Increased α_2-adrenergic binding sites and antilipolytic effect in adipocytes from genetically obese rats

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Abstract We have recently shown that functional α_2-adrenergic receptors, assessed by the α_2-agonist UK 14304, are present in rat white fat cells as in adipocytes of humans and other species. The aim of the present study was to further characterize rat fat cell α_2-adrenoceptors and to examine whether their number and biological effect were altered in fat cells from genetically obese Zucker rats. The maximal antilipolytic effect of UK 14304 was higher in obese than in lean littermates. Epinephrine, when its β-component was blocked by propranolol, also induced an antilipolytic response that was higher in the obese rats. Similarly, ^{3}H-labeled UK 14304 binding on adipocyte membranes was higher in obese than in lean animals. The radiolabeled α_2-antagonist [^{3}H]idazoxan also recognized a higher number of sites in obese animals. However, epinephrine only partially competed for the ^{3}H-labeled UK 14304 and [^{3}H]idazoxan, suggesting that these imidazolinic radioligands labeled not only α_2-adrenoceptors but also nonadrenergic binding sites. By contrast, ^{3}H-labeled RX 821002, an α_2-antagonist derived from the idazoxan family, did not recognize these sites and allowed accurate quantification of adipocyte α_2-adrenoceptors. The number of α_2-sites was higher in obese than in lean littermates (B_{max} = 64 ± 5 vs 39 ± 2 fmol/mg protein, $P < 0.01$) without change in affinity. The adipocyte α_2-adrenergic responsiveness showed a strong dependency on age and fattening between 5 and 10 weeks of age in both genotypes. At each age, obese rat adipocytes were larger and exhibited a greater α_2-antilipolytic response than those of lean littermates. However, enlarged fat cells from young obese rats exhibited a lower response than the smaller cells from the older lean animals. Thus, Zucker rat adipocytes possess α_2-adrenoceptors which are: *i*) able to mediate an antilipolytic response; *ii*) increased in number in the obese as compared to the lean; and *iii*) less numerous than those described on other species such as humans and the hamster. —**Carpéné, C., M-C. Rebourcet, C. Guichard, M. Lafontan, and M. Lavau.** Increased α_2-adrenergic binding sites and antilipolytic effect in adipocytes from genetically obese rats. *J. Lipid Res.* 1990. **31:** 811–819.

Supplementary key words lipolysis • α_2-adrenergic receptor • RX 821002 • idazoxan • obesity • adipose tissue • aging

It is now well established that, in humans and other species such as dog, rabbit, and hamster, catecholamines exert a dual control on white fat cell lipolysis: stimulation by acting on β-adrenergic receptors and inhibition mediated by α_2-adrenergic receptors (1,2). On the other hand, the rat fat cell is suspected to possess only a β-adrenergic regulation of lipolysis (3) since many investigators have concluded that rat adipocytes are devoid of an α_2-adrenergic receptor (4–6). This assumption, based mainly on the lack of effect of clonidine, a partial α_2-agonist which is clearly antilipolytic on other species, was recently reassessed (7) by using a very selective and fully efficient α_2-adrenoceptor agonist, UK 14304 (8).

The finding that functional α_2-adrenergic receptors are present in rat adipocytes prompted us to examine whether the number and biological effect of these receptors are increased in obese rats. In fact, such an increase in adipocyte α_2-adrenergic receptivity has already been described in fatter and/or older animals of other species well known for their dual α_2-/β-adrenergic control of lipolysis such as dog (9), rabbit (10, 11), and hamster (12, 13). As the rat is, of the various species investigated, the only model that possess strains exhibiting a genetic obesity, we carried out the present investigation in order to test whether, in the Zucker rat, obesity is connected with an increase of adipocyte α_2-receptivity.

The data reported here show that the α_2-adrenergic antilipolytic response (to UK 14304 or to epinephrine associated with propranolol) is stronger in adipocytes of the genetically obese rats than in the lean littermates. Also, the number of α_2-adrenoceptors, quantified with ^{3}H-labeled RX 821002, as well as the number of nonadrenergic

Abbreviations: HEPES, 4-[2 hydroxy ethyl)-1-piperazine-ethane sulfonic acid; Tris, tris(hydroxymethyl) aminomethane; EDTA, ethylene diamine tetraacetate; EGTA, ethylene glycol bis aminoethylene tetraacetate; ADA, adenosine deaminase; RX 821002, 2-(2-methoxy-1,4-benzodioxan-2yl)-2-imidazoline; idazoxan (imidazolinyl-2)2-benzodioxane 1,4; UK 14304, 5-bromo-6-[2-imidazolin]-2-ylamino-quinoxaline.

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sites, labeled by ^{3}H-labeled UK 14304 or [^{3}H]idazoxan are higher in obese rat fat cells than in lean rat fat cells.

MATERIALS AND METHODS

Animals

Male Zucker rats (lean Fa/fa or obese fa/fa) used in this study were littermates from known heterozygous (Fa/fa) female and obese homozygous (fa/fa) male crossings. They were bred in the laboratory and used at 10 weeks of age unless otherwise stated. They were fed ad libitum on laboratory chow (carbohydrate 65%, protein 24%, and fat 11% of total energy) up until the time of the experiments between 9:00 and 10:00 AM.

Lipolysis measurements

Isolated fat cells were obtained from epididymal fat pads according to the Rodbell's method (14). Duplicate aliquots of adipocytes were suspended in 2.5 ml of Krebs Ringer bicarbonate buffer (pH 7.4) containing 10 mM HEPES, 5 mM glucose, and 3% bovine serum albumin, and incubated at 37°C in an atmosphere of O_2/CO_2 (95:5) in stoppered Nalgene vials for 60 min. Then, an aliquot (1 ml) of the infranant incubation medium was removed, acidified (trichloroacetic acid), neutralized (potassium hydroxide), and assayed in duplicate for glycerol content using an enzymatic method (triglyceride kits from Boehringer Mannheim). The number of fat cells was estimated from the mean fat cell size determined by the method of Lavau et al. (15) and from the lipid content of each incubation vial, gravimetrically obtained by the method of Dole and Meinertz (16).

Binding experiments

Crude membranes were prepared from isolated adipocytes by hypotonic lysis at 20°C in 2 mM Tris, 2.5 mM $MgCl_2$, 1 mM $KHCO_3$, 0.1 mM phenylmethylsulfonyl fluoride, 0.1 mM benzamidine, and 3 mM EGTA (pH 7.4; 35 mosm/l) followed by centrifugation at 35,000 *g* for 10 min. Fat cakes and supernatants were discarded, pellets were resuspended in 4 ml of the fresh buffer described above and stored at −80°C until the binding experiments.

For α_2-adrenoceptor identification, ^{3}H-labeled UK 14304, [^{3}H]idazoxan, and ^{3}H-labeled RX 821002 binding studies were performed on membranes (200 μg protein) that were incubated 25 min at 25°C in 0.4 ml of 50 mM Tris-HCl, 10 mM $MgCl_2$, pH 7.4 (Tris-Mg buffer) at various radioligand concentrations. Separation of bound ligand was performed by vacuum filtration through Whatman GF/C glass fiber filters. After washing with 2 × 10 ml of ice-cold Tris-Mg buffer, the filters were counted for radioactivity in a Packard liquid scintillation spectrometer. Nonspecific binding determination is detailed in Results. The membrane protein content was determined by the technique of Lowry et al. (17).

Chemicals

^{3}H-labeled UK 14304 (3 Tbq/mmol) was purchased from NEN (Boston, MA) and [^{3}H]idazoxan (^{3}H-labeled RX 781094, 2.2 TBq/mmol) from Amersham (Buckinghamshire, UK). ^{3}H-Labeled RX 821002 (2-(2-methoxy-1, 4-benzodioxan-2yl)-2imidazoline; 1.6 Tbq/mmol) was a generous gift from Reckitt and Colman labs (Hull, UK). Unlabeled UK 14304 came from Pfizer Labs (Sandwich, UK) and idazoxan was a generous gift from Pr. Dabiré H. (Paris, France). Phentolamine was obtained from Ciba Geigy (Basel, Switzerland), clonidine from Boehringer (Ingelheim, FRG), epinephrine from Sigma (St. Louis, MO) and (−)propranolol from ICI labs (Macclesfield, UK). Enzymes came from Boehringer Mannheim and all other compounds were of reagent grade.

Statistical methods

Binding data were analyzed by a computer-aided technique as previously reported (18). Statistical significance was assessed by Student's *t* test for unpaired samples. A nonsignificant difference between lean and obese ($P > 0.05$) is indicated by NS; n values refer to the number of separate experiments.

RESULTS

Lipolysis measurements

In order to allow observation of any α_2-adrenergic antilipolytic response in rat adipocytes, it was necessary to stimulate the very low basal levels of spontaneous glycerol production. This was achieved by removal of endogenous adenosine; the addition to incubation medium of adenosine deaminase 1.6 μg/ml (ADA, 0.32 IU/ml) increased lipolysis to 2–3 μmol glycerol/10^6 cells/h. Further stimulation was obtained by the combination of ADA with 1.65 mM theophylline (**Table 1**). Under these conditions, glycerol production reached about 10 μmol/10^6 cells per h and the antilipolytic response was expressed in % inhibition of lipolysis (0% corresponding to the values in the presence of lipolytic agents alone and 100% corresponding to basal glycerol values, without any addition). This method was suitable for the estimation of the α_2-adrenergic inhibitory responsiveness as previously described (12, 18, 19).

The addition of increasing concentrations of clonidine did not noticeably modify the lipolysis of Zucker rat adipocytes, whatever the genotype, either in the presence of ADA (not shown) or in the presence of ADA + 1.65 mM theophylline (Table 1). These results, showing that clonidine is not antilipolytic on Zucker rat adipocytes, fit with

TABLE 1. Lack of antilipolytic response to clonidine in Zucker rat adipocytes

Addition to Medium	Lean Rats	Obese Rats
	μmol glycerol/10⁶ cells/h[a]	
None (basal)	0.03 ± 0.02	0.08 ± 0.04
ADA 1.6 μg/ml	2.59 ± 0.42	3.21 ± 0.43
ADA + theophylline 1.7 mM	7.43 ± 0.87	11.76 ± 1.15
ADA + clonidine 10^{-7} M[b]	7.24 ± 0.60	10.28 ± 0.87
ADA + clonidine 10^{-6} M[b]	7.02 ± 0.65	9.82 ± 1.04
ADA + clonidine 10^{-5} M[b]	6.90 ± 0.24	9.59 ± 0.82

[a]Lipolytic activity of isolated rat adipocytes was assessed by the determination of the amount of glycerol released in the incubation medium in the presence of adenosine deaminase (ADA), theophylline, and increasing concentrations of clonidine. Values are means ± SEM of three experiments.

[b]There is no significant difference between the values with and without clonidine (paired *t*-test).

previous observations from us (6) and others (4, 5) on Sprague Dawley or Wistar rat fat cells.

To further investigate the putative α_2-antilipolytic responsiveness of Zucker rat adipocytes, another α_2-agonist, UK 14304, that possesses full α_2-agonist properties on several models (8) including human adipocytes (20) was used. In both genotypes, adipocytes displayed a dose-dependent response to UK 14304 (**Fig. 1**). The accelerated rates of lipolysis obtained in the presence of adenosine deaminase were completely blocked at 10^{-6} M UK 14304. Under these conditions, the inhibition of glycerol production amounted to 1.66 and 2.68 μmol/10^6 cells per h in lean and obese rats, respectively (Fig. 1A). Half-maximal lipolysis inhibition was achieved at the same concentration of UK 14304 in lean and obese rats (EC_{50} = 7.1 ± 1.4 and 7.6 ± 1.7 nM, n = 4, NS). The lipolysis stimulated by the combination of ADA and 1.65 mM theophylline was higher in obese than in lean rats. However, the UK 14304-induced dose-dependent response was greater in obese than in lean rats (Fig. 1B). At the maximally effective concentration (10^{-5} M), UK 14304 inhibited lipolysis by 45 ± 3% in lean and 75 ± 1% in obese rat adipocytes (n = 3, $P < 0.001$, Fig. 1B). These results suggest the presence of functional α_2-adrenoceptors on Zucker rat adipocytes.

In order to eliminate a possible antilipolytic effect of UK 14304 other than that mediated by α_2-adrenoceptors, we studied the influence of α_2-antagonists on UK 14304 action in obese rat adipocytes. The effect of the α_2-agonist alone (93 ± 1% at 10^{-6} M, n = 4) was significantly reduced in the presence of 10^{-5} M yohimbine (4 ± 28%, $P < 0.02$), 10^{-5} M RX 821002 (17 ± 14%, $P < 0.02$) or 10^{-5} M idazoxan (29 ± 10%, $P < 0.001$, not shown).

To confirm the nature of the α_2-mediated antilipolytic responses of rat fat cells, it was of interest to verify whether epinephrine, the physiological agonist, was also

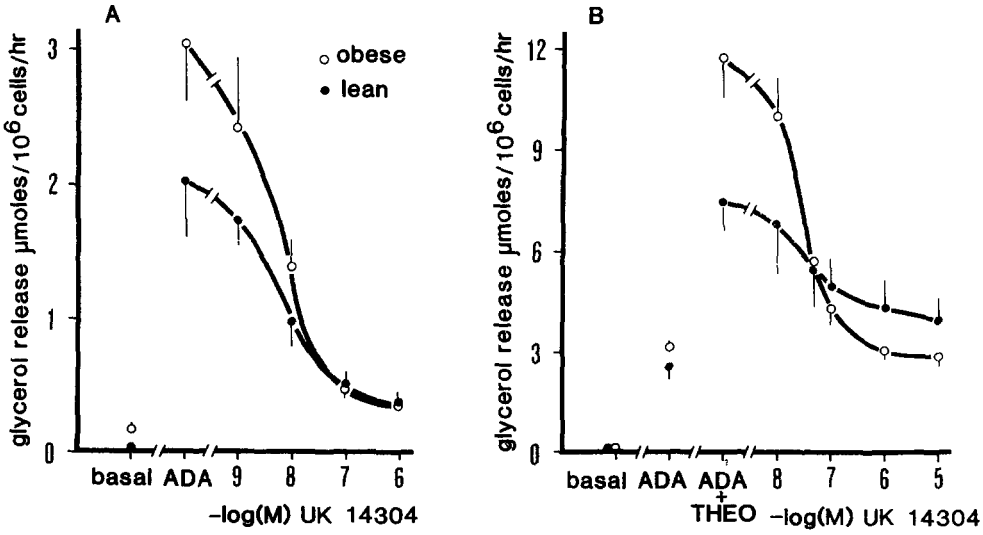

Fig. 1. Antilipolytic dose-response curves of UK 14304 in Zucker rat adipocytes. Epididymal adipocytes from 10-week-old lean (●) or obese (○) Zucker rats were incubated without any addition (basal) or with 1.6 μg/ml adenosine deaminase alone (ADA, Fig. 1A), or in combination with 1.7 mM theophylline (ADA + THEO, Fig. 1B). Release of glycerol in the incubation medium was measured in the presence of increasing concentrations of UK 14304. Results are in μmol glycerol released/10^6 cells per h. Each point represents mean ± SEM of three to seven experiments.

able to induce an α_2-antilipolytic effect in Zucker rat adipocytes. Under β-adrenergic blockade, using 2×10^{-5} M (−)propranolol, epinephrine induced a dose-dependent inhibition of glycerol release in both genotypes. However, the maximal antilipolytic effect was two times higher in obese than in lean rat adipocytes: 87 ± 3 vs 41 ± 8% inhibition at 10^{-5} M epinephrine ($P < 0.01$, n = 3, **Fig. 2**).

We further tested whether the greater amplitude of the antilipolysis elicited by UK 14304 and epinephrine plus propranolol observed in the obese Zucker rat adipocytes could be linked to differences in the number of α_2-adrenoceptors between the two genotypes.

Quantification of adipocyte α_2-adrenoceptors

In order to compare the α_2-adrenoceptor population in lean and obese Zucker rat adipocytes, binding experiments were first carried out using the α_2-agonist (8) UK 14304 and the α_2-antagonist (21, 22) idazoxan under their tritiated forms since, as previously reported, [^{3}H]clonidine labels very few sites in rat adipocytes (6).

However, the sites labeled by ^{3}H-labeled UK 14304 on rat adipocyte membranes (B_{max} = 797 ± 80 vs 1811 ± 227 fmol/mg protein of Percoll-purified membranes, $P < 0.02$, and K_D = 8.5 ± 1.3 vs 8.0 ± 0.9, for lean and obese respectively, n = 4) did not fulfill the classical characteristics of true α_2-adrenoceptors since, in both genotypes, more than 70% of the ^{3}H-labeled UK 14304 binding was not displaceable by epinephrine. Competition experiments showed that ^{3}H-labeled UK 14304, like [^{3}H]idazoxan, labeled not only α-adrenoceptors but also nonadrenergic binding sites which exhibited high affinity for some of the α_2-adrenergic agents that contain an imidazoline moiety in their chemical structure (not shown). These "imidazoline binding sites," which have been described for [^{3}H]idazoxan in various cell types (23–25) including adipocytes (18, 26), give rise to about 60 to 90% of [^{3}H]idazoxan or ^{3}H-labeled UK 14304 total binding and therefore impaired an accurate quantification of the α_2-adrenoceptors.

In an attempt to assess the real α_2-adrenoceptor potential of rat adipocytes, we used a novel radioligand, ^{3}H-labeled RX 821002. This derivative of idazoxan has been described to be highly selective for α_2-adrenoceptors (27, 28). Although we recently observed that ^{3}H-labeled RX 821002 had poor affinity for the "imidazoline binding sites" present on hamster (18) or Wistar rat adipocytes (Carpéné, C., unpublished results), we limited the definition of specific binding to adrenoceptors as the binding displaceable by epinephrine (24).

^{3}H-Labeled RX 821002 binding was rapid and reversible, and kinetic constants were, in membrane preparations of rat adipocytes, very similar to those reported by our group on human adipocytes (29). Thus, further binding experiments were carried out during 25 min at 25°C. Competition experiments showed that all the competitors tested inhibited around 70% of the total ^{3}H-labeled RX

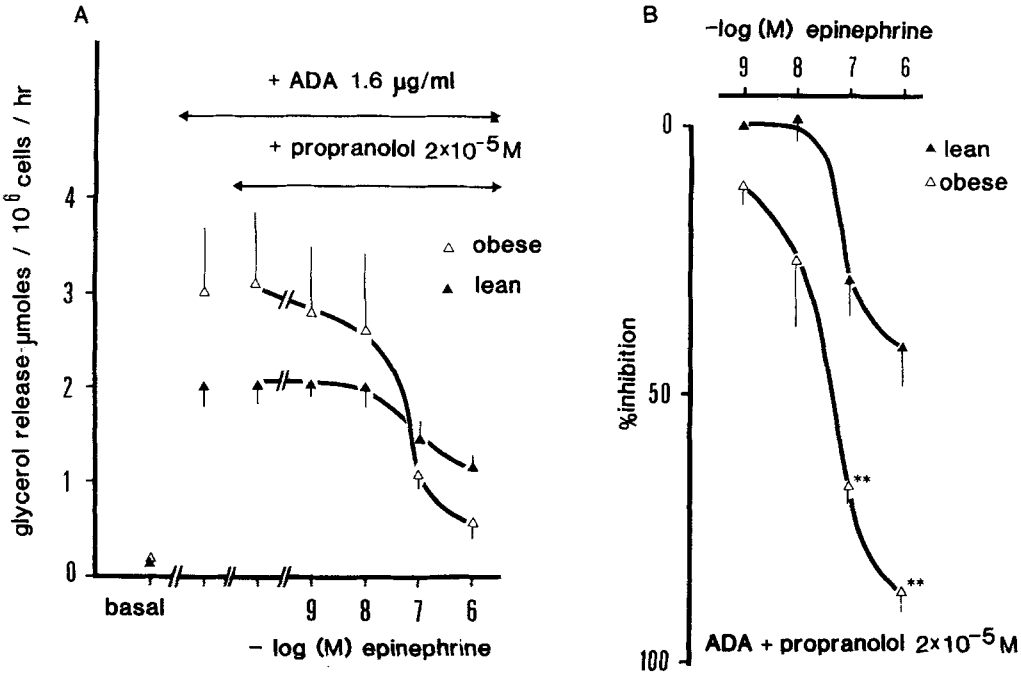

Fig. 2. Antilipolytic effect of epinephrine associated with propranolol. Adipocytes from 10-week-old lean (▲) or obese (△) Zucker rats were incubated with 1.6 µg/ml adenosine deaminase (ADA) in the presence of propranolol (2×10^{-5} M) and increasing epinephrine concentrations. Each point represents mean ± SEM of three (lean) or four (obese) experiments. Results are in µmol of glycerol released per 100 mg of cellular lipids (A) or in % inhibition of lipolysis (B); **, % inhibition significantly different from lean at $P < 0.01$.

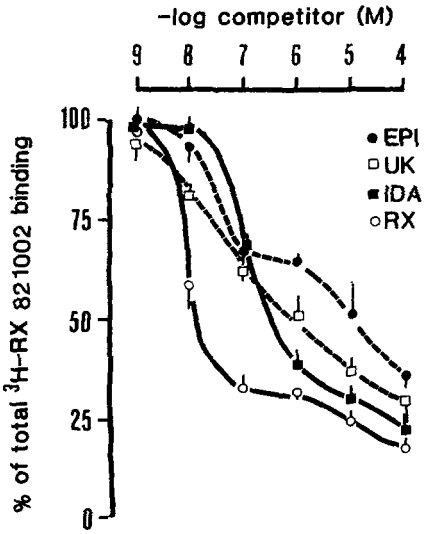

Fig. 3. Competition of ^{3}H-labeled RX 821002 binding on crude adipocyte membranes from obese Zucker rats. Inhibition of 12 nM ^{3}H-labeled RX 821002 binding on adipocyte membranes was measured in the presence of the α_2-antagonists RX 821002 (RX) and idazoxan (IDA) (solid lines), or the agonists UK 14304 (UK) and epinephrine (EPI) (broken lines). Data are expressed in % of total ^{3}H-labeled RX 821002 binding. Under our conditions, residual binding of ^{3}H-labeled RX 821002 on filters alone accounted for ≈ 10% of the total binding which was 79 ± 6 fmol/mg protein with a protein concentration of 1.6 ± 0.2 mg/ml. Mean of three experiments ± SEM.

821002 binding on crude membranes of obese rat adipocytes (**Fig. 3**). Residual binding in the presence of 10^{-4} M competitor ranged from 16 ± 1 (RX 821002) to 36 ± 3% (epinephrine) of total binding. When the portion of binding to irrelevant materials such as glass fiber filters (≈ 10% of total binding under our conditions) is subtracted, it can be assumed that most of the ^{3}H-labeled RX 821002 is epinephrine-displaceable, a result markedly different from that obtained with ^{3}H-labeled UK 14304 or [^{3}H]idazoxan (not shown). However, the highest concentrations (10^{-5}–10^{-4} M) of α_2-antagonists only appeared to compete for a small proportion (10%) of the ^{3}H-labeled RX821002 binding that was not recognized by epinephrine. Nevertheless, unlabeled RX 821002 had greater affiinity than the other α_2-agents tested in competition for the epinephrine-displaceable sites. The rank order of relative potencies was for antagonists: RX 821002 > idazoxan > phentolamine (K_i values were: 1.0 ± 0.3, 17 ± 8, 46 ± 16 nM, respectively, n = 3). In addition, the competition curves for the agonists were not as steep as that for the antagonists (n_H = 0.29 ± 0.10 and 0.29 ± 0.03 for UK 14304 and epinephrine, respectively), suggesting the presence of high and low affinity state for agonists (Fig. 3).

Saturation curves of ^{3}H-labeled RX 821002 binding show that nonspecific binding assessed in the presence of 2.10^{-4} M epinephrine is linear and represents less than 25–35% of total binding in obese and lean rats (**Fig. 4**). Addition of ascorbic acid (0.1 mM) (in order to prevent catecholamine degradation) to the incubation medium did not modify the results. Specific binding analysis showed linear Scatchard plots and Hill coefficients not dif-

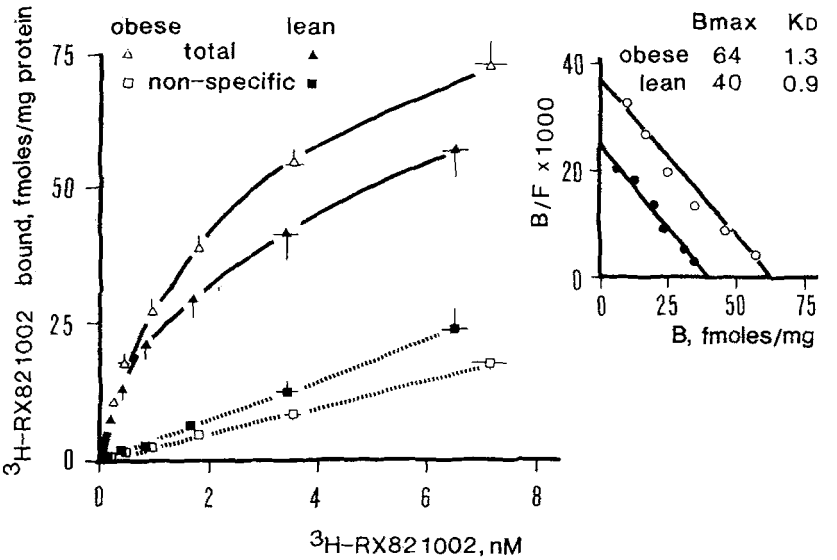

Fig. 4. ^{3}H-Labeled RX 821002 binding characteristics on crude membranes of Zucker rat adipocytes. Saturation curves of ^{3}H-labeled RX 821002 binding on membranes obtained from obese (open symbols) or lean (closed symbols) 10-week-old littermates. At this age, crude membranes prepared from epididymal + perirenal adipose depots (13 ± 2 g) of one obese animal (body weight = 355 ± 10 g) were sufficient to carry out a saturation experiment, whereas pools of five or six lean littermates (283 ± 15 g of body weight 2.2 ± 0.3 g of adipose tissues) were necessary to obtain sufficient membrane preparations. Total (▲, △) and nonspecific binding (■, □, measured in the presence of 2.10^{-4} M epinephrine) were carried out as described in Materials and Methods. Values are mean ± SEM of six (lean) or eight (obese) different determinations. Inset: Scatchard analyses of a representative experiment where B_{max} is expressed in nM (mean values = 65 ± 5 vs 39 ± 2) and K_D is expressed in nM (mean values = 1.3 ± 0.2 vs 1.2 ± 0.2 for obese and lean, respectively).

ferent from unity (0.997 ± 0.020 and 0.996 ± 0.018 for obese and lean, respectively, n = 6–8, NS) indicating the presence of one class of binding sites in each genotype. Moreover, maximal specific binding was different for lean and obese without change in affinity: B_{max} = 65 ± 5 vs 39 ± 2 fmol per mg of crude membrane protein for obese and lean, respectively (n = 6–8, $P<0.01$), K_D = 1.3 ± 0.2 vs 1.2 ± 0.2 nM (NS, Fig. 4).

The binding data obtained with ^{3}H-labeled RX 821002 indicated that obese rat fat cell membranes possessed a higher number of α_2-adrenoceptor sites than lean ones. This difference in α_2-receptor equipment could explain the difference in the α_2-antilipolytic responsiveness observed between obese and lean 10-week-old animals.

Influence of age and/or adipose tissue enlargement on α_2-mediated antilipolysis

With regard to the α_2-adrenergic response, fat cell size and aging are factors that must be considered if we refer to previously reported data in rabbit (10, 11) and dog adipocytes (9). Since, in these two species, aging and obesity are associated with a concomitant increase of α-adrenergic antilipolytic effect, we studied the influence of enlargement of adipose tissue on α_2-receptivity during the development of obesity at different ages in obese and also in lean Zucker rats.

Fig. 5 shows the evolution of both fat cell size and UK 14304-induced antilipolysis at three different ages. Within each genotype there was a steady increase in the response to UK 14304 with age and/or fat cell enlargement, at least during the life span studied. Whatever the age, the fat cell size and the amplitude of the α_2-adrenergic responses were greater in obese than in lean littermates. However, the very large adipocytes from 5-week-old obese rats were much less responsive to UK 14304 than the relatively smaller adipocytes from 10-week-old lean rats. Nevertheless, the greatest α_2-antilipolytic effect (46% inhibition of lipolysis) was observed in the larger cells (792 ng lipid/cell) of the older obese rats (Fig. 5).

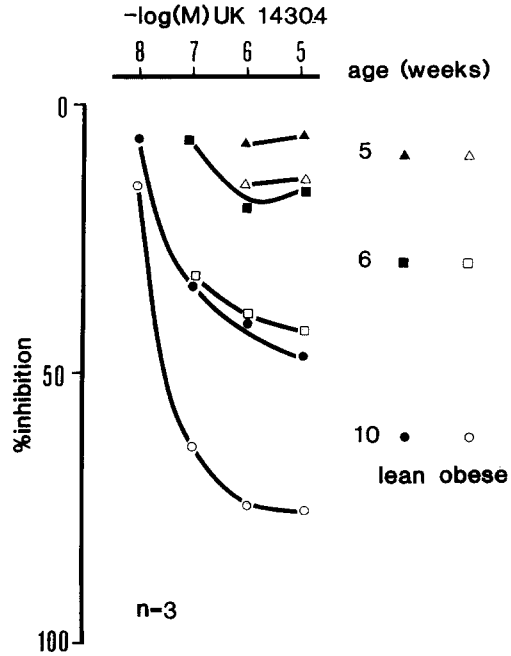

Fig. 5. Antilipolytic effect of UK 14304 on isolated fat cells from rats of different ages. Epididymal adipocytes from lean (closed symbols) or obese (open symbols) Zucker rats aged 5 weeks (triangles), 6 weeks (squares), or 10 weeks (circles) were incubated with 1.6 μg/ml adenosine deaminase + 1.7 mM theophylline. The antilipolytic effect of increasing concentrations of UK 14304 was expressed in % inhibition of stimulated lipolysis. Pools of five, four, and three animals were necessary to obtain sufficient amounts of adipocytes for one determination from 5-,6-, and 10-week-old lean rats, respectively. Mean fat cell weight was 167 ± 10, 262 ± 30, 792 ± 81 ng lipid/cell for obese and 45 ± 5, 60 ± 5, 104 ± 5 ng lipid/cell for lean rats at 5, 6, and 10 weeks of age, respectively. Each point is the mean of three experiments; SEM were deleted for clarity.

DISCUSSION

In many species excess of fat deposits and enlargement of adipocytes are associated with various alterations of the hormonal regulation of adipose tissue lipolysis. Increased α_2-adrenergic responsiveness is one of them. This enhanced capacity to respond to the α_2-adrenergic stimulation by antilipolysis could explain, in part, the impaired lipolytic activities of catecholamines observed with aging and fattening (2, 9, 10). In humans, the increase of α_2-responsiveness of adipose tissue is responsible for the inversion of the epinephrine effect; the physiological agonist, normally lipolytic, behaves as an antilipolytic hormone in adipocytes possessing a large number of α_2-sites as is the case for subcutaneous fat deposits of obese subjects (19, 30). Since we have recently demonstrated the presence of α_2-adrenoceptors on rat while fat cells (7), we conducted the present work in order to examine whether the genetic obesity of the Zucker rat is accompanied by an enhancement of fat cell α_2-adrenergic responses.

Until now, it has been widely claimed and accepted that rat adipocytes do not possess α_2-adrenoceptors mediating an antilipolytic effect since, on the one hand, Burns and Langley (3) showed that lipolysis of rat adipocytes is unaffected by α-antagonists, and on the other hand, Giudicelli et al. (31) reported that only "atypical" α-receptors could be detected on rat fat cells by binding studies. The use of the selective α_2-agonist, clonidine, in further experiments in rat adipocytes confirmed these results since neither metabolic effects (1, 4–6) nor α_2-receptor identification (6) were obtained with this α_2-agonist. The present results confirm the very weak action of clonidine on rat adipocytes since it was unable to modify the lipolysis stimulated by adenosine deaminase alone or in combination with theophylline (Table 1). But, with our present knowledge, it is misleading to conclude, from the lack of clonidine effect, that rat adipocytes are devoid of α-adrenoceptors

since clonidine has been demonstrated to be only a partial α_2-agonist on several cell types such as human platelets (32) and adipocytes (20). On these cells, clonidine is less effective in lowering cAMP levels than the full α_2-agonist UK 14304 (8) or than the physiological catecholamines. Thus, the absence of α_2-adrenergic sensitivity cannot be assessed by the weakness of the action of clonidine. Nevertheless, clonidine is fairly antilipolytic in human (2) and hamster (4, 6) white adipocytes and also in rat brown fat cells (33); these discrepancies are not easily explainable but the blunted effect of this molecule reported here lets us suppose that α_2-adrenoceptors of rat white adipocytes are less numerous and, in particular, less sensitive to clonidine than those of other models. However, the antilipolytic effect of UK 14304 (Figs. 1 and 5) and the blockade of its effect by α_2-antagonists are convergent proofs for the existence of an α_2-adrenergic responsiveness in Zucker rat adipocytes. Moreover, the antilipolytic effect of epinephrine, associated with the β-blocker propranolol, argues for a functional status of the rat adipocyte α_2-receptor (Fig. 2).

Whatever the type of lipolysis stimulation used (ADA alone or in combination with theophylline), UK 14304 and epinephrine induced stronger antilipolysis, in terms of absolute effect, in obese than in lean littermates (Figs. 1 and 2). Thus, increased α_2-adrenergic responsiveness could be demonstrated in adipocytes of the genetically obese rat. Such findings are in good agreement with those previously reported in fatter and/or older animals of other species (9–13). This enhanced α_2-adrenergic responsiveness in the obese rat, as compared to lean littermates, could be linked to an increased number of α_2-sites or to a better coupling efficiency of the adipocyte α_2-adrenoceptors to the adenylyl cyclase-inhibiting mechanisms.

In order to verify the status of the adipocyte α_2-adrenoceptors, we performed various binding experiments on fat cell membranes. Scatchard analyses showed that ^{3}H-labeled UK 14304 labeled about twice as many sites on membranes preparations in obese than in lean rats. However, ^{3}H-labeled UK 14304 binding appeared to be not only composed of α_2-sites but also of other sites that are nonadrenergic in nature (not recognized by epinephrine, even at high concentrations) (not shown). These sites, also labeled by [^{3}H]idazoxan in rat fat cells, have characteristics similar to those described in rabbit in kidney (23), brain cortex (24), or adipocyte membranes (26). They exhibit a high affinity for some imidazoline derivatives but their biological action is still unknown (see ref. 25 for a review). The significance of the increase in the density of the nonadrenergic "imidazolinic" sites observed in the adipocyte membranes of obese rats remains unclear. However, these sites did not appear to be directly involved in the regulation of lipolysis since: *i*) UK 14304-induced antilipolysis was mimicked by epinephrine which did not interact with the nonadrenergic binding sites recognized by imidazolinic compounds; *ii*) UK 14304 antilipolysis was blocked either by yohimbine or RX 821002, α_2-antagonists which are, unlike idazoxan, devoid of interaction with nonadrenergic sites. Thus, their presence on the rat adipocyte only impaired an accurate quantification of α_2-adrenoceptors with ^{3}H-labeled UK 14304 or [^{3}H]idazoxan, but did not appear to modify the α_2-antilipolytic effect.

Thus, it was necessary to both define the α_2-adrenergic receptors as sites "truly displaceable by epinephrine," and to use a radioligand that exhibits poor affinity for the nonadrenergic sites. The combined use of ^{3}H-labeled RX 821002 (28, 29) and of epinephrine (2×10^{-4} M for nonspecific determination) allowed us to fulfill these requirements and to accurately determine the α_2-adrenoceptor population of rat fat cells. Competition of ^{3}H-labeled RX 821002 binding showed that α_2-agents differed by their affinity but not by their maximal displacing capacities (Fig. 3). Most of ^{3}H-labeled RX 821002 binding on membranes of Zucker rat adipocytes was epinephrine-displaceable, arguing for the adrenergic nature of the labeled sites. In addition, ^{3}H-labeled RX 821002 appeared to label only a small proprotion of imidazoline binding sites (displaced by idazoxan or RX 821002, but not by epinephrine) even at relatively high concentrations (12 nM, i.e., tenfold the K_D values, see Fig. 3). The rank order of relative potencies of the α_2-antagonists tested on Zucker rat adipocytes is in good agreement with that obtained on hamster (18) or Wistar rat adipocytes (Carpéné, C., unpublished data) where a more detailed pharmacological characterization demonstrated that ^{3}H-labeled RX 821002 selectively labeled α_2-adrenoceptors. The shallow competition curves for agonists are also very like those reported for hamster or Wistar rat fat cells. In these latter models, the high- and low-affinity states for agonists were detected by both analysis of ^{3}H-labeled RX 821002 competition curves and GppNHp-induced shift to low affinity state (18). Thus, the α_2-selectivity of RX 821002 (28, 29) and the similarity of the pharmacological characteristics reported here with those reported for hamster or Wistar rat (18) support the α_2-adrenergic nature of the sites labeled by this ligand on Zucker rat adipocytes.

^{3}H-Labeled RX 821002 B_{max} values were significantly higher in adipocytes from obese than lean littermates (65 ± 5 vs 39 ± 2 fmol per mg protein) whereas no change in affinity was found. Thus, the increased α_2-adrenoceptor number found in the binding studies could contribute to the higher α_2-adrenergic responsiveness of adipocytes from obese rats. In addition, the ^{3}H-labeled RX 821002 binding data allowed the comparison of the adipocyte α_2-adrenoceptor density of the rat fat cell to that of other species possessing a well-defined adipocyte α_2-receptivity, since optimal conditions for ^{3}H-labeled RX 821002 binding have been found very similar in all the species so far studied. This comparative approach

shows that, in the obese rat, the adipocyte α_2-adrenoceptor number, even though it is twice as high as that of the lean littermate, represents only one tenth of that found on white fat cells from hamster or humans; the B_{max} values of ^{3}H-labeled RX 821002 reached 776 ± 60 fmol/mg protein in hamster (18) and 810 ± 78 fmol/mg protein in human adipocyte membranes (29). An additional difference between the adipocyte α_2-receptors of the rat and those of other species is the low capacity of clonidine to initiate an antilipolytic response or exhibit determinable binding on membrane preparations. Whether these discrepancies among species are due to the existence of different α_2-receptor subtypes (34) on the adipocytes remains to be elucidated.

Although less numerous and slightly different in nature, rat adipocyte α_2-adrenoceptors seem to be regulated in a manner similar to those of other species with regard to the increase in the number observed with aging and/or fattening (10–13). The slow ontogenesis of the α_2-antilipolytic responsiveness observed for the rat adipocyte resembles that observed in the hamster (13), dog (19), and rabbit (10) where young and lean animals possess only a weak α_2-adrenergic sensitivity which develops later in older and/or fatter animals. This late appearance of fully developed α_2-adrenergic responsiveness contrasts with the antilipolytic response to adenosine which is already present in young animals (12, 35). During this process, the fat cells from obese rats exhibited, at each age studied, markedly increased α_2-adrenergic responses as compared to those of lean littermates. Unfortunately, it was difficult to observe any further modifications of age-dependent α_2-adrenergic antilipolysis since adipocytes isolated from obese animals older than 10 weeks were subject to dramatic cell breakage.

In addition, data from Fig. 5 show that, within each genotype, the maximal α_2-adrenergic antilipolytic effect increased concomitantly with the fat cell size. However, larger fat cells (167 ng lipid/cell) from 5-week-old obese rats were less responsive to UK 14304 than the smaller (104 ng lipid/cell) adipocytes from 10-week-old lean rats. The influence of cell size on α_2-adrenergic responsiveness was thus not identical in the two genotypes. Although it is difficult to separate the respective influences of age and fattening on the regulation of adipocyte α_2-adrenergic receptivity, this study does indicate that the larger fat cells, which are found in the older obese rats, exhibit the higher α_2-adrenergic-dependent antilipolysis (Fig. 5) and the higher density of α_2-adrenoceptors. Even though this α_2-adrenergic dysfunction could be of significance for the development of obesity through a reduction in lipid mobilization, its role in the etiology of the disease seems unlikely inview of the late ontogenesis of the adipocyte α_2-adrenergic responsiveness. In this regard, the marked increase in both the binding and biological action of adenosine, exhibited by adipocytes from 4-week-old obese rats (35) and already present at 16 days of age (Rebourcet, M. C., and M. Lavau, unpublished observations), might be of greater importance.

In conclusion, the present findings show that *i*) clonidine is without any antilipolytic action in Zucker rat fat cells, whatever the genotype. *ii*) The antilipolytic effect of UK 14304, a selective α_2-agonist, and of epinephrine in the presence of propranolol is always stronger in obese than in lean Zucker rats. *iii*) ^{3}H-Labeled RX 821002 identifies sites that fulfill the characteristics of α_2-adrenoceptors and are more numerous in adipocyte membranes from obese animals than those of lean littermates. However, the dentisy of α_2-adrenoceptors is tenfold lower in Zucker rat adipocytes than in those of other species such as hamster or humans. *iv*) The α_2-adrenergic antilipolysis increases with aging and fattening in both genotypes.

These data provide compelling evidence that the adipocytes of the genetically obese rat possess more α_2-adrenergic receptors than those of the lean littermates. Thus, the rat is an additional species where an increased α_2-adrenergic receptivity of the fat stores is observed in the obese state, as described for humans, hamster, dog, and rabbit (9–12).

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REFERENCES

1. Fain, J. N., and J. A. García-Sainz. 1983. Adrenergic regulation of adipocyte metabolism. *J. Lipid Res.* **24:** 945–966.
2. Lafontan, M., and M. Berlan. 1981. Alpha-adrenergic receptors and the regulation of lipolysis in adipose tissue. *Trends Pharmacol. Sci.* **2:** 126–129.
3. Burns, T. W., and P. E. Langley. 1971. Differential effects of alpha- and beta-adrenergic blocking agents on basal and stimulated lipolysis of human and rat isolated adipose tissue cells. *Pharmacol. Res. Commun.* **3:** 271–277.
4. García-Sainz, J. A., B. B. Hoffman, S. Y. Li, R. J. Lefkowitz, and J. N. Fain. 1980. Role of alpha$_1$-adrenoceptors in the turnover of phosphatidyl inositol and of alpha$_2$-adrenoceptors in the regulation of cyclic AMP accumulation in hamster adipocytes. *Life Sci.* **27:** 953–961.
5. García-Sainz, J. A., and J. N. Fain. 1982. Regulation of adipose tissue metabolism by catecholamines: role of alpha$_1$, alpha$_2$, and beta-adrenoceptors. *Trends Pharmacol. Sci.* **3:** 201–203.
6. Carpéné, C., M. Berlan, and M. Lafontan. 1983. Lack of functional antilipolytic alpha$_2$-adrenoceptor in rat fat cell: comparison with hamster adipocyte. *Comp. Biochem. Physiol.* **74C:** 41–45.
7. Rebourcet, M. C., C. Carpéné, and M. Lavau. 1988. Evidence of functional α_2-adrenergic receptors in adult rats adipocytes by using the agonist UK. 14304. *Biochem. J.* **252:** 679–682.

8. Cambridge, D. 1981. UK 14304, a potent and selective α_2-agonist for the charactirization of α-adrenoceptor subtypes. *Eur. J. Pharmacol.* **72:** 413–415.
9. Berlan, M., C. Carpéné, M. Lafontan, and L. Dang Tran. 1982. α_2-Adrenergic antilipolytic effect in dog fat cells: incidences of obesity and adipose tissue localization. *Horm. Metab. Res.* **14:** 257–260.
10. Lafontan, M. 1979. Inhibition of epinephrine-induced lipolysis in isolated white adipocytes of aging rabbits by increased alpha-adrenergic responsiveness. *J. Lipid Res.* **20:** 208–216.
11. Lafontan, M. 1981. Alpha-adrenergic responses in rabbit white fat cells: the influence of obesity and food restriction. *J. Lipid Res.* **22:** 1084–1093.
12. Carpéné, C., M. Berlan, and M. Lafontan. 1983. Influence of development and reduction of fat stores on the antilipolytic α_2-adrenoceptor in hamster adipocytes: comparison with adenosine and β-adrenergic lipolytic responses. *J. Lipid Res.* **24:** 766–774.
13. Pecquery, R., and Y. Giudicelli. 1980. Ontogenic development of alpha adrenergic receptors and responsiveness in white adipocytes. *Biochem. J.* **192:** 947–950.
14. Rodbell, M. 1964. Metabolism of isolated fat cells. 1. Effects of hormones on glucose metabolism and lipolysis. *J. Biol. Chem.* **239:** 375–380.
15. Lavau, M., C. Susini, J. Knittle, S. Blanchet-Hirst, and M. R. C. Greenwood. 1977. A reliable photomicrographic method for determination of fat cell size and number: application to dietary obesity. *Proc. Soc. Exp. Biol. Med.* **156:** 251–256.
16. Dole, V. P., and H. Meinertz. 1960. Micro-determination of long chain fatty acids in plasma and tissues. *J. Biol. Chem.* **235:** 2529–2599.
17. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193:** 265–275.
18. Saulnier-Blache, J. S., C. Carpéné, D. Langin, and M. Lafontan. 1989. Imidazolinic radioligands for the identification of hamster adipocyte α_2-adrenoceptors. *Eur. J. Pharmacol.* **171:** 145–157.
19. Berlan, M., and M. Lafontan. 1985. Evidence that epinephrine acts preferentially as an antilipolytic agent in abdominal human subcutaneous fat cells: assessment by analysis of beta- and alpha$_2$-adrenoceptor properties. *Eur. J. Clin. Invest.* **15:** 341–348.
20. Galitzky, J., M. Lafontan, H. Paris, and M. Berlan. 1989. Human fat cell α_2-adrenoceptors. II. Comparative study of partial and full agonist binding parameters using [^{3}H]clonidine and [^{3}H]-UK 14304. *J. Pharmacol. Exp. Ther.* **249:** 592–600.
21. Dabiré, M. 1986. Idazoxan: a novel pharmacological tool for the study of α_2-adrenoceptors. *J. Pharmacol. (Paris).* **17:** 113–118.
22. Pushpendran, C. K., and J. A. Garciá-Sainz. 1984. RX 781094, a potent and selective α_2-adrenergic antagonist. Effects in adipocytes and hepatocytes. *Eur. J. Pharmacol.* **99:** 337–339.
23. Coupry, I., R. A. Podevin, J. P. Dausse, and A. Parini. 1987. Evidence for imidazoline binding sites in basolateral membranes from rabbit kidney. *Biochem. Biophys. Res. Commun.* **147:** 1055–1060.
24. Convents, A., D. Convents, J. D. De Backer, J. De Keyser, and G. Vauquelin. 1989. High affinity binding of [^{3}H]rauwolscine and [^{3}H]-RX 781094 to α_2-adrenergic receptors and nonstereoselective sites in human and rabbit brain cortex membranes. *Biochem. Pharmacol.* **38:** 455–463.
25. Michel, M. C., and P. A. Insel. 1989. Are there multiple imidazoline binding sites? *Trends Pharmacol. Sci.* **10:** 342–344.
26. Langin, D., and M. Lafontan. 1989. [^{3}H]Idazoxan binding at non-α_2-adrenoceptor in rabbit adipocyte membranes. *Eur. J. Pharmacol.* **159:** 199–201.
27. Stillings, M. R., C. B. Chapleo, R. C. M. Butler, J. A. Davis, C. D. England, M. Myers, P. L. Myers, N. Tweddle, A. P. Welbourn, J. C. Doxey, and C. F. C. Smith. 1985. α-Adrenoceptor reagents. Synthesis of some 2-substituted 1,4-benzodioxans as selective presynaptic α_2-adrenoceptor antagonists. *J. Med. Chem.* **28:** 1054–1062.
28. Langin, D., M. Lafontan, M. R. Stillings, and H. Paris. 1989. ^{3}H-RX 821002: a new tool for the identification of α_2-adrenoceptors. *Eur. J. Pharmacol.* **167:** 95–104.
29. Galitzky, J., D. Larrouy, M. Berlan, and M. Lafontan. 1990. New tools for human fat cell α_{2A}-adrenoceptor characterization. Identification on membranes and on intact cells using the new antagonist ^{3}H-RX 821002. *J. Pharmacol. Exp. Ther.* **252:** 312–319.
30. Mauriège, P., J. Galitzky, M. Berlan, and M. Lafontan. 1987. Heterogeneous distribution of beta- and alpha-adrenoceptor binding sites in human fat cells from various fat deposits: functional consequences. *Eur. J. Clin. Invest.* **17:** 156–165.
31. Giudicelli, Y., N. R. Thotakura, D. Lacasa, R. Pecquery, and B. Agli. 1981. Direct binding studies do not support the existence of true alpha-adrenoceptors in rat white fat cells. *Biochem. Biophys. Res. Commun.* **100:** 621–628.
32. Timmermans, P. B. M. W., and P. A. Van Zwieten. 1982. Alpha$_2$-adrenoceptors: classification, localization mechanisms and targets for drugs. *J. Med. Chem.* **25:** 1389–1401.
33. Sundin, U., and J. N. Fain. 1983. α_2-Adrenergic inhibition of lipolysis and respiration in rat brown adipocytes. *Biochem. Pharmacol.* **32:** 3117–3120.
34. Bylund, D. B. 1985. Heterogeneity of alpha$_2$-adrenergic receptors. *Pharmacol. Biochem. Behav.* **22:** 835–843.
35. Rebourcet, M. C., C. Guichard, M. Guerre-Millo, and M. Lavau. 1986. Increased sensitivity to the antilipolytic effect of adenosine in adipocytes from genetically obese rats. *Diabete & Metab.* **12:** 289–290 (Abstract).