Insulin binding in differentiating rat preadipocytes in culture

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Abstract Binding, degradation, and antilipolytic effect of insulin were studied during the differentiation of preadipocytes into unilocular adipocytes. The precursor cells were isolated from the stromal-vascular fraction of adult rat epididymal fat pads and were cultured according to methods previously described. Under appropriate conditions the cells attained full morphological maturation after 6 days. A gradual increase in insulin binding was found concomitant with the morphological development of the preadipocytes into adipocytes. This increase was due to an enhanced number of binding sites whether expressed per cell or per unit cell surface area. The presence of a high insulin concentration (1.67 μg/ml or 278 nM) in the culture medium did not prevent this effect. The receptor density, expressed per unit surface area, was higher in the newly developed univacuolar cells than in mature fat cells from the same rat. The increased receptor density was also reflected by a leftward shift in the dose-response curve for the antilipolytic effect of insulin. In parallel with the increased binding, insulin degradation also increased. The lipolytic response to catecholamine also showed a gradual increase with development. When expressed per unit surface area, newly formed cells exhibited a considerably greater response (~3.4 times) than mature cells from the same animals. The maximal antilipolytic effect of insulin in new cells was of the same order as in old cells when the data were expressed per unit cell surface area. Thus, the data show that developing adipocyte precursors gain membrane properties similar to those of mature fat cells. This cell system may serve as a useful model for studying receptor formation and factors that regulate hormone responsiveness. — **Pettersson, P., R. L. R. Van, P. Lönnroth, P. Björntorp, and U. Smith.** Insulin binding in differentiating rat preadipocytes in culture. *J. Lipid Res.* 1985. **26:** 1187–1195.

Supplementary key words fat cells • differentiation • insulin • lipolysis

During the past decade, techniques that allow the isolation and culture of adipocyte precursors from the stromal-vascular fraction of adipose tissue have been established (1–5). In rat and bovine as well as in human adipose tissue, cells that under certain culture conditions develop into mature adipocytes have been identified (6–9). Furthermore, the finding that cultured adipocyte precursors derived from adult rats can differentiate fully, not only in culture but also when implanted into the same donor animal from which they were derived, supports the physiological significance of these cells (10).

The development and regulation of the insulin receptors in preadipocytes of various origin are not clear, as differences seem to exist between the various cell lines studied. Several studies with 3T3-L_1 cell lines, which develop into adipocytes, have shown an increased insulin binding during differentiation (11–13). No direct insulin-mediated regulation of the number of binding sites has been found (11). However, in 3T3-C_2 cell lines, which do not develop into adipocytes, insulin-mediated down-regulation and dexamethasone-induced up-regulation of insulin binding have been demonstrated (14). Furthermore, insulin seems to induce a down-regulation of its own receptor number in the mouse Ob_{17} cell line, which was originally derived from delipidated adipocytes (15).

In this study, insulin binding and effect were investigated in developing rat preadipocytes in order to characterize this potentially useful system and also to evaluate the optimal conditions for the differentiation of the precursor cells in primary culture.

EXPERIMENTAL PROCEDURES

Cell culture

Fed male, Sprague-Dawley rats, about 45 days of age and weighing 200–220 g, were used. They were fed ordinary rat chow, containing by weight 5% fat, 55% carbohydrate, and 22.5% protein, plus vitamins and minerals (Ewos, Södertälje, Sweden) and water ad libitum. The rats were killed by a blow on the head and the epididymal fat pads were removed under sterile conditions.

The adipose cells were isolated from the stroma by collagenase digestion as previously described (3, 4). The resulting sedimented stromal-vascular fraction was fil-

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tered through successive 250- and 50-μm mesh filters. The filtrate was subsequently suspended in medium 199 supplemented with 20% fetal calf serum, insulin, 1.67 μg/ml (278 nM), and sodium cefalothin, 0.1 g/l and then cultured in Falcon T-flasks (25 cm^2). After allowing 24 hr for initial plating, the fetal calf serum was replaced by 20% pooled human serum according to previously described procedures (3, 4). The human serum was tested to ensure a consistency between experiments in insulin and triglyceride levels (average 263 ± 49 pM and 1.93 ± 0.06 mM, respectively, means ± SEM). An emulsion of 0.5% triolein and 1.2% lecithin (Intralipid) was added on the second day of culture. In order to evaluate the importance of this triglyceride fortification on the differentiation process, some experiments were performed without the addition of the emulsion. The importance of the insulin addition was studied in experiments where the cultures were either performed without or with insulin (1.67 μg/ml) during the entire culture process. The culture medium was changed every 24 hr.

Cell preparation

Insulin binding was studied after 2, 4, 6, and 9 days in the different incubation media. On assay days, cells were recovered by the gentle use of a rubber policeman and a Pasteur pipette. In the early stages of preadipocyte differentiation (i.e., culture days 2 and 4), the cells could simply be separated into light and heavy cell fractions depending on their lipid content, where the light cells (univacuolar newly formed adipocytes) floated and the heavier, lipid-poor adipocyte precursors sedimented after 5 min of centrifugation at 200 *g*.

Differentiating adipocyte cultures synthesize a collagen matrix (16). Therefore, in the case of preadipocyte cultures in the final stage of morphological differentiation (from culture day 4), where an extensive matrix had formed, cleavage of the intercellular collagen bridges was necessary. After incubation with collagenase (1 g/l) for 1 hr at 37°C and pH 7.4 (17) the resulting cells were separated into light and heavy cells by the centrifugation procedure described above. In some experiments, the data obtained with the newly formed cells were compared to those found with "old" mature fat cells. These latter cells were then prepared under exactly the same conditions as the cultured cells (17).

Insulin binding

Before the binding assay, the isolated cells were carefully washed four times in fresh buffer and collected each time with a 5-min centrifugation procedure at 200 *g*. Insulin binding was performed at 37°C in medium 199 (pH 7.4) containing 1 mM glucose, 1% bovine albumin, monocomponent ^{125}I-labeled insulin (0.7–1.1 ng/ml), and different concentrations of unlabeled insulin. Bacitracin was added (1 g/l) to minimize proteolysis. In some control experiments, the incubations were performed at 16°C for 2 hr under the same conditions. Following incubation with labeled insulin for the indicated period of time, the incubation was stopped by adding 5 ml of ice-cold medium. The cells were then sedimented by a 5-min centrifugation at 200 *g*, rapidly resuspended in cold medium, and collected by filtration on paper discs, using a Millipore vacuum extractor. The paper discs were then dried and counted. The radioactivity bound in the presence of 4.17 μg/ml (0.695 μM) unlabeled insulin was considered nonspecific and was subtracted from the total binding. In order to evaluate whether the insulin present in the culture medium could influence the binding results in spite of the long isolation procedure, some control experiments were carried out where ^{125}I-labeled insulin (0.7 ng/ml) was added to the culture medium 2 hr before the harvesting procedure and the amount of tracer bound to the isolated cells was determined.

Following binding at 37°C, the hormone receptor complex is rapidly internalized (18) and a proportion of the hormone and receptors is degraded intracellularly (19). However, most of the receptors seem to recycle back to the plasma membrane (19). Thus, the cell-associated ^{125}I-labeled insulin recovered after the incubation period represents both the surface-bound and the intracellularly accumulated radioactivity. In order to overcome this problem and to verify that a changed insulin binding truly represents an alteration of the membrane receptors, control experiments were performed at 16°C. The internalization of the insulin receptor, subsequent to the binding of the hormone, is inhibited by low temperatures (18, 19). As affinity changes may appear during the recycling process (20), the Scatchard plot (21) for the insulin binding is characteristically curvilinear, and as the exact proportion of nonspecific binding for each insulin concentration used is unknown, no calculation of the total receptor number was attempted. However, when differences in insulin binding are found in the face of parallel individual Scatchard plots, differences in receptor number are taken to occur. By evaluating the binding data in this way, the errors involved in estimating the total number of binding sites are avoided.

Insulin degradation

Insulin degradation was determined during the normal binding assay by determining the precipitability of radioactivity in 10% trichloracetic acid.

Lipolysis

Following 30 min preincubation in medium 199, isolated mature fat cells or floating, newly formed cells from the same animals were incubated for 2 hr in medium 199 with 4% albumin at 37°C and pH 7.4. The cell volume

fraction was 2%. Norepinephrine and insulin were added at the indicated concentrations. The glycerol content of the medium after 2 hr incubation was analyzed according to Laurell and Tibbling (22) and taken as an index of lipolysis.

Cell counting

The number of cells in an aliquot of the cell suspension was counted in a Fuchs-Rosenthal cell chamber with 0.2 mm depth and 1/16 mm^2 area. The geometry of the cultured cells changed during their maturation in vitro. The comparison between the cell size of cultured and "old" mature fat cells was carried out after at least 6 days culture as the cells were by then truly spherical and could be accurately measured. The diameter of the spheres was measured as described by Smith, Sjöström, and Björntorp (17) and the cell surface area (CSA) was then calculated according to the formula: $CSA = \pi (\bar{d}2 + SD^2)$ where $\bar{d}$ = mean cell diameter and SD = standard deviation. After 6 days in the enriched culture medium, the mean size was 19.1 ± 5.5 μm (± SD).

Materials

HEPES (hydroxy ethyl-piperazine-ethane-sulfonic acid), norepinephrine, collagenase type I, and bovine serum albumin, Fraction V, were obtained from Sigma (St. Louis, MO). Culture flasks were obtained from Falcon Plastics (Los Angeles, CA), and medium 199 was from Statens Bakteriologiska Laboratorium (Stockholm, Sweden). Intralipid was from Vitrum (Stockholm, Sweden) and sodium cephalothin was from Eli Lilly (Indianapolis, IN). Glucagon-poor insulin was generously supplied by Eli Lilly (Indianapolis, IN) and ^{125}I-labeled monocomponent insulin (sp act about 8 MBq/μg) was a generous gift from Dr. S. Ivarsson, Malmö, Sweden.

RESULTS

Cell development

Adipocyte precursors isolated from the stromal-vascular fraction of adult rat epididymal fat pads developed morphologically into adipocytes in the culture. Initially, the preadipocytes replicated (**Fig. 1A**) and upon reaching confluency began to accumulate multilocular lipid inclusions relatively quickly (Fig. 1B). Full morphological differentiation, characterized by a monolocular cytoplasmic lipid droplet, was obtained after 6 days in culture (Fig. 1C). In all cultures confluence was reached within 48 hr. The full development of adipocyte precursors in culture, however, depended upon the incubation conditions used. In the absence of the lipid supplementation but in the presence of insulin, the adipocyte precursors did not fully differentiate into fat cells over the observation period. Although after 6 days in culture they had accumulated appreciable multilocular lipid inclusions, they were still only recoverable in the heavy fractions of cells after centrifugation. In the triglyceride-enriched system, however, differentiation was far more complete. Two days following the introduction of the triolein emulsion (day 4) more than half the cell population was recovered in the floating fraction (light cells) after centrifugation. After 6 days in culture (i.e., 4 days after the addition of the enriched medium), most of the cultured adipocyte precursors (80–90%) were recovered in the light fraction of cells. **Table 1** shows the increase in cell number with time in the presence of the different additions to the culture medium. It is clear that neither insulin nor triglyceride addition further increased the cell number or the doubling time.

Insulin binding

Initial experiments (not shown) were carried out to establish the time course for insulin binding to the cells at different stages of development. Adipocyte precursors cultured in the presence of the triolein emulsion for 2 or 4 days and recovered in the heavy fraction reached steady-state binding after 2 hr, whereas light cells reached maximum binding after 1 hr at 37°C and after 2 hr at 16°C. These incubation times were used in the subsequent studies.

Adipocyte precursors cultured without lipid supplementation for 6 days had a low specific insulin binding which was only about 30% of that of the heavy cells grown in the enriched culture system for 2 days (not shown). In the insulin- and lipid-enriched culture system there was a gradual increase in the specific insulin binding concomitant with growth and lipid accumulation of the cells (**Fig. 2A**). Scatchard analyses of these results show that the increased binding was due to an increase in both the apparent high- and low-affinity binding sites of the adipocyte precursors (Fig. 2A). Similar results were obtained when the binding assay was performed at 16°C which further supports this concept. Under these conditions, insulin binding to cells cultured for 6 days was about 3.4 times higher than that to cells cultured for 2 days (Fig. 2B). Insulin dissociation was slightly reduced (about 10%) in the heavy cells as compared to the light cells (data not shown). This small difference cannot make any substantial contribution to the increased insulin binding during development supporting the concept that new binding sites appear.

Fig. 3 shows the binding of ^{125}I-labeled insulin to cells grown in the enriched culture medium but in the absence of insulin. This system also provides a clear increase in insulin binding with growth. The importance of the

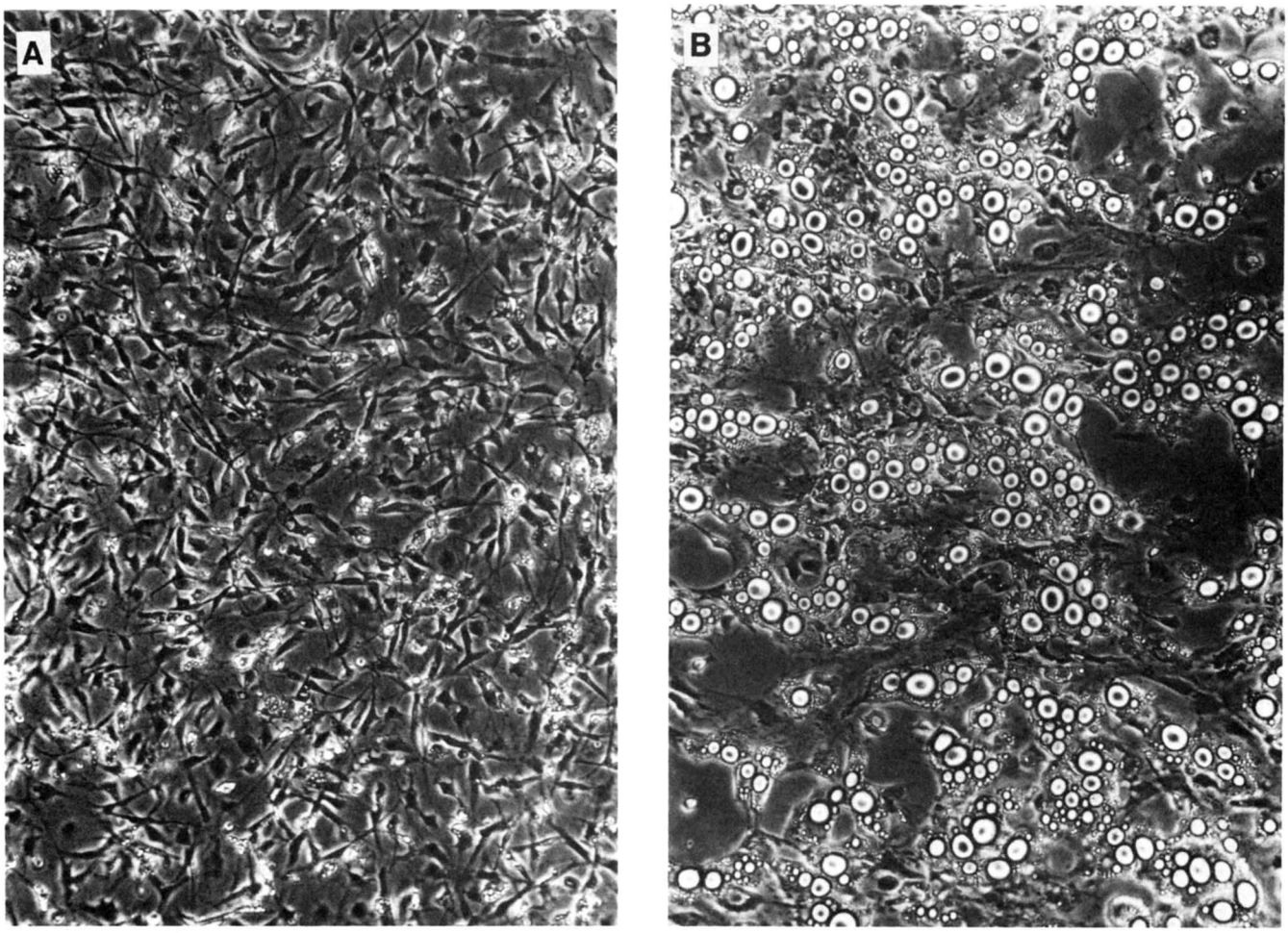


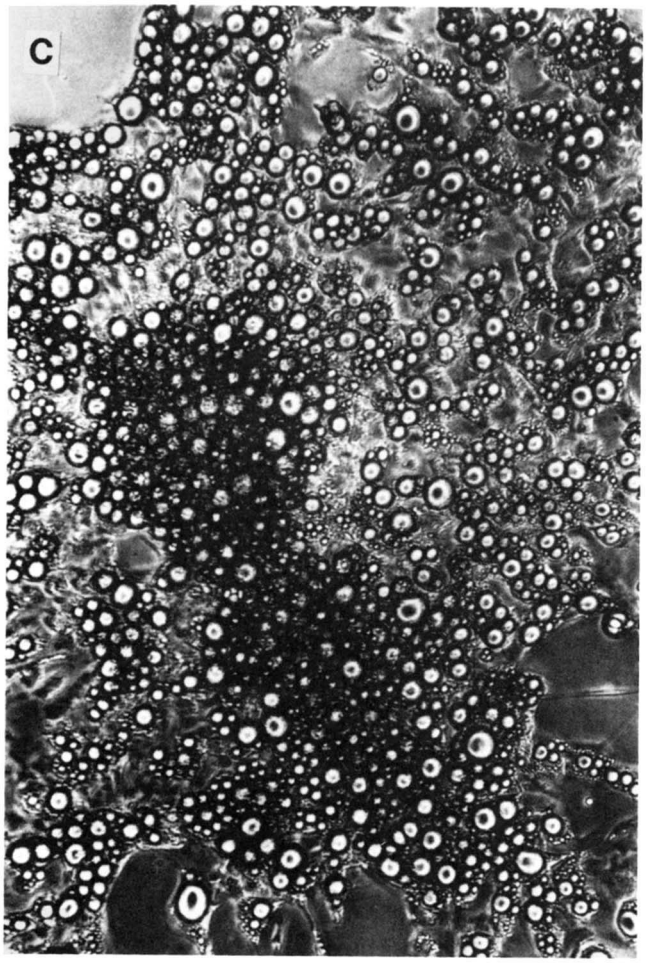
Fig. 1. Rat epididymal adipocyte precursors in primary culture. A, Cells after 2 days in culture (proliferation stage) prior to the introduction of the lipid-rich medium. B, Cells at confluency (day 4), 2 days after the addition of the lipid-rich medium.

insulin in the culture medium for the insulin binding was further studied in four experiments where cells were cultured for 9 days in the enriched medium with or without 1.67 μg/ml (278 nM) insulin. There was no evidence that this high insulin concentration caused a down-regulation of the number of binding sites (^{125}I-labeled insulin binding 103% of control cells). The increase in insulin binding did not parallel the expansion of the cells since the receptor density, expressed per unit surface area, was also increased. This was shown in experiments where insulin binding was measured both to epididymal precursor cells that had been allowed to develop into unilocular cells following 9 days culture in the enriched medium but without insulin and to "old" mature fat cells isolated from the same rats (Fig. 3). Although the average surface area of the "new" cells was only about 10% of that of the "old" cells (1.2 and 11.3 × 10^3 μm^2, respectively), insulin binding to the new cells was about 36% of that to the "old" cells. Consequently, the newly developed cells bound significantly more insulin (~340%) when the binding was expressed per unit surface area (Fig. 3).

The nonspecific binding remained essentially unchanged throughout the culture period (~20%) and was not significantly altered by the addition of insulin to the culture medium. After 6 days of culture in the enriched medium, insulin degradation by the new light cells was also similar to that of the "old" mature fat cells from the same animals (13% and 20%, respectively).

Lipolysis

Lipolytic studies were performed on both the "old" and the new fat cells from the same animals. The new cells were obtained by culturing the preadipocytes in the enriched medium but in the absence of insulin for 9 days. Basal lipolysis, expressed per cell, was similar in the new and the "old" mature cells. However, when expressed per



C, Cells at monolayer confluency (day 6) in the enriched culture system.

unit cell surface area, lipolytic rate was ~8-fold higher in the new cells (**Table 2**). Catecholamine-stimulated lipolysis was less in the new as compared to the "old" fat cells but, when the differences in cell surface area were corrected for, the lipolytic rate was 4-fold higher in the newly developed cells (Table 2).

The antilipolytic effect of insulin is depicted in **Fig. 4.** The dose-response curve for insulin (Fig. 4A) was shifted to the left in the newly developed cells, presumably reflecting the increased receptor density. Half-maximal antilipolytic effect was obtained with 0.5 and 3 μU/ml (3 and 20 pM) in the new and the "old" fat cells, respectively. The maximal antilipolytic effect of insulin was markedly decreased in the newly developed cells when expressed per cell, but not after correcting for the differences in cell surface area (Fig. 4B).

DISCUSSION

This study shows that precursor cells obtained from adult rat epididymal adipose tissue increase their number of insulin binding sites during development in culture. When the cells reached morphological maturation (i.e., univacuolar floating cells), total insulin binding, expressed on a per cell basis, was about 36% of that to "old" mature fat cells. Per unit cell surface area, however, the preadipocyte binding was 3.4 times as high as in the mature adipocytes isolated from the same tissue and animal. Thus, these data suggest that an "adult" number of insulin receptors is established early during the differentiation and growth of the cells. Following futher cellular enlargement, total receptor binding is only slightly increased and not in parallel to the expansion of the cell size. This is demonstrated by the observation that insulin binding per unit cell surface area decreased concomitant with the cellular enlargement. Decreased binding per unit cell surface area has also been described in "old" mature fat cells from obese rats (23–25) as well as in obese humans (26–29).

Over a 6-day culture period there is an increased binding to both the apparent high- and the low-affinity binding sites and insulin degradation becomes similar to that of "old" mature adipocytes. Thus, it seems that in this enriched culture system the preadipocytes gain functional characteristics similar to those of "old" mature fat cells.

TABLE 1. Increase in cell number with time

Additions to Medium	Days of Culture 2	4	7	9
	cells × 10^5/2 cm^2			
Basal	0.62 ± 0.12	2.86 ± 0.17	3.31 ± 0.25	2.92 ± 0.07
+ Insulin	0.88 ± 0.01	3.05 ± 0.47	3.56 ± 0.60	2.62 ± 0.02
+ Triglyceride	0.68 ± 0.05	2.86 ± 0.15	3.07 ± 0.15	3.12 ± 0.06
+ Insulin + triglyceride	0.65 ± 0.03	3.14 ± 0.36	3.56 ± 0.32	3.0 ± 0.25

The adipocyte precursor cells (54,000 ± 4,000) were plated and then incubated with 1.67 μg/ml insulin (278 nM) and/or triglyceride (Intralipid) as shown and the cell numbers were determined after the indicated times. The results are means ± SEM of three different experiments.

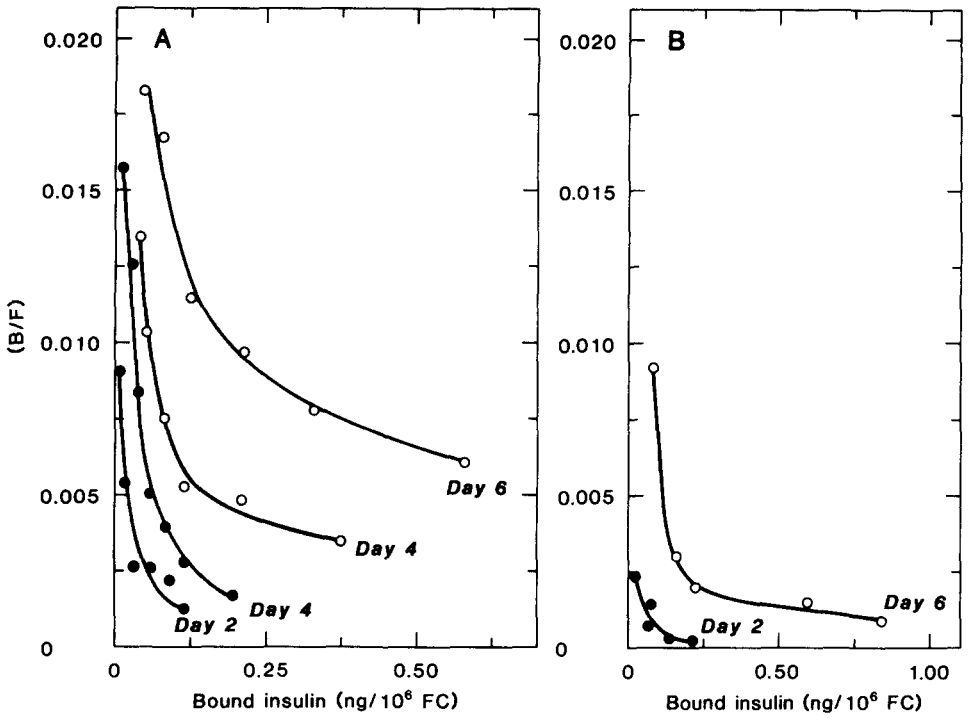


Fig. 2. A, Scatchard analyses of the insulin binding to differentiating adipocyte precursors grown in the enriched culture medium in the presence of 1.67 μg/ml insulin (278 nM) for the indicated times. After washing and isolating the cells as indicated in Methods, the cells were incubated at 37°C with ^{125}I-labeled insulin in the presence of various concentrations of unlabeled insulin. The radioactivity in the presence of 4.17 μg/ml unlabeled insulin was considered nonspecific and was subtracted from the results. The data are the means of three different experiments. Light, floating cells (○—○); heavy, non-floating cells (●—●). B, Scatchard analyses of ^{125}I-labeled insulin binding in the presence of various concentrations of unlabeled insulin at 16°C. The cells were cultured for 2 days (heavy cells) or 6 days (light cells). Note the differences in scale as compared to Fig. 2A.

These findings corroborate and further strengthen previous reports for the relatedness of these two cell types (3, 4, 30–32).

The functional importance of the apparent additional insulin binding sites was evaluated by measuring the antilipolytic effect of insulin. Due to the presence of spare receptors in adipocytes, only 2–5% of the total number of insulin binding sites have to be occupied to elicit a maximal insulin effect (33). Thus, the number of binding sites usually mirrors the insulin sensitivity (insulin concentration yielding half-maximal effect) rather than the insulin responsiveness (maximal insulin effect) (33, 34).

According to this concept, an increased number of insulin receptors leads to a shift to the left in the dose-response curve for insulin. However, it has been suggested that the receptor density in fat cells, expressed per unit surface area, influences the insulin sensitivity (25, 29). In agreement with this, the dose-response curve for the antilipolytic effect of insulin was shifted to the left while the insulin responsiveness was similar in preadipocytes and in "old" mature fat cells when the differences in cell surface area were taken into account.

Although some insulin binding was found in the heavy cells prior to confluency, it was not until post-confluency that there was a marked increase in binding. Thus, it would seem that any important metabolic effect of insulin exerted over the receptor occurs after confluency has been reached. This is also true for the 3T3-L_1 cells, which only accumulate lipid after confluency has been reached even in the presence of insulin (12). The dramatic increase of lipoprotein lipase activity at confluency is certainly an important event for the lipid accumulation at this point (4, 9).

The mechanism for the increase in the insulin binding concomitant with the expression of the adipocyte phenotype in lipid-rich medium is unknown. During adipose conversion, numerous enzymes and proteins have been shown to change (35–38). One possibility is that the enriched culture medium, regardless of the stage of adipocyte development, induces an increase in these proteins at the time of confluency. However, this possibility is unlikely since the appearance of the insulin binding sites paralleled the growth of the univacuolar fat cells. Insulin binding remained low in the non-floating cells even several

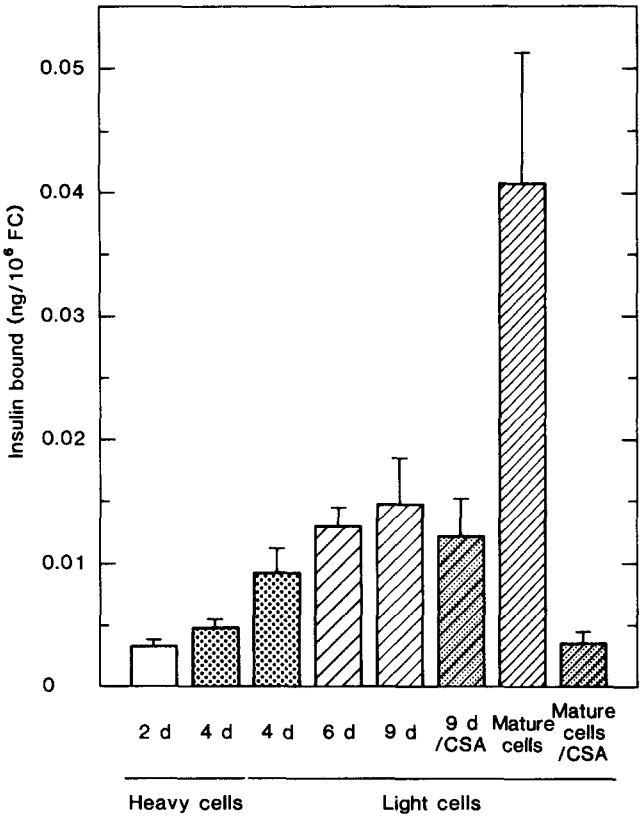


Fig. 3. Specific binding of ^{125}I-labeled insulin to mature fat cells and adipocyte precursors from the same animals during various stages of cell differentiation in the lipid-enriched culture medium but in the absence of insulin. Following harvesting and washing procedures, the cells were incubated with ^{125}I-labeled insulin as indicated in Methods for the indicated number of days. The binding in the presence of 4.17 μg/ml unlabeled insulin was considered nonspecific and was subtracted from the results. The data represent the mean ± SEM of three to five experiments. Insulin binding to "old" mature cells and to cells grown in vitro for 9 days is also expressed per unit cell surface area (CSA).

days after confluency had been reached. The fact that these heavy cells had the property of true preadipocytes was shown by their transformation into univacuolar cells in the lipid-enriched medium. It is also clear that insulin alone could not replace the triglyceride emulsion in this respect. In addition, insulin had no effect on the cell replication process as shown in the growth curves. This finding differs from the recent report by Grimaldi et al. (38) with the Ob_{17} cell line. However, in order to definitely exclude any mitogenic effect of insulin in our system, specific studies evaluating the effect of the low insulin concentration present in the serum that was added to the cultures would have to be performed.

Thus, the enhancing effect of the triglycerides in the medium on the insulin binding is associated with the expansion and growth of the cells. In this context, it is interesting to note that Grunfeld, Baird, and Kahn (39) reported that saturated fatty acids reduced insulin binding to differentiated 3T3-L_1 cells. This discrepancy from the present study, where triglycerides in the medium were associated with enhanced insulin binding, demonstrates that different regulatory processes are operative in differentiating and multiplying cells as compared to mature cells.

The presence of a very high insulin concentration in the lipid-containing medium did not prevent the increase in the insulin binding during the development of the cells. This is in contrast to the findings that a high ambient insulin concentration reduces the number of insulin receptors in cultured mature adipocytes (40) as well as in other cell types (41–43). However, it has been shown that physiological insulin concentrations can also exert a regulatory effect on the number of insulin receptors in some cells (42). Thus, the present design cannot completely exclude the observation that the insulin in the 20% human serum added to the medium, producing an average insulin concentration of ~56 pM, exerted some slight regulatory effect.

Other studies on established preadipose cell lines have reported different results with respect to the regulation of insulin on its cellular binding sites. Insulin can induce a down-regulation of the number of binding sites in the Ob_{17} dedifferentiated adipocyte cell line and in 3T3-C_2 cells (14, 15), whereas experiments with the 3T3-L_1 cell

TABLE 2. Basal and noradrenaline-stimulated glycerol release in mature fat cells and newly developed cells

Cells	Basal		+ Noradrenaline	
	Per Cell	Per Unit Surface Area (μm²)	Per Cell	Per Unit Surface Area (μm²)
	glycerol release, nmol/10⁵cells			
Preadipocytes (n = 3)	19 ± 4	16 ± 4	268 ± 113	224 ± 94
"Old" mature cells (n = 5)	16 ± 2	2 ± 1	613 ± 56	55 ± 5

The preadipocytes were cultured for 9 days in the lipid-enriched medium before assay. After harvesting and washing, the floating newly developed cells or the mature adipocytes from the same animals were incubated for 2 hr in medium 199 with or without noradrenaline (10^{-5} M) as indicated and the glycerol release was determined. Data are means ± SEM.

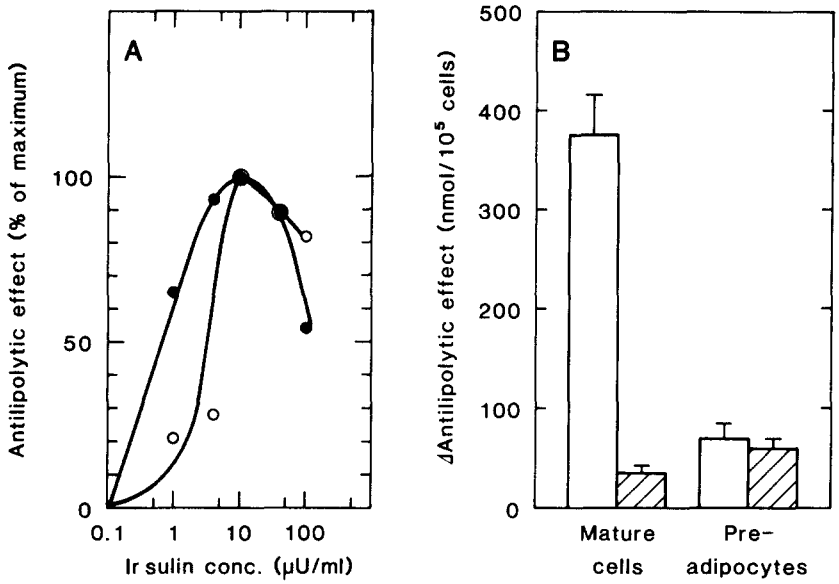


Fig. 4. A, The antilipolytic effect (% of maximum) of different concentrations of insulin in preadipocytes cultured for 9 days in the lipid-enriched medium in the absence of insulin (●—●) and in mature fat cells from the same animals (○—○). The cells were incubated for 2 hr in medium 199 with 4% bovine albumin in the presence of noradrenaline (10^{-5} M). The results are the means of three and five experiments, respectively. B, The maximal antilipolytic effect of insulin expressed per cell (□) and per unit cell surface area (▨).

lines have not demonstrated this phenomenon (13). This inconsistency in the effect probably reflects the inherent differences in cell origin between these lines.

In conclusion, the present investigation demonstrates the appearance of specific binding sites during adipocyte precursor differentiation. This increase appears to be dependent on the growth of the cells rather than on the presence of a specific factor in the culture medium. This system should, therefore, provide a valuable tool to study the mechanisms operating during the differentiation process. Such a system should also be widely useful to elucidate factors involved in different insulin-resistant states as well as to allow a systematic exploration of the influence of nutrients, hormones, and other growth factors.

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