Insulin Regulation of Lipoprotein Lipase Activity and Release in 3T3-L1 Adipocytes

SEPARATION AND DEPENDENCE OF HORMONAL EFFECTS ON HEXOSE METABOLISM AND SYNTHESIS OF RNA AND PROTEIN*

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Insulin regulation of lipoprotein lipase was studied in fully differentiated 3T3-L1 fat cells. Insulin at low concentrations (10^{-11} to 10^{-10} M) elicited a rapid and maximal increase (~10-fold) in lipoprotein lipase release from the cells to the medium within 30 min and caused a 2- to 10-fold increase in total cellular lipoprotein lipase activity over a 2-day period. Insulin had no effect on cell lipoprotein lipase activity during the first 4 h of treatment. Prolonged exposure to insulin (2 to 4 days) resulted in additional increases in enzyme release rate, and increased the fractional release rate per h approximately 10-fold.

Stimulation of cellular lipoprotein lipase activity was dependent on the presence of glucose in the incubation medium. Although half-maximal increases in cell activity required 12 h of insulin treatment in glucose-containing medium, removal of either glucose or insulin resulted in loss of more than 50% of the stimulation in enzyme activity within 90 min. Addition of glucose to cells incubated 20 h with insulin in carbohydrate-free medium elicited a rapid and full response to insulin in 2 h. Fructose, mannose, and glucosamine could substitute for glucose in supporting this rapid response in insulin-pretreated cells, but only glucose, mannose, and fructose would support the effect in 24-h incubations with hormone.

Induction of the increase in lipoprotein lipase activity by glucose in insulin-pretreated cells was prevented by cycloheximide, but not by actinomycin D. In contrast, inhibitors of either protein (cycloheximide, puromycin) or RNA (α-amanitin, actinomycin D) synthesis completely blocked the response when added with insulin to cells incubated in medium containing glucose.

We propose that insulin regulates lipoprotein lipase from fibroblasts into fat cells in culture. Development of the adipocyte phenotype, and the biochemical and functional properties of the enzyme were similar to that found in adipose tissue. Appearance of the enzyme in the tissue is directly related to plasma insulin levels during fasting and feeding and is increased manyfold by injection of insulin (7-9).

Studies with segments of adipose tissue and isolated adipocytes showed that insulin and glucose are both required to maintain enzyme activity in vitro (9-13). Although these findings support the concept that insulin may regulate lipoprotein lipase activity in adipose tissue, a direct stimulatory effect of insulin on the activity in isolated mature adipocytes has not, to our knowledge, been reported.

We and others (14-17) recently documented the appearance of lipoprotein lipase in ADULT 3T3-L1 cells as they differentiate from fibroblasts into fat cells in culture. Development of the enzyme was found to be a primary characteristic of the adipocyte phenotype, and the biochemical and functional properties of the enzyme were similar to that found in adipose tissue. Appearance of the enzyme early in the differentiation sequence was enhanced by several hormones and agents that promote adipose conversion, but it was most strongly affected by insulin (14). These results suggested that mature, fully differentiated 3T3-L1 fat cells might provide a useful system for examining hormonal control of the enzyme. This report describes a study of effects of insulin on lipoprotein lipase activity in such cells, and presents a model of mechanisms involved in the regulation of the enzyme in fat cells.

EXPERIMENTAL PROCEDURES

Preparation of Cells—Starter cultures of 3T3-L1 cells were generously supplied by Dr. Howard Green, Massachusetts Institute of Technology, Cambridge, Mass. Cells were grown to confluence in 100-mm culture dishes with DME medium1 containing 20% calf serum, 50

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1 The abbreviations used are: DME medium, Dulbecco’s modified Eagles medium; PGF_2α, prostaglandin F_2α, MSA, multiplication stimulating activity.

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Lipoprotein lipase activity in cell extracts and incubation media was measured by the rate of release of [³H]oleic acid from a serum-activated emulsion of tricaprylin and lecithin as described earlier (14). Different aliquots of each sample, generally 40 and 100 μl, were assayed in duplicate. Lipolytic activity was linear with respect to both time and amount of sample assayed. One unit of lipolytic activity represents release of 1 nmol of fatty acid/min at 37°C. It should be noted that lipoprotein lipase activity in cells was measured in aqueous extracts of defatted cells, whereas the activity in media was measured directly in filtrates of the media. Consequently, direct comparison of the amounts of activity in the two compartments may not be valid. The lipase activity present in cell extracts and that released into the incubation medium had a pH optimum of 8.3 and was inhibited >80% by inclusion of 1 M NaCl or omission of serum (a source of the activator peptide, apolipoprotein C_II) in the assay mixture. These properties are identical with those of partially purified lipoprotein lipase from differentiating 3T3-L1 cells (14).

Chemical Analyses—DNA was measured by the method of Hinegardner (18), using calf thymus DNA as standard, and triacylglycerol by the method of Chernick (19). Insulin radioimmunoassays were performed as described earlier (20). Insulin radioimmunoassays were kindly performed by Dr. Emmanuel Van Obberghen, National Institute of Arthritis, Metabolism, and Digestive Diseases (21). Measurement of ³H-labeled fatty acid was made with a Beckman LS-8000 B spectrometer using Beckman GP scintillation solvent and automatic counting correction techniques.

**Figure 1.** Short term effects of insulin on cell lipoprotein lipase (LPL) and release of enzyme into the medium. Mature 3T3-L1 fat cells were preincubated in 5% serum-DME medium for 5 days. Cells were then incubated in 5% serum-DME medium with 10⁻⁷ M insulin for the periods indicated. Control cells were incubated without insulin for 4 h. A, cell lipoprotein lipase activity. B, rate of lipoprotein lipase release from the same cells was determined by measuring enzyme activity in the medium at the end of incubation and dividing by the incubation time. Results are means ±S.E. of five determinations.
**RESULTS**

**Effect of Various Hormones on Cell Lipoprotein Lipase Activity**

The effect of various hormones on lipoprotein lipase activity in mature 3T3-L1 fat cells is shown in Table I. All cells were treated for 4 days. Insulin and the insulin-like growth factor, MSA, increased lipoprotein lipase activity in mature cells 2- to 3-fold. Neither insulin nor MSA had any effect on cell triacylglycerol content or total DNA content of cultures during 4-day treatment. Serum at a high concentration (20%), and other agents (prolactin, PGF2a, and 1-methyl-3-isobutylxanthine) which enhanced development of lipoprotein lipase activity during differentiation of 3T3-L1 cells (14), had no effect on enzyme activity in mature cells. This suggests that the increase in activity previously observed with these agents in developing cells was probably related to accelerated differentiation rather than to a direct effect of the agents on lipoprotein lipase activity. Estrogen and progesterone, reported to alter lipoprotein lipase activity of adipose tissue in vivo (22, 23), had no effect on the enzyme in these cells. Triiodothyronine, dexamethasone, and growth hormone, sub-

![Graph](image1)

**Fig. 2.** Long term effects of insulin on cell lipoprotein lipase (LPL) and release of enzyme into the medium. Mature 3T3-L1 fat cells were preincubated in 5% serum-DME medium for 5 days. Cells were then incubated in 5% serum-DME medium for up to 4 days with $10^{-7}$ M insulin. Control cells were incubated 4 days without insulin. A, cell lipoprotein lipase activity. B, rate of release of lipoprotein lipase from the same cells during the last 2 h of incubation. Results are means ±S.E. of three determinations.

**Materials**—Cell culture media were prepared by the National Institutes of Health Media Unit. Calf serum was obtained from GIBCO, Grand Island, N. Y. (Lots C-473412 and E-181011). Intralipid was purchased from Cutter Laboratories, Oakland, Calif. Crystalline porcine zinc insulin (25 units/mg) was provided by the Eli Lilly Co., Indianapolis, Ind. Oxine prolactin, NIH-o-PRL-S11, and ovine growth hormone, NIH-o-GH-S11, were gifts from the National Institute of Arthritis, Metabolism and Digestive Diseases Hormone Distribution Program. Human placental lactogen was purchased from Nutritional Biochemicals, Cleveland, Ohio. PGF2a, trimethylamine salt was a gift of Dr. John Pike, Upjohn Co., Kalamazoo, Mich. MSA was a gift from Dr. Matthew Rechler, National Institute of Arthritis, Metabolism and Digestive Diseases. 1-Methyl-3-isobutylxanthine was purchased from Aldrich Chemicals, Milwaukee, Wis. Estradiol-17β, progesterone, paromycin di-HCl, actinomycin D, cycloheximide, 3,3',5-triiodothyronine, dexamethasone, l-a-phosphatidylcholine, glucosamine, 2-deoxyglucose, fucose, N-acetylglucosamine, fructose, galactose, mannose, mannosamine, dihydroxycetone, and pyruvate were obtained from Sigma, St. Louis, Mo. Crystalline bovine serum albumin (Lot N 72905) was from Armour Pharmaceuticals, Phoenix, Ariz. Trioleoylglycerol (Lot Z-IT) was obtained from the Hormel Institute, Austin, Minn. Heparin (150 units/mg) and a-amanitin were purchased from Calbi-ochem, La Jolla, Calif. Trypsin (Trp[9,10-H]troleoylglycerol (591 mCi/mmol) was purchased from Amersham/Searle Corp., Arlington Heights, Ill. Conventional reagents were of analytic or spectral quality.

![Graph](image2)

**Fig. 3.** Relationship between insulin concentration and its effects on cell lipoprotein lipase (LPL) and release of enzyme into the medium. Mature 3T3-L1 fat cells were preincubated 4 days in 5% serum-DME medium. A, cell lipoprotein lipase activity was determined after incubating cells for 24 h with insulin at the concentrations indicated. B, rate of release of lipoprotein lipase was determined in separate experiments during a 2-h incubation with hormone. Results are means ±S.D. of duplicate determinations.
Insulin Regulation of Lipoprotein Lipase

Time Course—Rapid effects of insulin were assessed by incubating cells with the hormone for 4 h and measuring lipase activity in extracts of dried, defatted cell powders and in the incubation medium. Insulin treatment had little or no effect in 4 h on enzyme activity in cells (Fig. 1A), whereas it immediately increased the release of lipase into the medium (Fig. 1B). The rate of enzyme release increased from ~1 to ~10 milliunits·mg DNA⁻¹·h⁻¹ in 30 min and remained at that level during the next 3 h.

An effect of insulin on lipoprotein lipase activity in cells...
occurred after longer periods of treatment (Fig. 2A). Lipase activity increased from ~0.2 to 1.2 units-mg DNA⁻¹ in 2 days, and was unchanged during the next 2 days. A half-maximal effect occurred after ~18 h. This response was accompanied by a parallel increase in rate of release of enzyme to the medium measured during the last 2 h of incubation (Fig. 2B). The rate of lipase release was ~1 milliunit-mg DNA⁻¹·h⁻¹ in cells incubated without hormone and ~80 milliunits-mg DNA⁻¹·h⁻¹ in cells incubated with insulin for 2 to 4 days. The time required for a half-maximal increase in enzyme release, ~24 h, was similar to that required for a half-maximal increase.

![Fig. 7. Effect of glucose withdrawal on lipoprotein lipase (LPL) in insulin-treated cells. Mature 3T3-L1 fat cells were preincubated 4 days in 5% serum-DME medium. Cells were then incubated 24 h in 5% serum-DME medium with (O) or without (●) 10⁻⁷ M insulin. At zero time, media were removed from insulin-treated cultures and the cells were washed twice with 10 ml of DME medium containing no glucose. Cells were then incubated up to 12 h in DME medium containing 10⁻⁷ M insulin, but no glucose. The decay in activity between 0 and 1½ h is fitted to the regression lipoprotein lipase = 1.73 units e⁻³₆₅ (r = 0.97). Values are means ±S.E. of three to four determinations.](http://www.jbc.org/)

**Table II**

Carbohydrate specificity of the 24-h response to insulin in lipoprotein lipase activity

Mature 3T3-L1 fat cells were preincubated in 5% serum-DME medium for 3 days. Cells were then incubated in DME medium containing 5% dialyzed serum, 10⁻⁷ M insulin, and the indicated concentration of each carbohydrate. Lipoprotein lipase activity of cells was determined after 24 h. Results are means ±S.D. of two experiments.

<table>
<thead>
<tr>
<th>Carbohydrate added</th>
<th>Insulin</th>
<th>Lipoprotein lipase activity</th>
<th>units-mg DNA⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-Glucose</td>
<td>10</td>
<td>0.75 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>D-Glucose</td>
<td>10</td>
<td>2.31 ± 0.21</td>
<td></td>
</tr>
<tr>
<td>D-Fructose</td>
<td>10</td>
<td>2.13 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>D-Mannose</td>
<td>10</td>
<td>2.41 ± 0.06</td>
<td></td>
</tr>
<tr>
<td>D-Galactose</td>
<td>10</td>
<td>0.79 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>Fructose</td>
<td>10</td>
<td>0.66 ± 0.06</td>
<td></td>
</tr>
<tr>
<td>Dihydroxyacetone</td>
<td>20</td>
<td>0.24 ± 0.09</td>
<td></td>
</tr>
<tr>
<td>D-Glucosamine</td>
<td>10</td>
<td>0.17 ± 0.06</td>
<td></td>
</tr>
<tr>
<td>D-Fucose</td>
<td>10</td>
<td>0.16 ± 0.04</td>
<td></td>
</tr>
</tbody>
</table>

![Fig. 8. Effect of glucose addition to cells incubated with or without insulin in carbohydrate-free medium. Mature 3T3-L1 fat cells were preincubated in 5% serum-DME medium for 6 days. Cultures were divided into three groups and incubated in glucose-free DME medium containing 5% dialyzed serum with (●) or without (○), (△) 10⁻⁷ M insulin for 20 h. At time zero, the media of the three groups (●), (△) were replaced with 5% dialyzed serum-DME medium containing 10⁻⁷ M insulin plus 25 mM glucose. Media of the third group (○) were replaced with 5% dialyzed serum-DME medium containing 25 mM glucose, but not insulin. Lipoprotein lipase (LPL) activity in these cell groups was compared with that in cells incubated 24 h with (●), or without (○) 10⁻⁷ M insulin in complete DME medium containing 5% dialyzed serum and 25 mM glucose. Values are means ±S.E. of three to four determinations.](http://www.jbc.org/)

![Fig. 9. Dependence of the insulin effect on lipoprotein lipase (LPL) in carbohydrate-deprived cells on glucose concentration. Mature 3T3-L1 fat cells were preincubated in 5% serum-DME medium for 4 days. Cells were then incubated for 20 h with 10⁻⁷ insulin in glucose-free 5% dialyzed serum-DME medium. Cells were then incubated in 5% dialyzed serum-DME medium containing 10⁻⁷ M insulin and the indicated concentrations of glucose for 2 h and lipoprotein lipase activity determined. Results are means ±S.D. of two experiments.](http://www.jbc.org/)
Insulin Regulation of Lipoprotein Lipase

Lipoprotein Lipase—Requirement of serum, amino acids, and glucose for the effect of insulin on cell lipoprotein lipase was examined in cells incubated with insulin for 24 h. Omission of serum or amino acids had little effect on the response to insulin, whereas omission of glucose blocked completely the response to hormone (Fig. 5). Omission of amino acids from glucose-free medium had no additional effect. Omission of serum, amino acids, or glucose for 24 h had no effect on basal enzyme activity.

A maximal effect of insulin on cell lipoprotein lipase occurred in 24 h with glucose concentrations in excess of 5 mM and a half-maximal effect occurred at 0.5 mM glucose (Fig. 6). The activity in control cells increased only slightly when glucose was increased from 0 to 25 mM. The results in Fig. 7 show that maintenance of lipoprotein lipase in insulin-treated cells was also dependent on glucose. Removal of glucose from the medium of cells incubated with insulin for 24 h caused a biphasic loss of enzyme activity. The initial rapid decline ($t_{1/2} = 1.6$ h) reduced the activity from 1.7 to 0.7 units·mg DNA$^{-1}$ in 1 h, and the second slower decline ($t_{1/2} = 11$ h) reduced the activity to the same level as in control cells (0.4 unit·mg DNA$^{-1}$) in 6 h. The slower decline during the second phase may reflect utilization of endogenous glycogen or other carbohydrate stores.

The ability of other hexose and triose metabolites to substitute for glucose was examined in cells incubated with insulin for 24 h (Table II). Fructose and mannose, sugars which can enter the glycolytic pathway and can be metabolized in fat,

<table>
<thead>
<tr>
<th>Carbohydrate added</th>
<th>Lipoprotein lipase activity</th>
</tr>
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<tbody>
<tr>
<td>D-Glucose</td>
<td>0.10 ± 0.02 0.52 ± 0.04 0.82 ± 0.05</td>
</tr>
<tr>
<td>D-Gluoseamine</td>
<td>0.50 ± 0.04 1.00 ± 0.12</td>
</tr>
<tr>
<td>D-Mannose</td>
<td>0.51 ± 0.05 0.91 ± 0.16</td>
</tr>
<tr>
<td>D-Fructose</td>
<td>0.38 ± 0.04 0.64 ± 0.06</td>
</tr>
<tr>
<td>D-Mannosamine</td>
<td>0.19 ± 0.06</td>
</tr>
<tr>
<td>N-Acetyl-d-glucosamine</td>
<td>0.14 ± 0.04 0.15 ± 0.06</td>
</tr>
<tr>
<td>2-Deoxy-d-glucose</td>
<td>0.10 ± 0.03 0.12 ± 0.04</td>
</tr>
<tr>
<td>D-Galactose</td>
<td>0.13 ± 0.06 0.17 ± 0.07</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>0.14 ± 0.07 0.19 ± 0.08</td>
</tr>
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</table>

Addition of cycloheximide and actinomycin D on the lipoprotein lipase (LPL) response to insulin. Mature 3T3-L1 fat cells were preincubated in 5% serum-DME medium for 3 days. Cells were then incubated in 5% serum-DME medium containing 25 mM glucose with $10^{-7}$ M insulin and inhibitors as indicated for 24 h. Lipoprotein lipase activity was determined at 24 h. Results are means ± S.E. of three to four determinations.

Effect of cycloheximide and actinomycin D on the lipoprotein lipase response to glucose in insulin-pretreated, glucose-deprived cells.

Mature 3T3-L1 fat cells were incubated in 5% serum-DME medium for 5 days. Cells were then incubated 20 h in glucose-free DME medium containing 5% dialyzed calf serum and insulin ($10^{-7}$ M). Culture media were then replaced with a similar solution containing 25 mM glucose with $10^{-7}$ M insulin and inhibitors as indicated for 24 h. Lipoprotein lipase activity was determined at 24 h. Results are means ± S.E. of two experiments.

Addition | Lipoprotein lipase activity
<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td>D-Glucose</td>
<td>0.11 ± 0.03 0.32 ± 0.04 0.54 ± 0.06 0.96 ± 0.03</td>
</tr>
<tr>
<td>D-Glucose + cycloheximide</td>
<td>0.11 ± 0.02 0.09 ± 0.02 0.08 ± 0.03</td>
</tr>
<tr>
<td>D-Glucose + actinomycin D</td>
<td>0.37 ± 0.04 0.78 ± 0.09 0.94 ± 0.05</td>
</tr>
</tbody>
</table>
cells (23, 26), were equivalent to glucose in supporting the response to insulin. Substitution of either fructose or mannose for glucose, at 10 mM, did not increase the activity of untreated cells (data not given). The action of insulin on cell lipoprotein lipase was not supported by galactose, whose transport, but not metabolism, is increased by insulin (27). Pyruvate or dihydroxyacetone, or glycoprotein precursors (glucosamine and fucose) were also ineffective.

The requirement for sugars which are actively taken up and metabolized via the glycolytic pathway raised the possibility that the action of insulin on lipoprotein lipase was dependent on hexose or secondary to the effects of insulin on hexose metabolism. To evaluate this, cells were incubated in glucose-free medium, with or without insulin, for 20 h and then in medium containing glucose. Fig. 8 shows that lipoprotein lipase activity increased immediately in insulin pretreated cells when glucose (25 mM) was added to the medium. Enzyme activity 1 h after glucose addition was equal to that in cells incubated with both insulin and glucose for 24 h. Glucose addition produced only a small rise in lipase activity of untreated cells. Addition of insulin with glucose had little effect in 4 h on lipoprotein lipase in cells deprived of both insulin and glucose prior to the experiment.

Fig. 9 shows the dependence on glucose concentration of the increase in lipoprotein lipase activity in cells pretreated with insulin in the absence of carbohydrate for 20 h. Cell lipase activity increased considerably in 2 h when medium glucose concentration was raised from 0 to 0.02 mM and it increased further as the concentration was raised to 1.0 mM. Half-maximal response for the overall biphasic increase occurred at 0.15 mM glucose.

Because of the possibility of extensive metabolic interconversions during prolonged incubations (e.g. 24 h in Table II), chemical specificity of carbohydrate support for the insulin effect on lipase activity was reassessed in short term incubations with insulin pretreated, carbohydrate-deprived cells. Table III shows that addition of 0.5 mM glucose increased enzyme activity ~5-fold in 45 min and ~8-fold in 90 min. Similar increases were observed with either 0.5 mM glucosamine or 0.5 mM mannose, and a smaller increase with 0.5 mM fructose. The response at 5 mM fructose, however, was identical with that at 5 mM glucose (data not shown). Mannosamine, 2-deoxyglucose, N-acetylglucosamine, galactose, and pyruvate, all at 0.5 mM, did not support the response. Support for the increase in lipoprotein lipase activity in response to insulin thus appears limited to glucose, fructose, and their potential precursors, mannose and glucosamine.

Dependence of the Response to Insulin on Protein and RNA Synthesis—To assess whether the enzyme response to insulin and its support by glycolytic metabolites involves new protein and RNA synthesis, the effects of inhibitors of these processes were examined. Fig. 10 shows that addition of low concentrations of either cycloheximide (0.05 µM·ml⁻¹) or puromycin (1 µg·ml⁻¹) blocked completely the effect of insulin on lipoprotein lipase in cells incubated in 25 mM glucose for 24 h. Low concentrations of either a-amanitin (10 µg·ml⁻¹), a specific inhibitor of RNA polymerase II (28), or actinomycin D (0.005 µg·ml⁻¹), also inhibited the response to insulin. The data in Table IV show that the increase in lipoprotein lipase activity in insulin pretreated, glucose-deprived cells following addition of glucose (25 mM) was blocked by cycloheximide, but not by actinomycin D. Although both protein and RNA synthesis appear to be required for the insulin effect on lipoprotein lipase, only protein synthesis was required for insulin-pretreated cells to respond to glucose.

This study demonstrates that insulin exerts two major regulatory effects on lipoprotein lipase in mature 3T3-L1 fat cells: A rapid increase in release of the enzyme from cells to the medium and a slower increase in cell enzyme activity (Figs. 1 and 2). Both effects occurred at low, physiologically relevant concentrations of insulin (Fig. 3) and mimic previously observed effects of the hormone on lipoprotein lipase in adipose tissue.

Adipocytes isolated from rats injected with insulin released increased amounts of lipoprotein lipase during incubation (9). Release of enzyme from such cells was maximal 20 min after hormone injection and persisted over a 4-h period. Enzyme activity in the cells remained unchanged throughout this response. In the present work, release of lipoprotein lipase from mature 3T3-L1 cells was maximal within 30 min after insulin addition, and also occurred without a significant effect on cell enzyme activity (Fig. 1). Incubation of the 3T3-L1 cells with insulin for prolonged periods (½ to 4 days) resulted in further increases in lipase release in parallel with a large rise in cell enzyme activity (Fig. 2). This was accompanied by a 10-fold increase in the fraction of cell enzyme activity released to the medium per h. These results indicate that insulin acts acutely to liberate a small fraction, or pool, of enzyme which increases as a function of the total enzyme activity of the cell. Vanhove et al. (29) recently reported that the amount of enzyme in plasma membranes of fat cells was increased in fed rats, which could be expected to have increased plasma insulin levels. These observations suggest that interaction of insulin with its cell surface receptors may result in changes in membrane conformation or chemical activities which alter binding of the enzyme to the membrane and increase enzyme release to the medium.

The mechanisms by which lipoprotein lipase is synthesized and released from parenchymal cells are presently unknown. Earlier authors have proposed that release of enzyme from rat adipocytes involves conversion or "activation" of a high molecular weight cellular form to a smaller, more active, "secreted" form (30, 31). Although one report (32) suggested that such forms could represent artifacts of enzyme preparation, it seems possible that insulin could act to stimulate release by enhancing such an enzymatic conversion process. However, the fact that insulin was also reported to have no effect on the relative proportion of enzyme present in each form argues against this possibility (9). Since lipoprotein lipase is believed to be a glycoprotein, it is also possible that insulin could enhance release by stimulating exocytotic secretion processes similar to those involved in the secretion of other exoenzymes (33). More definitive resolution of the actual processes involved will probably await definition of the basic nature of the release mechanism itself. The physiological significance of enzyme release by isolated fat cells is, therefore, difficult to evaluate at this time.

In contrast to its effects on enzyme release, insulin stimulation of lipoprotein lipase in 3T3-L1 fat cells required extended periods of exposure to the hormone (cf. Figs. 1 and 2). Little effect was seen during the first 4 h, and maximal effects occurred after 2 days. Maximal increases in lipoprotein lipase activity of rat adipose tissue were observed in vivo 3 to 4 h after injection of the hormone and half-maximal effects were seen in 1 to 2 h (9). This difference may be related to the continuous exposure of adipocytes to insulin and serum factors in vivo, as opposed to the withdrawal of insulin and high serum levels required for demonstrating the response in 3T3-
L1 cells in vivo (see "Experimental Procedures"), or it may indicate that different mechanisms are involved in the responses observed in the two systems.

Insulin stimulates glucose transport and metabolism in 3T3-L1 fat cells as in fat cells derived from adipose tissue (24, 34). Our data (Figs. 5 and 6) indicate that insulin's effect on cellular lipoprotein lipase is dependent on its effects on hexose uptake and metabolism. Incubation with glucose at concentrations between 0.5 and 5 mM for 24 h resulted in large responses to the hormone, whereas glucose at even high concentrations, up to 25 mM, had little effect on the activity of untreated cells. Treatment of cells with insulin for 20 h in the absence of glucose did not alter lipoprotein lipase activity, while addition of glucose to such cells evoked a rapid and full response in 2 h and a half-maximal increase in 30 min (Fig. 8). Thus, the rapid response observed in adipose tissue in vivo following injection of insulin may simply reflect the time course of insulin's effects on glucose transport into the intact tissue (35).

The presence of glucose was essential not only for induction of the response to insulin, but also for maintenance of activity in insulin-treated cells. Removal of either insulin or glucose resulted in a decrease of insulin-stimulated lipase activity with half-times much shorter than those required for enzyme induction (Figs. 4 and 7). This implies that expression of activity may depend on increased levels of glucose or its metabolites within the cells. In prolonged 24-h incubations support for the response was limited to hexoses which enter the glycolytic sequence, and whose transport and metabolism are increased by insulin (glucose, fructose, and mannose). In short term (i.e. 45 to 90 min) incubations of insulin-treated, carbohydrate-deprived cells, the same requirements prevailed, with the exception that glucosamine, which was inhibitory during prolonged experiments, was also effective (Tables I and III). Although the identity of the glucose metabolite(s) which could mediate this effect is not yet known, two speculative interpretations can be offered. Since a number of different sugar residues have been identified in lipoprotein lipase preparations obtained from various sources (36-38) it is possible that hexose is required as a structural component for synthesis of the enzyme or protein essential to its activity. Glycoprotein glucosamine is derived in animal cells primarily from fructose 6-phosphate with glucosamine 6-phosphate being the direct intermediate (39). As each of the hexoses which support the response are fructose phosphate precursors, formation of glucosamine and its incorporation into proteins may be rate limiting in carbohydrate-deprived cells. Alternatively, increased levels of this or other metabolites within the glycolytic sequence could enhance activity of newly synthesized proteins directly by processes analogous to those by which insulin-induced increases in glucose 6-phosphate levels appear to regulate glycogen synthetase activity in fat cells (40), or they could regulate activity indirectly by interacting with factors that control protein synthesis at the ribosomal level (41, 42). Since cycloheximide inhibited the response to glucose (Table IV), it seems most likely that the mechanism of this effect does involve new protein, possibly enzyme, synthesis. It is of interest in this regard that recent immunotitration studies by Jansen et al. (43) have shown that the increase in adipose tissue lipoprotein lipase which occurs in fed rats is due to increased enzyme protein. It will be of considerable interest to determine whether a similar mechanism exists in 3T3-L1 cells.

Ashby et al. (44) recently suggested that glucose may exert post-translational controls on the activity of lipoprotein lipase. They observed that addition of glucose to intact epidydimal fat bodies of fasted rats results in an increase in enzyme activity which is blocked by respiratory poisons, but not by cycloheximide. Their data suggest that full expression of the lipase response in the tissue may also depend on cellular energy balance. Insulin was reported to have no effect on the cycloheximide-insensitive response to glucose, suggesting that the origin of this effect is different from that observed in our studies of insulin-treated, carbohydrate-deprived cells (Fig. 8). In our experiments, glucose addition to carbohydrate-deprived cells or to glucose-supplemented cells had little effect on basal enzyme activity (Figs. 6 and 8). Thus, the phenomena they observed may not be involved in the regulation of lipoprotein lipase activity in the 3T3-L1 cell line.

In contrast to acute effects of insulin on various aspects of membrane and metabolic function, continuous exposure of adipose and other cells to insulin promotes a more delayed anabolic response characterized by increased synthesis of many different proteins. We reported earlier (14) that induction of the enzyme in differentiating 3T3-L1 cells requires prolonged treatment with insulin and noted that its appearance coincided with a large increase in protein content per cell. Others have shown that a variety of different enzymes is also increased by insulin during this period (45-47). These findings, together with the prolonged latent period and extended time course of the response in the mature cell (Fig. 2), suggest that the effect is not specific for induction of lipoprotein lipase, but rather, involves a generalized increase in proteins characteristic of the adipocyte phenotype. α-Amanitin, actinomycin D, puromycin, and cycloheximide blocked induction of this response (Fig. 10), indicating that these effects appear to be mediated through increased synthesis of both RNA and protein.

The cellular processes by which insulin might increase nuclear expression remain unknown (48). The present results indicate that the absence of glucose does not inhibit these effects. The insulin response occurring 2 h after glucose ad-

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**Fig. 11.** Schematic model for insulin regulation of lipoprotein lipase (LPL) in fat cells. Interaction of insulin (I) with its receptor-effector system (R-E) is postulated as having three major effects: 1) direct stimulation of enzyme release by an energy- and protein synthesis-independent mechanism which may involve decreased enzyme binding to the plasma membrane or the conversion of a large precursor to a more active form which is then released; 2) stimulation of gene expression which leads to increased enzyme formation by processes dependent on new RNA synthesis; and 3) stimulation of the hexose transport system (TS) and increased formation of regulatory glycolytic intermediates which are required for expression of the increase in enzyme activity (see text).
dition to cells preincubated with hormone for 20 h, but without carbohydrate, was equivalent to that in cells incubated with insulin plus glucose for 24 h (Fig. 8). Rosen et al. [24] reported that prolonged incubation of 3T3-L1 fat cells with insulin in the absence of glucose leads to an increase in Vmax of the hexose transport system when glucose was added, consistent with an increase in the number or activity of hexose transport units. Cycloheximide completely blocked this response and actinomycin D inhibited it approximately 50%. Both studies imply that effects of the hormone which involve increased RNA synthesis may not result simply from increased transport of substrate into the cells.

Fig. 11 presents a schematic model of our interpretation of present and previous findings concerning regulation of lipoprotein lipase in fat cells by insulin. Interaction of insulin with its receptors is considered to exert three major effects which affect enzyme activity. Stimulation of enzyme release appears to result from rapid alterations in enzyme binding to the plasma membrane, or perhaps from the “activation” and release of a less active precursor enzyme form (30). We suggest that this effect is energy-independent, does not require new protein synthesis, and is probably not dependent on function of cytoskeletal elements because it is unaffected by NaN3, 2,4-dinitrophenol, cycloheximide, colchicine, and cytochalasin E, and it does not require glucose.3 Stimulation of lipoprotein lipase activity in the cells is depicted as the result of interactions which initiate a generalized increase in nuclear expression, including genes coding for the enzyme and perhaps for other proteins which modify its activity. This effect apparently requires increased RNA synthesis and occurs in the absence of glucose or other carbohydrate substrates. We have recently observed that antibodies to human insulin receptors duplicate insulin’s stimulation of lipoprotein lipase in these cells (49). These findings indicate that cytoplasmic or membrane actions of the ligand-receptor complex itself may be sufficient to initiate this nuclear response, but the mechanism of this effect remains unknown. The results of the present study indicate that effective translation of these more primary nuclear events into a realized increase in enzyme activity depends upon insulin’s ability to increase glucose transport and thus increase the levels of as yet unidentified glycolytic intermediates that enhance protein synthesis in the cell.

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