Proc. Natl. Acad. Sci. USA
Vol. 78, No. 2, pp. 732–736, February 1981
Biochemistry

Hormone-sensitive lipase in differentiated 3T3-L1 cells and its activation by cyclic AMP-dependent protein kinase

(lipolysis)

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Communicated by Howard Green, October 6, 1980

ABSTRACT **Differentiation of 3T3-L1 fibroblasts to adipocyte-like cells was accompanied by a 19-fold increase in neutral triglyceride lipase activity, a 12-fold increase in diglyceride lipase activity, a 10-fold increase in monoglyceride lipase activity, and a 280-fold increase in cholesterol esterase activity. In contrast, acid acylhydrolase activities did not increase during differentiation. The rate of glycerol release from unstimulated intact cells increased by more than 1 order of magnitude upon differentiation. Isoproterenol (1 μM) and 1-methyl-3-isobutylxanthine (0.1 mM) further stimulated this rate of glycerol release 3-fold. The neutral triglyceride lipase activity in cell-free preparations of differentiated cells was activated 105% by cyclic AMP-dependent protein kinase. Neutral cholesterol esterase, diglyceride lipase, and monoglyceride lipase were also activated (117%, 10%, and 37%, respectively) by cyclic AMP-dependent protein kinase. In contrast, protein kinase had no effect on any of the four lysosomal acid acylhydrolase activities. Thus, hormone-sensitive lipase, the most characteristic and functionally important enzyme of adipose tissue, has been characterized in differentiated 3T3-L1 cells. The 3T3-L1 cell should be a valuable model system in which to study regulation of hormone-sensitive lipase, particularly its long-term regulation.**

3T3-L1 fibroblasts, a subclone of Swiss mouse embryo 3T3 fibroblasts isolated by Green and coworkers (1–4), are capable of differentiating into adipocyte-like cells engorged with cytoplasmic lipid droplets. The differentiation can be accelerated by treating 3T3-L1 fibroblasts with serum (4), insulin (2), biotin (5), or 1-methyl-3-isobutylxanthine (MIX) (6). Rubin *et al.* (7) found the combination of MIX and dexamethasone (DEX) to be highly effective. The accumulation of triglyceride is accompanied by a marked increase in the specific activities of several enzymes involved in fatty acid synthesis and triglyceride synthesis (2, 5, 7–10). There is also an increase in lipoprotein lipase which allows the cells to utilize efficiently the fatty acids from lipoproteins in the medium (11–15). The differentiated 3T3-L1 cells acquire hormone receptors for insulin (7, 16–18), β-adrenergic agonists, and adrenocorticotropic hormone (corticotropin) (19). The increase in levels of these enzymes and receptors, together with the accumulation of stored triglycerides, strongly supports the conclusion of Green and coworkers that the 3T3-L1 cell can serve as a model for studies of adipose tissue metabolism.

However, thus far there is no evidence for the expression of the most characteristic and functionally important enzyme of adipose tissue—hormone-sensitive lipase, the neutral lipase activated by cyclic AMP (cAMP)-dependent protein kinase that controls rates of lipid mobilization (20–25). This enzyme appears to be unique to the adipocyte. The only other tissue in which it has been reported is the adrenal (26, 27). Attempts in this laboratory to demonstrate it in rat heart, skeletal muscle, and liver under various conditions have been negative (unpublished results). Severson (28) reported negative results in rat heart. It has been shown that treatment with dibutyryl cAMP and epinephrine over a period of 2 weeks reduces the accumulation of lipid in 3T3-L1 cells (1), suggesting the presence of a hormone-sensitive lipase. However, the observed reduction in lipid accumulation could be due to inhibition of the biosynthesis of fatty acid and triglyceride.

The studies reported here demonstrate a striking increase (19-fold) in neutral triglyceride lipase during differentiation. Concurrently, basal rate of glycerol release increased by more than 1 order of magnitude and glycerol release was further stimulated 3-fold by isoproterenol. Finally, the neutral triglyceride lipase activity in differentiated cells was increased 2-fold by treatment with cAMP-dependent protein kinase.

MATERIALS AND METHODS

Materials. Labeled triolein, diolein, and monoolein (containing [1-^{14}C]oleic acid distributed randomly among the acylated positions) and cholesteryl [1-^{14}C]oleate were purchased from Rosechem (Los Angeles, CA). Dulbecco's modified Eagle's medium, fetal calf serum, penicillin, and streptomycin were obtained from Irvine Scientific (Santa Ana, CA). MIX was from Aldrich, isoproterenol hydrochloride was from Breon Laboratories (New York, NY), and sodium heparin was from Invenex (San Francisco, CA). DEX, nucleotides, glycerokinase, α-glycerophosphate dehydrogenase, cAMP-dependent protein kinase (bovine heart), and nonradioactive triolein, diolein, monoolein, and cholesteryl oleate were obtained from Sigma.

Heparin-Sepharose affinity gel was prepared according to the method of Iverius (29). Protein kinase inhibitor was purified from rabbit skeletal muscle through the DEAE-cellulose chromatography step by the method of Walsh *et al.* (30).

Cell Culture. The 3T3-L1 and 3T3-C2 fibroblasts from Swiss mouse embryo (generously provided by Howard Green, Massachusetts Institute of Technology, Cambridge, MA) were maintained in 150-mm culture dishes in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 50 units of penicillin and 50 μg of streptomycin per ml, and 2 mM glutamine. The experimental plates were seeded with 7×10^5 cells in 150-mm dishes and reached confluency in about 4 days. To initiate differentiation, the cells were treated with 0.25 μM DEX and 0.5 mM MIX for 2 days as described by Rubin *et al.* (7). After withdrawal of DEX and MIX, the cells were allowed to differentiate for an additional 4–6 days during which they developed the characteristic accumulation of multilocular fat drop-

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Abbreviations: MIX, 1-methyl-3-isobutylxanthine; DEX, dexamethasone; cAMP, adenosine 3′,5′-cyclic monophosphate.

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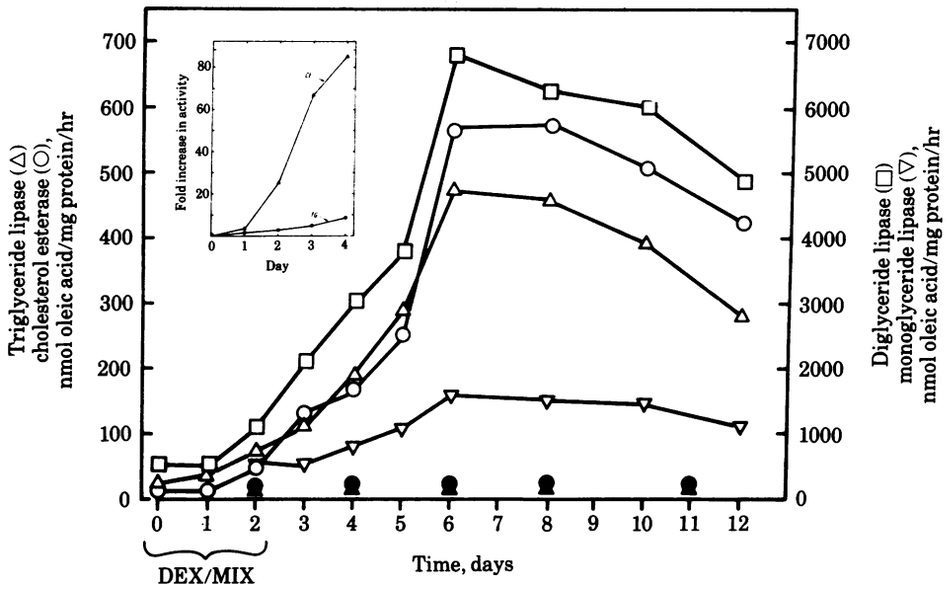

FIG. 1. Development of triglyceride lipase (△), diglyceride lipase (□), monoglyceride lipase (▽), and cholesterol esterase (○) activities in the S_{40} fraction of differentiating 3T3-L1 cells and of triglyceride lipase (▲) and cholesterol esterase (●) in 3T3-C2 cells. The assays were carried out with various substrate mixtures containing 50 mM phosphate buffer (pH 7) and incubated at 30°C for 30 min. (*Inset*) Triglyceride lipase (TG) and cholesterol esterase (CE) activities in the S_{40} fraction of 3T3-L1 cells during the first 4 days of differentiation.

lets. Unless otherwise indicated, the cultures were incubated with heparin (2 units/ml) for 1 hr before harvesting, to release lipoprotein lipase.

Preparation of Subcellular Fractions. Cells in monolayers were washed twice with Dulbecco's phosphate-buffered saline at pH 7.4 and once with 10 mM Tris·HCl/1 mM EDTA/0.25 M sucrose, pH 7.4 (medium I). Cells were scraped with a rubber policeman and centrifuged at 1000 × *g* for 1 min. The packed cells were homogenized in 2 vol of medium I and centrifuged at 40,000 × *g* for 1 hr. The supernatant fraction was designated the S_{40} fraction and the sedimented fraction was designated the P_{40} fraction. The P_{40} fraction was resuspended and homogenized in medium I. It was then subjected to freezing and thawing 10 times to disrupt lysosomes.

Activation of Acylhydrolases. The activation mixture (final volume, 0.1 ml) consisted of 5 mM magnesium acetate, 0.5 mM ATP, 0.01 mM cAMP, 1 mM EDTA, 25 mM Tris·HCl (pH 8), and S_{40} fraction. Activation was carried out for 5 min at 30°C. In control tubes, both ATP and cAMP were omitted.

Lipase Assays. Emulsions of tri[^{14}C]oleoylglycerol, di[^{14}C]oleoylglycerol, mono[^{14}C]oleoylglycerol, and cholesteryl [^{14}C]oleate were prepared as described by Khoo *et al.* (22). The substrate mixture contained 0.1 mM radioactive triolein, diolein, monoolein, or cholesteryl oleate, 50 mM sodium phosphate, bovine serum albumin at 5 mg/ml, and 2 mM EDTA at pH 7. Substrate mixture (0.7 ml) and enzyme (0.1 ml) were mixed and incubated at 30°C for 30 min. The reaction was stopped by the addition of 3 ml of a fatty acid extraction mixture consisting of chloroform/methanol/benzene, 1:2.4:2 (vol/vol). The [^{14}C]oleic acid was separated from unhydrolyzed esters by using liquid/liquid partition and assayed for radioactivity as described (31). One unit was defined as 1 nmol of oleic acid released per hr.

The apparent degree of activation of acylhydrolases by cAMP-dependent protein kinase was greater when the acylhydrolase assays were carried out in buffer of low ionic strength and at high pH. Therefore, in studies involving measurement of protein kinase activation the substrate mixture contained 50 mM Tris·HCl at pH 7.4 for triglyceride hydrolase and at pH 8 for cholesterol ester, diglyceride, and monoglyceride hydrolase assays. Acylhydrolase activities obtained under these conditions were about one-third of those found by using a buffer containing 50 mM phosphate at pH 7.

Protein was determined by the Coomassie blue dye-binding method of Bradford (32). Glycerol was measured by using a coupled enzyme reaction (glycerokinase plus α-glycerophosphate dehydrogenase) and determining the NADH formed fluorometrically (33).

RESULTS

Development of Triglyceride, Diglyceride, Monoglyceride, and Cholesterol Ester Hydrolase Activities in Differentiating 3T3-L1 Cells. Before differentiation, the triglyceride, diglyceride, monoglyceride, and cholesterol ester hydrolase activities in the S_{40} fraction assayed at pH 7 were 25, 548, 154, and 2 units/mg of protein, respectively. After treatment of confluent 3T3-L1 fibroblasts with 0.25 μM DEX and 0.5 mM MIX to pro-

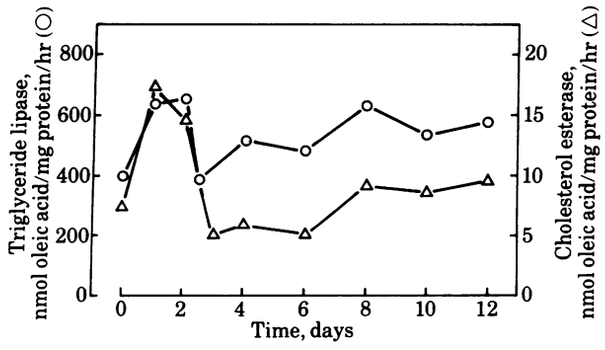

FIG. 2. Acid triglyceride lipase and cholesterol esterase activities in the P_{40} fraction of 3T3-L1 cells during differentiation. The particulate fractions were derived from the same homogenates used to prepare the S_{40} fractions shown in Fig. 1. Assays were carried out with substrate mixture containing 50 mM acetate buffer (pH 5) and incubated at 30°C for 30 min. Note difference in scales.

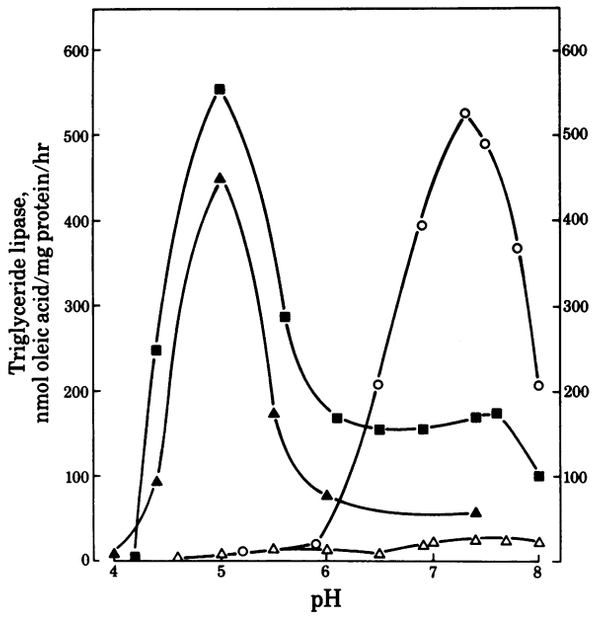

FIG. 3. Activity–pH profiles for triglyceride lipase of the supernatant (S_{40}) and particulate (P_{40}) fractions of 3T3-L1 cells before and after differentiation. △, ○, S_{40} before and after differentiation; ▲, ■, P_{40} before and after differentiation (4 days after exposure to DEX/MIX). Between pH 4 and pH 6, assays were carried out with substrate mixtures containing 50 mM acetate buffer; between pH 6.5 and 8, assays were carried out with substrate mixtures containing 50 mM phosphate buffer.

mote differentiation, all of these acylhydrolase activities increased markedly (Fig. 1). At day 6, triglyceride, diglyceride, monoglyceride, and cholesterol ester hydrolase activities were 475, 6750, 1600, and 560 units/mg protein, respectively, representing 19-fold, 12-fold, 10-fold, and 280-fold increases in activity. The rate of increase of cholesterol esterase activity was much greater than that of triglyceride lipase activity during the first 4 days of differentiation (Fig. 1 *Inset*). At day 4, the specific activity of cholesterol esterase had increased 86-fold but the specific activity of triglyceride lipase had increased only 8-fold. Diglyceride lipase and monoglyceride lipase activity had increased about 5-fold at day 4 (not shown). These high levels of activity were not fully sustained but declined by about 30% over the next 6 days.

Because differentiation is known to induce high levels of lipoprotein lipase in these cells (11–15), steps were taken to eliminate any contribution of that enzyme to the neutral acylhydrolase activities assayed. First, the cells were incubated with heparin (2 units/ml) for 1 hr prior to harvesting, to release lipoprotein lipase. Second, the S_{40} fraction was passed through a heparin-Sepharose affinity column to remove any residual lipoprotein lipase. Addition of serum or of purified apoprotein C-II as activator did not increase lipase activity in the S_{40} fraction, confirming the absence of significant level of lipoprotein lipase.

Parallel studies were done using 3T3-C2 cells, a subclone with a very low frequency of conversion to adipocyte-like form. The neutral triglyceride lipase and cholesterol esterase activities in the S_{40} fraction of 3T3-C2 cells were 11 and 7 units/mg of protein, respectively, and showed no measurable increase when the cells were subjected to the same treatment as 3T3-L1 cells (Fig. 1).

In contrast to the neutral acylhydrolase activities in the S_{40} fraction, which increased by at least 1 order of magnitude during differentiation, there was little or no change in acid acylhydrolase activities during differentiation (Fig. 2). Activities were assayed in the P_{40} fractions at pH 5 after freezing and thawing and presumably represent predominantly lysosomal hydrolase activities. As shown in Fig. 2, there was a transient increase in both acid triglyceride lipase and acid cholesterol esterase over the first day or two of differentiation, but both activities returned to their original levels over the next day or two. Acid cholesterol esterase activity then never exceeded the day 0 value; acid triglyceride lipase activity drifted upward but increased by no more than 50% above the day 0 value. The difference in scales in Fig. 2 should be noted; the absolute acid triglyceride lipase activity was much greater than that of acid cholesterol esterase (397 vs 7.3 units/mg of protein). There was no significant change in either acid diglyceride lipase or monoglyceride lipase activities in the P_{40} fractions during differentiation (data not shown).

Activity–pH Profiles of Triglyceride Lipase and Cholesterol Esterase Activities. Prior to differentiation the triglyceride lipase activity in the soluble fraction (S_{40}) was very low at all pH values (Fig. 3). The differentiated cells showed a 19-fold increase in soluble neutral triglyceride lipase activity (optimal at pH 7.4) whereas there was no change in soluble acid lipase activity. The triglyceride lipase activity in the P_{40} fraction prior to differentiation exhibited only a single peak of activity at pH 5. In the differentiated cells this activity increased by only about 25% (in contrast to the 19-fold increase in neutral lipase) and the major activity was still at pH 5. The small increase of activity in the P_{40} fraction assayed in the neutral region after differentiation may be due to some extent to the presence of soluble triglyceride lipase trapped in the pellet, which was not washed.

The cholesterol esterase activity in the soluble fraction (S_{40}) of differentiated cells showed a single optimum at pH 6.8 and there was virtually no activity at acidic pH values (Fig. 4). There appeared to be a significant amount of neutral cholesterol esterase activity in the P_{40} fraction as well, but again some of this may be due to cytosolic activity trapped in the pellet.

The level of cholesterol esterase activity in the undifferentiated cells was extremely low in both the S_{40} and P_{40} fractions. In fact, it was less than 5% of that found at neutral pH values. The little cholesterol esterase activity that was present in the P_{40} fraction prior to differentiation showed pH optima at both 5 and 7 (Fig. 4 *Inset*) and activities at these two pH values were about the same. This supports the interpretation that the apparent increase in neutral cholesterol esterase in the P_{40} fraction does not

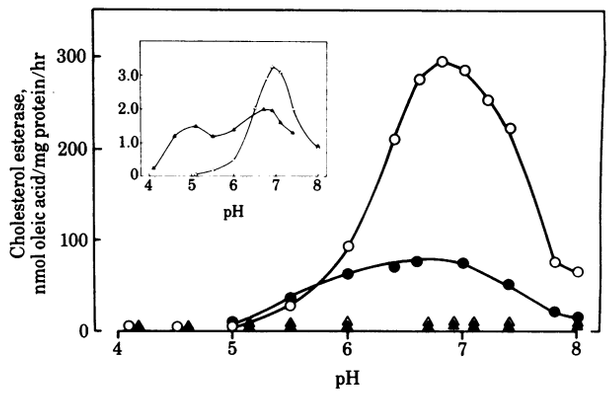

FIG. 4. Activity–pH profiles for cholesterol esterase activity of 3T3-L1 cells before and after differentiation. Conditions as described in Fig. 3. △, ○, S_{40} before and after differentiation; ▲, ●, P_{40} before and after differentiation. (*Inset*) Cholesterol esterase activity in S_{40} fraction (△) and P_{40} fraction (▲) of undifferentiated 3T3-L1 cells replotted with expanded scale to show pH optima.

represent induction of neutral cholesterol esterase in the particulate fraction but rather is due to some contamination from the large amount of activity present in the soluble fraction.

Glycerol Release from Intact Cells and Effects of Isoproterenol Stimulation. Basal and isoproterenol-stimulated glycerol release were measured in suspensions of 3T3-C2 cells, undifferentiated 3T3-L1 cells, and differentiated 3T3-L1 cells. The suspended cells were incubated in the absence or in the presence of 1 μM isoproterenol plus 0.1 mM MIX at 37°C under a gas phase of 95% O_2/5% CO_2. At time intervals, 0.5-ml aliquots of the cell suspensions were removed for measurement of glycerol. The release of glycerol from 3T3-C2 cells and undifferentiated 3T3-L1 cells was extremely low (Fig. 5). After differentiation, the basal glycerol release rate from 3T3-L1 cells was 36 nmol/mg of protein per hr (in contrast with the value of 2.6 nmol/mg of protein per hr prior to differentiation). In the presence of isoproterenol/MIX the glycerol release rate from the differentiated 3T3-L1 cells increased 3-fold (to 106 nmol/mg of protein per hr).

Activation of Acylhydrolase Activities in Cell-Free Preparations by cAMP-Dependent Protein Kinase. The S_{40} fraction of differentiated 3T3-L1 cells was incubated with Mg^{2+}, ATP, and cAMP for 5 min at 30°C. Both the neutral triglyceride lipase activity and the neutral cholesterol esterase activity were activated, by 105% and 117%, respectively (Fig. 6). When any one of the three cofactors was omitted, activation was abolished. The degree of activation in this relatively crude fraction was not further enhanced by addition of exogenous protein kinase, implying the presence of sufficient endogenous protein kinase to support maximum activation. (The level of cAMP-dependent protein kinase activity in 3T3-L1 cells did not increase during differentiation.)

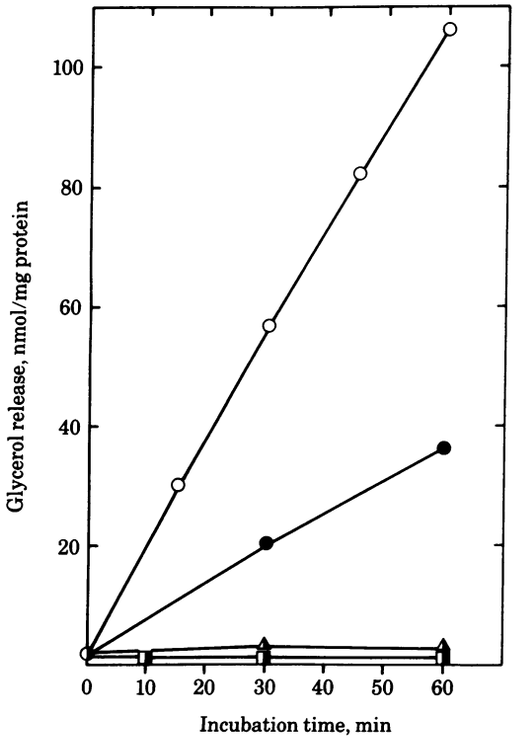

FIG. 5. Effect of isoproterenol and MIX on glycerol release from intact 3T3-L1 cells. Suspensions of differentiated 3T3-L1 cells, undifferentiated 3T3-L1 cells, and 3T3-C2 cells in Krebs–Ringer bicarbonate buffer (pH 7.4) containing 2% bovine serum albumin were incubated in the presence and in the absence of 1 μM isoproterenol/0.1 mM MIX at 37°C. Aliquots of cell suspensions (0.5 ml) were removed at time intervals and assayed for glycerol content. ○, ●, Differentiated 3T3-L1 cells in the presence (○) and in the absence (●) of isoproterenol and MIX; △, undifferentiated 3T3-L1 cells in the presence and in the absence of isoproterenol and MIX; ▯, 3T3-C2 cells in the presence and in the absence of isoproterenol and MIX.

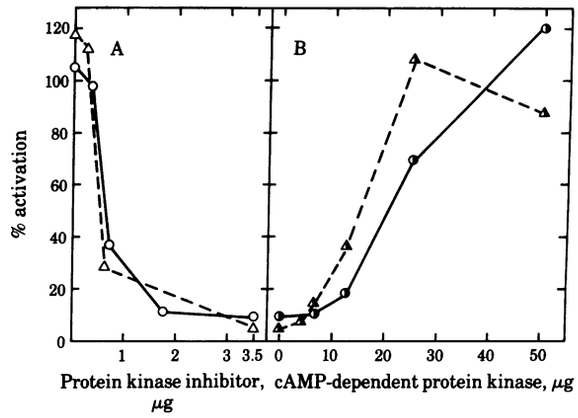

FIG. 6. Effects of protein kinase inhibitor and protein kinase on triglyceride lipase and cholesterol esterase activities. Aliquots of S_{40} fraction prepared from differentiated 3T3-L1 cells were incubated with MgATP and cAMP or with Mg^{2+} only in 25 mM Tris·HCl (pH 8) for 5 min at 30°C. Triglyceride lipase activity was assayed with a substrate mixture containing 50 mM Tris·HCl at pH 7.4. Cholesterol esterase activity was assayed with a substrate mixture containing 50 mM Tris·HCl at pH 8. Cells in this experiment were not incubated with heparin but the S_{40} fraction was treated with a heparin-Sepharose affinity gel to adsorb lipoprotein lipase. (*A*) Percentage activation of triglyceride lipase activity (○) and cholesterol esterase activity (△) with increasing concentrations of protein kinase inhibitor. (*B*) Percentage activation of triglyceride lipase activity (◑) and cholesterol esterase activity (▲) with increasing concentrations of purified protein kinase in the presence of a constant amount of protein kinase inhibitor (3.5 μg).

The addition of protein kinase inhibitor (Fig. 6*A*) blocked activation. This inhibition could be overcome (Fig. 6*B*) by adding back progressively increasing concentrations of purified protein kinase, thus demonstrating the dependence of the reaction on protein kinase.

Monoglyceride lipase activity in the S_{40} fraction of differentiated 3T3-L1 cells was also activated by cAMP-dependent protein kinase but only by about 40%. Diglyceride lipase activity showed little or no activation (less than 10%).

DISCUSSION

Hormonal stimulation of the release of fatty acids and glycerol from adipose tissue is correlated with an increase in tissue levels of cAMP and an increase in the activity of a neutral triglyceride lipase, hormone-sensitive lipase (20). This enzyme, partially purified from rat (23, 24) and chicken (25) adipose tissue, is phosphorylated/activated by cAMP-dependent protein kinase and dephosphorylated/deactivated by phosphoprotein phosphatases of various origins (34). Hormone-sensitive lipase has not been isolated or characterized from tissues other than adipose tissue [the adrenal is the only exception to date (26, 27)] and thus provides a unique marker for this cell type. The availability of a cultured cell system expressing hormone-sensitive lipase should facilitate further studies of this enzyme and its regulation, particularly its long-term regulation by hormones such as corticosteroids and growth hormone.

Differentiation of the 3T3-L1 cells was accompanied by a 19-fold increase in neutral triglyceride lipase activity. The undifferentiated cells contained only very low levels of neutral triglyceride lipase activity and basal rates of glycerol release were almost unmeasurable. After differentiation, the basal rate of

glycerol release increased by more than 1 order of magnitude. Isoproterenol stimulation increased glycerol release rate further by 3-fold. Differentiation was accompanied by only a very small increase in the acid triglyceride lipase activity in the particulate fraction (presumably referable to lysosomes). Thus, the increase in glycerol release rate and sensitivity to lipolytic hormone parallel the increase in neutral triglyceride lipase activity. Some investigators have suggested that hormone-stimulated lipolysis might be attributable to some extent to the activity of lysosomal triglyceride lipase (35) but the present studies give no support to that suggestion.

The cytosolic neutral triglyceride lipase activity in fully differentiated 3T3-L1 cells reached levels comparable to those found in adult mouse adipose tissue. The enzyme in 3T3-L1 cells was activated 2-fold by cAMP-dependent protein kinase; the enzyme in mouse adipose tissue was activated to a lesser extent (60–80%).

Differentiation was also accompanied by large increases in the activities of diglyceride and monoglyceride lipases. The diglyceride lipase activity in the differentiated cells was again close to that found in adult mouse adipose tissue, and the enzyme activity from both cell types was activated less than 10% by protein kinase. The level of monoglyceride lipase activity in differentiated 3T3-L1 cells was only 20% of that found in mouse adipose tissue. Unlike the enzyme in mouse adipose tissue and rat adipose tissue (36), it was activated about 40% by cAMP-dependent protein kinase. It should be noted that the absolute activities of diglyceride and monoglyceride lipases were considerably higher than the activity of triglyceride lipase, a pattern similar to that found in mouse and rat adipose tissue. Thus, it appears that the hydrolysis of the first ester bond in the triglyceride is generally rate-limiting in triglyceride mobilization.

Differentiation was also accompanied by a marked increase in neutral cholesterol esterase activity and this activity was increased about 2-fold by treatment with protein kinase. These findings parallel those previously reported in rat adipose tissue (37) and in chicken adipose tissue (22). As in adult adipose tissue, the level of neutral cholesterol esterase activity was much higher than that of the acid (lysosomal) cholesterol esterase activity (38).

The relationship among the four acylhydrolase activities studied here remains uncertain. In the course of recent studies on the purification of hormone-sensitive lipase from chicken adipose tissue, evidence was found that the monoglyceride lipase represents a distinct enzyme protein and some partial resolution of cholesterol esterase from triglyceride lipase was obtained (25). Further studies are needed to characterize these activities in 3T3-L1 cells.

We gratefully acknowledge the gift of 3T3-L1 and 3T3-C2 cells from Dr. Howard Green. This project was supported by the National Institutes of Health Grant HL 22053 and a grant from the American Diabetes Association. M.K. is the recipient of a fellowship from The Jikei University School of Medicine, Tokyo, Japan.

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