Acquisition of Increased Hormone Sensitivity during in Vitro Adipocyte Development*

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SUMMARY

Murine 3T3-L1 fibroblasts enter a differentiation program subsequent to prolonged maintenance in the confluent state and develop into adipocytes. The hormone sensitivity of adenylate cyclase and the physiological responsiveness to insulin were compared in 3T3-L1 preadipocytes and adipocytes. The following observations, comprising several distinct categories of hormone responsiveness, were made. (a) (2.5 μM) isoproterenol stimulated adenylate cyclase 15-fold in adipocyte homogenates, but only 2.5-fold in preadipocyte preparations, suggesting a considerable magnification in β-adrenergic responsiveness during development. (b) A totally new control element, adrenocorticotropic hormone responsiveness, was incorporated into the adenylate cyclase system of the adipocytes. (c) Sensitivity to prostaglandin E1 was observed in both preadipocytes and adipocytes, but no change in responsiveness could be detected in the differentiated cells. (d) Glucagon-sensitive adenylate cyclase could not be detected in either preadipocytes or adipocytes. (e) Both preadipocytes and adipocytes possess considerable insulin binding activity, but near physiological levels of insulin stimulate the conversion of glucose to CO2 and lipid only in the differentiated cells.

Green and Kehinde (1-3) established and cloned several sublines of mouse 3T3 fibroblasts (4) that are capable of differentiating into adipocytes in vitro. During the course of exponential growth and the approach to confluence, preadipose 3T3-L1 cells are indistinguishable from previously established 3T3 lines with respect to growth rate, sensitivity to density-dependent inhibition, cell morphology (1-4), and the specialized biosynthesis of collagen (5). When these cells achieve confluence and are subsequently maintained in the resting state by replenishing the medium every 2 days, a significant proportion spontaneously enters a differentiation program that parallels the development of mammalian adipose tissue (1, 3, 5). The cells accumulate lipid droplets that eventually ring the nucleus and ultimately coalesce to constitute the large triglyceride aggregates that occupy nearly all the intracellular volume. Concomitant with triglyceride accumulation, the preadipose cells retract their processes, pass through several stages of morphological differentiation that mirror mammalian adipocyte development, and finally emerge as enlarged and rounded adipocytes that remain attached to the substrate (3).

Several biochemical correlates of elevated triglyceride formation have been established. 3T3-L1 adipocytes incorporate palmitate, glucose (2), and acetate (2, 6) into triglycerides at rates that are 10- to 100-fold greater than those observed in preadipose 3T3-L1 cells and other 3T3 fibroblasts. Moreover, the 50-fold increment in de novo lipogenesis indicated by [14C]acetate incorporation into triglycerides appears to be accomplished by a coordinate 40- to 50-fold increase in the activities of ATP-citrate lyase, acetyl-CoA carboxylase, and fatty acid synthetase (6).

The spontaneous adipocyte conversion process occurs over a period of 2 to 4 weeks after the cells reach confluence. Treatment of confluent 3T3-L1 cells with either 20 to 30% serum (5), 0.18 μM insulin (2), 0.5 mM 1-methyl-3-isobutylxanthine (7), 0.3 μM prostaglandin F2α (7), 33 μM biotin (6), or combinations of insulin and any of the other agents (6, 7) significantly accelerates the expression of the fat cell phenotype. Conversely, adipocyte differentiation and development are blocked when the cells are grown in the presence of 5 μM bromodeoxyuridine (2, 5).

Since (a) mature mammalian fat cells contain adenylate cyclases that are activated by a number of lipolytic polypeptide hormones and β-adrenergic agents (see Ref. 8 for review) as well as insulin receptors that control lipogenesis (9) and (b) triglyceride synthesis in 3T3-L1 adipocytes is enhanced by insulin and inhibited by epinephrine (2), we commenced investigations on the development, regulation, and physiological coupling of hormone receptors and effector systems in 3T3-L1 cells. In this report, the hormone sensitivity of adenylate cyclase in intact and broken cell preparations and the physiological responsiveness to insulin are compared in confluent, preadipose cells, and differentiated 3T3-L1 adipocytes.

EXPERIMENTAL PROCEDURES

Materials

(-)-Isoproterenol bitartrate, ATP, cyclic AMP, creatine phosphate, creatine phosphokinase, chromatographic alumina (neutral), and Hepes were purchased from Sigma. Dowex AG 50W-X2 was obtained from Bio-Rad. [3H]PITP (12 to 20 Ci/mmol) was purchased from ICN. [U-14C]Glucose (1 μCi/μmol) and carrier-free Na14C in 0.1 N NaOH was obtained from New England Nuclear. 1-Methyl-3-isobutylxanthine was obtained from Aldrich.

Insulin and glucagon were generously supplied by Dr. M. Root, Eli Lilly, PGE, was a gift from Dr. J. Pike, Upjohn, and ACTH and its 1-24 fragment were donated by Organon.

Cell Culture

Dr. H. Green, Massachusetts Institute of Technology generously provided 3T3-L1 cells. Cells were grown in 100-mm culture dishes in Dulbecco's Modified Eagle's Medium (GIBCO) containing 10% fetal calf serum (GIBCO) and were supplemented with 2 mM glutamine.

1 The abbreviations used are: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PGE, prostaglandin E; ACTH, adrenocorticotropic hormone; FSH, follicle-stimulating hormone; LH, leutinizing hormone; TSH, thyroid-stimulating hormone.

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Hormone Sensitivity in Adipocyte Development

Cultures were fed with 8 ml of medium every 2 days and were maintained in an atmosphere of 10% CO₂, 90% air at 37°C. Adipocyte differentiation was accelerated according to the procedure of Russell and Ho (7). Confluent 3T3-L1 cells were exposed to 0.5 mM 1-methyl-3-isobutylxanthine and 0.2 μM insulin in standard medium for 48 h. Subsequently, the medium was removed, the cells were washed with isotonic phosphate-buffered saline, pH 7.4, and were then refed with medium containing 0.2 μM insulin. Cells were refed with medium containing insulin every 2 days. Differentiation was monitored by phase contrast microscopy. Cells containing multiple fat droplets were scored as differentiated. Accumulation of lipid in the droplets was verified by staining with oil red O (1). At least 80% of the cells in differentiating cultures could be identified as adipocytes 7 to 8 days after the termination of treatment with 1-methyl-3-isobutylxanthine. Unless otherwise indicated, experiments on adipocytes were carried out on cultures that were 85 to 95% differentiated.

Adipocytes were compared to confluent preadipose 3T3-L1 cells (preadipocytes) that had not been treated with 1-methyl-3-isobutylxanthine or insulin, but were fed every 2 days with standard medium.

Adenylate Cyclase Assay

The standard assay conditions are essentially those described by Salomon et al. (10). Assays were performed for 10 min at 37°C in 50 μl of a mixture containing 25 mM Tris/HCl buffer, pH 7.4, 5 mM MgCl₂, 1 mM cyclic AMP, 0.6 mM [α-32P]ATP (150 cpmpmol), 20 mM creatine phosphate, 2.5 μg of creatine phosphokinase (0.4 units), and 40 μg of cell homogenate protein. Cyclic AMP was isolated by chromatography on Dowex A550 and alumina and data were corrected for recovery of added cyclic AMP (10).

Determination of Cyclic AMP Content

Trichloroacetic acid-free cell extracts were acetylated (11) and the amount of cyclic AMP present was determined by radioimmunoassay (12). 3H-2'-O-succinyl cyclic AMP tyrosine methyl ester, anti-cyclic AMP antibody, and goat anti-rabbit γ-globulin were obtained from Collaborative Research.

Iodination of Insulin

Insulin (1 μg) was iodinated by the limiting chloramine-T method of Roth (13) using 0.5 mM of [125I]NaI. The iodinated product was purified by chromatography on cellulose (14) and had a specific activity of 586 μCi/μg.

Insulin Binding Assay

Assessment of insulin binding by intact cells was carried out according to Thomopoulos et al. (15) using 5 × 10⁶ cells/assay and 3H-insulin at a concentration of 0.33 nM. The absolute values obtained using the preceding assays varied as much as 100% from one preparation of cells to another. However, the degree of adenylate cyclase stimulation and alteration in insulin binding during differentiation were highly consistent.

Protein Determination

Protein was measured by the method of Lowry et al. (16) using bovine serum albumin as the standard.

Preparation of Cell Suspensions

To obtain single cell suspensions, monolayer cultures were incubated with trypsinics’ Spinner medium (17) containing 1 mM EDTA for 30 min at 37°C. Cells were then gently pipetted off the surface of the dish, washed four times with Krebs-Ringer phosphate buffer, pH 7.4, containing 40 mg of bovine serum albumin/ml and resuspended in this buffer.

Measurement of CO₂ Production—The ability of cells to oxidize glucose was determined by incubating 0.2 to 0.5 × 10⁶ cells in a final volume of 0.4 ml of the Krebs-Ringer phosphate buffer containing bovine serum albumin in triplicate at 37°C for 90 min with 0.2 mM [U-14C]glucose (1.2 × 10⁶ cpmpmol). The 14CO₂ was collected and counted as described by Rodbell (18). Under these conditions, 14CO₂ production was linearly related to cell number and time of incubation.

Conversion of Glucose to Lipid—The 14C incorporation into total cell lipid was measured on the same samples by the method of Rodbell (18). Although the differentiated cells responded to as little as 10 microunits of insulin/ml (70 pm), the comparisons to be presented here were made at the near saturating concentration of 360 microunits/ml (2.5 mM). All studies on insulin sensitivity were performed on cells which had either never been exposed to added insulin (preadipocytes) or were withdrawn from insulin for 24 h prior to assay (adipocytes).

RESULTS AND DISCUSSION

Hormone-sensitive Adenylate Cyclase—Possible changes in hormone responsiveness during adipose conversion were evaluated by comparing basal, fluoride-stimulated, and hormone-stimulated adenylate cyclase activities in undifferentiated (preadipocytes) and differentiated (adipocytes) cells. Preadipocyte and adipocyte homogenates exhibited approximately the same basal adenylate cyclase activity, but the specific activity of the adipocyte enzyme was twice that of the preadipocytes in the presence of 20 mM F⁻ (Table I). The same relationship is observed when the data are expressed on a per cell basis. Since the optimal, fluoride-stimulated adenylate cyclase activity is believed to be independent of hormone receptors and a function of the status of the active site (19), these results suggest that the catalytic capacity of adenylate cyclase is increased 2-fold in the differentiated cells.

In preadipocytes, 2.5 μM isoproterenol and 3 μM PGE, enhanced adenylate cyclase activity 2- to 2.5-fold, but a series of polypeptide hormones, including ACTH, glucagon (Table I), FSH, LH, and TSH (data not shown), were without effect. Assessment of hormone-sensitive adenylate cyclase activity in adipocyte homogenates showed that two dramatic changes occurred during differentiation (Table I). First, the adipocyte enzyme was stimulated 14-fold by 2.5 μM isoproterenol, yielding a specific activity 35% higher than that observed in the presence of 20 mM F⁻. Secondly, adipocyte cyclase had become highly responsive to ACTH, the active NH₂-terminal fragment ACTH 1-24 elevating adenylate cyclase activity 4- to 6-fold. PGE-stimulated adenylate cyclase activity rose approximately 2-fold in adipocytes, but this increase may simply reflect the doubling of total adenylate cyclase activity in the differentiated cells (Table I). In parallel with the preadipocytes, adipocyte cyclase was not affected by glucagon (Table I), FSH, LH, and TSH (data not shown).

Regulation of Cyclic AMP Levels in Intact Cells—The pattern of hormone sensitivity of adenylate cyclase observed in broken cell preparations (Table I) should correlate with cyclic AMP production by intact preadipocytes and adipocytes. The intracellular cyclic AMP content in differentiated and undifferentiated cells subsequent to treatment with the agents listed in Table I is presented in Table II. In general, the results are in agreement with the data on the hormone activation of adenylate cyclase in cell homogenates (Table I). Isoproterenol and PGE, raised the cyclic AMP concentration 2- to 3-fold in preadipocytes, while treatment with ACTH 1-24 and glucagon resulted in no change. In adipocytes, however, both 2.5 μM isoproterenol and 2 μM ACTH 1-24 increased the cyclic AMP content more than 25-fold (Table II). PGE, was also quite effective in adipocytes, causing a 5-fold elevation in cyclic AMP. Glucagon did not alter the cyclic AMP content of adipocytes.

Two inconsistencies are apparent when the results in Tables I and II are compared. Basal adenylate cyclase activity is somewhat higher in adipocyte homogenates, but the cyclic AMP phosphodiesterase activities in the two types of cells.

2 C. S. Rubin and O. M. Rosen, unpublished observations.

3 Assuming approximately constant and equal levels of cyclic AMP phosphodiesterase activities in the two types of cells.
AMP content in untreated intact adipocytes is 35% lower than in preadipocytes. This could arise by several mechanisms including an increased level of cyclic AMP phosphodiesterase in adipocytes or a differential responsiveness of adenylate cyclase to serum factors, but these hormone-dependent changes are clearly apparent only in the differentiated cells. When the specific binding of insulin was measured in intact cells (Table IV), confluent preadipocytes that were physiologically unresponsive (Table III) exhibited 53% the specific binding activity observed in cultures containing 25% morphologi-
production varied between 10 and 25 cpm/10^6 cells/min of incubation.

The data are presented as stimulation by insulin (360 microunits/ml) above basal (control) values which were taken as 1.0. Basal ^14C incorporation into lipid was 1.5 to 2.0 cpm/10^6 cells/min of incubation for preadipocytes and 4.0 to 5.0 cpm/10^6 cells/min of incubation for adipocytes.

| Stage of cell differentiation | ^14CO_2 (cpm/10^6 cells/min) | ^14C-lipid (cpm/10^6 cells/min) |
|-----------------------------|-----------------------------|--------------------------------|
| Undifferentiated^a          | 0.3                         | 0.2                             |
| Differentiated^b             | 3.3                         | 2.4                             |

^a Three independent experiments on confluent preadipocytes.
^b Five independent experiments; each culture was approximately 70 to 80% differentiated.

Effects of insulin on the conversion of glucose to CO_2 and lipid

Preparation of cell suspensions and methods for determining CO_2 and lipid synthesis are given under "Experimental Procedures." The basal ^4C incorporation into lipid was 1.5 to 2.0 cpm/10^6 cells/min of incubation for preadipocytes and 4.0 to 5.0 cpm/10^6 cells/min of incubation for adipocytes.

Basal ^14C incorporation into lipid was 1.5 to 2.0 cpm/10^6 cells/min of incubation for preadipocytes and 4.0 to 5.0 cpm/10^6 cells/min of incubation for adipocytes.

Differentiated^a 3.3 2.4-4.5 3.8 3.0-5.0
Undifferentiated^a 0.3 0.2-0.4 0.2 0.1-0.5

The 3T3-L1 cells should provide an excellent system for studying insulin receptor synthesis and the coupling of these receptors to physiological effectors. The initial data presented here suggest that the relationship between binding and insulin responsiveness in these cells may be quite complex.

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In several additional experiments, insulin binding activity consistently peaked when the cultures were 25 to 40% differentiated and slowly diminished thereafter; no obvious direct correlation between physiological responsiveness and binding activity was found. However, more detailed analyses of insulin binding may reveal differences not apparent from our measurements. The 3T3-L1 cells should provide an excellent system for studying insulin receptor synthesis and the coupling of these receptors to physiological effectors. The initial data presented here suggest that the relationship between binding and insulin responsiveness in these cells may be quite complex.
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