Targeted Gene Disruption Reveals a Leptin-independent Role for the Mouse β_3-Adrenoceptor in the Regulation of Body Composition

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Abstract

Targeted disruption of mouse β_3-adrenoceptor was generated by homologous recombination, and validated by an acute in vivo study showing a complete lack of effect of the β_3-adrenoceptor agonist CL 316,243 on the metabolic rate of homozygous null (−/−) mice. In brown adipose tissue, β_3-adrenoceptor disruption induced a 66% decrease ($P < 0.005$) in β_1-adrenoceptor mRNA level, whereas leptin mRNA remained unchanged. Chronic energy balance studies in chow-fed mice showed that in −/− mice, body fat accumulation was favored (+41%, $P < 0.01$), with a slight increase in food intake (+6%, NS). These effects were accentuated by high fat feeding: −/− mice showed increased total body fat (+56%, $P < 0.025$) and food intake (+12%, $P < 0.01$), and a decrease in the fat-free dry mass (−10%, $P < 0.05$), which reflects a reduction in body protein content. Circulating leptin levels were not different in −/− and control mice regardless of diet. The significant shift to the right in the positive correlation between circulating leptin and percentage of body fat in high fat–fed −/− mice suggests that the threshold of body fat content inducing leptin secretion is higher in −/− than in control mice. Taken together, these studies demonstrate that β_3-adrenoceptor disruption creates conditions which predispose to the development of obesity. (*J. Clin. Invest.* 1997. 100:1098–1106.) Key words: β-adrenoceptors • adipose tissue • obesity • thermogenesis • leptin

Introduction

The β_3-adrenoceptor, first isolated from a human genomic library (1) and then from rat brown adipose tissue (BAT)[1] cDNA libraries (2, 3), is the predominant β-adrenoceptor in rodent BAT and white adipose tissue (WAT) (4). One major difference between β_3- and β_1/β_2-adrenoceptors is the much lower affinity of the former for catecholamines. This led to the postulate that the β_1/β_2-adrenoceptors mediate the effects of circulating catecholamines, whereas the β_3-adrenoceptor mediates only the effects of the much higher concentrations of norepinephrine present in the sympathetic synaptic cleft during nerve stimulation of lipolysis in WAT and BAT and of thermogenesis in BAT (4).

The concept that a defect in the β_3-adrenoceptor might be involved in the pathogenesis of obesity and diabetes in rodents was suggested by the observation that in BAT and WAT of obese *fa/fa* Zucker rats and *ob/ob* mice, the β_3-adrenoceptor expression was decreased (2, 5). The β_3-adrenoceptor is also involved in the relaxation of the esophagus (6) and the stomach fundus (7) as well as in the control of colon motility (8), and it has been detected recently in rat brain hippocampus, cerebral cortex, and striatum (9). In human heart, it was found to be responsible for unexpected negative inotropic effects (10).

The various postulated biological roles for the β_3-adrenoceptor and the lack of conclusive evidence in favor of its association with obesity or diabetes were strong incentives to attempt its targeted disruption in mice. A targeted disruption of the β_3-adrenoceptor has been reported recently by Susulic et al. (11). It was accomplished by direct injection of the targeting DNA construct into mouse zygotes. Mice homozygous for the disrupted allele (−/−) responded normally to chronic cold exposure and, although they did not become overtly obese, they had significantly higher body fat content than control mice (11).

An interesting interplay has been described between the β_3-adrenoceptor and leptin, the product of the recently cloned *ob* gene (12). This newly described protein secreted by the adipocyte has been proposed to function as a signal linking adiposity to the control of food intake and metabolic rate. Leptin expression and secretion are positively correlated with the adipose tissue mass (12–14), and administration of leptin inhibits food intake and increases energy expenditure in rodents (15–17). These findings have led to the hypothesis that leptin could stimulate the sympathetic nervous system via the hypothalamus (18), thus exerting β_3-adrenoceptor–mediated effects in BAT and WAT. In these tissues, leptin mRNA expression is also downregulated by stimulation of the β_3-adrenoceptor (19). This finding supports a specific role played by the β_3-adrenoceptor in the control of energy balance through leptin.

In an attempt to clarify the possible physiological role of the β_3-adrenoceptor, we generated mice with a targeted disruption of the cognate gene by classical homologous recombination. This study describes the consequences of β_3-adrenoceptor deficiency on energy balance, uncoupling protein (UCP), leptin and β-adrenoceptor expressions, and on circulating leptin levels.

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1. *Abbreviations used in this paper:* BAT, brown adipose tissue; ES, embryonic stem; UCP, uncoupling protein; WAT, white adipose tissue.

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Methods

All organic and inorganic chemicals were of analytical or molecular biology grade and were purchased from Merck (Darmstadt, Germany), Boehringer Mannheim (Mannheim, Germany), Sigma Chemical Co. (St. Louis, MO), and Fluka AG (Buchs, Switzerland). Restriction endonucleases and modification enzymes were purchased from Promega Corp. (Madison, WI), Hybond N and N^+ membranes, [α-^{32}P]dCTP (3,000 Ci/mmol) from Amersham International (Bucks, United Kingdom), and tissue culture reagents from GIBCO BRL (New York, NY), except for fetal bovine serum which was purchased from Seromed Fakola (Basel, Switzerland), and newborn calf serum from Amimed Produkte AG (Basel, Switzerland). CL 316,243 was the generous gift of Wyeth-Ayerst Research (Princeton, NJ).

Cloning of the mouse β_3-adrenoceptor gene. Genomic β_3-adrenoceptor fragments were obtained by screening a BglII-digested genomic DNA library derived from a 129 Sv mouse strain (made in λ FIX II Stratagene, kindly provided by P. Vassalli, Centre Médical Universitaire, Genéve, Switzerland), with the rat β_3-adrenoceptor cDNA probe described by Revelli et al. (20). From one of the strongly hybridizing phage clones obtained during this screening, a 10-kb fragment resulting from a partial PstI-BglII digestion was isolated. A restriction map of this fragment was performed, and its comparison with the nucleotide sequence of the mouse gene (21) showed that it contained the complete β_3-adrenoceptor gene flanked by 7.5 and 0.3 kb of its upstream and downstream sequences, respectively (Fig. 1 *a*).

Construction of the targeting plasmid. The 10-kb PstI-BglII fragment was cloned into a pGEM5 vector. The targeting plasmid was constructed by inserting a neomycin resistance gene, driven by a PGK promoter (PGK-NEO) in the XhoI site of the first exon of the β_3-adrenoceptor gene. In the β_3-adrenoceptor, this restriction site corresponds to the end of the fifth transmembrane domain. The third intracellular loop which immediately follows this transmembrane domain is of vital importance, since it is involved in the interaction of the receptor with G proteins (22). Translation from the initiation codon, if it should occur, would thus result in the expression of a short peptide with no biological activity. The targeting plasmid contains 8.0 and 1.6 kb of uninterrupted homologous sequence upstream and downstream, respectively, of the PGK-NEO gene. Before electroporation, the plasmid was digested at the SalI site of pGEM5 directly upstream of the PstI site, and at the SfiI site of the third exon. The insert was gel purified, and splinkers (adaptators with hairpin structure) were ligated to both ends to protect the fragment against exonuclease activity (Fig. 1 *b*), as described by Bernet-Grandaud et al. (23).

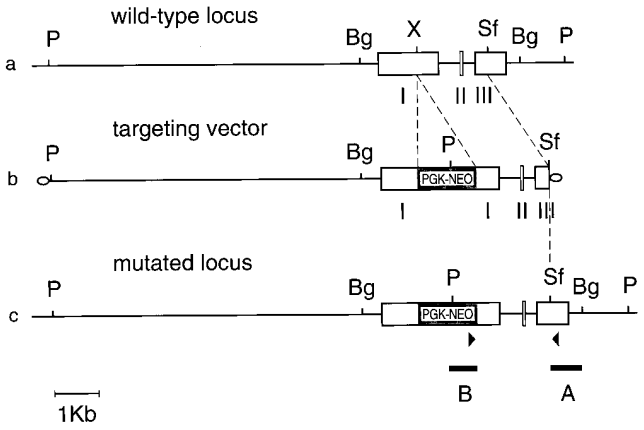

Figure 1. The wild-type β_3-adrenoceptor gene (*a*) was interrupted in the first exon by insertion of a neomycin resistance gene driven by a PGK promoter (*PGK-NEO, hatched box*). The targeting vector is described in *b*; it carries splinkers at both ends, herein depicted as loops. The resultant recombinant gene is schematized in *c.* Numbers I to III (*open boxes*) represent the β_3-adrenoceptor exons. *A* and *B*, Probes used for Southern blotting detection. *Arrowheads*, Positions of the primers used for PCR screening. Restriction sites: *Bg*, BglII; *P*, PstI; *Sf*, SfiI; *X*, XhoI.

Cell culture and electroporation. An embryonic stem (ES) cell line, PCJ1 (established in our laboratory from blastocysts of the 129 Sv mouse strain, i.e., isogenic with the library from which the gene was cloned), was used for all experiments. Cells were cultured in Dulbecco's modified medium, containing FBS 10%, newborn calf serum 10%, penicillin G 100 μg/ml, streptomycin 100 U/ml, β-mercaptoethanol 0.1 mM, L-glutamine 2 mM, Hepes 20 mM, pH 7.4, and recombinant murine leukemia inhibitory factor 10^3 U/ml. 10^8 cells were collected by trypsinization, resuspended in 800 μl PBS with 30 μg targeting DNA, and electroporated using a Gene Pulser (220 V, 960 μF, electrode distance 0.4 cm; Bio-Rad Labs., Hercules, CA). Cells were reseeded in the above described medium on gelatin-coated 100-mm-diameter dishes at a density of 10^7 cells per dish. 24 h after electroporation, the medium was replaced, and geneticin at 400 μg/ml was added to select for neomycin-resistant clones.

Screening of ES cells. ES cells carrying a β_3-adrenoceptor gene disrupted by homologous recombination were identified by PCR and by genomic Southern blotting. Briefly, 10–14 d after electroporation and selection by geneticin, individual colonies were picked up and expanded in duplicate on 10-mm-diameter dishes. DNA from one of the duplicates was prepared for PCR analysis by a 2-h incubation of the cells at 55°C with Proteinase K 400 μg/ml followed by a 15-min boiling to inactivate Proteinase K. Screening for homologous recombinants was performed with a 5′ primer (5′-GTTGGCTACCCGTGATATTGC-3′) in the NEO gene, and 3′ primer (5′-ATACAGCACAAACATAGGCAGG-3′) (nucleotides 2833–2854) in the third exon of the β_3-adrenoceptor gene (Fig. 1 *c*). The PCR reaction was performed on an aliquot of the inactivated Proteinase K digestion mixture under standard conditions using Pfu DNA polymerase (Stratagene Inc., La Jolla, CA) on a thermal cycler (Cetus 480; Perkin-Elmer Corp., Norwalk, CT) according to the following protocol: denaturation for 1 min at 95°C, annealing for 1 min at 67°C, extension for 1 min at 72°C during 20 cycles (with a 0.5°C decrease in annealing temperature at each cycle), followed by 1 min at 94°C, 1 min at 57°C, and 1 min at 72°C for another 20 cycles. Out of a total of 107 clones screened, three homologous recombinants were identified by the presence of a 1.7-kb amplification product.

Screening of chimeric progeny. Two ES cell lines carrying the disrupted β_3-adrenoceptor gene were expanded, microinjected into C57BL/6J blastocysts, and transferred into tribrom-ethanol–anesthetized mice as described by Hogan et al. (24). The progeny of both lines showed a strong coat color chimerism and readily transmitted the mutated allele when mated with C57BL/6J mice. DNA was isolated from the tails of the F1 progeny. Heterozygous mice (129 Sv × C57BL/6J) were interbred to generate homozygous −/− mice. Genotyping was performed by Southern blot analysis on BglII-digested tail DNA using an SfiI-BglII external probe (Fig. 1 *c*, *A*) or PstI-digested DNA with an NEO internal probe (Fig. 1 *c*, *B*) to rule out tandem repeat integrations.

Northern blots. Experiments were performed on tissues from 8-wk-old male mice housed in groups of six at a temperature of 22°C with free access to tap water and to a standard laboratory chow diet (low fat) composed of 20% protein, 60% carbohydrate, and 4% fat (wt/wt), and with a metabolizable energy content of 12.1 kJ/g.

Interscapular BAT and epididymal WAT were dissected, with BAT carefully trimmed from contaminating WAT by microdissection. RNA was isolated using a Micro RNA Isolation Kit (Stratagene Inc.). 10 μg of total RNA was electrophoresed on a 1% agarose gel containing formaldehyde as described by Lehrach et al. (25) and transferred to Hybond N membranes by capillary blotting. Hybridizations were performed with rat probes (^{32}P-labeled with a Random Priming Kit; Amersham International) having a high homology to the corresponding mouse DNA region. The rat cDNA β_1-, β_2-, and

β_3-adrenoceptor probes used were those previously described by Revelli et al. (20). The rat UCP probe was that described by Bouillaud et al. (26). The *ob* gene probe of 354 bp was that described by Moinat et al. (27). Size estimates for the RNA species were established by comparison with an RNA ladder. RNA levels were quantified by scanning photodensitometry of the autoradiograms. Subsequent hybridization of the blots with a [γ-^{32}P]ATP-labeled synthetic oligonucleotide specific for the 18S rRNA subunit was used to correct for differences in the amounts of RNA loaded onto the gels.

Metabolic rate measurements. Evaluation of metabolic rates was performed on mice fed a chow diet. 8-h energy expenditure was measured using open-circuit indirect calorimetry with computerized equipment. The calorimeter includes six chambers maintained at 29°C. The values for O_2 consumption were automatically and sequentially recorded every minute in each chamber. Two unrestrained mice were housed per chamber and, $\sim$ 3 h later, when the O_2 consumption was stable, the mice were injected with either NaCl or the β_3-adrenoceptor agonist CL 316,243 dissolved extemporaneously in sterile saline (1 μg/g, subcutaneous). The metabolic rate was calculated offline using Weir's equation and normalized to body weight to the 0.75 power. 15 successive 1-min sampling periods were averaged, and the mean metabolic rate during the control period for each group of mice was that obtained for 60 min before the administration of CL 316,243.

Body composition. Six +/+ and six −/− male mice (20-wk-old) were killed by cervical dislocation followed by decapitation, and the whole carcasses were incised, dried to a constant weight in an oven at 60°C, and then homogenized. Total body fat content was determined by the Soxhlet extraction method using light petroleum ether; the results are presented as absolute weight (g) and as percentages of total body weight. The fat-free dry mass was obtained by subtraction of body fat content from dry weight.

High fat feeding experiment. Nine +/+ and 10 −/− male mice (6-wk-old) housed individually were fed during an additional 5 wk a pelleted high fat diet composed of 20% protein, 60% carbohydrate, and 20% corn oil (wt/wt) purchased from ICN Biomedicals Inc. (Costa Mesa, CA) and stored at 4°C. This diet had an energy density of 19.8 kJ/g with a percentage of energy derived from fat of 40%. Mice were weighed, and food intake was determined, after correction for spillage, five times per week for the duration of the study. At 11 wk of age, the mice were killed and processed as described above for the mice on a chow diet.

Circulating compounds. Serum was obtained by retroorbital bleeds from fed or 7-h–fasted mice. Circulating leptin was measured using a mouse leptin RIA kit (Linco, Inc., St. Charles, MD), insulin using a ^{125}I insulin tracer from Sorin Diagnostics (Saluggia, Italy) and guinea pig anti–rat insulin serum (Linco, Inc.), glucose using a Glucose GOD Perid Kit (Boehringer Mannheim) and FFA using a NEFA C kit (Wako Chemicals GmbH, Neuss, Germany).

Statistical analysis. Statistical analyses were performed with the Statistix 4.0 program (Analytical Software, St. Paul, MN). Comparisons between groups were made by paired or unpaired Student's *t* test for equal or unequal variances for normally distributed variables, or Wilcoxon's test for not normally distributed variables. Correlation coefficients were obtained by the Pearson method.

Results

Generation and validation of mice with a targeted disruption of the β_3-adrenoceptor. Homologous recombination in two ES cell clones was confirmed by Southern blot analysis as described below for tail genomic DNA (not shown).

ES cells carrying the disrupted β_3-adrenoceptor gene were microinjected into blastocysts which were transferred into female mice. The progeny was interbred to obtain homozygous mutant mice. The identification of the wild type (+/+) or β_3-adrenoceptor–deficient heterozygous (+/−) and homozygous (−/−) mice was performed by Southern blot analysis of

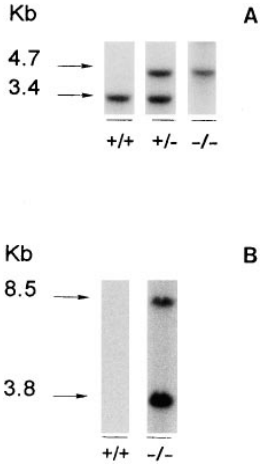

Figure 2. (*A*) Southern blot of wild-type β_3-adrenoceptor (+/+), heterozygous (+/−), or homozygous (−/−) mouse-tail genomic DNA digested with BglII and hybridized with the SfiI-BglII external probe shows the 3.4-kb wild-type allele and the 4.7-kb recombinant allele. (*B*) Southern blot of mouse-tail genomic DNA digested with PstI and hybridized with the NEO internal probe: no signal for the wild type, two fragments of 3.8 and 8.5 kb for the recombinant allele.

mouse-tail genomic DNA digested with BglII and hybridized with the ^{32}P-labeled SfiI-BglII external probe (Fig. 1 *c*, *A*). The mutated allele was identified as a 4.7-kb fragment, and the wild-type allele as a 3.4-kb fragment (Fig. 2 *A*). Digestions with PstI followed by hybridization with the ^{32}P-labeled 620-bp NEO probe (Fig. 1 *c*, *B*) gave the expected mutated allele fragments of 3.8 and 8.5 kb (Fig. 2 *B*). These results showed that both the 3′ and 5′ ends of the targeting plasmid have recombined in the correct position in the genome. The chimeras transmitted the mutated allele to their pups, and F1 mice were mated to obtain homozygous mutant mice.

The validation of the β_3-adrenoceptor disruption was initially performed by Northern blot analysis of BAT and WAT mRNA. In wild-type (+/+) mouse BAT, one β_3-adrenoceptor transcript of 2.1 kb, and in WAT, three major transcripts of 2.1, 2.8, and 3.6 kb are present. In −/− mice, no β_3-adrenoceptor mRNA could be detected (Fig. 3), confirming the lack of expression of the disrupted β_3-adrenoceptor gene in both adipose tissues.

The β_3-adrenoceptor disruption was also validated in vivo by measurement of the metabolic rates of +/+ and −/− male mice before and after injection of saline or of the selective β_3-adrenoceptor agonist, CL 316,243. This experiment is illustrated in Fig. 4, in which the metabolic rate of chow-fed mice is expressed in Watts/kilogram body weight to the power 0.75. Before the injection, the metabolic rates were similar in +/+ and −/− mice, 6.3±0.4 and 6.5±0.3 W/kg$^{0.75}$, respectively. Saline-injected mice were used as controls. CL 316,243 induced a 56±14% increase in the metabolic rate of +/+ mice, which remained stable throughout the 3-h measurement period, whereas it had no significant effect on their −/− counterparts.

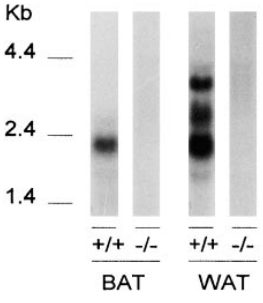

Figure 3. Northern blot of wild type (+/+) or β_3-adrenoceptor–deficient (−/−) 8-wk-old male mouse BAT and WAT RNAs, hybridized with β_3-adrenoceptor ^{32}P-labeled DNA probe as described in Methods. The positions of the molecular size markers are indicated in kilobases.

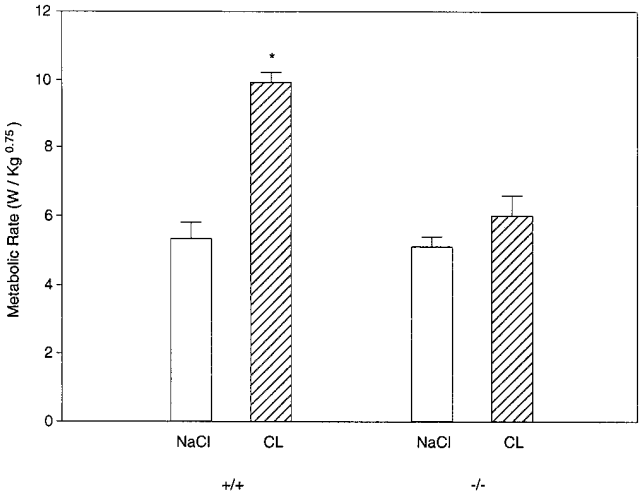

Figure 4. Metabolic rates of wild-type (+/+) or β_3-adrenoceptor–deficient (−/−) 8-wk-old chow diet–fed male mice. The animals were injected subcutaneously with either saline (NaCl) or CL 316,243 (1 μg/g) at time 0, and oxygen consumption was measured during 3 h and expressed as *metabolic rate* ($W/kg^{0.75}$). The metabolic rates were stable during the 3-h measurement period. The results are the mean±SE of three to five experiments. *NaCl*, saline. *CL*, CL 316,243. $*P < 0.025$ vs. control value.

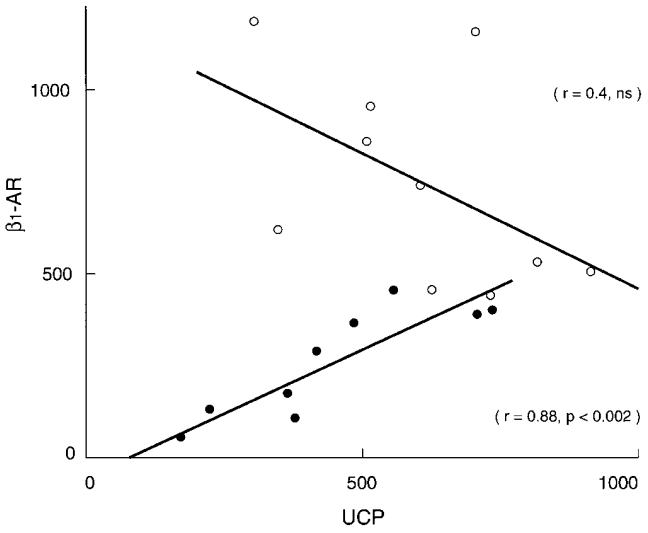

Figure 6. UCP mRNA level expressed as a function of β_1-adrenoceptor mRNA level in +/+ (○) and −/− (●) mice. Each point represents the values obtained from each BAT.

Northern blot analysis. The degree of expression of various genes implicated in the regulation of energy balance was compared in BAT and WAT of −/− and +/+ mice. As shown in Fig. 5, the targeted disruption of the β_3-adrenoceptor changed neither the level of UCP mRNA in BAT nor that of leptin mRNA in both BAT and WAT. The β_1-adrenoceptor mRNA level of −/− mice was markedly decreased (−66%) in BAT, whereas it tended to be slightly reduced in WAT. The β_2-adrenoceptor mRNA level in +/+ mouse BAT and WAT was ∼ 25% that of the β_1-adrenoceptor, and was not affected by the targeted disruption of the β_3-adrenoceptor (not shown).

Therefore, the only effect of the β_3-adrenoceptor disruption observed in these experiments was to decrease β_1-adrenoceptor mRNA level in BAT. However, as shown in Fig. 6, an analysis of a possible relationship between UCP and β_1-adrenoceptor mRNA levels in BAT of individual mice revealed a positive correlation ($r = 0.880$; $P < 0.002$) in −/− but not +/+ mice.

Energy balance, body composition, and circulating leptin. Male mice, which are hormonally more stable than females, were used in this study. They were fed a chow diet ad libitum, killed at the age of 20 wk, and their body composition was analyzed. As shown in Table I, body fat content and fat expressed as a percentage of total body weight increased by 41 and 34%, respectively, in −/− as compared to +/+ mice. The fat-free dry mass was similar in −/− and +/+ mice, and the ratio of

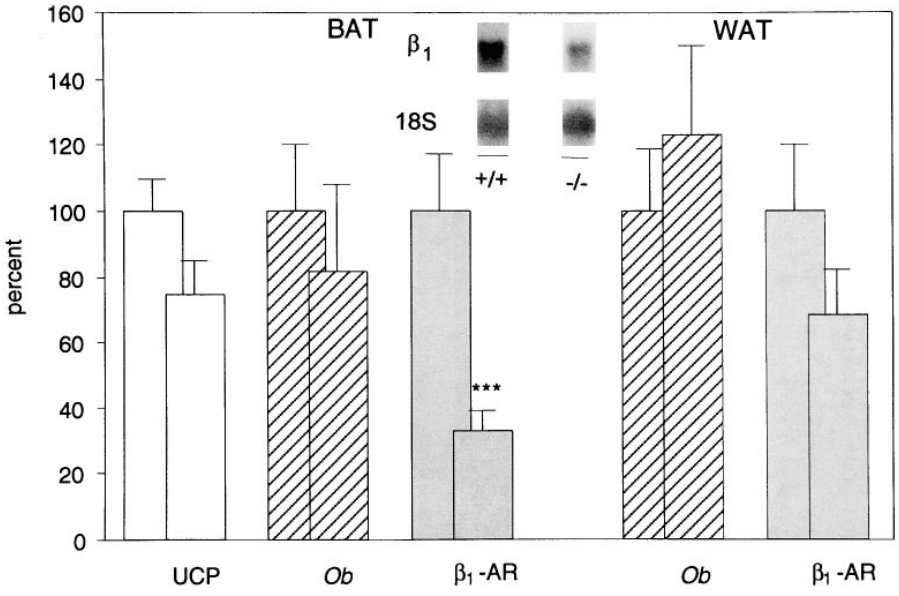

Figure 5. Photodensitometric comparisons of signals obtained from wild-type (*back columns*) or β_3-adrenoceptor–deficient (*front columns*) 8-wk-old chow-fed male mouse BAT and WAT total RNA probed with UCP, *ob* gene (*Ob*), or β_1-adrenoceptor (*AR*) cDNAs. The results are expressed as percentage±SE of the mean value in +/+ mice taken as 100%±SE; $n = 9$–10 experiments. The signals were quantified by scanning photodensitometry and normalized using the corresponding 18S rRNA values. Only signals obtained on the same Northern blot were compared. (*Inset*) Representative autoradiograms. Signals obtained in BAT with β_1-adrenoceptor and 18S rRNA (*18S*) probes are shown. $***P < 0.005$ −/− vs. +/+ mice. The sizes of the RNAs were for UCP, 1.4 kb; *ob* gene (leptin) mRNA, 3.3 kb; and β_1-adrenoceptor, 3.1 kb.

Table I. Energy Balance and Body Composition of Control (+/+) and β_3-Adrenoceptor–deficient (−/−) Mice Maintained on a Chow Diet

	+/+ $n = 6$	−/− $n = 6$	P value (−/− vs. +/+)
Final body wt (g)	31.6±0.87	33.4±1.02	NS
Food intake (g/d)	5.04±0.12	5.36±0.32	NS
Body fat content (g)	2.99±0.20	4.23±0.25	$P < 0.01$
Fat % of total body wt	9.50±0.72	12.67±0.65	$P < 0.01$
Fat-free dry mass (g)	7.26±0.22	7.41±0.24	NS
Fat-free dry mass/ body fat content	2.48±0.18	1.78±0.09	$P < 0.001$

Mice fed a chow diet ad libitum were weighed and killed at the age of 20 wk, and total carcass fat content and fat-free dry mass were determined as described in Methods. All results are means±SE (n = number of animals).

Table II. Energy Balance and Body Composition of Control (+/+) and β_3-Adrenoceptor–deficient (−/−) Mice Fed a High Fat Diet for 5 wk

	+/+ $n = 9$	−/− $n = 10$	P value (−/− vs. +/+)
Final body wt (g)	27.75±0.78	29.67±1.31	NS
Body wt gain (g)	4.40±0.57	7.29±0.94	$P < 0.05$
Food intake (g/d)	2.96±0.10	3.32±0.05	$P < 0.01$
Body fat content (g)	3.56±0.31	5.56±0.84	$P < 0.025$
Fat % of total body wt	12.30±0.85	18.07±1.87	$P < 0.02$
Fat-free dry mass (g)	7.28±0.30	6.52±0.23	$P < 0.05$
Fat-free dry mass/ body fat content	2.10±0.11	1.34±0.14	$P < 0.0005$

Mice were fed a high fat diet ad libitum between the ages of 6 and 11 wk. Body weight and food intake were measured five times per week. Mice were killed at the age of 11 wk, and total carcass fat content and fat-free dry mass were determined as described in Methods. All results are means±SE (n = number of animals).

fat-free dry mass to body fat content decreased by 28% in −/− as compared to +/+ mice. Thus, the disruption of the β_3-adrenoceptor in chow-fed mice induces a significant change in body composition. Circulating leptin levels were compared in −/− and +/+ mice under two different metabolic conditions, i.e., fed ad libitum or after a 7-h fast. Fig. 7 shows circulating leptin concentrations. It can be seen that a 7-h fast induced decreases in circulating leptin of 35 and 40% in +/+ and −/− mice, respectively. In none of the two conditions tested were the circulating leptin levels of +/+ and −/− mice significantly different.

High fat feeding experiments. Male mice were fed a high fat diet ad libitum for 5 wk, between the ages of 6 and 11 wk. They were then killed, and their body composition was analyzed. As shown in Table II, body weight gain and food intake increased by 66 and 12%, respectively, in −/− as compared to +/+ mice. Body fat content and percent body fat increased by 56 and 47%, respectively, in −/− as compared to +/+ mice. The fat-free dry mass and the ratio of fat-free dry mass to body fat content decreased by 10 and 36%, respectively, in −/− as compared to +/+ mice. Fig. 8 shows the evolution of body weight and food intake during the 5 wk of the high fat diet on a week-by-week basis. Variance analysis indicates a significantly higher body weight and food intake in −/− as compared to +/+

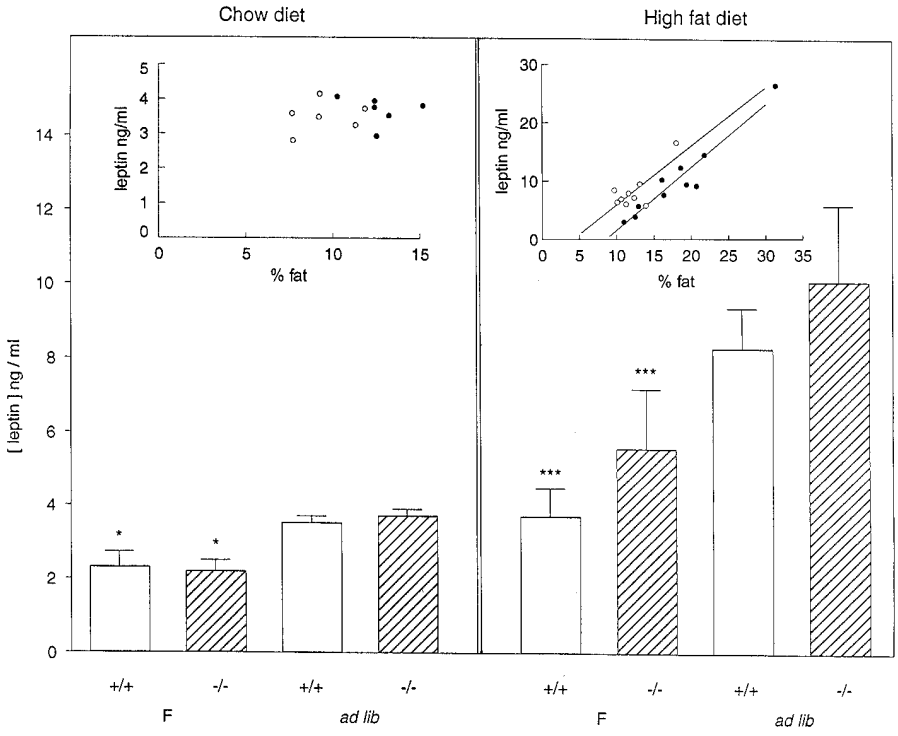

Figure 7. Radioimmunoassay determination of circulating leptin in +/+ and −/− 8-wk-old chow-fed (*left*) or 11-wk-old high fat diet–fed (*right*) male mice. The blood samples were collected under anesthesia by retroorbital bleeds either in ad libitum (*ad lib*)–fed or in 7-h–fasted (*F*) mice. The results are expressed in nanograms/milliliter of serum: mean±SE of 6–10 experiments. $*P < 0.05$, $***P < 0.005$ fasted vs. respective ad libitum values (two-tail paired t tests). In two-factor ANOVA analysis with mouse type (+/+, −/−) and treatment (fasted, ad libitum), only the effect of treatment was found to be significantly different, $P < 0.01$ in each study. (*Insets*) Circulating leptin level expressed as a function of percentage of body fat in +/+ (○) and −/− (●) mice.

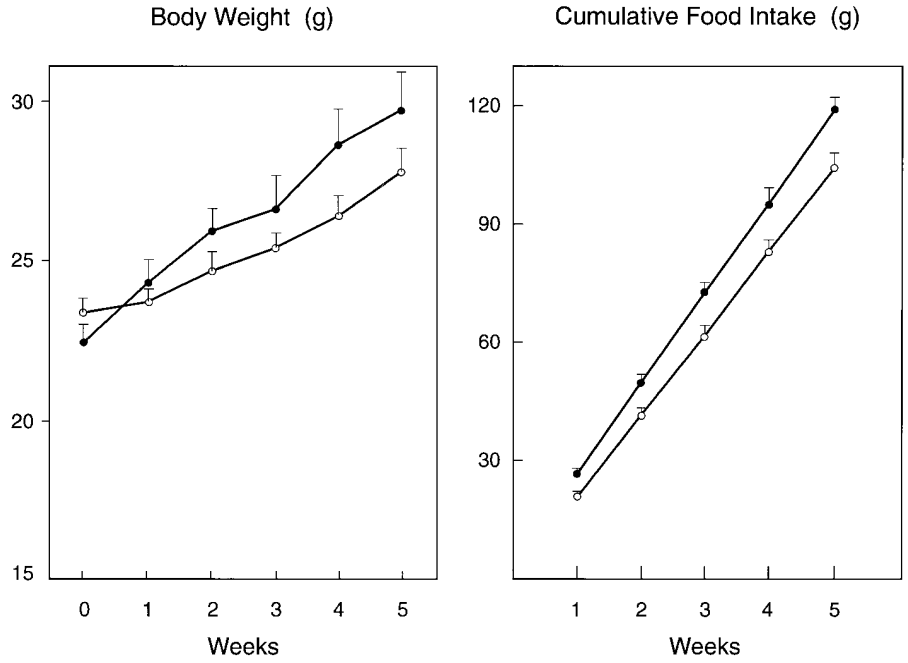

Figure 8. Body weight and food intake of +/+ (○) and −/− (●) mice expressed during 5 wk of high fat diet on a week-by-week basis. Body weight is expressed in grams and food intake in grams/wk.

mice ($P = 0.05$ and 0.01, respectively). As illustrated in Fig. 7, in mice on a high fat diet, fasting induced a significant decrease in circulating leptin of 56 and 45% in +/+ and −/− mice, respectively. In neither of the two conditions were the circulating leptin levels of +/+ and −/− mice significantly different. The disruption of the β_3-adrenoceptor is therefore without effect on circulating leptin levels. In the insets of Fig. 7, circulating leptin levels are plotted against percentage of body fat for individual mice in both diet groups. There is no correlation in mice on a chow diet. By contrast, a positive correlation is found in both +/+ and −/− mice on a high fat diet ($r = 0.75$, $P < 0.025$ and $r = 0.95$, $P < 0.0001$, respectively). Furthermore, variance analysis showed that the regression lines are parallel ($P = 0.01$), and that the regression of circulating leptin as a function of percentage of body fat for −/− mice is significantly shifted to the right ($P = 0.01$) as compared to +/+ mice.

In the course of the high fat diet experiments, circulating insulin, glucose and FFA levels were also measured; these values were similar in +/+ and −/− mice. These parameters have already been reported as unchanged in the −/− mice on a standard diet (11, and see 39).

Discussion

This study presents a targeted disruption of the mouse β_3-adrenoceptor obtained by homologous recombination. Northern blot analysis of BAT and WAT demonstrated a total disappearance of the β_3-adrenoceptor mRNA in −/− mice; moreover, an in vivo study showed a lack of stimulatory effect of the specific β_3-adrenoceptor agonist CL 316,243 on the metabolic rate of −/− mice. These results confirm the complete functional disruption of the β_3-adrenoceptor gene.

Susulic et al. (11) reported in their β_3-adrenoceptor knockout mice on a standard chow diet a significant increase in body fat content as compared with +/+ mice, similar to that shown in this study (+34% in male mice at the age of 15 wk compared to +41% at the age of 20 wk, respectively). These results are in line with those of Dulloo et al. (28), who reported that mice treated with sympatholytic drugs, which reduce norepinephrine concentration in the synaptic cleft, experience an excess of fat deposition similar to that described in this study. This suggests that the β_3-adrenoceptor plays a pivotal role in the regulation of energy balance under sympathetic neural control. Since there is no significant increase in food intake under these conditions, the increase in body fat content should reflect decreased energy expenditure. This contention is in line with the blunted thermogenic response to CL 316,243.

New findings concerning the β_3-adrenoceptor–knocked-out phenotype follow.

β_3-adrenoceptor disruption favors lipid accumulation in response to a high fat diet. Mice on a high fat diet have a higher body fat content than mice on a chow diet: this effect is more prominent in −/− mice. The percentage of fat reached 18% of total body weight in −/− mice on a high fat diet. A value of 23% has been reported in 8-wk-old transgenic mice which develop obesity after genetic ablation of their BAT (29). These results show that, in the absence of β_3-adrenoceptor, mice on a high fat diet cannot cope as well as control mice for the maintenance of body fat content, and suggest that β_3-adrenoceptor is crucial for full effectiveness of catecholamines on lipolysis and thermogenesis.

β_3-adrenoceptor disruption decreases lean body mass. It has been reported that treatment with selective β_3-adrenoceptor agonists tends to spare lean tissues (30). The β_3-adrenoceptor knockout model presented in this study provides evidence for a physiological role of the β_3-adrenoceptor in protein metabolism. Indeed, in high fat–fed mice, the fat-free dry mass, i.e., mostly protein, was found to be 10% lower in −/− than in +/+ mice. The β_3-adrenoceptor might therefore directly or indirectly exert anabolic effects on skeletal muscle. In this context, it is interesting to note that skeletal muscle contains an atypical β-adrenoceptor (31) which, among other effects, mediates

stimulation of glucose uptake by β_3-adrenoceptor agonists (32, 33). The possible links between the β_3-adrenoceptor and the muscle atypical β-adrenoceptor are currently under study in our laboratory. The presence of a β_3-adrenoceptor in skeletal muscle would widen the variety of tissues reported for β_3-adrenoceptor expression (6–10).

β3-adrenoceptor disruption increases food intake. The β_3-adrenoceptor may also play a role in the control of food intake. On a chow diet, the −/− mice tended to have a higher food intake than the +/+ mice. In high fat–fed −/− mice, this increase was accentuated (12%) and reached statistical significance. Several explanations can be proposed for these results.

First, the β_3-adrenoceptor might specifically control the activity of the satiety center of the hypothalamus by a yet unknown mechanism.

Second, since administration of a high fat diet is postulated to induce a leptin resistance (characterized by increased circulating leptin concentrations without any decrease in food intake [34]), the higher food intake of the −/− mice on a high fat diet without significant change in circulating leptin level suggests that the activity or the sensitivity to leptin of the hypothalamic satiety center are decreased in −/− mice.

A third possible explanation is based upon the notion that appetite is driven by the need to lay down lean tissues during growth (35) or during weight recovery (36); the observed increase in food intake could thus be a consequence of decreased lean body mass in −/− mice.

β_3-adrenoceptor disruption might change the set point linking body fat content and circulating leptin level. A correlation between circulating leptin concentrations and body fat content has been consistently described in humans as well as rodents (19, 34). In −/− as compared to +/+ mice, there is an increase in body fat content without a concomitant increase in circulating leptin. Even in high fat–fed −/− mice, with a percentage of body fat of 18 vs. 12% in +/+ mice, no increase in the level of circulating leptin was observed. The shift to the right of the regression line observed when circulating leptin levels were plotted against percentage of body fat in mice on a high fat diet corroborates this observation. A similar shift was observed when circulating leptin was plotted as a function of body fat content, although with a lower statistical significance ($P = 0.05$). This indicates that the set point of the control of leptin secretion by body fat content is shifted to a higher level in −/− than in +/+ mice, a shift which could contribute to the increase in fat deposition.

It has been shown, both in vivo and in vitro, that β_3-adrenoceptor agonists induce a decrease in the expression of leptin in BAT and WAT (19). This effect can be mimicked in cultured differentiated brown adipocytes by β_1- and β_2-adrenoceptor agonists, albeit with a lower affinity (37). The finding that BAT expresses leptin has been challenged by Cinti et al. (38), who suggested that leptin expression in BAT is due to the presence of contaminating white adipocytes. However, a genuine leptin expression in brown adipocyte is more likely, on the basis of evidence that leptin is expressed and secreted at a high level in brown adipocytes differentiated in culture (37), and that, in addition, its modulation in vivo in BAT differs in various aspects from that in WAT (4, 27). The results of this study show that the disruption of the β_3-adrenoceptor did not modify the degree of expression of leptin mRNA in BAT and WAT or the level of circulating leptin. These results are in agreement with those obtained by Mantzoros et al. (39) in their mouse with a disruption of the β_3-adrenoceptor. These findings suggest that, if an increase in β_3-adrenoceptor stimulation is known to decrease leptin expression in both BAT and WAT (19, 37), the inverse is not true: i.e., a decrease in β_3-adrenoceptor function does not induce an upregulation of leptin in these tissues. Our results are therefore inconsistent with the hypothesis that, in hereditary obese rodents, the consistently reported β_3-adrenoceptor defect (2, 5) might be responsible for leptin overexpression (19). The results of this study showing that the β_3-adrenoceptor disruption does not change the level of circulating insulin, glucose, and FFA confirm those of previous reports (11, 39). They also extend them by showing that in mice on a high fat diet or 7-h fasted, the β_3-adrenoceptor disruption also does not change these parameters. Therefore, under the various conditions tested, no sign of insulin resistance can be detected in −/− mice, and the in vivo lipolytic activity is not limited by the decrease in adipose tissue β-adrenoceptors.

In mice with a β_3-adrenoceptor disruption, the β_1-adrenoceptor becomes rate limiting for thermogenesis. The lack of β_3-adrenoceptor was not compensated for by an increase in β_1- or β_2-adrenoceptor mRNA expression. In fact, in BAT, a sharp decrease in β_1-adrenoceptor mRNA expression was observed in −/− mice. This result is in conflict with that obtained by Susulic et al. (11), who reported a compensatory upregulation of the β_1-adrenoceptors in the BAT and WAT of −/− mice. The genetic background of the mice used in that study (FVB/N, FVB/N × 129/SvJ, 129/SvJ) is different from that of the mice used in this study (129 Sv × C57BL/6J). This could explain the discrepancy observed in the expression of the β_1-adrenoceptor, since genetic background may affect the penetrance of a mutation (40). Preliminary data from our laboratory also show a decrease in both BAT and WAT β_1-adrenoceptor mRNA in mice housed individually at 6°C for 3 wk, indicating that β_1-adrenoceptor downregulation is a consistent feature of our mutant phenotype. It suggests that, if a cross talk exists between β_3- and β_1-adrenoceptors in adipose tissues, it is of a negative nature in BAT. UCP, its level of expression under the control of the sympathetic nervous system, is responsible for heat production through uncoupled mitochondrial respiration, and is a measure of the BAT thermogenic capacity. The disruption of the β_3-adrenoceptor did not decrease UCP mRNA levels significantly in BAT. The finding of a positive correlation in −/− mice between UCP and β_1-adrenoceptor mRNA levels in BAT suggests that, in these mice, the β_1-adrenoceptor becomes rate-limiting in the control of UCP expression and thermogenesis in BAT. Therefore, the −/− mice with the lowest level of β_1-adrenoceptor in their BAT have the lowest level of UCP, and should be prone to be the fattest, and vice versa. Studies are currently underway in our laboratory to test this hypothesis. The decrease in β_1-adrenoceptors may confound interpretation of the role of β_3-adrenoceptors in physiological regulation. It is a general observation that targeted disruption of a gene can induce counterregulations which must be taken into account in the interpretation of the results (41).

Conclusions. Targeted disruption of the β_3-adrenoceptor does not induce rapid development of obesity in mice. However, it creates conditions for a gradual increase in body fat content. Recently, Walston et al. (42) described a Trp64Arg missense mutation of the human β_3-adrenoceptor. The possibility that this mutation has clinical consequences is still con-

troversial. Gagnon et al. (43) reported no evidence of a linkage between the mutation and obesity-related phenotypes. However, in studies on Pima Indian, Finnish, and Japanese patients, the mutant genotype was associated significantly with non–insulin-dependent diabetes mellitus (44), with increased body mass index (44), increased capacity to gain weight (45), and earlier age of non–insulin-dependent diabetes mellitus onset (42, 44). It is interesting to note the analogy between the β_3-adrenoceptor knockout model described in this study and the discrete phenotypic changes observed in humans with a Trp64Arg mutation. This suggests that changes in the lean tissue mass and in the threshold of body fat content inducing leptin production might also be observed in humans with the β_3-adrenoceptor mutation.

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