Characterization of rat preadipocytes from normal rat adipose tissue by their effector response

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We isolated from normal rat adipose tissue, by a Percoll-density-gradient procedure, two populations of adipocyte precursors. These preadipocytes undergo morphological and biochemical adipose conversion in primary culture. For full adipose conversion, these precursor cells, in addition to the adipogenic factor present in fetal-calf serum, require other effectors differentially. One population completes terminal differentiation in the presence of physiological concentrations of insulin. The second population requires a pre-sensitization with isobutylmethylxanthine at a critical period of the culture in order to respond to insulin. The fact that dibutyl cyclic AMP could not be substituted for isobutylmethylxanthine suggests that the effect of the latter agent is not through its inhibition of particulate phosphodiesterase activity. These two populations further differ in their response to exogenously added haemin. Thus the existence of at least two developmentally regulated rat adipose-precursor compartments is demonstrated.

INTRODUCTION

For 20 years it has been known that the stromal vascular cell fraction of rat adipose tissue comprises cells which are able to proliferate and differentiate into adipocytes (Hollenberg & Vost, 1968; Greenwood & Hirsch, 1974; Björntorp *et al.*, 1978; Gaben-Cogneville & Swierczewski, 1979). Given the cellular heterogeneity of this fraction, the response of preadipocytes to various effectors could not be clearly assessed. We refined the analysis by isolating density-defined cell populations and studying in primary culture their morphological and biochemical characteristics during lipogenic expression. Two of the light-density fractions underwent adipose differentiation after reaching confluence, and the frequency of morphological and biochemical conversion was judged to be almost 100% in the presence of foetal-calf serum and physiological concentrations of insulin (Swierczewski & Gaben-Cogneville, 1985). A third fraction, of higher density, free of endothelial cells (as checked by optical and electron microscopy) but again possessing lipoprotein lipase activity like the other two fractions, was refractory to conversion under identical culture conditions (Gaben-Cogneville *et al.*, 1984). The data led us to hypothesize the existence of different adipose-precursor compartments in normal adipose tissue. In the present paper we investigate this possibility by submitting density-separated cell subfractions to various effectors in primary culture and monitoring for adipose conversion.

We found that at least two types of normal adipose precursors do exist and are recruited to adipose differentiation under the influence of different sets of effectors.

EXPERIMENTAL

Cell preparation for culture

For each batch, inguinal fat pads from 40–50 rats (3 days old) were removed under sterile conditions,

pooled, weighed, thoroughly minced and digested with collagenase as described previously (Gaben-Cogneville *et al.*, 1983, 1984). After isolation from the whole tissue, the stromal cells were filtered through a 80 μ m-mesh nylon filter, washed with Hanks solution and fractionated on a preformed Percoll density gradient under conditions previously reported (Gaben-Cogneville *et al.*, 1983) with the following modifications. Instead of dividing the gradient into three cell fractions as reported previously, ten fractions (3 ml each) were collected from density 1.018–1.075 g/ml. The cells were centrifuged (600 *g*), washed with Hanks solution and plated separately (5×10^5 cells/dish). They were grown to confluence in Parker medium 199 supplemented with 20% (v/v) fetal-calf serum, penicillin (100 units/ml) and streptomycin (100 ng/ml). In later experiments, as described below, relevant fractions were pooled together and were plated as above. Once the monolayer reached confluence (day 4 after plating), 10% fetal-calf serum was substituted for 20% and the cells were treated with the following effectors either alone or in various combinations, as mentioned in the text: insulin (3 nM), 3-isobutyl-1-methylxanthine (IBMX; 0.5 mM), dexamethasone (0.25 μ M), dibutyl cyclic AMP (dbcAMP; 1 mM) and haemin (60 μ M). IBMX (or dbcAMP) and dexamethasone were removed 48 h after addition. The other drugs, if present, remained in the medium from confluence until the end of culture. Control cultures in standard medium (medium 199, 10% fetal-calf serum) were run in parallel. Culture media were changed every second day after plating.

Cell extracts for enzyme assays

Cell extracts were prepared as described by Gaben-Cogneville *et al.* (1983). Glycerol-3-phosphate dehydrogenase (G3PDH) was assayed by the method of Wise & Green (1979) and ATP citrate lyase by the procedure of Srere (1962). Results were expressed as nmol of substrate/min per mg of protein at 37 °C. Protein content was

Abbreviations used: IBMX, 3-isobutyl-1-methylxanthine; G3PDH, glycerol-3-phosphate dehydrogenase; dbcAMP, dibutyl cyclic AMP.

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Fig. 1. Effect of treatment with different agents on the expression of G3PDH in preadipose-cell fractions of increasing density

Ten cell fractions, F1–F10 (each 3 ml), were collected from the top of a Percoll gradient and plated separately. At confluence, insulin (3 nM) alone or with dexamethasone (0.25 μ M) and IBMX (0.5 mM) were added to the cultures. Control cultures containing basal medium (Parker 199 + 10% fetal-calf serum) were run in parallel. Dexamethasone and IBMX were removed after 48 h, whereas insulin was freshly added every 2 days until the end of culture. At day 11 after plating, cells were harvested and the activity of G3PDH was measured under different experimental conditions: C, control culture; I, insulin; IDX, insulin, dexamethasone and IBMX. Each point represents the mean value obtained from at least six independent cultures. Variability between mean values of independent experiments did not exceed 15%. Mean G3PDH activity in non-differentiated cells (exponential growth) was 36 ± 4 nmol/min per mg of protein. The gradient curve represents the densities of the fractions plotted against the height of the liquid column, expressed in mm (total height = 75 mm).

measured as described by Lowry *et al.* (1951). All enzyme assays were performed after pooling the cells from at least two culture dishes (about 1×10^6 – 2×10^6 cells) for each independent experiment. The statistical analyses were carried out by the Fisher *t* test.

Materials

Pig monocomponent insulin was obtained from Novo Research Institute; IBMX, dexamethasone, dihydroxyacetone phosphate, coenzymes and 3-amino-1,2,4-triazole were from Sigma. Parker medium 199 with Hanks salts and 20 mM-Hepes buffer was obtained from Flow Laboratories. Fetal-calf serum, collagenase and dbcAMP were from Boehringer. 4,6-Dioxoheptanoic

acid was a product from Calbiochem. Recrystallized haemin was a laboratory preparation.

RESULTS

Effector response distinguishes precursor cell compartments

In the first set of experiments all the ten subfractions of cells in culture were exposed at confluence either to insulin or to a combination of insulin (3 nM), dexamethasone (0.25 μ M) and IBMX (0.5 mM). These concentrations were chosen as optimal from a series of pilot experiments (E. Swierczewski, Y. Aron & J. Y. Pello, unpublished work) which gave maximal peak activities for the

Table 1. Effect of different agents on the expression of G3PDH and ATP citrate lyase in rat precursor cell fractions of higher density (HCF) in culture

Effectors were added when the cultures became confluent. Insulin (3 nM) was included in the medium at each re-feeding until the end of culture. Dexamethasone (0.25 μ M) and IBMX (0.5 mM) were included in the medium only for 48 h after confluence. At day 11 of culture, cell extracts were prepared and the activity of both enzymes was measured as described in the Experimental section. G3PDH and ATP citrate lyase activities in undifferentiated cells (growing phase) were respectively 36 ± 4 and 2.4 ± 0.3 nmol/min per mg. The data are means $\pm$ S.E.M. of duplicates from three to five independent cultures.

Effectors	G3PDH (nmol/ min per mg of protein)	ATP citrate lyase (nmol/ min per mg of protein)
None (control medium)	62 ± 9	6 ± 0.51
Insulin	393 ± 70	11 ± 1.8
Dexamethasone	180 ± 31	8 ± 1.7
IBMX	305 ± 71	7 ± 0.6
Insulin and dexamethasone	639 ± 54	24 ± 2.8
Insulin and IBMX	1523 ± 58	31 ± 6
Insulin, IBMX and dexamethasone	2024 ± 315	52 ± 7

lipogenic enzymes. Three observed facts were: (i) that the change in the activity of lipogenic enzymes did not occur until after IBMX had been removed from cultures (day 6 after plating); (ii) that the presence of IBMX and dexamethasone for 48 h after confluence was largely sufficient to promote cell differentiation; (iii) that the continuous presence of IBMX led to premature cell death.

Adipose conversion was monitored both by phase-contrast microscopic examination and by measuring the activity of G3PDH. The results obtained at day 11 after plating (peak activity) are shown in Fig. 1. In the cell fractions F1–F6 (density range 1.018–1.048 g/ml), insulin alone enhanced the enzyme activity markedly about 10-fold above the equivalent control and 30-fold of the values obtained with non-differentiated cells (values given in legend for Fig. 1). IBMX and dexamethasone added to medium containing insulin did not significantly affect G3PDH activity of this cell fraction, but a slight inhibition was observed consistently. However, in the cell fractions of higher density (F6–F9), IBMX, dexamethasone and insulin together were much more effective than insulin alone in promoting adipose differentiation. The activity of G3PDH was increased 10–20-fold above the values observed with control cells, whereas with insulin alone the enzyme activity was not significantly different from that of the corresponding control cultures. These results led us to delineate with precision two precursor cell compartments differing in their responsiveness to insulin. In the following experiments these ten fractions were pooled into two distinct cell populations, named respectively LCF (fractions including cells from F1–F6) and HCF (cells from F7–F9).

Adipose conversion of precursors in HCF compartment

As shown above, precursors from the HCF compartment are refractory to efficient adipose conversion without the simultaneous addition of insulin, IBMX and dexamethasone at confluence. The effect of these agents either individually or in various combinations was examined. Adipose differentiation was evaluated by determining the specific activity of two lipogenic enzymes,

G3PDH and ATP citrate lyase, at day 11 of culture (peak activity).

Results are shown in Table 1, and can be summarized as follows: presensitization with IBMX alone is sufficient for an efficient conversion of these cells by insulin (a significant difference was observed in specific activity of both enzymes between the values obtained with insulin alone on the one hand and with insulin + IBMX on the other; $P \leq 0.01$). Dexamethasone is not essential, since no difference was noted in the specific activity of G3PDH when dexamethasone was present or absent with the combination of insulin and IBMX. However, an enhancing effect of dexamethasone was observed on ATP citrate lyase activity in presence of insulin and IBMX ($P \leq 0.01$).

Mechanism of action of IBMX

To answer the question whether alteration in cyclic AMP concentration plays a role in the mechanism whereby IBMX in conjunction with insulin enhances and maintains differentiation signals, IBMX was replaced by dbcAMP, the latter being added to the cultures at confluence and maintained for 48 h as with IBMX. For dbcAMP a dose-dependent response of G3PDH activity was observed up to 1 mM in preliminary assays. As shown in Table 2, individual effects of insulin, dexamethasone and dbcAMP become additive when these three agents are added together, whereas the specific activity of G3PDH obtained with insulin, dexamethasone and IBMX in combination was 2-fold that with insulin, dexamethasone and dbcAMP ($P \leq 0.01$), strongly suggesting that at least part of the potentiating effect of IBMX is not through the action of cyclic AMP.

Differential haemin effect on LCF and HCF compartments

In order to distinguish further the precursor cells from the LCF and HCF compartments, haemin, another agent of adipose conversion, was tested (at 60 μ M) on these cells at confluence, as reported for 3T3 cell lines (Chen & London, 1981). The results of these experiments are shown in Table 3. Haemin alone, present throughout

Table 2. Effect of dbcAMP alone or in combination with other effectors on the expression of G3PDH in rat precursor cell fractions of higher density (HCF) in culture

dbcAMP (1 mM) was maintained for 48 h in culture instead of IBMX, when indicated. The other effectors, when present, were added as described in the legend of Table 1. At day 11 of culture, cell extracts were prepared and the activity of G3PDH was measured as described previously. The data are means $\pm$ S.E.M. of duplicates from at least four to five independent cultures.

Effectors	G3PDH activity (nmol/min per mg of protein)
None (control medium)	79 $\pm$ 17
Insulin	331 $\pm$ 38
dbcAMP	230 $\pm$ 31
Dexamethasone	216 $\pm$ 55
Insulin, dbcAMP and dexamethasone	823 $\pm$ 48
Insulin, IBMX and dexamethasone	1493 $\pm$ 110

all the culture period, did not enhance G3PDH activity significantly with either LCF or HCF cells. Haemin in combination with insulin was not more effective in enhancing adipose differentiation of LCF cells than was insulin alone. However, the addition of haemin to cultures of HCF cells containing insulin, dexamethasone and IBMX at confluence markedly potentiated the effect of these agents. The peak activity of G3PDH increased by nearly 100% at day 11 after plating ($P \leq 0.05$). Inhibitors of haem biosynthesis (Chen & London, 1981) were also tested under relevant conditions. Both compounds effectively inhibit adipose conversion, as shown by a 70% decrease in G3PDH activity in LCF cells,

whereas in HCF cells 3-aminotriazole was more effective (70% inhibition) than 4,6-dioxoheptanoic acid (40% decrease). Since these experiments were designed to discriminate further between the LCF and HCF precursors, no concentration-dependence of either haemin or inhibitors was studied. Consequently only a qualitative difference in the response of LCF and HCF cells to haemin is demonstrated.

DISCUSSION

The present results provide evidence for the existence of two types of rat adipocyte precursor cells, characterized by their differential response to several effectors which are known to induce adipose conversion in preadipose cell lines (Green & Kehinde, 1974, 1976; Rubin *et al.*, 1978; Chen & London, 1981). We could delineate precisely the limits of the density range encompassing each of these cell populations. One population (LCF), as was shown in our previous studies (Swierczewski & Gaben-Cogneville, 1985), was susceptible to the modulation effect of insulin for enhancing lipogenic expression, whereas the other (HCF), as demonstrated here, can undergo adipose conversion only when IBMX is present in addition to insulin. Such a difference between LCF and HCF cells incited us to study the features that distinguish these two compartments, and also the mechanism by which IBMX sensitizes the HCF cells to insulin.

Two major known pharmacological effects of IBMX are the inhibition of interaction of adenosine with its receptor and the inhibition of cyclic nucleotide phosphodiesterases (Elks & Manganiello, 1985). The first possibility is remote, since in our earlier experiments we showed that the addition of adenosine deaminase at different stages of culture, or frequent changes of culture medium in both the presence and the absence of various effectors, did not modify the results significantly (Gaben-Cogneville *et al.*, 1984). Accordingly, the endogenous

Table 3. Effect of treatment by haemin or by inhibitors of haem biosynthesis on the expression of G3PDH in rat precursor cell fractions of lower (LCF) and higher density (HCF) in culture

Haemin (60 μ M), AMT (3-amino-1,2,4-triazole) (5 mM) and/or Hepta (4,6-dioxoheptanoic acid) (1 mM) were added at confluence and maintained in the medium until the end of culture. The other effectors, when present, were added as described in the legends of Tables 1 and 2. At day 11 of culture, cell extracts were prepared and the activity of G3PDH was measured as described previously. The data are means $\pm$ S.E.M. of duplicates from at least four to five independent cultures.

Effectors	G3PDH activity (nmol/min per mg of protein)	
	LCF	HCF
None (control medium)	233 $\pm$ 82	88 $\pm$ 16
Haemin	342 $\pm$ 148	113 $\pm$ 24
Insulin	1194 $\pm$ 298	—
Haemin and insulin	1394 $\pm$ 216	—
Insulin, IBMX and dexamethasone	—	1160 $\pm$ 189
Haemin, insulin, IBMX and dexamethasone	—	1921 $\pm$ 363
AMT	162 $\pm$ 28	83 $\pm$ 15
AMT and insulin	317 $\pm$ 56	—
AMT, insulin, IBMX and dexamethasone	—	330 $\pm$ 133
Hepta	184 $\pm$ 82	90 $\pm$ 12
Hepta and insulin	459 $\pm$ 161	—
Hepta, insulin, IBMX and dexamethasone	—	651 $\pm$ 48

production of adenosine might not play a significant role in differentiating preadipocytes in culture. The second possibility was examined by using dbcAMP instead of IBMX. We observed that the effect of IBMX on cyclic AMP phosphodiesterase activity could not totally account for the results obtained. Hence it appears that the role of IBMX in growth and differentiation of HCF cells is more complex than expected. In this context, it is relevant to mention the mixed role of IBMX, which, depending on the cell stage, can induce either a loss of adipocyte phenotype or a mitogenic response in a preadipocyte 3T3 stem-cell line (Hoerl *et al.*, 1984). From these considerations we hypothesize that the normal precursor cells isolated on density criteria are probably arrested at two distinct states of the cell maturation program. IBMX would drive HCF cells to another step of this differentiation program, thus making them more responsive to other effectors.

Yet another difference between LCF and HCF cells is the potentiating effect of haemin on IBMX dexamethasone insulin-induced HCF-cell differentiation, but not on LCF cells. However, experiments depleting endogenous haem with inhibitors of haem biosynthesis demonstrated the importance of haem *in vivo* for adipose differentiation in both cell populations.

In conclusion, our data are compatible with a model that the cells in the HCF compartment could be the precursors of cells in the LCF compartment, the confirmation of which requires the study of other molecular markers. The HCF compartment might be the one that may be recruited *in vivo* under special and/or pathological conditions to enter the adipogenic program.

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