Late expression of α2-adrenergic-mediated antilipolysis during differentiation of hamster preadipocytes

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Abstract In order to study the ontogenesis of the β- and α2-adrenergic control of lipolysis during the adipose conversion process, a model based on preadipocytes isolated from the stromal-vascular fraction of hamster adipose tissue was developed. When cultured in an ITT (insulin, transferrin, triiodothyronine) medium supplemented with 2% fetal calf serum, adipose precursors differentiated into adipose-like cells. On 8-day-post-confluent differentiating preadipocytes, the rank order of potency of activation of lipolysis by various β-adrenergic agonists (BRL37344 > norepinephrine = isoproterenol > epinephrine > fenoterol) was equivalent to that determined in mature adipocytes isolated from adult hamster adipose tissue. On 8-day-post-confluent differentiating preadipocytes, phenylisopropyladenosine (A1-adenosine agonist) and prostglandin E1 evoked a strong antilipolytic repsonse whereas that evoked by UK 14304 and clonidine (α2-adrenergic agonists) remained undetectable at this step of differentiation. The activity of UK 14304 and clonidine only appeared on 20- to 25-day-post-confluent differentiating preadipocytes. They induced dose-dependent antilipolysis with a maximal effect reaching 80–85% inhibition of adenosine deaminase-stimulated lipolysis. Their action was blocked by increased concentrations of different α2-adrenergic antagonists with the following order of potency, RX 821002 > phentolamine >> yohimbine. This order of potency was similar to that determined on mature adipocytes isolated from adult hamsters. Both the density of the α2-adrenoceptors, identified with the selective α2-adrenergic radioligand [^{3}H]RX-821002 (19 ± 1 vs. 30 ± 1 fmol/mg protein: $P < 0.01$) and the amount of Gi proteins identified by pertussis toxin-catalyzed ADP-ribosylation (31 ± 4 vs. 43 ± 4% of the amount defined in mature fat cells from adult hamsters: $P < 0.05$) were significantly increased between 8 days and 20–25 days after confluence, explaining the late emergence of the α2-adrenergic control of lipolysis during preadipocyte differentiation. In conclusion, the late emergence of the α2-adrenergic control of lipolysis, which is also supported by previous data obtained in vivo that demonstrated the age and/or the fat cell size dependence of α2-adrenoreceptor expression in mature adipocytes, allows the α2-adrenoceptor to be considered as a marker of adipocyte hypertrophy. —**Saulnier-Blache, J. S., M. Dauzats, D. Daviaud, D. Gaillard, G. Ailhaud, R. Négrel, and M. Lafontan.** Late expression of α2-adrenergic-mediated antilipolysis during differentiation of hamster preadipocytes. *J. Lipid Res.* 1991. **32:** 1489–1499.

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The lipolytic response induced by catecholamines in adipocytes relies on the balance between β- and α2-adrenergic responsiveness. The activation of the β-adrenergic receptor (β-AR) induces: *i*) an activation of adenylate cyclase via a cholera toxin-sensitive GTP-binding protein (Gs); *ii*) an increase in the intracellular level of cAMP; and *iii*) a phosphorylation of the hormone-sensitive lipase (HSL) by cAMP-dependent protein kinase A (PKA). Conversely, the activation of the α2-adrenergic receptor (α2-AR) induces an inhibition of adenylate cyclase via a pertussis toxin-sensitive GTP-binding protein (Gi), leading to an antilipolytic effect that counteracts the β-adrenergic-mediated responses (1).

Previous studies of human adipose tissue by our group and others have focused attention on the major role played by the adipocyte α2-AR through its involvement in the resistance to the lipolytic action of catecholamines in the femoral adipose tissue of women (2). Expression of the adipocyte α2-AR is also correlated to adipose tissue mass and fat cell size increment, as shown in animal models

Abbreviations: G3PDH, glycerol-3-phosphate dehydrogenase; ITT, medium containing insulin, transferrin, and triiodothyronine; SVF, stromal-vascular fraction; β-AR, β-adrenergic receptor; α2-AR, α2-adrenergic receptor; HSL, hormone-sensitive lipase; Gs-cholera toxin-sensitive GTP-binding protein; Gi, pertussis toxin-sensitive GTP-binding protein; PKA, protein kinase A; FCS, fetal calf serum; ADA, adenosine deaminase.

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(3–7). Moreover a fat mass-independent regulation of the α2-AR with photoperiod was also recently observed and appears to be related to plasma testosterone levels (8). Adipocyte α2-AR turnover was also defined by our group in hamster fat cells ($t_{1/2}$ = 46 h) (9).

In order to identify the neurohumoral and/or nutritional factors directly involved in the fat mass-dependent or fat mass-independent control of the adipocyte α2-AR, an in vitro adipocyte model appeared useful for long-term studies.

Previous work has described the use of adipose tissue explants (10) or primary culture of mature adipocytes (11) for investigations of the regulation of fat cell metabolism. These models appeared more appropriate for short-term than for long-term regulation studies because of the limited viability of such cell preparations (no more than 48 h). Our own experience revealed the stability of the adrenergic control of lipolysis on cultured mature hamster adipocytes over 48 h with a rapid disappearance thereafter (Saulnier-Blache, J. S., unpublished data).

Preadipocytes isolated from the adipose tissue of various species, as well as cells from established preadipocyte clonal lines, have been extensively used to delineate the main events involved in adipose cell differentiation and appear to be appropriate tools for long-term regulation studies (12). The β-adrenergic control of lipolysis, as well as the effects of insulin (13), adenosine (14), and prostaglandins (15), have already been described in the different types of preadipocyte, whereas no data regarding their α2-adrenergic control of lipolysis are as yet available. It is notable that most of the cellular models so far described have been established using the rat or mouse, in which the α2-adrenergic control of lipolysis in mature adipocytes is poorly efficient [rat (16) and mouse (Carpéné, C., unpublished data)] when compared to man, dog, rabbit, and hamster (17). The species-specific differences could explain, at least in part, the lack of information with the respect to the α2-AR in the commonly used rat and mouse preadipocyte models.

Thus, in order to delineate the ontogenesis of the α2-adrenergic control of lipolysis during adipose differentiation as well as to define an in vitro model of adipose cells on which long-term regulation studies could be performed, we decided to develop a model of cultured hamster preadipocytes isolated from the stromal-vascular fraction of white adipose tissue. Indeed, unlike in the rat or mouse, the hamster expresses a fully functional mature adipocyte α2-AR previously defined pharmacologically with appropriate radioligands such as the α2-antagonist [^{3}H]RX821002 (18). After the demonstration of a β-adrenergic control of lipolysis in these cells, the occurrence of an α2-adrenergic control was estimated by functional studies (i.e., the antilipolytic effect of α2-adrenergic agonists and by binding assays, using the selective radioligand [^{3}H]RX821002). Moreover, the transducing system of the α2-AR-mediated responses was also delineated by the identification of the Gi GTP-dependent proteins.

MATERIALS AND METHODS

Animals

All studies were performed on 4- to 7-week-old male Syrian hamsters (*Mesocricetus auratus*) weighing between 70 and 100 g, housed at 20–22°C under a controlled photoperiod (16 h light:8 h dark) with free access to water and fed ad libitum on a standard pellet diet (UAR, Paris, France).

Cell culture

All cultures were performed in a mixture of Dulbecco modified Eagle's (DME) medium and Ham's F12 medium (1:1; v:v), supplemented with 15 mM $NaHCO_3$, 15 mM HEPES buffer, pH 7.4, 33 μM biotin, 17 μM calcium pantothenate, 62 mg/l penicillin, and 50 mg/l streptomycin, as previously described (19); this is referred to as DME-F12 medium.

The animals were killed by cervical dislocation and the pooled white adipose tissue depots (periepididymal, perirenal, and subcutaneous) were immediately removed under sterile conditions and the blood vessels were dissected out. The tissue was cut into small pieces, weighed, and digested at 37°C with shaking in DME-F12 medium (3 ml/g of tissue) supplemented with 1 mg/ml collagenase and 20 mg/ml bovine serum albumin (BSA). The digestion was stopped after 30 min by dilution with DME-F12 medium and the suspension was filtered through a 100-μm nylon screen. After centrifugation for 8 min at 600 *g*, a first pellet, corresponding to the stromal-vascular fraction (SVF), was collected. The floating adipocyte fraction was resuspended and centrifuged again in order to collect the remaining stromal-vascular cells. Pelleted cells were pooled, resuspended in DME-F12 medium supplemented with 10% fetal calf serum (FCS), filtered through a 25-μm nylon screen, and centrifuged at 600 *g* for 8 min. The final pellet was resuspended in DME-F12 medium supplemented with 10% FCS, the cells were counted with a hemocytometer and seeded at a density of 10^5 cells/cm^2 into 2, 24, or 60 cm^2 culture dishes at 37°C in a humidified 95% air–5% CO_2 atmosphere. After 24 h for attachment, the cells were extensively washed with DME-F12 medium to eliminate contaminating erythrocytes and grown to confluence (4–6 days) in DME-F12 medium supplemented with 10% FCS and 850 nM insulin.

When confluent, the cells were washed twice, maintained in DME-F12 medium for 1 h, washed again, and then maintained in DME-F12 medium supplemented with 20 nM sodium selenite, 100 μM sodium ascorbate, 850 nM insulin, 10 μg/ml transferrin, and 200 pM

triiodothyronine (T3). This chemically defined medium previously established for the differentiation of rat adipose precursor cells (16) was referred to as ITT medium. For all the experiments concerning the pharmacological evaluation of the adrenergic control of lipolysis, the ITT medium was supplemented with 1.5 mg/ml BSA and 2% FCS to avoid the detachment of the adipose-like cells containing a large amount of unilocular lipid droplets (after 15–17 days post confluence).

The culture medium was changed every 3 days before cells were harvested for enzymatic, biological, or binding assays. The differentiation of hamster adipose precursor cells was assessed by assays of a late marker of adipose conversion, i.e., glycerol-3-phosphate dehydrogenase (GPDH; E.C. 1.1.1.8). For that purpose, cells washed twice with phosphate-buffered saline were homogenized in 20 mM Tris-HCl, pH 7.3, containing 1 mM EDTA, 1 mM β-mercaptoethanol. The spectrophotometric assay of G3PDH activity was performed according to Wise and Green (20).

Lipolytic assays

The lipolytic assays were performed in DME-F12 medium supplemented with 35 mg/ml BSA and adjusted to pH 7.4 (DME-F12-BSA medium).

Isolation of mature fat cells by collagenase digestion, optical sizing, and lipolytic activity measurements was performed as previously described (8).

Assays with differentiated cells in culture were performed as follows. After washing twice, the cells were maintained for 2 h in DME-F12-BSA medium, washed, and scraped off with a rubber policeman. The cell suspension was collected in polypropylene tubes (to limit cell breakage) and gently centrifuged (100 g × 3 min) in order to separate the floating lipid-containing cells from undifferentiated cells. The infranatant was discarded and the lipid-containing cells were washed twice in DME-F12-BSA medium by centrifugation. The cells were then dispersed in Eppendorf tubes in 300 μl of DME-F12-BSA medium (40,000 cells per assay) containing 3 μl of pharmacological agents added at suitable concentrations. After 60 min at 37°C, the reactions were stopped in ice; glycerol released by the cells was measured as previously described (4), and the results were expressed in μmol glycerol/h per 10^6 cells.

Radioligand binding studies

Binding assays were performed using crude membrane preparations obtained from isolated mature adipocytes or cultured differentiated cells after hypotonic lysis (8). Membrane preparations were kept frozen no longer than 2–3 days at −80°C before assays. After thawing, the membranes were rinsed twice in a large volume (30 ml) of 50 mM Tris-HCl, 0.5 mM $MgCl_2$, pH 7.4, by centrifugation (40,000 g, 10 min at 4°C). The pellet was homogenzied, filtered, and adjusted to a final protein concentration of 4–6 mg/ml.

The α2-adrenergic binding sites were identified with the α2-antagonist [^{3}H]RX821002. An extended validation of binding assay conditions with this radioligand on hamster fat cell membranes has recently been described by our group (18). Briefly, the incubation was carried out at 25°C in a water bath for 30 min under constant shaking in a medium consisting of 100 μl of radioligand and 100 μl of membrane suspension made up to a final volume of 400 μl with 50 mM Tris-HCl, pH 7.5, containing 0.5 mM $MgCl_2$. The specific binding was determined as the total radioactivity bound to the membranes minus the radioactivity bound in the presence of 200 μM (−)epinephrine (nonspecific binding).

The β-adrenergic binding sites were identified with the β-antagonist radioligand [^{125}I]cyanopindolol ([^{125}I]CYP), as previously described (4). The incubation was carried out at 37°C for 60 min in a medium consisting of 50 μl of radioligand and 50 μl of membrane suspension made up to a final volume of 200 μl with 25 mM Tris-HCl, pH 7.5, containing 0.5 mM $MgCl_2$. The specific binding was determined as the total radioactivity bound to the membranes minus the radioactivity bound in the presence of 100 μM isoproterenol (nonspecific binding).

In all binding assays, bound and free radioligand were separated by filtration of the membrane suspension through GF/C Whatman filters using a Skatron cell harvester. The filters were washed with ice-cold 10 mM Tris-HCl buffer, pH 7.5, containing 0.5 mM $MgCl_2$. For binding assays using [^{3}H]RX821002, the filters were put into minivials containing 2 ml of a scintillation cocktail and counted in a Packard beta counter (efficiency 45–50%). For [^{125}I]CYP binding studies the filters were directly counted in a Packard gamma counter (efficiency 80%). The binding parameters (B_{max}, K_D) were determined from equilibrium binding studies by a computer-assisted linear transformation of the saturation binding data (Scatchard analysis) (21).

The protein content of the membrane preparation was measured by the method of Lowry et al. using BSA as standard (22).

ADP-ribosylation assays

Pertussis toxin-catalyzed ADP-ribosylation was performed as described by Ribeiro-Neto et al. (23) with minor modifications according to the method of Sternweis and Robishaw (24). Crude membranes from various sources were suspended in Tris-HCl buffer, pH 8.0, containing 1 mM EDTA, 1 mM DTT, and 0.05% Lubrol. Membranes (100 μg) or a calf brain preparation (0.6 μg) containing purified α1, α2, α3, and α0 subunits of Gi (generous gift from Dr. Bruno Rouot, Montpellier, France) were then incubated for 60 min at 30°C with 0.5 μM NAD, 0.5–1 μCi of [α-^{32}P]NAD 800 Ci/mmol, 1 mM

ATP, 100 μM GTP, 10 mM thymidine, 1 mM EDTA, 0.1 mM $MgCl_2$, 1 mg/ml L-myristyl phosphatidylcholine, 10 mM nicotinamide, 25 mM dithiothreitol (DTT), 70 mM Tris-HCl, pH 8.0, and 100 ng of pertussis toxin, in a final volume of 60 μl. The reaction was stopped by adding 20 μl of a 100 μg/ml BSA solution containing 2% SDS. Proteins were precipitated overnight at 4°C with 70 μl of 10% trichloroacetic acid. After centrifugation for 5 min at 13,000 *g*, the pellets were washed twice with diethyl ether, dried, and analyzed by SDS-PAGE. The pellet was resuspended in 20 μl of 50 mM Tris-HCl buffer, pH 6.8, containing 10% SDS and 50 mM DTT. The suspension was incubated for 5 min at 90°C, and treated with 20 μl of 100 mM N-ethylmaleimide for 15 min at room temperature. Before electrophoresis, 20 μl of 100 mM Tris-HCl buffer, pH 6.8, containing 10% SDS, 10% β-mercaptoethanol, 20% glycerol, and 0.1% bromophenol blue was added. Electrophoresis was performed on a 10% acrylamide/SDS gel under constant current. Six hours later, the gels were stained in a solution of 0.1% Coomassie blue, destained in the presence of methanol–acetic acid–water 5:10:85 (v/v/v), dried, and autoradiographed for 24 h at −80°C. The autoradiograms were analyzed by measuring the density of the spots with a Biocom analyzer.

Chemicals

Fetal calf serum was obtained from I.B.F (Villeneuve la Garenne, France). [^{125}I]CYP was obtained from Amersham International (Buckinghamshire, UK).

[α-^{32}P]NAD was obtained from New England Nuclear (Boston, MA), Isoproterenol, (−)epinephrine, norepinephrine, transferrin, insulin, sodium ascorbate, triiodothyronine, prostaglandin E1, pertussis toxin, and bovine serum albumin (Fraction V) were purchased from Sigma (St. Louis, MO) RX 821002 and [^{3}H]RX 821002 were kindly given by Reckitt and Colman, (Hull, UK). UK 14304 was obtained from Pfizer (Sandwich, UK). Clonidine was from Boehringer (Ingelheim, Germany). Collagenase, (−)phenylisopropyladenosine, adenosine deaminase, NADH, NADP, and enzymes for glycerol assays were purchased from Boehringer Mannheim (Mannheim, Germany). Sodium selenite was from Gibco (Grand Island, NY). All other chemicals and organic solvents were of reagent grade.

RESULTS

Evaluation of the adipose conversion

Fibroblast-like cells isolated from the stromal-vascular fraction of hamster adipose tissue were first allowed to reach confluence within 4 to 6 days in DME-F12 medium supplemented with 10% FCS and 850 nM insulin (we had observed that the percentage of cells that undergo differentiation was slightly increased when insulin was added during the growth period; not shown). After confluence, exposure to ITT medium (see Materials and Methods) allowed the cells to enter their differentiation program. Within 2 weeks, the fibroblast-like cells acquired a round shape and began to accumulate triacylglycerol droplets. In order to avoid cell detachment at later times (15 to 17 days after confluence) due to increased cellular triacylglycerol content, ITT medium was supplemented with 2% FCS in order to supply certain attaching factors present in the serum. Under these conditions, the differentiation process was not modified and allowed us to maintain the differentiating preadipocytes until 25–30 days after confluence. At that stage, lipid droplets underwent coalescence and gave a few or a single fat globule(s). The cells reaching diameters between 15 and 20 μm exhibited a morphological aspect similar to that described for other differentiating preadipocyte models such as that based on the rat (19). The percentage of cells that underwent differentiation was roughly evaluated as 40–60%.

The specific activity of G3PDH, which is commonly used as an index of adipose conversion, increased regularly from confluence and reached a maximal value 7–8 days after confluence. The maximal activity obtained in these differentiated cells was equivalent to that determined in mature adipocytes isolated from adult hamsters (mean diameter of 80 μm) (**Table 1**). The presence or absence of 2% FCS in the ITT medium was without influence of the G3PDH activity (not shown).

It was observed that the recovery of precursor adipocytes extracted from the adipose tissue decreased with aging; however, when the density of cells seeded was checked, the differentiation rate evaluated from the G3PDH activity level was similar between 4-week and 7-week-old hamsters (not shown). Thus, it appeared reasonable in these conditions to compare experiments performed on 4-week-old hamsters with experiments per-

TABLE 1. G3PDH specific activity in differentiating hamster preadipocytes and mature adipocytes

Cells	n	G3PDH Specific Activity
		mU/mg protein
Differentiating preadipocytes		
Days after confluence		
1 Day	4	89 ± 12[a]
8 Days	5	831 ± 54**
20 Days	3	650 ± 53**
Mature adipocytes	3	740 ± 30

[a]G3PDH activity was measured as described in Materials and Methods. The means ± SE correspond to n separate experiments. A Student's *t* test was performed to compare the G3PDH activity between 1-day and 8-day or 20-day post-confluent differentiating preadipocytes, and between 20-day post-confluent differentiating and mature adipocytes; **, $P < 0.01$.

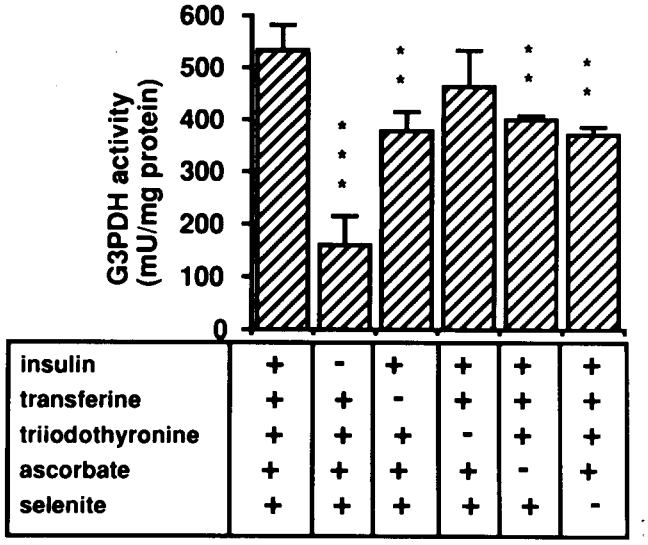

Fig. 1. Effect of the components of ITT medium on their ability to induce G3PDH activity in differentiating adipose precursor cells. The activity was determined in 4-day post-confluent cells cultured in ITT medium or in the same medium deprived of one of its components. Student's *t* test was performed in order to compare each mean ± SE with the complete ITT medium;**, $P < 0.01$;***, $P < 0.001$. These results correspond to the mean of three separate experiments.

formed on 5-, 6-, or 7-week-old hamsters. Moreover, slight differences were observed in the differentiation rate of the preadipocytes extracted from the different adipose tissue deposits. Indeed, the preadipocytes extracted from the epididymal fat pads exhibited about 30% lower GPDH activity than preadipocytes from the subcutaneous or perirenal fat pads 8 days after confluence (not shown). However, in the preadipocyte pool, approximately 20% of the cells originated from the epididymal fat pad. In these conditions and because of the amount of cells necessary to perform pharmacological and functional studies, it appeared reasonable to work on the pooled adipose tissue deposits. This choice was supported by the fact that although intersite differences exist in the number of mature adipocyte β- and α2-AR and also in the maximal lipolytic responses of the different fat deposits of standard adult hamsters, these receptors are pharmacologically equivalent and the differences in the actual lipolytic response are not so striking (3).

In order to define the relative importance of the various factors present in the serum-free hormone-supplemented medium, the G3PDH activity was measured in 4-day post-confluent differentiating cells maintained in a medium deprived of one of its components. Removal of insulin, transferrin, triiodothyronine, sodium ascorbate, or sodium selenite led to a 70%, 29%, 14%, 15%, and 31% decrease, respectively, of the G3PDH activity as compared to complete medium (**Fig. 1**). Under these culture conditions, insulin appeared as the main component of the ITT medium. Owing to the high concentration of insulin required, this hormone probably acts through an insulin-like growth factor receptor, as already observed in other preadipocyte cell systems (19); the other components of the culture medium appeared to behave merely as modulators.

Lipolytic activity of differentiating hamster preadipocytes

The lipolytic activity of the cells was determined by glycerol release in the presence or absence of different stimulatory agents. The lipolytic activity of the cultured cells at different stages of differentiation was compared to that of mature adipocytes isolated from the adipose tissue of adult hamsters fed ad libitum (mean fat cell diameter, 79 ± 3 μm; n = 5) or fasted for 6 days (mean fat cell diameter, 34 ± 7 μm; n = 7). The lipolytic responsiveness of adipocytes from fasted hamsters was similar to that of adipocytes isolated from young 10- to 15-day-old hamsters (not shown).

The basal lipolytic activity of 8-day post-confluent differentiating cells and that of mature fat cells were not significantly different (**Fig. 2**). Furthermore, there were no significant differences between the maximal lipolytic responses initiated by 10^{-5} M forskolin, in 8-day post-confluent differentiating cells and small adipocytes from fasted hamsters. Nevertheless, a 4-fold increase in the maximal lipolytic response was observed in large adipocytes from adult hamsters fed a standard diet (80 μm) (Fig. 2).

Other agents such as adrenocorticotropic hormone (ACTH), alkylated xanthines (isobutylmethylxanthine and theophylline), and cAMP-phosphodiesterase inhibi-

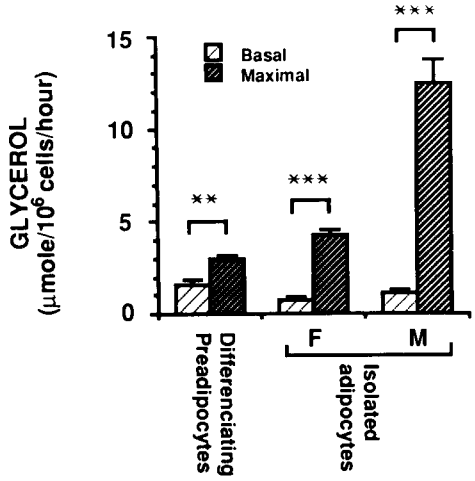

Fig. 2. Comparative lipolytic responsiveness of 8-day post-confluent differentiating cells, 30-μm mature adipocytes from fasted hamsters (F), and 80-μm mature adipocytes from fed animals (M). The maximal lipolytic response corresponds to that obtained in the presence of 10^{-5} M forskolin. The results are the mean ± SE of three separate experiments. Basal and maximal lipolysis were compared using Student's *t*-test (**, $P < 0.01$;***, $P < 0.001$).

TABLE 2. Half-maximal effective concentrations (EC_{50}) of various β-adrenergic agonists in 8-day post-confluent cells and mature isolated adipocytes

β-Adrenergic Agonists	Differentiating Preadipocytes n = 3	Isolated Mature Adipocytes n = 5
Isoproterenol	28 ± 1[a]	28 ± 3
(−)Norepinephrine	27 ± 4	18 ± 6
(−)Epinephrine	126 ± 27	227 ± 26*
BRL 37344	1.0 ± 0.1	0.5 ± 0.1
Fenoterol	113 ± 4	185 ± 14

[a] EC_{50} (nM) values were determined from the concentration-effect curves obtained with different β-adrenergic agonists using a computerized curve-fitting procedure. Each value is the mean ± SE of n separate experiments. Mean values are compared using Student's *t* test; *, $P < 0.05$.

tors (RP 55464 and cilostamide) were also assessed with respect to lipolysis; all these agents proved to be potent lipolytic activators in 8-day post-confluent differentiating cells (not shown).

β-Adrenergic control of lipolysis. The profile of β-adrenergic-responsiveness was defined as the lipolytic response obtained with different selected β-adrenergic agonists. The half-maximal effective concentrations, EC_{50} (nM), of the different agonists were determined using a computerized curve-fitting procedure shown in **Table 2**. It is interesting to note that instead of slight differences in the EC_{50} values of epinephrine and fenoterol, the order of potency (BRL 37344 > norepinephrine = isoproterenol > epinephrine > fenoterol) of the different β-adrenergic agonists was similar for 8-day post-confluent differentiating cells and mature adipocytes (**Fig. 3**), suggesting the involvement of the same kind of β-AR.

In order to confirm the presence of β-ARs, saturation binding studies were performed with the antagonist radioligand [^{125}I]CYP, in 8-day post-confluent cells and mature adipocytes. It was impossible to detect any [^{125}I]CYP binding in membranes of 8-day post-confluent cells, whereas [^{125}I]CYP binding was clearly detected in membranes of mature adipocytes (**Table 3**).

α2-Adrenergic control of lipolysis. The antilipolytic action of two α2-adrenergic agonists, UK14304 and clonidine, was investigated in differentiating cells 8 days and 20–25 days after confluence. The efficiency of the α2-agonists was compared to that of the antilipolytic effectors known to act by binding to other receptors such as the A1-adenosine receptor (phenylisopropyladenosine:PIA) and the prostaglandin E1 (PGE1) receptor.

In 8-day post-confluent cells, 10^{-6} M PIA and 10^{-6} M PGE1 were able to completely suppress the lipolytic effect induced by 4 μg/ml adenosine deaminase (ADA). In contrast, clonidine and UK 14304 were devoid of any antilipolytic effect (**Fig. 4**). The lack of antilipolytic effect was also observed when lipolysis was stimulated either by 10^{-8} M isoproterenol or 3 mM theophylline instead of

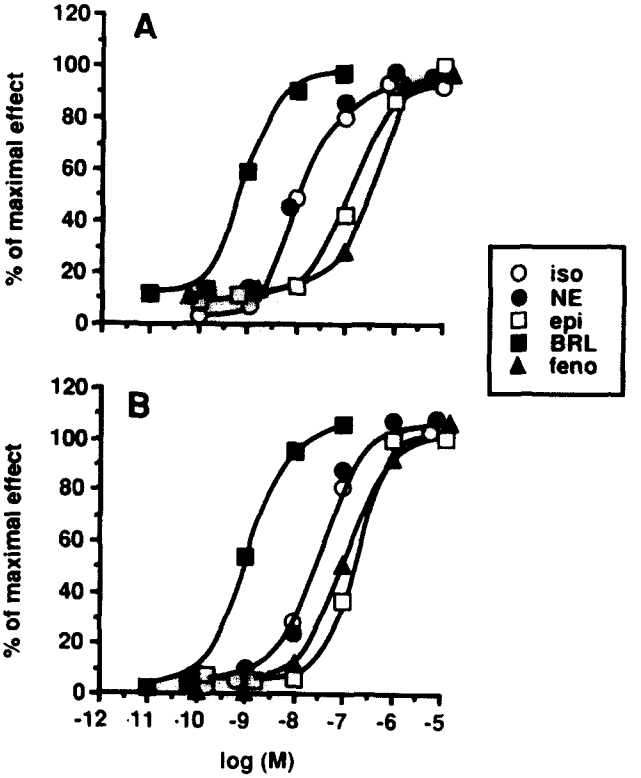

Fig. 3. Lipolytic response initiated by various β-adrenergic agonists; iso, isoproterenol; NE, (−)norepinephrine; epi, (−)epinephrine; BRL, BRL 37344; feno, fenoterol. (A) 8-Day post-confluent differentiating cells; (B) mature hamster adipocytes (80 μm mean fat cell diameter). The maximal lipolytic effect (100%) corresponds to the response induced by 10^{-6} M isoproterenol. It was equivalent to that observed with all the other lipolytic agents. Each curve corresponds to a typical response found in three (A) or five (B) separate experiments for each agonist. Computerized determinations of the EC_{50} values (nM) are reported in Table 2.

ADA (not shown). The presence or absence of 2% FCS in the culture medium had no effect on the α2-responsiveness (not shown).

However, in 20–25-day post-confluent differentiating preadipocytes, UK14304 and clonidine were able to inhibit the ADA-stimulated lipolysis in a dose-dependent

TABLE 3. Identification of the β-adrenoceptors with [^{125}I]CYP in membranes of 8-day post-confluent cells and adipocytes

Cells	n	B_{max}	K_d
		fmol/mg protein	*pM*
Differentiating preadipocytes	3	Und	Und
Isolated mature adipocytes	4	81 ± 2	20.6 ± 1.5

The saturation specific binding was determined by incubating the membranes with increasing concentrations of [^{125}I]CYP (2–100 pM) and using 100 μM isoproterenol for the definition of nonspecific binding. The binding parameters were determined as described in Materials and Methods. The results correspond to the mean ± SE of n separate experiments; Und, undetectable.

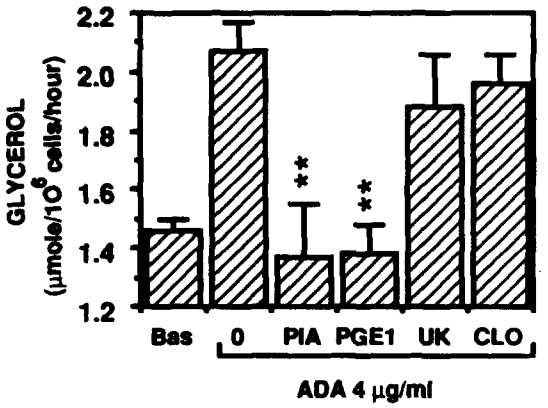

Fig. 4. Antilipolytic action of 10^{-6} M phenylisopropyladenosine (PIA), prostaglandin E1 (PGE1), 10^{-5} M UK14304, and 10^{-5} M clonidine on ADA (4 μg/ml)-stimulated lipolysis in 8-day post-confluent differentiated preadipocytes. The results correspond to the mean ± SE of three different experiments. Student's *t* test was performed to compare the effect of the different inhibitors on ADA-stimulated lipolysis;**, $P < 0.01$.

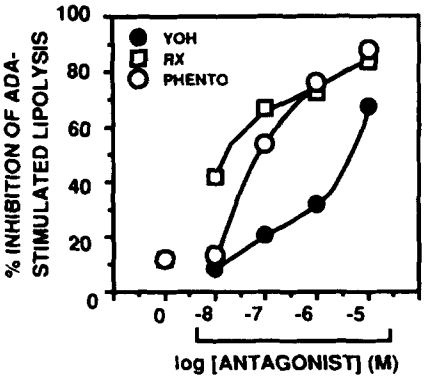

Fig. 6. Dose-dependent antagonism of UK14304-induced antilipolysis by different α2-adrenergic antagonists. The ADA (4 μg/ml)-stimulated lipolysis was inhibited by 10^{-6} M UK14304 alone (O) or in the presence of increasing concentrations of yohimbine (YOH), R821002 (RX), or phentolamine (PHENTO). This result is representative of three separate experiments.

manner. UK14304 was more potent than clonidine, as already shown for mature adipocytes (18). At this stage of differentiation, the amplitude of the ADA-stimulated lipolysis was identical to that obtained in 8-day post-confluent cells. The dose-response curve of UK14304 was clearly shifted to the right in the presence of 10^{-5} M RX821002, a highly selective α2-adrenergic antagonist (**Fig. 5**). The antilipolytic effect of 10^{-6} M UK14304 was inhibited by increasing concentrations of three α2-adrenergic antagonists with the following order of potency: RX-821002 > phentolamine >> yohimbine (**Fig. 6**). These results were in good agreement with those obtained on mature hamster fat cells (18) confirming the occurrence of specific binding sites.

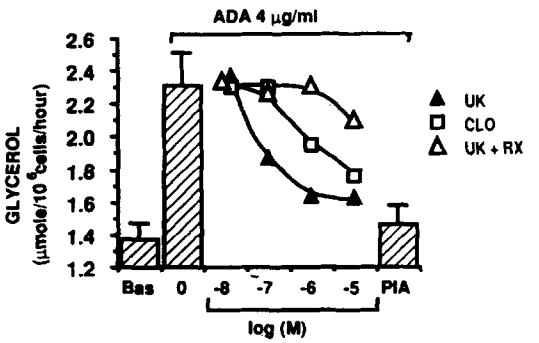

Fig. 5. Dose-dependent inhibition of ADA-induced lipolysis by UK14304 and clonidine on 20–25-day post-confluent differentiated preadipocytes. The ADA (4 μg/ml)-stimulated lipolysis was inhibited by increasing concentrations of clonidine (CLO) and UK14304 (UK) and with 10^{-5} M phenylisopropyladenosine (PIA). The effect of UK14304 was antagonized by 10^{-5} M of the α2-antagonist RX821002. These results are representative of at least three separate experiments.

α2-Adrenergic binding studies

The presence of the α2-AR was investigated in membranes of 8-day and 20–25-day post-confluent cells using the highly selective α2-adrenergic antagonist [^{3}H]RX-821002 (18). The binding parameters were compared with those obtained in adipocytes of 30 and 80 μm diameter isolated from adipose tissue of fasted and fed adult hamsters (see Fig. 2).

Specific binding was already detected in 8-day post-confluent preadipocytes (**Table 4**). The binding was saturable and of high affinity similar to that determined on mature fat cells (K_d about 1 nM) (18). Scatchard plot analysis revealed the occurrence of a single class of binding site (Hill coefficient not different from unity). (**Fig. 7**). Determination of B_{max} values revealed the existence of a lower density of α2-adrenoceptors in the differentiating cells than in mature fat cells from young and adult hamster (Table 4). However, the density in the α2-AR was significantly increased between 8-day and 20–25-day post-confluent preadipocytes.

Identification of the GTP-binding Gi proteins

The Gi proteins were identified by incorporation of [α^{32}P]NAD into the cell membranes by pertussis toxin-catalyzed ADP-ribosylation (**Fig. 8**). ADP-ribosylation of purified brain Gi proteins containing αi1, αi2, αi3, and α0 subunits was performed in parallel (lane 4). As previously described in the rat white adipose tissue (25) by Western blot analysis, αi1 and αi3 have the same molecular masses of 41 kDa, whereas αi2 and α0 exhibited molecular masses of 40 and 39 kDa, respectively. In both 8-day and 20–25-day post-confluent differentiating preadipocytes two bands of 41 and 40 kDa (lanes 1 and 2) were observed (αi1 and/or αi3 and αi2). The α0 subunit

TABLE 4. Binding parameters of [^{3}H]RX821002 in 25- to 30-day post-confluent differentiating preadipocytes and small and large mature adipocytes

Cells	n	B_{max}	K_d
		fmol/mg protein	*nM*
Differentiating preadipocytes			
8 Days	5	19 ± 1[a]	0.9 ± 0.1
20–25 Days	5	30 ± 1***	1.1 ± 0.1
Mature adipocytes			
30 μm	5	179 ± 54***	0.8 ± 0.1
80 μm	10	719 ± 67***	0.9 ± 0.1

[a]Binding studies, B_{max} and K_d value determinations, were performed as described in Materials and Methods. Saturation binding studies were performed with increasing concentrations of [^{3}H]RX821002 (0.2–5 nM) using 200 μM (−)epinephrine for the determination of nonspecific binding. Student's t test was performed to compare the mean values between 20–25 days post-confluent preadipocytes and mature adipocytes. A paried t-test was performed to compare the mean values between 8-day and 20–25-day post-confluent preadipocytes; ***, $P < 0.001$.

was absent. This qualitative profile was exactly the same as that observed in mature adipocyte membranes (lane 3) (Fig. 8). Quantification of the Gi proteins (αi1 plus αi2) revealed a slight but significant increase between 8-day and 20–25-day post-confluent preadipocytes (31 ± 4 vs. 43 ± 4% of the Gi proteins existing in mature adipocytes isolated from adult hamsters; n = 3, P < 0.05; Student's t test comparison).

DISCUSSION

In the present study we have developed a model using preadipocytes extracted from the hamster white adipose tissue. When these cells differentiate into adipose-like cells their lipolytic activity can be controlled by functional β-

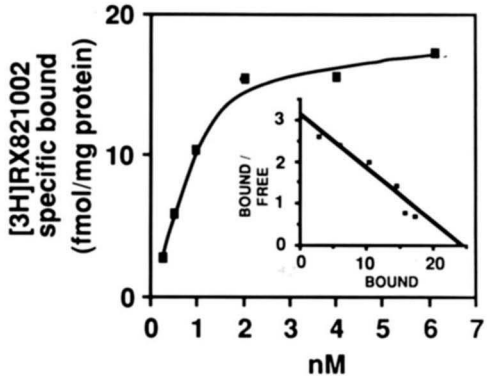

Fig. 7. Representative saturation binding curve of [^{3}H]RX821002 on 15-day post-confluent differentiating preadipocyte membranes. The nonspecific binding, determined in the presence of 200 μM epinephrine, was subtracted. The insert represents the Scatchard plot analysis corresponding to the specific binding curve: B_{max}, 23.5 fmol/mg protein; K_d, 1.5 nM.

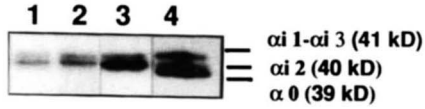

Fig. 8. Pertussis toxin-catalyzed ADP-ribosylation of Gi α-subunit proteins in 8-day and 20–25-day post-confluent preadipocyte membranes (lanes 1 and 2) and in mature adipocyte membranes (lane 3). Lane 4 corresponds to the ADP-ribosylation of a mixture of calf brain G0, Gi1, Gi3, and Gi2 α-subunits. The same amount of total protein was added in each lane.

and α2-adrenergic receptors as well as by A1-adenosine and PGE1 receptors. But whereas the β-adrenergic, the A1-adenosine, and the PGE1-dependent control of lipolysis were already present during early adipose differentiation (8 days after confluence), the α2-adrenergic control of lipolysis appeared later (20–25 days after confluence) indicating different kinetics of appearance of the receptors expressed in the adipocytes.

These conclusions were obtained on the hamster whereas for the rat or mouse, from which most preadipocyte models have been developed until now (12), no data concerning the α2-adrenoceptors were available. Indeed, unlike the rat or mouse, the hamster expresses a high density of fully functional α2-adrenoceptors in the mature adipocytes isolated from adult animals. Moreover, the late appearance of the α2-AR during the adipose differentiation process shows that it is necessary to maintain the cell for a long time (20–30 days) to observe the α2-adrenergic control of lipolysis. So, we supplemented the culture medium with 2% of FCS, which presumably brings some attachment factors and which prevents cells filled with triacylglycerol from floating off.

We observed that the maximal lipolytic response obtained in the differentiating preadipocytes 8 days after confluence was lower than that obtained on mature fat cells. This discrepancy could be attributed to the lower triglyceride content of the differentiating preadipocytes and associated with a lower activity of the hormone-sensitive lipase that has been described to be a late marker of adipose differentiation (12).

With respect to the β-adrenergic control of lipolysis, our study shows that full β-adrenergic response was observed in cells that had accumulated lipids and that were able to express G3PDH at a maximum level. This finding is not surprising since a similar pharmacological profile of lipolytic responsiveness promoted by the β-agonists has also been observed in differentiated BFC-1 and Ob17 adipose like-cells (26) as well as in mature adipocytes (27, the present study). This profile is characteristic of the presence of an "atypical" β-AR, i.e., pharmacologically different from the classical β1- and β2-AR found, for example, in the human adipose tissue (28). This "atypical" β-AR exhibited a high affinity for the β-adrenergic agents, BRL37344 and CGP12177, and could

correspond to a mixed β1-β2-β3-AR populaion (29). But, unlike in mature hamster adipocytes, it was not possible to detect any [^{125}I]CYP binding in cultured hamster differentiating preadipocytes. Previous studies had shown that [^{125}I]CYP has a lower affinity for the β3 (490 pM; ref. 30) than for the β1- and β2-adrenoceptors (Table 2) and, within the range of concentration used in [^{125}I]CYP binding studies (2 to 100 pM), only the population of β1-β2-AR was detectable but not the β3-AR. Consequently, it might well be that in 8-day post-confluent differentiating preadipocytes the β1-β2-ARs were absent or in a very low proportion as compared to mature adipocytes. That can explain the impossibility of detecting them with [^{125}I]CYP. Moreover, since the profile of the response evoked by the β-agonists was identical in both types of cells, it is likely that the "atypical" β-receptor might play a major role in β-AR-dependent lipolysis. Therefore, the development of new β-adrenergic radioligands able to identify and quantify the β3-AR specifically will be of utmost importance.

With respect to the α2-adrenergic control of lipolysis, which plays an important role in inducing the antilipolytic response of catecholamines, our study gives evidence of a different developmental pattern as compared to the β-AR. The α2-adrenergic control of lipolysis was absent in the early stages (8 days after confluence) of adipose differentiation, but became effective (80–85% inhibition of the ADA-stimulated lipolysis) at a later stage (20–25 days after confluence) in spite of a lower α2-AR density as compared to that of mature fat cells isolated from hamster adipose tissue. This can be attributed to the lower maximal stimulation of lipolysis induced by ADA. Indeed, as demonstrated in various mature fat cell preparations with ADA or isoproterenol stimulation, the α2-dependent antilipolysis is stronger when the stimulation is weaker. The pharmacology of the α2-AR defined on differentiating preadipocytes appeared similar to that defined in mature hamster adipocytes. In particular, yohimbine shows a lower affinity for the α2-AR than other antagonists such as RX821002 or phentolamine. This characteristic has previously been described in mature adipocytes and could be related to an "atypical" α2-AR (18). This suggests the existence, in differentiating preadipocytes, of the same α2-adrenoceptor as in mature hamster adipocytes. It must be noted that no specific binding of [^{3}H]RX821002 was detectable in the differentiated 3T3F442A preadipose cell line (12 days post-confluence) (not shown). The present results allow the differentiated hamster preadipocytes to be considered close to terminally differentiated cells. This is the first demonstration of the existence of α2-adrenoceptors on a cultured preadipose cell model.

As previously described in 3T3-L1 cells and rat and human fat cells (31, 32) differentiating adipose precursor cells from the hamster have qualitatively the same Gi protein subunit equipment as mature adipocytes, as illustrated by the occurrence of Giα1 (and/or Giα3) and Giα2 subunits and the absence of the Gα0 subunit. However, the total amount of Gi proteins is lower in the 20–25-day post-confluent differentiating preadipocytes than in mature adipocytes, but the level seems to be sufficient to allow the antilipolytic effects induced by A1-adenosine receptors, prostaglandin receptors, and α2-adrenoceptors to be observed. But the difference between 8 days and 20–25 days post-confluence shows that a steady-state level has to be reached to observe an α2-adrenergic-dependent antilipolytic effect, whereas the A1-adenosine- or PGE1-dependent antilipolysis can be measured. So, the late appearance of the α2-adrenergic control of lipolysis during the adipose differentiation process can be explained, at least in part, by the combination of two parallel events: increase in the α2-AR density and increase in the amount of Gi proteins.

The hamster preadipocyte model decribed herein appears to be a valuable tool for the study of the ontogenesis of both the β- and the α2-ARs during adipose conversion as well as their short- and long-term regulations. Indeed, the expression of these receptors can be controlled by some regulating factors such as androgens (8, 33). The hamster preadipocytes would allow the putative direct effect of these regulating factors to be confirmed in vitro on the fat cells. The interest of this model is strengthened by the fact that, in contrast to other preadipocyte models (34, 35), adipose conversion proceeds in the absence of glucocorticoid hormones, which have been reported to regulate β-ARs (35) and probably also α2-ARs (36) independently of their promoting effect on the adipose differentiation process. However, concerning neurohumoral or nutritional regulation studies of the control of the adipocyte α2- and β-AR expression, it will be necessary to develop specific pharmacological or genetic probes.

It can be concluded that, during the early stages of adipose differentiation, the control of lipolysis by catecholamines relies solely on β-adrenoceptors. It is only in the later stages that the α2-adrenoceptor can exert its antilipolytic action, counteracting the β-adrenergic response. Previous studies have presented evidence in favor of a relationship between α2-AR expression and fat mass enlargement correlated to adipose cell size, i.e., triacylglycerol content (3, 4, 7). This was confirmed in the present paper. We have demonstrated that the expression of the α2-AR increases with the level of triacylglycerol stored in the fat cells (8-day post-confluent preadipocytes > 20–25-day post-confluent preadipocytes > small mature adipocytes isolated from young hamsters > large mature adipocytes isolated from adult hamsters). Thus in vivo and in vitro studies have shown that the α2-AR can be considered as a marker of adipose cell hypertrophy but the actual demonstration of such a direct relationship re-

mains to be shown. Recently, we demonstrated by in vivo studies on male hamsters that the expression of the α2-AR can be controlled by androgens without modification of either the fat mass of the animals or the fat cell size (8), a result suggesting that the increase of the adipocyte α2-AR expression observed with fat mass increment is rather a consequence than a cause of fat cell size hypertrophy.

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