Development of Hormone Receptors and Hormonal Responsiveness in vitro

INSULIN RECEPTORS AND INSULIN SENSITIVITY IN THE PREADIPOCYTE AND ADIPOCYTE FORMS OF 3T3-L1 CELLS

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Green and Kehinde (Green, H., and Kehinde, O. (1974) Cell 1, 113-116; (1975) Cell 5, 19-27; (1976) Cell 7, 105-113) isolated and cloned murine fibroblasts (3T3-L1) which spontaneously generate foci of adipocytes when the cells are maintained at confluence for several weeks. When confluent cultures of 3T3-L1 cells were treated for 48 hr with a combination of 0.5 mM methylisobutyrate and 0.25 mM dexamethasone, rapid and uniform differentiation was elicited in the absence of added insulin. This new procedure permitted an analysis of insulin binding activity and cellular responsiveness to insulin during adipocyte conversion in vitro. Although preadipocytes possess cell surface receptors for insulin, metabolic sensitivity to physiological concentrations of insulin first appeared 2 to 3 days after the initiation of treatment with dexamethasone and methylisobutyrate. Three or four days following the onset of drug treatment, specific insulin binding activity also increased. Four to five days after drug treatment, the cells appeared to be adipocytes by morphological and biochemical criteria and the specific insulin binding was 6- to 10-fold greater than in undifferentiated control cells.

The insulin receptors in undifferentiated (preadipocyte) and differentiated (adipocyte) forms of 3T3-L1 cells were characterized by equilibrium binding and kinetic measurements. Curvilinear Scatchard plots of equilibrium binding data were resolved into high affinity-low capacity and low affinity-high capacity components. Preadipocytes possess approximately 7,000 high affinity binding sites/cell with a Kd value of 0.8 nM. During the course of differentiation, the number of high affinity receptor sites rises 35-fold to 250,000/cell with a concomitant increase in Kd to 4 nM. In kinetic studies, association rate constants were estimated to be 4.1 x 10^7 min^-1 M^-1 for preadipocytes and 0.7 x 10^7 min^-1 M^-1 for adipocytes; first order dissociation rate constants were 0.014 and 0.025 min^-1 for preadipocytes and adipocytes, respectively. Kd values calculated from kinetic studies were in agreement with the equilibrium binding results. Prolonged incubation of the physiologically responsive adipocytes with 0.2 uM insulin did not induce hormone-mediated receptor depletion ("down regulation").

We now report that treatment of confluent preadipocytes with 0.25 uM dexamethasone and 0.5 mM 1-methyl-3-isobutylxanthine for 48 hr, followed by subsequent refeeding with standard medium compresses the differentiation program into 7 days and consistently yields 85 to 90% adipocytes. Preadipocytes bind insulin but are relatively unresponsive to the acute effects of the hormone on hexose transport; 3 days after initiating treatment with dexamethasone and 1-methyl-3-isobutylxanthine, insulin stimulates deoxylucose uptake and the conversion of glucose to CO2 and lipid 3- to 5-fold, with little increase in insulin binding activity. During the subsequent 72 h, apparent insulin binding activity increases 6- to 10-fold and the magnitude and sensitivity of cellular responsiveness to insulin are further elevated to maximum values. Since the 3T3-L1 preadipocyte cell line presents a unique model system for studying the development of insulin receptors in vitro,
kinetic and equilibrium binding studies were used to characterize the insulin receptors in preadipocytes and adipocytes and to assess the possibility of hormone-mediated receptor depletion (11).

**EXPERIMENTAL PROCEDURES**

**Materials**—Dexamethasone, deoxy corticosterone, progesterone, dihydrotosterone, 2-deoxy-D-glucose, ATP, NAD, triolein, and [125I]-insulin were purchased from Sigma. 1-methyl-3-isobutylxanthine was from Aldrich. 4-(1-Butoxy-4-methoxybenzyl)-2-imidazolidione was a gift from Dr. H. Sheppard, Hoffmann La Roche. Cytocerinase and glycerol-3-phosphate dehydrogenase were acquired from Boehringer Mannheim. [1-14C]-Glucose (1.0 mCi/µmol), 2-deoxy-D-[6-3H]glucose (6.3 Ci/mmol), and carrier-free Na[125I] in 0.1 M NaOH were obtained from New England Nuclear. Bovine serum albumin was purchased from Armour Pharmaceutical. Insulin and glucagon were generously supplied by Dr. M. Rood, Eli Lilly and Co., and human anti-insulin receptor antisera was a gift from Drs. C. R. Kahn and J. Roth, Diabetes Section, NIAID, National Institutes of Health, ACTH was 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 µM glutamine. Cultures were fed with 8 ml of medium every 2 or 3 days during exponential growth and according to the schedule described below during differentiation. The cells were maintained in an atmosphere of 10% CO₂, 5% O₂, and 95% air at 37°C.

**Differentiation of 3T3-L1 Cells to Adipocytes**—Preadipocytes were grown to confluence on 100-mm tissue culture dishes (Falcon). The cells were then fed with 8 ml of fresh standard medium containing 0.5 mM 1-methyl-3-isobutylxanthine and 0.25 µM dexamethasone. After 48 h, the medium containing steroid and 1-methyl-3-isobutylxanthine was aspirated, the cells were given 8 ml of standard medium and allowed to differentiate for an additional 5 days.

**Iodination of Insulin**—Insulin (13 µg) was iodinated by the procedure of Roth (12) using 0.5 mCi of Na[125I] and near-stoichiometric quantities of chloramine-T. The iodinated product was purified by chromatography on cellulose (13) and had specific activities in the range of 160 to 220 pCi/pg.

**Insulin Binding Assay**—Cells suspensions were prepared by incubating monolayer cultures (100-mm dishes) with 4 ml of Joklik's Spinner medium (14) containing 1 nM [125I]-insulin for 20 min at 37°C. Cells were then gently pipetted off the surfaces of the dishes and were harvested by sedimentation at 100 x g for 5 min. Subsequently, cells were vigorously washed with Hepes (pH 7.4, containing 100 mM NaCl, 1.2 mM MgCl₂, 5 mM KCl, and 1.0 mM glucose) and suspended in 0.2 ml of 100 mM Hepes, 120 mM NaCl, 1.2 mM MgCl₂, 5 mM KCl, 10 mM glucose, 15 mM sodium acetate, 10% bovine serum albumin, pH 7.9, 0.4 ml/plate). The final cell concentration was adjusted to 2 x 10⁶/ml and 250-µl aliquots were incubated with 0.1 nM [125I]-insulin for 10 min at 37°C. The reaction was terminated by the sequential addition of 0.65 ml of 0.15% bovine serum albumin and 0.1 ml of 50% (w/v) trichloroacetic acid at 0°C. After 30 min at 0°C and subsequent washing, centrifugation at 1000 x g for 5 min in the supernatant and pellet fractions was determined in an Auto-Gamma spectrometer. Cleavage products derived from [125I]-insulin are acid-soluble while the iodinated hormone is completely precipitated under these conditions. Acid-soluble [125I]-labeled peptides were readily separated from native insulin by gel filtration on Sephadex G-50 in 6 M urea, 1 M acetic acid.

**Triglyceride Determinations**—Culture dishes (100 mm) of confluent cells (0.7 to 1.2 x 10⁶ cells) were exposed to standard medium containing 0.175 µM insulin plus varying concentrations of dexamethasone, progesterone, deoxy corticosterone, or dihydrotosterone, insulin, or combinations of these steroids. The cells were incubated for 48 h, the medium containing steroid and 1-methyl-3-isobutylxanthine was then changed to standard medium and differentiation was allowed to continue until 40 to 50% of the cells differentiated (as monitored by phase contrast microscopy, see Fig. 1) at the highest concentration of drug. The medium was then aspirated and the plates were washed twice with 5 ml of phosphate-buffered saline (0.15 M NaCl, 5 mM potassium chloride, 0.1 mM sodium azide, pH 7.3) and then treated with 0.2 mM deoxyglucose (25,000 cpm/nmol) for 4 min at 37°C in 0.2 ml of H₂O and the resulting aqueous methanol phase was removed. The chloroform layer was then evaporated under reduced pressure at 30°C. The triglyceride residue was resuspended in 0.1 ml of 0.05 M tetrathylammonium hydroxide (Eastman) and saponified by heating at 60°C for 30 min (16). Samples were then acidified with 0.1 ml of 0.1 N HCl and the free fatty acids were extracted with 1.5 ml of heptane (Fisher) and the hexane layer was discarded. Aliquots (3 to 40 µl) of the remaining aqueous phase were withdrawn and assayed for glycerone content in the coupled enzyme assay described by Wieland (17) in which glycerol is converted to glycerol 3-phosphate via glycerol kinase and the compound is subsequently oxidized to dihydroxyacetone phosphate by glycerol-3-phosphate dehydrogenase. Reduction of NAD in the second step was quantitated by measuring the relative fluorescence of NADH at a Hitachi-Perkin Elmer MFP-3 fluorometer using 340 nm as the exciting wavelength and 457 nm for emission.

**Measurement of Deoxyglucose Uptake**—Cell suspensions (1 to 2 x 10⁶ cells/ml) were incubated in Krebs-Ringer phosphate buffer, pH 7.4, containing 10 mg/ml bovine serum albumin in the presence or absence of 1 to 5 nM insulin for 10 min at 37°C. Subsequently, aliquots (10⁶ cells) were removed and incubated in the same medium containing 0.2 mM deoxyglucose (25,000 cpm/nmol) for 4 min at 23°C in a final volume of 0.2 ml. Reactions were terminated by the addition of 0.25 ml of cold Krebs-Ringer phosphate buffer, pH 7.4, containing 20 mM glucose. Aliquots (10 µl) were then withdrawn, combined with 500 µl of 6 M HCl, and the radioactivity was counted in a liquid scintillation spectrometer. Treatment of differentiated cells (Day 5) with 0.25 µM dexamethasone for 48 h did not affect either basal or insulin-stimulated uptake of deoxyglucose for 5 min and the presence of insulin was not determined.

**[14C]Glucose Metabolism**—Cell suspensions were incubated at a density of 1 to 3 x 10⁶/ml in a final volume of 0.4 ml of Krebs-Ringer phosphate buffer, pH 7.4, containing 10 mg/ml bovine serum albumin, 1.5 mM Bactracin, and 2.0 mM [1-14C]glucose (1.2 x 10⁶ cpm/ml) for 4 h. Incubations were performed with continuous shaking at 37°C for 60 to 90 min in the presence or absence of 10% bovine serum albumin. [14C]CO₂ and [14C]-lipid were assayed according to Rodbell (18) as previously described (8). Under the conditions of these experiments, the addition of sufficient Bactracin (1.5 µg) to maintain 95 to 97% of the added insulin in acid-precipitable form, had no adverse effect on either basal- or insulin-stimulated [14C]glucose metabolism.
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Although differentiated cells responded to as little as 0.025 ng/ml of insulin, the studies depicted in Fig. 4 utilized saturating insulin concentrations.

RESULTS

Differentiation of 3T3-L1 Cells in Absence of Insulin—Since the plasma membrane content of insulin receptors is subject to insulin-mediated receptor depletion (i.e., “down regulation”) in some cells (19) and because chronic exposure to insulin may alter the physiologic state of cells, conditions were sought which would permit rapid conversion of preadipocytes to adipocytes in the absence of insulin. Among the compounds tested mixtures of glucocorticoids and the cyclic nucleotide phosphodiesterase inhibitor, 1-methyl-3-isobutylxanthine, proved to be highly effective in promoting differentiation. When confluent monolayers of 3T3-L1 cells were treated with 0.5 mM methylisobutylxanthine and 0.25 μM dexamethasone in standard culture medium for 48 h and then transferred to medium free of drugs for an additional 96 h, 80 to 90% of the cells clearly exhibited the differentiated phenotype (Fig. 1). During the 48-h exposure to methylisobutylxanthine and dexamethasone, the cells assume a spindle shape and significantly decrease their surface area, thereby leaving a major fraction of the surface of the culture dish free of cells (Fig. 1, d1 and d2). After the cells are returned to standard medium they regain their fibroblastic morphology and begin to accumulate small lipid droplets (Fig. 1, d3). One day later, many cells display polygonal shapes and triglyceride droplets ring the nucleus (Fig. 1, d4). These droplets then coalesce and ultimately occupy a major fraction of the internal cell volume as the cells become spherical in shape (Fig. 1, d5 to d7). When confluent cells were exposed to either 0.5 mM methylisobutylxanthine (9) or 0.25 μM dexamethasone in standard medium for 48 h and then maintained an additional 96 h in standard medium without drug, approximately 90 to 95% of the cells differentiated. Thus, either agent alone appears to enhance adipocyte differentiation and the effects seem to be at least partially additive when a mixture of both compounds is employed. Untreated confluent cells exhibited no differentiation during this period of time. During the course of differentiation cell number increases 2- to 3-fold, but the protein to DNA ratio remains constant.

The methylisobutylxanthine-dexamethasone differentiation procedure has several advantages: adipocyte differentiation is controlled and consistent, the time course of differentiation is compressed into a 6- to 7-day period, and the elimination of insulin from the medium during differentiation permits detailed analyses of the development of insulin binding and degradative capacities and cellular responsiveness at physiologic concentrations of hormone. Differentiation also occurs quite uniformly in the monolayer rather than in a focal distribution as originally described (1). In addition to the morphological criteria of differentiation (Fig. 1), cells differentiated with dexamethasone and methylisobutylxanthine exhibited the same dramatic increases in the enzymes of fat biosynthesis as those reported for cells differentiated spontaneously or in the presence of high concentrations of insulin.

Although the mechanism(s) by which methylisobutylxanthine and dexamethasone facilitate differentiation is unknown, the foregoing results exclude an absolute requirement for supraphysiological levels of insulin in the medium during adipocyte conversion. A comparable level of differentiation was obtained when 0.25 μM dexamethasone was replaced by higher concentrations of deoxycorticosterone or progesterone (see below) or when a non-purine cyclic nucleotide phosphodiesterase inhibitor, 4-(3-butoxy-4-methoxybenzyl)-2-imidazolidinone (0.2 mM (20)), was substituted for methylisobutylxanthine. Since dexamethasone and other glucocorticoids inhibit prostaglandin E₂ synthesis in a number of normal and transformed cell lines of fibroblasts by limiting the availability of unsaturated fatty acid precursors (21-23) and Williams and Polakis (10) have recently shown that indomethacin, a potent inhibitor of prostaglandin endoperoxide synthetase (24), potentiates insulin-mediated differentiation in 3T3-L1 preadipocytes it is possible that dexamethasone-mediated inhibition of the biosynthesis of prostaglandin E₂ or its precursors or products may occur during the 48-h dexamethasone-methylisobutylxanthine treatment. This inhibition might play a role in triggering the activation of the differentiation program. Williams and Polakis (10) also found that cyclic AMP stimulated the onset of differentiation, thus suggesting a further correlation that is consonant with our observation of the synergistic effects of methylisobutylxanthine and dexamethasone in facilitating adipocyte conversion.

Effect of Steroids on Adipocyte Differentiation—Dose-response curves were obtained for several steroids representative of glucocorticoids (dexamethasone), mineralocorticoids (deoxycorticosterone), and sex steroids (progesterone, dihydrotestosterone) in order to obtain a preliminary notion of the relative potencies of these subclasses of steroids in eliciting differentiation of 3T3-L1 cells. In these experiments confluent cells were treated with steroid for 48 h and then transferred to standard medium until 40 to 50% differentiation was achieved at the highest concentration of drug. Differentiation was monitored by phase microscopy and the direct determination of triglyceride content (Fig. 2). Dexamethasone was the most potent inducer of differentiation, having a half-maximal effect at 65 nM and an optimal enhancement of fat cell development at 0.85 μM. Deoxycorticosterone and progesterone also promoted expression of adipocyte differentiation with half-maximal effects at 1.3 and 6.4 μM, respectively. Treatment with dihydrotestosterone (7 μM) or insulin (0.175 μM) alone failed to induce adipocyte differentiation within the time course of these experiments.

The dose-response data (Fig. 2 and text) represent measurements on the rate of appearance of differentiated cells rather than end points of maximal adipocyte development. Doses of dexamethasone as low as 10 nM cause the emergence of 10 to 20% differentiated cells when the cells are maintained for extended periods (>7 days). Furthermore, fully stimulated cells will ultimately accumulate concentrations of triglyceride that exceed the maximum level shown in Fig. 2 by a factor of 10 when the cells are maintained for 7 to 10 days and provided with fresh medium on Days 2 and 6.

Development of Insulin Binding Activity and Insulin Responsiveness—3T3-L1 preadipocytes provide a system for examining the degree of coordination and possible interdependence between insulin binding and metabolic control during fat cell maturation. A preliminary assessment of insulin degrading capacity during development indicated that insulin cleaving activity in cell suspensions increases 2- to 3-fold after day 2. This activity reaches a plateau when nearly 40% of the added insulin (0.1 nM) can be destroyed in 10 min by 10⁶ cells on Days 6 and 7. For this reason insulin inactivation must be effectively blocked in differentiating adipocytes in order to obtain meaningful data on the development of insulin binding activity. Addition of 1.5 mM Bacitracin ³ completely blocks insulin breakdown under the conditions of the binding assay at 18°C and limits degradability to 1 to 3% when tested at 37°C.

³ The concentration is calculated using an average molecular weight of 1400 for Bacitracin.

² Some insulin is likely to be supplied by the fetal calf serum in the culture medium. Radioimmunoassays for insulin in the medium suggest concentrations <10⁻¹⁰ M are normally present.
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FIG. 1. Photomicrographs of differentiating 3T3-L1 cells. Confluent monolayers (Day 0 (d0)) were treated with dexamethasone and methylisobutylxanthine (dl, d2) as described under “Experimental Procedures.” Typical areas were photographed with phase optics at a total magnification of × 160. Following 48 h of treatment, cells were refed with fresh medium lacking drugs and differentiation was allowed to proceed without any further manipulation (d3 to d7).

FIG. 2. Effects of dexamethasone on triglyceride accumulation in differentiating adipocytes. Confluent 3T3-L1 cells (100-mm dishes) were exposed to the indicated dexamethasone concentrations in the presence of 0.175 μM insulin in 8 ml of medium for 48 h. Subsequently, cells were maintained in standard medium until 40 to 50% of the preadipocytes differentiated at the highest concentration of drug (total time = 96 h). Cells were then washed, harvested, and extracted as described under “Experimental Procedures.” Following saponification, the triglyceride content of the extract was determined in a coupled enzymic assay as indicated under “Experimental Procedures.” Methylisobutylxanthine was eliminated from the differentiation procedure because it independently stimulates some adipocyte differentiation, thus obscuring the effects of steroid alone. Insulin was included during the first 48 h because it potentiates the effects of the steroids but induces no differentiation when the same procedure is carried out in the absence of steroid. Each point is the average of triplicate determinations that did not differ from each other by greater than 5%. The data reported in this figure are within 20% of those obtained in two other independent experiments of this kind.

FIG. 3. Development of insulin binding activity in differentiating 3T3-L1 adipocytes. Control cells received fresh medium on Days 0 and 2 while differentiating cells received fresh medium containing 0.5 mM methylisobutylxanthine (MIX) and 0.25 μM dexamethasone (DEX) on Day 0 and fresh medium alone on Day 2 (see “Experimental Procedures” for additional details). The ^125I-insulin concentration was 0.022 nM. Nonspecific binding was equal to 7 to 10% of specific insulin binding and was subtracted from the total amount bound. Data are presented as per cent specific binding per 10^6 cells, where per cent specific binding is calculated as (total ^125I-insulin bound - nonspecific binding) / total ^125I-insulin present in the assay × 100. Each point is the average of triplicate determinations that differed from each other by less than 10%. The pattern presented is typical of many experiments. From one experiment to another, insulin binding in the adipocytes may vary as much as 100% during Days 1 to 3; following Day 3, the interexperimental variability is reduced to 20 to 30%.

for 30 min. In studies on undifferentiated preadipocytes, which exhibit minimal insulin inactivating activity, addition of 1.5 mM Bacitracin to the binding assay had no effect on the affinity or number of binding sites for insulin.

The specific binding of insulin in developing adipocytes was similar to that determined in parallel, control cultures for 3 days after the initiation of methylisobutylxanthine-dexamethasone treatment (Fig. 3). The increase in insulin binding activity in untreated, control 3T3-L1 preadipocytes that is detected on Day 2 is compatible with an earlier observation (25) that depletion of nutrients in the medium of confluent fibroblasts results in an augmentation in the number of insulin receptors. After the addition of fresh medium on Day 2, insulin binding in undifferentiated 3T3-L1 cells declines to a stable level of 4% bound/10^6 cells (Fig. 3).

In contrast, specific insulin binding activity rises precipitously in developing adipocytes after Day 3. The initial appearance of increased binding capacity (Figs. 3 and 4) lags behind the development of hormone responsiveness. However, the enhanced insulin binding activity seen in developing adipocytes on Days 4 and 5 appears to be linked to the expression of heightened cellular responsiveness to hormone. In the experiment shown in Fig. 4, the ability of insulin to stimulate CO₂ production and lipid synthesis from exogenously added glucose was evident following the 2-day exposure to dexamethasone and methylisobutylxanthine (Day 2) at a time when insulin binding had not yet changed. Extension of these experiments to Days 5 and 6 indicated that the degree of stimulation by insulin was maintained at the level seen on Day 4. Studies using cellular uptake of 2-deoxy D-[14C]glucose as a parameter of insulin sensitivity gave essentially the same...
results as those employing [14C]glucose. Uptake of 2-deoxyglucose was enhanced 2- to 3-fold by insulin during Days 2 to 3 of the differentiation program; by Days 6 and 7 it could be stimulated as much as 9-fold. Undifferentiated confluent monolayers of the 3T3-L1 cells exhibited only 20 to 30% increases in deoxyglucose uptake in response to insulin.

The development of increased insulin binding activity appears to correlate specifically with adipocyte differentiation since the treatment of nondifferentiating 3T3-C cells with dexamethasone and 1-methyl-3-isobutylxanthine failed to affect insulin receptor activity (Table I).

**Characterization of the Insulin Receptor in Undifferentiated and Differentiated 3T3-L1 Cells: Attainment of Equilibrium and Specificity** — The kinetics of insulin binding by undifferentiated (preadipocytes) and differentiated (adipocytes) 3T3-L1 cells at 18°C are shown in Fig. 5. For both types of cells, receptors are filled slowly under the assay conditions employed. The times for half-maximal binding were approximately 2.5 and 12 min for preadipocytes and adipocytes, respectively. All samples attained equilibrium by 65 min and all equilibrium binding experiments were carried out for 100 min to assure steady state conditions.

The specificity of hormone binding was demonstrated by the competitive displacement of [125I]insulin with native insulin, desalanine-desasparagine insulin, and fish insulin (data not shown). The displacement profile obtained with native insulin was similar to curves reported for insulin receptors in a variety of tissues and species (11, 26-30). Competition by desalanine-desasparagine insulin and tuna fish insulin was in all equilibrium binding experiments were carried out for 100 min at 18°C in Hepes binding buffer adjusted to pH 6.0. After bound insulin was released by incubating adipocytes for 60 min at 18°C as described under “Experimental Procedures.” Cells were assayed for insulin binding activity using 0.02 and 0.2 nM [125I]insulin 4 days after the removal of dexamethasone and 1-methyl-3-isobutylxanthine.

**TABLE I**

<table>
<thead>
<tr>
<th>Cells</th>
<th>Treatment</th>
<th>0.02 nM insulin</th>
<th>0.2 nM insulin</th>
</tr>
</thead>
<tbody>
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<td>2.1</td>
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<td>Control</td>
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<td>2.0</td>
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<td>Dexamethasone, 1-methyl-3-isobutylxanthine</td>
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<td>2.4</td>
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**Fig. 4.** Effects of insulin on the conversion of glucose to CO2 and lipid during the early phase of differentiation. Cells were differentiated and assays performed as described under “Experimental Procedures.”

**Fig. 5.** Kinetics of association of insulin with 3T3-L1 preadipocytes and adipocytes. Preadipocytes (5.5 × 10⁶/ml) and adipocytes (4 × 10⁵/ml) were incubated in the presence of 0.53 nM [125I]insulin. Samples (0.15 ml) were removed at the indicated times and binding was determined by the centrifugation assay. Duplicate tubes containing the same concentration of [125I]insulin and 5 μM nonradioactive insulin were assayed in parallel to determine the extent of nonspecific binding. Nonspecific binding was less than 9% of total binding at all points. The data presented have been corrected for nonspecific binding. The specific activities of [125I]insulin were 1332 cpm/fmol in the preadipocyte experiment and 940 cpm/fmol in the adipocyte study.

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A. 14CO₂ (— — —) and 14C-lipid (— — —) formation in response to 0.8 munits insulin was assayed daily for the first 4 days of differentiation. Basal 14CO₂ production was 450 to 1350 cpm/10⁶ cells/90-min incubation and basal 14C-lipid formation varied between 700 and 450 cpm/10⁶ cells/90-min incubation. Fold stimulation by insulin was calculated from points representing the average of triplicate incubations which differed from each other by less than 10%. Although the pattern depicted in this figure is typical, the data obtained in independent experiments using different batches of cells has varied as much as 50 to 75%. B, specific insulin binding of the differentiating cells measured at 0.4 nM [125I]insulin. Binding is expressed as per cent specific binding per 10⁶ cells.
analyzed subsequent to a 100-min incubation. Similar results were obtained when these experiments were performed with preadipocytes.

Effect of pH on Insulin Binding—Differentiated and undifferentiated 3T3-L1 cells bind little insulin below pH 7.0. Binding activity increases sharply above pH 7.0, attaining a maximal value at pH 7.9. A near-optimal level of binding is maintained over the pH range 7.6 to 8.2, but receptor occupancy declines at more alkaline pH values. Similar pH profiles for insulin binding have been described for lymphocytes, liver plasma membranes, and mature rat adipocytes (34). All binding studies described in this communication were performed at pH 7.9 unless otherwise noted.

Equilibrium Binding Studies—A comparison of the insulin receptors in undifferentiated and differentiated 3T3-L1 cells was made by determining specific insulin binding at equilibrium over the range 0.01 to 100 nM insulin. Typical equilibrium data, plotted according to the method of Scatchard (35), are presented in Fig. 6. Both preadipocytes and adipocytes yield curvilinear plots suggestive of heterogeneity of sites (27, 36) or negative cooperativity (37, 38). Since the most marked curvature is observed at insulin concentrations (≥10 nM) that exceed the physiological range of response of these cells (0.01 to 5 nM) and because Pollet et al. (36) have recently reported that the enhancement of 125I-insulin dissociation by native insulin and 131I-insulin in lymphocytes is not correlated with binding site occupancy and is inconsistent with negative cooperativity, the data have been analyzed by a graphical resolution (39) of the Scatchard plot into independent high and low affinity components (Fig. 6). We have focused operationally on the high affinity, low capacity sites since they should participate in the mediation of the physiological actions of insulin (40), while the low affinity sites may reflect the association of insulin with receptors for other hormones and growth factors (e.g. multiplication stimulating activity, somatomedin A, see Rechler et al. (30). The numbers of binding sites were calculated from the intercept on the abscissa and the cell density in the assays, while the equilibrium dissociation constant (Kd) was determined from the slope as indicated in the Scatchard equation (35).

Preadipocytes (Fig. 6B) exhibit approximately 7,000 high affinity sites/cell with a dissociation constant (Kd) of 0.8 nM. During differentiation, the number of insulin binding sites increases dramatically to a level of approximately 250,000 sites/cell, while the apparent Kd also rises to 4 nM (Fig. 6A). The composite result is a 7- to 10-fold increase in apparent insulin binding capacity over the range 0.01 to 1 nm insulin.

In another typical set of equilibrium experiments, the data were plotted by the method suggested by Gammeltoft and Gliemann (26). Fig. 7 shows that the experimental points provide a reasonable fit to the equation.

\[
\text{bound insulin} = \frac{\text{free insulin}}{K_d + \text{total insulin concentration}}
\]

Data obtained in studies on the differentiated adipocytes are consistent with 250,000 sites/cell and a Kd of 4 nM (Fig. 7A), while results in preadipocytes indicate 6,500 sites/cell and a Kd of 0.9 nM (Fig. 7B).

Kinetic Studies—The association rate constant kₐ was estimated from the initial velocities of insulin binding (see Fig. 5 for typical data) and the equation \( v = k_a \) (insulin concentration) (receptor concentration). Values obtained were 4.1 \( \times 10^7 \) min⁻¹ m⁻¹ for preadipocytes (average of four experiments) and 0.7 \( \times 10^7 \) min⁻¹ for adipocytes (average of four experiments).

The first order dissociation rate constant, kₜ, was determined by following the dissociation of cell-bound 125I-insulin (Fig. 8) subsequent to equilibrating the cells with 0.5 nM 131I-insulin at 18°C. No cell-bound insulin was released during the brief exposure of the cells to Heps binding buffer at 0°C. The average kₜ values (three experiments) determined for preadipocytes and adipocytes were 0.014 and 0.025 min⁻¹, respectively. Calculations of the equilibrium Kd from the kinetic data yield values of 3.6 nM for adipocytes and 0.34 nM for preadipocytes. Thus, the kinetic and equilibrium binding results are in agreement and are also consistent with a single set of noninteracting, high affinity insulin receptor sites.

Effect of Insulin on the Dissociation of Cell-bound 125I-insulin—As in a number of other systems (36–38), the rate of dissociation of cell-bound 125I-insulin is significantly enhanced by the addition of a high concentration of native insulin (Fig. 9). This effect is especially large when the percentage of receptors occupied at the initiation of dissociation is low. In the experiments depicted in Fig. 9, approximately 3 to 4% of the high affinity receptors were occupied at zero times (cells equilibrated with 0.03 nM 125I-insulin). Under these conditions, a constant rate of dissociation is observed for approximately 10 min (Fig. 9, A and B), but the initial rate is comparable to the rate observed when a higher occupancy is attained at zero time (Fig. 8, cells equilibrated with 0.5 nM insulin). In the absence of added ligand, adipocytes dissociate 125I-insulin more rapidly (Fig. 9B) than preadipocytes (Fig. 9A). However, in the presence of 200 nM insulin, rates of dissociation in differentiated and undifferentiated cells are nearly equal. When the ratio of 131I-insulin bound (dilution alone) to 125I-insulin bound (plus 200 nM insulin) is determined as a function of time (Fig. 9C), it can be seen that the ratio plateaus at 1.75 for differentiated cells and reaches approximately 3.3 for preadipocytes. Enhanced rates of dissociation have been attributed to negatively cooperative interactions induced by the occupancy of a large proportion of available sites by unlabeled

\[ k_a \]

4 Kinetic studies were performed using 0.5 and 0.03 nM insulin. Under these conditions, contributions of the low affinity sites to overall insulin binding were sufficiently low to be neglected in estimating the rate constants.

5 In the determination of kₜ, values the standard error of the mean was ±23% of the mean value; the standard error in kₕ measurement was ≦12% of the average value.
Equilibrium ratios of bound to free insulin were determined for differentiated (8 × 10⁶ cells/ml) and undifferentiated (2.0 × 10⁶ cells/ml) 3T3-L1 cells as described under "Experimental Procedures" and in Fig. 6. The data are presented according to the format suggested by Gammeltoft and Gliemann (26). The data points were determined experimentally while the curves were calculated as indicated in the text. Parameters used for adipocytes (A) were \( K_d = 4 \text{nM} \), number of sites = 250,000/cell; for preadipocytes (B) \( K_d = 0.9 \text{nM} \), number of sites = 6,500/cell.

Studies on Effect of Prolonged Insulin Treatment on Insulin Binding Capacity—Adipocytes and preadipocytes were incubated with 200 nM insulin for 5, 16, and 48 h and were then examined for insulin binding activity (Table II). Both preadipocytes and adipocytes exhibited the same binding capacity in control and treated cells, suggesting that differentiated cells and preadipocytes are not susceptible to hormone-mediated receptor depletion.

DISCUSSION

Equilibrium and kinetic binding studies on the insulin receptor in 3T3-L1 preadipocytes and adipocytes indicate a 35-fold increase in receptor number during the course of adipocyte development. Simultaneously, the \( K_d \) also increased from approximately 0.8 to 4 nM, yielding a net 6- to 10-fold increase in insulin binding capacity at physiologically relevant concentrations of hormone (0.01 to 5 nM). Equilibrium data plotted according to Scatchard (35) produced curvilinear plots (Fig. 6) which were resolved into high affinity and low affinity components by graphical analysis (39). Since insulin stimulates deoxyglucose uptake and the conversion of glucose to...
Insulin Receptors in Preadipocytes and Adipocytes

CO₂ and lipid over the range 0.01 to 5 nM and the deviation of the Scatchard plots from linearity is predominantly observed above 10 nM insulin, it appears that the high affinity-low capacity sites play the more important role in mediating the acute, metabolic effects of hormone action. Measurement of association and dissociation rate constants provided \(K_d\) values in agreement with equilibrium measurements. Given the complexities involved in determining the binding of insulin at the surface of intact cells, the consistency of the kinetic and equilibrium measurements and the near linearity of the Scatchard plots in the physiological range, we tentatively conclude that single sets of noninteracting binding sites with the parameters summarized in Table III, provide a reasonably accurate description of physiologic insulin receptors in both undifferentiated and differentiated 3T3-L1 cells. Cuatrecasas (41), Gammeltoft and Gliemann (27), and Pollet et al. (36) have suggested that the insulin receptors in mature mammalian fat cells and lymphocytes may be adequately described as a single set of noninteracting sites, while DeMeyts and colleagues (37, 38) have proposed a scheme based on negatively cooperative interactions.

In addition to the increase in insulin receptors during differentiation, several other interesting phenomena have been observed. The decrease in receptor affinity during adipocyte development may provide a fine-tuning control by shifting the \(K_d\) to a value that may permit maximum responsiveness to insulin in the physiological range. It will be important to determine whether the change in affinity reflects a new type of receptor characteristic of fat cells or whether this stage is a transient phenomenon with the \(K_d\) value being influenced nonspecifically by newly active biosynthetic programs involved in the rearrangement of the plasma membrane for expression of the adipocyte phenotype. Similarly, the value of 250,000 sites/adipocyte is 5- to 8-fold greater than that reported for mature rat fat cells (26). Further studies in 3T3-L1 adipocytes aged in culture may permit the discernment of additional alterations in the \(K_d\) and number of insulin receptors.

Native insulin stimulated \(^{125}\)I-insulin dissociation from receptors in both preadipocytes and adipocytes (Fig. 9), but the effect is greater in undifferentiated cells. Although the mechanism underlying this action is unknown, the quantitatively smaller effect in insulin-responsive cells results in a near equalization of dissociation rates in differentiated and undifferentiated cells in the presence of 200 nM insulin.

Studies (Table II) suggest that neither preadipocytes nor adipocytes are susceptible to down regulation by 2 × 10⁻⁷ M insulin. Furthermore, in adipocytes, the basal level of hexose transport is highly elevated, while the level of insulin-mediated enhancement of uptake is severely diminished following exposure to such concentrations of insulin (43).

In a recent report, Reed et al. (42) described an increase in insulin binding capacity from 35,000 sites/cell in 3T3-L1 preadipocytes to 170,000 sites/cell in adipocytes. No change in receptor affinity was noted in these studies. At present it is not possible to directly compare these findings to the observations delineated here because Reed and colleagues employed markedly different conditions for adipocyte differentiation and insulin binding assays. These differences include: assaying insulin activity on cell monolayers where nonspecific binding may reach 29% of the total binding and accessibility to receptors may be limited; the utilization of different buffers (Krebs-Ringer phosphate), pH (7.4), temperature (4°C), and time (6 h) conditions; differentiating the cells in the presence of 2 × 10⁻⁷ M insulin. Furthermore, in adipocytes, the basal level of hexose transport is highly elevated, while the level of insulin-mediated enhancement of uptake is severely diminished following exposure to such concentrations of insulin (43).

In conclusion, 3T3-L1 cells offer a unique model system for assessing both the development and physiological perturbation of insulin receptors. Using a new method to induce rapid adipocyte conversion, the absence of insulin in the studies presented in this communication give the following indications. 1) Preadipocytes contain 7,000 high affinity binding sites/cell with a \(K_d\) of 0.8 nM. 2) Concurrent with differentiation adipocytes become insulin-sensitive and raise their insulin receptor concentration to 250,000/cell; a concomitant increase in \(K_d\) to 4 nM results in a 6- to 10-fold increase in hormone binding activity in the physiological range for insulin. 3) Kinetic studies confirm and support the equilibrium binding results, while parallel physiological studies (43) indicate that

<table>
<thead>
<tr>
<th>Cells</th>
<th>Time of treatment with 0.2 μM insulin</th>
<th>% 125I-insulin specifically bound/10⁶ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preadipocyte</td>
<td>Control (-insulin)</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>10 min</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td>5 h</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>16 h</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>48 h</td>
<td>3.0</td>
</tr>
<tr>
<td>Adipocytes</td>
<td>Control (-insulin)</td>
<td>14.4</td>
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<td>16 h</td>
<td>14.9</td>
</tr>
<tr>
<td></td>
<td>48 h</td>
<td>12.9</td>
</tr>
</tbody>
</table>

| Parameters of insulin binding in preadipocytes and adipocytes |
|---------------------|---------------------|-------------------------------|------------------|-----------------------|
| \(K_d\) (equilibrium) | Sites/cell | \(k_+\) \(\text{min}^{-1} \text{M}^{-1}\) | \(k_d\) \(\text{min}^{-1}\) | \(K_d/(k_d/k_+))\) |
| Preadipocytes       | 0.8 nM | ~7,000 | 4.1 × 10⁻⁴ | 0.014 min⁻¹ | 0.34 nM |
| Adipocytes          | 4.0 nM | ~250,000 | 0.7 × 10⁻⁷ | 0.025 min⁻¹ | 3.6 nM |
only the insulin receptors of differentiated cells are effectively coupled to the hexose transport system and glucose-metabolizing enzyme systems. 4) Prolonged treatment of insulin-sensitive adipocytes with 0.2 μμ insulin failed to induce any alteration in insulin binding capacity.

REFERENCES

Development of hormone receptors and hormonal responsiveness in vitro. Insulin receptors and insulin sensitivity in the preadipocyte and adipocyte forms of 3T3-L1 cells.

C S Rubin, A Hirsch, C Fung and O M Rosen


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