fa/fa*) and obese (*fa/fa*) rats respectively. These quantities of RNA were co-amplified with the following quantities of the corresponding mutated RNA: 3×10^4, 1×10^5, 3×10^5, 1×10^6 molecules; the expected sizes of amplification products were 308 bp and 317 bp for wild type and mutant respectively. To distinguish the wild type from synthetic mutants, aliquots of the PCR reaction were digested for 2 h at 37 °C with 5 units of *Bam*HI (Boehringer Mannheim) in a total volume of 10 μl of PCR buffer. Products were run in ethidium bromide-stained 2% agarose gels, and the fluorescence associated with DNA bands was measured using a high-speed video camera (Imager, Appligene, France) together with the NIH image program (Twilight Clone BBS, Silver Spring, MD, U.S.A.). Results were expressed as log(mutant cDNA signal/wild-type cDNA signal) as a function of log(amount of standard RNA). With such an analysis, linear curves were obtained with regression coefficients greater than 0.98. The quantity of wild-type RNA was calculated at the equivalence point when the ordinate equalled zero.

[^{3}H]CGP12177 binding to plasma membranes

Membrane aliquots (50 μg of protein) were incubated for 30 min at 37 °C in a final volume of 200 μl containing 10 mM $MgCl_2$, 1 mM ascorbic acid, 100 μM GTP, 50 mM Tris/HCl (pH 7.5) and various concentrations of [^{3}H]CGP12177 (TRK 751; Amersham) ranging from 0.2 to 120 nM. After incubation, the reaction mixture was immediately diluted with 4 ml of cold reaction buffer and bound [^{3}H]CGP12177 was separated from free radioligand by filtration over glass-fibre disks (Whatman GF/C) pretreated with polyethylenimine (0.3% for 3 h) and washed three times. Radioactivity of the filters was measured in a liquid scintillation counter. Non-specific binding was defined as the amount of [^{3}H]CGP12177 bound in the presence of 100 μM propranolol, and represented about 15 and 35% of total binding at the high- and low-affinity binding sites respectively. Receptor density ($B_{max.}$) and dissociation constant (K_d) values were determined by Scatchard analysis.

Statistical analysis

Results are means ± S.E.M. The level of significance in the difference between groups was calculated according to the analysis of variance (ANOVA) test.

RESULTS

Results presented in Table 1 show that membrane characteristics of preparations from lean and pre-obese rats were not changed by *fa* gene expression. In both genotypes, purified plasma membranes have a 5'-nucleotidase specific activity 30-fold higher than in crude membranes. Whatever the membrane preparations, contamination with mitochondria (assessed by cytochrome *c* oxidase activity) was identical in *fa/fa* and *Fa/fa* rats. Thus it could be concluded that membrane preparations from the two genotypes were of similar purity.

Activity of the AC catalytic subunit (estimated in the presence of Mn^{2+} and without Mg^{2+}) was not different between the two genotypes either in basal conditions (9.7 ± 1.2 and 13.2 ± 2.56 pmol of cyclic AMP produced/min per mg of protein, for six *Fa/fa* and six *fa/fa* rats respectively) or after stimulation by 1×10^{-4} M forskolin (134 ± 23.2 and 106 ± 22.0 pmol of cyclic AMP produced/min per mg of protein, for six *Fa/fa* and six *fa/fa* rats respectively).

NaF or non-metabolizable GTP analogues, such as GTP[S] and p[NH]ppG, are known to have both stimulatory and inhibitory effects on AC. The ultimate effect of these compounds depends on their concentration and on the nature and concentration of cations present in the incubation medium.

Ability of G_s to stimulate AC activity was assessed either at 10 mM NaF or at 1×10^{-4} M GTP[S] (Table 2). With both drugs, AC activity was significantly reduced in *fa/fa* rats compared with

Table 1 Marker enzyme activities in membranes obtained from BAT of 14-day-old Zucker rats

Crude and purified plasma membrane extracts were prepared as described in the Materials and methods section. Results are means ± S.E.M.; (*n*) number of experiments. One unit of enzyme activity was defined as the amount of enzyme needed to transform 1 mol of substrate/min.

	(*n*)	5′-Nucleotidase specific activity (*μ*-unit/mg of protein)	Cytochrome *c* oxidase specific activity (m-unit/mg of protein)
Lean	(3)		
Crude membranes		5.70 ± 0.52	44.30 ± 4.52
Plasma membranes		174.20 ± 19.51	16.50 ± 1.57
Obese	(3)		
Crude membranes		6.20 ± 0.54	54.30 ± 5.48
Plasma membranes		183.80 ± 19.30	19.30 ± 1.66

Table 2 Stimulation of AC activity by NaF and GTP[S] in BAT of 14-day-old Zucker rats

Results are means ± S.E.M. of eight different experiments. $^{**}P < 0.01$ *fa/fa* versus *Fa/fa*.

	AC activity (pmol of cyclic AMP produced/min per mg of protein)		
	(*n*)	Lean (*Fa/fa*)	Obese (*fa/fa*)
NaF (10 mM)	(7)	350 ± 30	177 ± 30**
GTP[S] (100 *μ*M)	(6)	323 ± 64	116 ± 23**

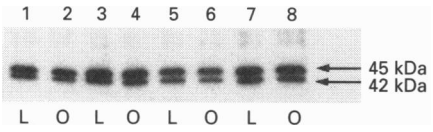

Figure 1 Immunoblotting of αG_s protein isoforms in BAT plasma membranes from lean and pre-obese animals

Detection of the 42 kDa and 45 kDa forms of G_s in membranes (15–30 *μ*g of protein) from lean (tracks 1, 3, 5 and 7) and pre-obese (tracks 2, 4, 6 and 8) animals.

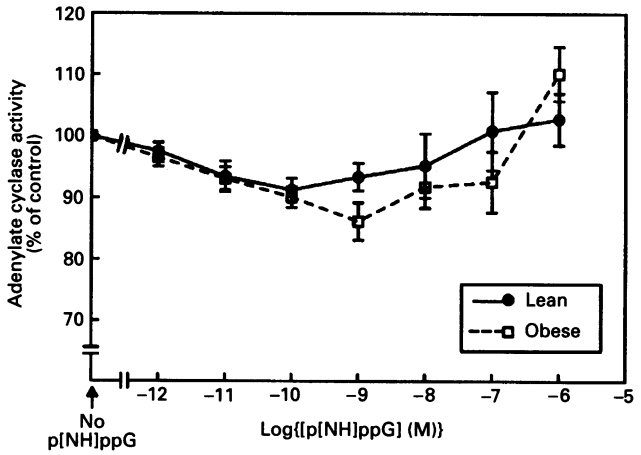

Figure 2 Inhibition of AC activity with low concentrations of p[NH]ppG

AC activity was assayed in the presence of 100 *μ*M forskolin and increasing concentrations of p[NH]ppG. Results are means ± S.E.M. of six experiments.

Table 3 AC activity in basal conditions and after maximal stimulation by different *β*-agonists

AC activity in BAT membranes from lean and pre-obese rats was determined in the absence or in the presence of 100 *μ*M agonist: NA, Iso, BRL37344, or CGP12177. Results are means ± S.E.M.; (*n*) number of experiments; $^{**}P < 0.01$ *fa/fa* versus *Fa/fa*.

	AC activity (pmol of cyclic AMP produced/min per mg of protein)			
	(*n*)	Lean (*Fa/fa*)	(*n*)	Obese (*fa/fa*)
Basal	(27)	51.50 ± 4.42	(26)	28.00 ± 2.18**
NA	(6)	247.80 ± 19.75	(4)	110.70 ± 9.04**
Iso	(6)	325 ± 85	(6)	124.00 ± 18.80**
BRL37344	(7)	221.00 ± 39.05	(8)	85.30 ± 11.75**
CGP12177	(8)	105.00 ± 15.23	(8)	40.80 ± 5.25**

their *Fa/fa* littermates (−50% and −60% for NaF and GTP[S] respectively).

Western-blot experiments were therefore performed to examine the possibility that decreased G_s activity could have resulted from decreased α subunit expression level. Figure 1 clearly shows that the concentration of αG_s in BAT plasma membranes was identical in *Fa/fa* and in *fa/fa* Zucker rats.

Because G_i is believed to be at least as abundant as G_s in membranes [35], AC activity might depend upon the functional activity of G_i. Analysis of G_i functioning was therefore assessed by measuring the action of p[NH]ppG upon 1×10^{-4} M forskolin-stimulated AC activity (Figure 2). Due to the higher affinity of p[NH]ppG for G_i compared with G_s, inhibition of AC activity observed at low concentrations of p[NH]ppG, could be attributed to G_i action. At 10^{-9}–10^{-8} M p[NH]ppG, a slight but significant inhibitory effect was observed in *Fa/fa* as well as in *fa/fa* pups, and this effect was of similar magnitude in both genotypes.

In conclusion, these experiments demonstrate that coupling activity of G_s is lower in pre-obese Zucker rats compared with their lean counterparts. Such alterations can be attributed neither to decreased αG_s concentrations nor to modifications of the activities of αG_i or of AC catalytic subunits.

To examine the status of the *β*-AR component of the AC complex, dose–response curves were constructed using various *β*-agonists. Table 3 presents AC activity in basal conditions and after maximal stimulation by different *β*-adrenergic agonists: NA, Iso, BRL37344 and CGP12177. In *fa/fa* rats compared with their lean *Fa/fa* littermates, basal AC activity was significantly decreased (−50%). Saturating concentrations (10^{-4} M) of NA,

Table 4 Comparative analysis of the effector-stimulated AC activities in BAT membranes of 14-day-old Zucker rats

AC activity was determined in BAT crude membranes in the presence of various concentrations of indicated ligands. Kinetic parameters ($K_{act.}$ and $V_{max.}$) of AC activity were estimated by non-linear regression analysis (Inplot4). Values are means ± S.E.M.; (*n*) number of experiments. $K_{act.}$ values correspond to concentration of ligand giving 50% of the maximal stimulation. $V_{max.}$ values represent the maximal production of cyclic AMP in pmol/min per mg of protein.

	Lean (*Fa/fa*)			Obese (*fa/fa*)		
Ligand	(*n*)	$K_{act.}$ (μM)	$V_{max.}$ (pmol/min per mg of protein)	(*n*)	$K_{act.}$ (μM)	$V_{max.}$ (pmol/min per mg of protein)
NA	(13)	3.50 ± 0.37	253 ± 39	(12)	1.70 ± 0.33**	122 ± 16**
Iso	(6)	2.40 ± 0.31	282 ± 66	(6)	1.10 ± 0.34*	119 ± 15*
BRL37344	(6)	0.110 ± 0.018	179 ± 14	(5)	0.240 ± 0.024**	103 ± 7**
CGP12177	(5)	0.720 ± 0.050	105 ± 8	(3)	1.40 ± 0.33*	41.0 ± 0.5**

** $P < 0.01$; * $P < 0.05$ *fa/fa* versus *Fa/fa*.

Table 5 Kinetic parameters of AC activation by NA and Iso in BAT membranes from 14-day-old lean and pre-obese Zucker rats

AC activity was determined in BAT crude membranes in the presence of various concentrations of NA or Iso. Activation of the high- and low-affinity components could be modelled by a two-site mass action equation. Site 1 and site 2 correspond to ($\beta1 + \beta2$) and $\beta3$ components respectively. Proportion of total catecholamine-stimulated activity mediated by each site is given in parentheses. $V_{max.}$ values represent the maximal production of cyclic AMP in pmol/min per mg of protein. $K_{act.}$ values correspond to concentration of ligand giving 50% of the maximal stimulation. Values are means ± S.E.M.; (*n*) number of experiments.

	Lean (*Fa/fa*)			Obese (*fa/fa*)		
Ligand	(*n*)	$K_{act.}$ (nM)	$V_{max.}$ (pmol/min per mg of protein)	(*n*)	$K_{act.}$ (nM)	$V_{max.}$ (pmol/min per mg of protein)
NA						
Site 1	(7)	142.0 ± 16.7	80 ± 17 (34%)	(7)	87.0 ± 11.7*	36.0 ± 8.5 (30%)
Site 2		1703 ± 147	163 ± 32 (66%)		2260 ± 151*	83.0 ± 18.6 (70%)
Iso						
Site 1	(6)	62.0 ± 11.5	93.0 ± 27.2 (29%)	(6)	9.50 ± 2.56**	41.0 ± 6.7 (30%)
Site 2		1608 ± 76	231 ± 67 (71%)		1953 ± 70**	96.0 ± 14.9 (70%)

** $P < 0.01$; * $P < 0.05$ *fa/fa* versus *Fa/fa*.

Iso, the $\beta3$-selective agonist BRL37344 and the $\beta3$-partial agonist CGP12177 (and $\beta1/\beta2$-specific antagonist) were able to exert potent stimulation of AC activity in membranes from lean and pre-obese Zucker rats. However, AC activity remained significantly lower (40–60%) in *fa/fa* than in *Fa/fa* pups.

$K_{act.}$ and $V_{max.}$ values calculated after analysis of dose–response curves are shown in Table 4. The concentration of the non-selective β-agonists Iso and NA required for half-maximal stimulation of AC ($K_{act.}$ values, Table 4) was 2–3-fold lower in *fa/fa* than in *Fa/fa* rats. In contrast, stimulation of AC with the $\beta3$-selective agonists BRL37344 and CGP12177 resulted in $K_{act.}$ values 2-fold higher in *fa/fa* than in *Fa/fa* pups.

For each agonist, the calculated $K_{act.}$ values were in good agreement with those obtained on 3T3-F442A adipocytes [36], suggesting that, as for these cells, a major population of $\beta3$-AR is also present in BAT adipocytes. For NA- and Iso-stimulated AC activity, kinetics parameters ($K_{act.}$ and $V_{max.}$) were thus estimated by fitting concentration–response data to one- or two-site mass action equations with non-linear regression analysis (Table 5). Taking into account the fact that $\beta2$-ARs are undetectable in BAT [8], the high-affinity component mainly corresponds to $\beta1$-AR, whereas the low-affinity component represents $\beta3$-AR. This analysis revealed that 70% of total activity was mediated by $\beta3$-adrenoceptors. It also showed that at the low-affinity site, Iso and NA were less potent in *fa/fa* than in *Fa/fa* pups, whereas the contrary was observed at the high-affinity site.

These results suggest that in BAT of pre-obese rats, the $\beta3$-AR component activity of AC was decreased, while that of $\beta1$ was increased. To test this possibility, we explored the effect of selective $\beta1$-receptor blockade with CGP20712A on the Iso-stimulated AC activity (Figure 3). Results showed that the concentration of CGP20712A required to exert half-maximal inhibition of Iso-stimulated AC activity (IC_{50}) was 2-fold lower in *fa/fa* than in *Fa/fa* rats (41.3 versus 89.2 μM, $n = 6$, for *fa/fa* versus *Fa/fa* respectively; $P < 0.05$).

In order to study the molecular mechanism at the basis of decreased $\beta3$-AR-stimulated AC activity, quantitative RT-PCR analysis was performed to compare receptor mRNA steady-state levels in lean and pre-obese pups (Figure 4). Results clearly showed that, in BAT of 14-day-old Zucker rats, a significant decrease in $\beta3$-mRNA concentration was already present in *fa/fa* compared with *Fa/fa* pups [$(1062 \pm 170) \times 10^3$ and $(303 \pm 33) \times 10^3$ molecules/μg of RNA for lean and pre-obese rats respectively, $n = 6$; $P < 0.05$, for *fa/fa* versus *Fa/fa*]. It could be speculated that an increase in the absolute amount of total RNA could compensate for the relative decrease of $\beta3$ mRNA observed in BAT of pre-obese pups. However, measurement of total RNA in tissues from *fa/fa* and *Fa/fa* pups revealed that there was no difference between the two genotypes (125 ± 10.28 and 124.7 ± 7.53 μg per whole tissue for seven *Fa/fa* and seven *fa/fa* rats respectively).

Hydrophilic radioligand [^{3}H]CGP12177 binding to β-ARs in BAT plasma membranes was performed to compare binding characteristics of *Fa/fa* and *fa/fa* pups. The binding of [^{3}H]CGP12177 to membranes from lean and pre-obese rats

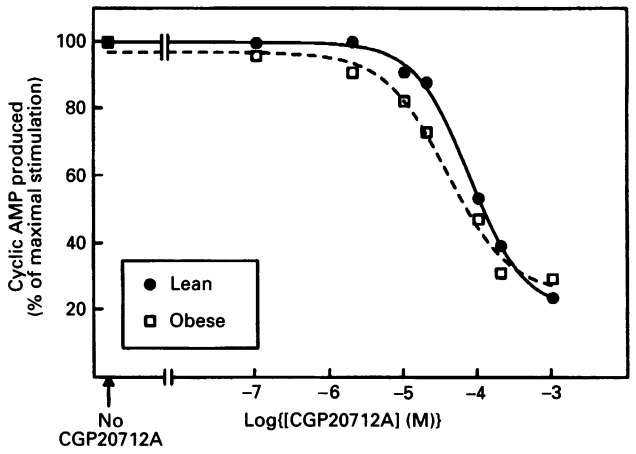

Figure 3 One example of an inhibition curve of Iso-stimulated AC activity by the β1-selective antagonist CGP20712A

Inhibition of Iso-stimulated (10^{-4} M) AC activity was performed in the presence of increasing concentrations of CGP20712A. IC_{50} values (the negative logarithm of the inhibitory constant for agonist) have been calculated from dose–response curves for the antagonist versus constant agonist concentration.

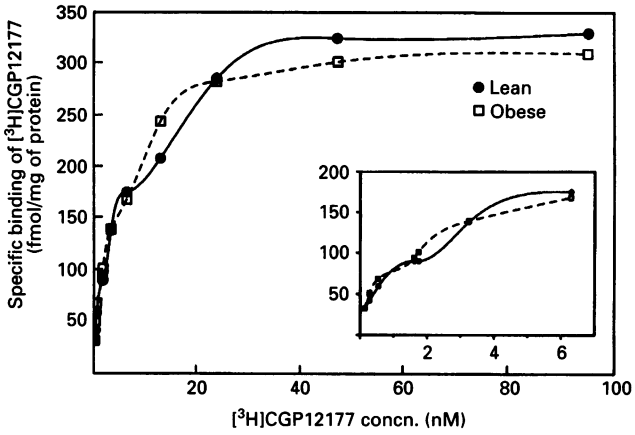

Figure 5 Saturation curves of a typical [³H]CGP12177 binding experiment performed on BAT plasma membranes from lean and pre-obese Zucker rats

Data were analysed according to the method of Scatchard using the EBDA/LIGAND computer program. For the two genotypes, data fit well with a two-site model. Inset shows details of binding data at low concentrations of radioligand.

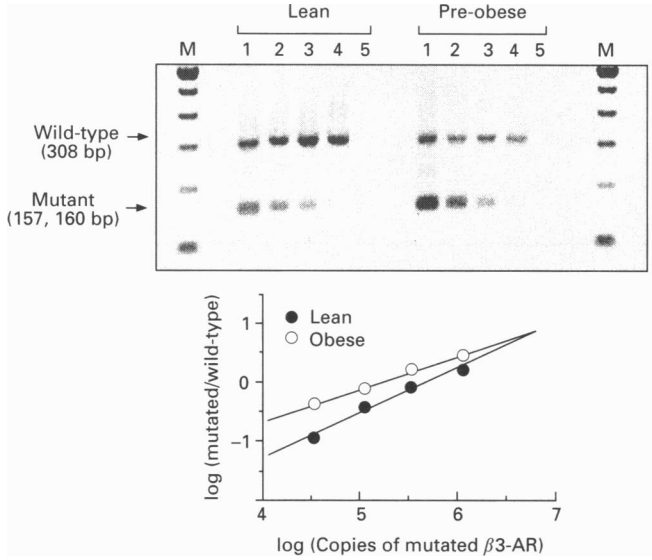

Figure 4 β3-AR mRNA steady-state level analysis by a quantitative RT-PCR assay

Following amplification of β3-AR cDNA, reaction products were digested with *Bam*HI and separated by agarose gel electrophoresis (top panel). Analysis has been performed on 50 ng and 100 ng samples of wild-type RNA for lean and pre-obese rats respectively and lanes 1–4 contained four different quantities of synthetic mutated RNA (see the Materials and methods section). Negative control of PCR reaction was performed using wild-type RNA without the initial reverse transcriptase step (lanes 5). Fluorescence signals associated with each band were measured by video-densitometry scanning. In the graphic representation of the results (lower panel), log(mutated/wild type) represents the logarithm of the ratio of signals corresponding to mutant and wild-type RNA. Quantities of sample mRNA were calculated at the equivalence point when log(mutated/wild type) = 0 (see the Materials and methods section).

showed an initial plateau of saturation at about 2 nM of the radioligand and a second saturable binding component at higher concentrations (Figure 5). Scatchard analysis of saturation isotherms allowed the determination of the $B_{max.}$ and K_d values of both the high- and the low-affinity [³H]CGP12177 sites, corresponding to β1- plus β2-AR and β3-AR, respectively. Results (Table 6) showed that in BAT membrane from Zucker pups, β3-ARs represented 80% of total β-ARs. K_d and $B_{max.}$ values of the two affinity AR populations were not modified by the genotype. Furthermore, K_d values of the high-affinity β1-adrenoceptors and low-affinity β3-adrenoceptors for [³H]CGP12177 were in the same range as those observed in CHO cells expressing the rat β3-adrenoceptor [37], in 3T3-F442A adipocytes [38] and in BAT membranes of adult Zucker rats [37].

DISCUSSION

In the present study we have shown that αG_s and β3-AR coupling to AC are already altered in the BAT of 14-day-old pre-obese Zucker rats (*fa/fa*) compared with their lean counterparts. The coupling activity of β1-AR is on the contrary increased in the pre-obese animals.

Similar observations have also been made in the liver and adipose tissue AC system of obese adult rats and mice [12,15,17,39]. However, this situation in adults may relate to the profound modifications of body hormonal status which accompany obesity [19]. Indeed, both the activity and expression levels of receptors and G-proteins of the AC cascade are regulated by factors such as insulin, thyroid hormones, glucocorticoids and catecholamines [16,18,40]. In 14-day-old *fa/fa* rats, even if endocrine status is not strongly modified by *fa* gene expression, it cannot be excluded that the changes observed here in BAT were secondary to slight hormonal alterations. However, the increase in insulin level reported in *fa/fa* pups [24] would be expected to have an effect on the activity of G_i rather than on that of G_s [17,39]. Thus, altered αG_s and β3-AR coupling to AC could in itself play a role in the development of this obesity.

Our experiments using forskolin, NaF and GTP[S] clearly show that the *fa* mutation does affect the αG_s subunit rather than the catalytic component of AC. However, concentrations of both the small and the large forms of αG_s were not modified by the genotype, indicating that decreased G_s activity could not be accounted for by a diminution of protein content or a modi-

Table 6 Characteristics of [^{3}H]CGP12177 binding sites in BAT plasma membranes from lean and pre-obese Zucker rats

Saturation experiments were performed within a range of concentrations of [^{3}H]CGP12177 (0.10–100 nM). Data were analysed according to the method of Scatchard using the EBDA/LIGAND computer program. Results are means ± S.E.M.; (*n*) number of experiments. Percentage of total binding for high- and low-affinity sites is given in parentheses.

		High-affinity sites		Low-affinity sites	
	(*n*)	K_d (nM)	$B_{max.}$ (fmol/mg of protein)	K_d (nM)	$B_{max.}$ (fmol/mg of protein)
Lean (*Fa/fa*)	(4)	0.600 ± 0.150	114 ± 9 (24%)	9.80 ± 1.54	352 ± 28 (76%)
Obese (*fa/fa*)	(4)	0.470 ± 0.144	100 ± 19 (24%)	8.40 ± 1.24	315 ± 11 (76%)

fication of the ratio of these two forms. Indeed, modifications of G_s activity with no changes in αG_s concentration nor in β-AR density have already been reported [13,39].

G_i activity was also compared between pre-obese and lean pups since it is now accepted that increased activity of this G-protein component, resulting in increased levels of β/γ dimers, may also reduce G_s stimulation of AC. A similarly weak G_i activity was, however, detected in both types of animals. Furthermore, expression levels of αG_{i1} and αG_{i2} were found to be identical in *Fa/fa* and *fa/fa* genotypes (P. de Mazancourt, personal communication).

In pre-obese Zucker rats, besides a decreased G_s activity, we also observed a diminished capacity of β3-AR, together with a higher efficiency of the β1-AR to stimulate AC. No changes, either in the affinity of these two β-AR subtypes for CGP12177 or in their absolute density could, however, be measured between lean and pre-obese pups. In white adipose tissue and/or BAT of obese adult rats [7] and mice [41] a decreased responsiveness to β3-agonists has been associated with a decreased β3-AR mRNA level rather than with a decreased receptor number. In pre-obese Zucker rats, a diminution of β3-AR mRNA levels is already present, but seems to have no bearing on receptor number.

Decreased steady-state levels of β3-AR transcripts in the BAT of *fa/fa* pups could result from insulin action [32,42,43], since at 14 days of age a slight hyperinsulinaemia has already developed [24]. Longer duration and/or higher levels of hyperinsulinaemia could, however, be able to down-regulate β3-AR protein and mRNA in obese adults.

From these observations, it remains possible that the lower efficiency of β3-AR, in pre-obese versus lean animals, to stimulate AC is due to modifications in the coupling properties of αG_s with the β3-AR. Since we showed here that coupling of β1-AR to AC is, on the contrary, increased in *fa/fa* rats, this suggests two hypotheses. Either β1- and β3-AR interact with the same G_s protein, but the coupling properties of the G-protein and of the two β-AR subtypes are differentially affected by the *fa* mutation; or alternatively it is possible that, as recently suggested for G_i [44], several combinations of heterotrimeric G_s proteins exist with which interactions with β-AR subtypes are differentially regulated. Modulation of β-AR high- and low-affinity states for agonists should probably result from both situations. In systems as complex as adipose cells, which express three β-AR subtypes (and by consequence six affinity states), such phenomena are, however, almost impossible to quantify.

In conclusion, whatever the mechanisms, decreased activity of the β3-AR/AC cyclic AMP signalling pathway in BAT of pre-obese *fa/fa* pups, together with the low sympathetic stimulation of the tissue [23], is certainly involved in, and possibly the cause of, the development of obesity in Zucker rats.

We thank Dr. Ph. de Mazancourt from the Laboratory of Biochemistry (CHI Poissy, France) for immunoblot analysis of G_i proteins. This work was supported by grants from the Association pour la Recherche sur le Cancer, the Ligue Nationale Française contre le Cancer, the Bristol–Myers–Squibb Company (Princeton, NJ, U.S.A.), and the University Paris VII.

REFERENCES

1 Levitzki, A. (1988) Science **241**, 800–806
2 Lafontan, M. and Berlan, M. (1993) J. Lipid Res. **34**, 1057–1091
3 Arch, J. R. S., Ainsworth, A. T., Cawthorne, M. A. et al. (1984) Nature (London) **309**, 163–165
4 Emorine, L. J., Marullo, S., Briend-Sutren, M. M. et al. (1989) Science **245**, 1118–1121
5 Strassheim, D., Palmer, T. M., Milligan, G. and Houslay, M. D. (1991) Biochem. J. **276**, 197–202
6 Nahmias, C., Blin, N., Elalouf, J. M., Mattei, M. G., Strosberg, A. D. and Emorine, L. J. (1991) EMBO J. **10**, 3721–3727
7 Muzzin, P., Revelli, J.-P., Kuhne, F. et al. (1991) J. Biol. Chem. **266**, 24053–24058
8 D'Allaire, F., Atgié, C., Mauriège, P., Simard, P.-M. and Bukowiecki, L. J. (1995) Br. J. Pharmacol. **114**, 275–282
9 Granneman, J. G. and Lahners, K. N. (1992) Endocrinology **130**, 109–114
10 Zhang, Y., Proenca, R., Maffei, M., Barone, M., Leoplod, L. and Friedman, J. M. (1994) Nature (London) **372**, 425–432
11 Truett, G. E., Bahary, N., Friedman, J. and Leibel, R. L. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 7806–7809
12 Begin-Heick, N. (1985) J. Biol. Chem. **260**, 6187–6193
13 Granneman, J. G. and MacKenzie, R. G. (1988) J. Pharmacol. Exp. Ther. **245**, 1068–1074
14 Mitchell, F. M., Griffiths, S. L., Saggerson, E. D., Houslay, M. D., Knowler, J. T. and Milligan, G. (1989) Biochem. J. **262**, 403–408
15 Houslay, M. D., Gawler, D., Milligan, G. and Wilson, A. (1989) Cell. Signal. **1**, 9–22
16 Houslay, M. D. (1991) Cell. Signal. **3**, 1–9
17 Begin-Heick, N. (1990) Biochem. J. **268**, 83–89
18 Ros, M., Northup, J. K. and Malbon, C. C. (1988) J. Biol. Chem. **263**, 4362–4368
19 Bray, G. A. and York, D. A. (1979) Physiol. Rev. **59**, 719–809
20 Krief, S. and Bazin, R. (1991) Proc. Soc. Exp. Biol. Med. **198**, 528–538
21 Planche, E., Joliff, M. and Bazin, R. (1988) Int. J. Obesity **12**, 353–360
22 Bazin, R., Eteve, D. and Lavau, M. (1984) Biochem. J. **221**, 241–245
23 Krief, S., Bazin, R., Dupuy, F. and Lavau, M. (1989) Biochem. J. **263**, 305–308
24 Marie, V., Dupuy, F. and Bazin, R. (1992) Am. J. Physiol. **263**, E115–E120
25 Charon, C., Dupuy, F., Marie, V. and Bazin, R. (1995) Am. J. Physiol. **268**, E1039–E1045
26 Körtner, G., Petrova, O., Vogt, S. and Schmidt, I. (1994) Am. J. Physiol. **267**, E947–E953
27 Lavau, M. and Bazin, R. (1982) J. Lipid Res. **23**, 941–943
28 Giacobino, J. P. (1979) J. Supramol. Struct. **11**, 445–449
29 Avruch, J. and Wallach, D. F. H. (1971) Biochim. Biophys. Acta **233**, 334–347
30 Yonetani, T. and Ray, G. (1965) J. Biol. Chem. **240**, 3392–3398
31 Bradford, M. M. (1974) Anal. Biochem. **72**, 248–254
32 Engfeldt, P., Hellmer, J., Wahrenberg, H. and Arner, P. (1988) J. Biol. Chem. **263**, 15553–15560
33 Chaudry, A. and Granneman, G. (1991) Am. J. Physiol. **261**, R403–R411
34 Krief, S., Fève, B., Baude, B., Zilberfarbe, V., Strosberg, A. D., Pairault, J. and Emorine, L. J. (1994) J. Biol. Chem. **269**, 6664–6670
35 Gilman, A. G. (1987) Signals Annu. Rev. Biochem. **56**, 615–649

36 Feve, B., Emorine, L. J., Lasnier, F. et al. (1991) J. Biol. Chem. **266**, 20329–20336
37 Muzzin, P., Revelli, J. P., Fraser, C. M. and Giacobino, J. P. (1992) FEBS Lett. **298**, 162–164
38 Feve, B., Emorine, L. J., Briend-Sutren, M. M., Lasnier, F., Strosberg, A. D. and Pairault, J. (1990) J. Biol. Chem. **265**, 16343–16349
39 Begin-Heick, N. (1992) Am. J. Physiol. **263**, C121–C129
40 Ros, M., Northup, J. K. and Malbon, C. C. (1989) Biochem. J. **257**, 737–744
41 Collins, S., Kiefer, W. D., Rohlfs, E. M., Ramkumart, V., Taylor I. L. and Gettys, T. W. (1994) Mol. Endocrinol. **8**, 518–527
42 Feve, B., Elhadri, K., Quignard-Boulangé, A. and Pairault, J. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 5677–5681
43 Olansky, L., Jacobsson, B. and Pohl, S. (1981) Clin. Res. **29**, 871A
44 Chaudry, A., MacKenzie, R. G., Georgic, L. M. and Granneman, J. G. (1994) Cell. Signal. **6**, 457–465

Received 30 May 1995/10 July 1995; accepted 1 August 1995