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Relationship between Hormone-Sensitive Lipolysis and Lipase Activity in Rat Fat Cells[1]

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An assay for total hormone-sensitive lipase (HSL) in rat fat cells was devised in which fat-associated HSL was solubilized with ether, and triolein or cholesteryloleate was used as substrate. Norepinephrine (NE) caused marked release of glycerol from fat cells but did not activate HSL as estimated using triolein or cholesteryloleate as substrate. Propranolol, a β-blocker, inhibited NE-induced lipolysis in fat cells without a concomitant reduction in HSL activity. The antilipolytic action of insulin on NE-induced lipolysis could not be explained by a decrease in HSL activity. Neither ACTH-induced lipolysis in fat cells nor its inhibition by insulin was accompanied by matching fluctuations in HSL activity. These results indicate that neither NE and ACTH-induced lipolysis in fat cells, nor the antilipolytic actions of propranolol and insulin, involve fluctuations in HSL activity.

Key words: fat cell, insulin, lipase, lipolysis, norepinephrine.

It is well known that lipolytic hormones such as catecholamines and ACTH stimulate lipolysis in fat cells and cause them to release glycerol and fatty acids. The mechanisms of action of these lipolytic hormones are believed to be mediated by the cAMP cascade. Lipolytic hormones activate adenylate cyclase, thus increasing cAMP formation (*1, 2*). cAMP then promotes lipolytic activity by activating cAMP-dependent protein kinase, which phosphorylates hormone-sensitive lipase (HSL) (*3*), resulting in the hydrolysis of stored triglyceride to glycerol and free fatty acids (FFA). Although the effect of the cAMP cascade on hormone-sensitive lipolysis is widely accepted, some contradictory results have been reported. In a previous study, we found that cAMP-dependent activation of HSL stimulated the lipolysis of [^{3}H]triolein emulsified with gum arabic, but not that of endogenous lipid droplets from rat fat cells (*4*). The lack of responsiveness of these endogenous lipid droplets to activated HSL was shown to be due to the presence of phosphatidylcholine in the droplets. These results suggested that hormone-induced lipolysis in fat cells was not mediated by the activation of HSL, or by phosphorylation of this enzyme.

In 1992, Egan *et al.* reported that Western blotting of adipocyte homogenate fractions with polyclonal antiserum raised againt HSL caused a shift of the enzyme from the supernatant of control cells to the floating fatty cake of lipolytically stimulated cells (*5*). Based on these findings, they proposed that the critical event in lipolytic activation of adipocytes is not an increase in HSL activity but a translocation of the lipase to its substrate at the surface of the lipid droplets.

Despite its key role in hormone-induced lipolysis in fat cells, little is known about HSL activity during the lipolytic stimulation of these cells. Lipolysis in fat cells is not only due to their HSL activity, which needs to be estimated with an artificial substrate such as triolein emulsified with gum arabic in the presence of the enzyme extracted from fat cells. In this system, lipolysis is not affected by factors such as the content and nature of the substrate, but solely by the activity of the enzyme. In contrast, total hormone-induced lipolysis in fat cells is affected by the nature of the lipid droplets.

In 1964, Vaughan *et al.* found that hydrolytic activity in a rat epididymal adipose tissue homogenate was increased by exposure of the tissue to catecholamines and ACTH for as little as 3 min prior to homogenization (*6*). Although they termed this hydrolysis HSL activity, it was not true HSL activity but rather hormone-sensitive lipolysis, because there was no evidence that the endogenous lipid droplets in the homogenate did not limit the lipolysis. Treatment of endogenous lipid droplets by sonication or with phospholipase C is known to cause increased hydrolytic activity in a cell-free system consisting of HSL and such lipid droplets (*7, 8*). These results indicate that the lipid droplets have a limiting effect on lipolysis in fat cells.

The present investigation was designed to elucidate the

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Abbreviations: HSL, hormone-sensitive lipase; cAMP, cyclic adenosine monophosphate; NE, norepinephrine; ACTH, adrenocorticotropic hormone; FFA, free fatty acids; A-kinase, cAMP-dependent protein kinase; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; BES, *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis.

relationship between hormone-sensitive lipolysis and lipase activity in rat fat cells.

MATERIALS AND METHODS

Animals—Young male Crj:Wistar rats, weighing between 150 and 200 g, were given a standard laboratory diet (Oriental Yeast) and water *ad libitum*. They were cared for in the Laboratory Animal Center at Ehime University, School of Medicine. They were killed by cervical dislocation and their epididymal adipose tissues were quickly removed.

Materials—Collagenase (type IV) was purchased from Worthington Biochemical (Freehold, NJ, USA). Norepinephrine was obtained from Sankyo (Tokyo) and ACTH from Daiichi Seiyaku (Tokyo). Propranolol was supplied by Nacalai Tesque (Kyoto). Triolein and insulin were purchased from Sigma (St. Louis, MO, USA). [^{3}H]Triolein (glycerol tri-[9,10-^{3}H]oleate) was obtained from Amersham Japan (Tokyo). TES, BES, and bovine serum albumin were purchased from Wako Pure Chemical Industries (Osaka). The albumin was extracted by the method of Chen (*9*) to remove free fatty acids.

Measurement of Lipolysis in Fat Cells by Glycerol Release—Isolated fat cells were obtained from rat epididymal adipose tissues by the method of Rodbell (*10*). The fat cells (200 μl packed volume) were incubated at 37°C in 500 μl of buffer A (25 mM TES, pH 7.4, containing 135 mM NaCl, 5 mM KCl, and 1 mM $MgCl_2$) supplemented with 2.5% bovine serum albumin and the test samples. After incubation, the reaction mixture was centrifuged at $100 \times g$ for 30 s to separate the medium from the fat cells. The medium and fat cells were used for lipolysis and lipase measurements, respectively. The glycerol content of the medium was estimated by the method of Warnick (*11*). Briefly, the medium was heated at 70°C for 10 min, then a 50-μl aliquot was incubated at 37°C for 5 min with 1 ml 0.1 M HEPES buffer (pH 7.5) containing 2 mM ATP, 0.5 mM 4-aminoantipyrine, 1 mM EDTA, 0.5 U glycerol kinase, 4 U glycerol-3-phosphate oxidase, 2 U peroxidase, 2.7 mM *p*-chlorophenol, 0.04% Triton X-100, and 2 mM $MgSO_4 \cdot 7H_2O$. The glycerol content was then determined from the absorption at 505 nm. Lipolysis was expressed as nmol glycerol released per ml of packed fat cells.

Measurement of HSL Activity in Fat Cells—Fat cells (200 μl packed volume) separated from the reaction mixture as described above were added to 450 μl of buffer B (50 mM Tris-HCl, pH 7.0, containing 250 mM sucrose, 1 mM EDTA, 2 μg/ml leupeptin, and 1 μM okadaic acid) in a plastic tube and agitated 20 times using a hand-held plastic pestle. The homogenate was centrifuged at $5,500 \times g$ for 10 min at 4°C. Then 100 μl of diethyl ether was placed onto the fat layer in the centrifuge tube, which was shaken for 3 s and centrifuged at $1,200 \times g$ for 5 min at 4°C. The upper ether layer was aspirated, and an aliquot of the supernatant was used for measuring HSL. As described later, the HSL activity in the fat layer was solubilized into buffer solution without loss of activity after ether treatment. Since there was no HSL activity in the precipitate, the supernatant, which included HSL extracted from the fat layer, was used as an enzyme solution for HSL assay (*5*). HSL activity was determined with [^{3}H]triolein as a substrate using a previously described procedure (*7*).

The [^{3}H]oleic acid released was measured by the method of Belfrage and Vaughan (*12*). Lipase activity was expressed as μmol of oleic acid released per ml of packed fat cells/h. The lipase activity was neither inhibited by 1 M NaCl nor activated by human serum, indicating that it did not include lipoprotein lipase activity. NaF reduced the lipase activity to 50% at 25 mM and to 30% at 100 mM, indicating that most of the lipase activity was not due to monoglyceride lipase but to HSL. Monoglyceride lipase is not inhibited by NaF (*13*).

In the present experiment, HSL activity was also estimated with cholesteryloleate as substrate using the method of Khoo *et al.* (*14*), because cholesterol esters are known to be more specific substrates for HSL than triolein (*15*). Incubation was carried out for 1 h at pH 6.8, and the [1-^{14}C]-oleic acid released was measured by the method of Belfrage and Vaughan (*12*). Lipase activity was expressed as μmol of oleic acid released per ml of packed fat cells/h.

Preparation of Antiserum against HSL and Western Blotting—HSL antiserum was raised in rabbits using a synthetic peptide, GPRLELRPRPQQAPRS, based on rat HSL (amino acid sequence from 326 to 341) (*16*). For Western blotting, proteins were separated by SDS-PAGE and transferred to PVDF membrane (BIO-RAD Laboratories, CA, USA). The membrane was blocked with 5% skin milk and incubated with the mono-specific antiserum prepared as described above. Immunoreactivity was visualized with alkaline phosphatase-conjugated goat anti-rabbit IgG (ICN Pharmaceuticals, OH, USA) and Fluorlmager (Amersham Pharmacia Biotech UK, Bucks., UK).

Analysis of Data—Statistical analysis was performed by one-way ANOVA. Student's *t* test was used to determine the significance of differences.

RESULTS

Fat cells (200 μl packed volume) were homogenized as described in "MATERIALS AND METHODS." The homogenate was centrifuged at $5,500 \times g$ for 10 min at 4°C and separated into fat layer, supernatant, and precipitate fractions. HSL activity was found in the supernatant fraction (0.87 ± 0.03 μmol/ml packed cells/h) when triolein was used as the substrate, whereas no HSL activity was detected in the precipitate fraction, as reported by Egan *et al.* (*5*).

The fat layer was transferred to a centrifuge tube. One portion of the fat layer was added to 450 μl of buffer B and centrifuged at $1,200 \times g$ for 5 min at 4°C. Then 100 μl of ether was placed onto the fat layer, and the tube was shaken for 3 s, then recentrifuged. The ether layer was then aspirated, and an aliquot of the supernatant was assayed for HSL with triolein as the substrate. Another fat layer prepared from the same fat cell fraction was homogenized with 450 μl of buffer B, and a 100-μl aliquot of the homogenate was incubated in 50 μl of BES buffer, pH 6.8, containing bovine serum albumin and 100 μl of water at 37°C for 1 h. After incubation, FFA release was estimated as an index of lipolytic activity.

As shown in Table I, almost identical triacylglycerol-hydrolyzing activities were found in the homogenized fat layer and the ether-treated fat layer with triolein as the substrate. The latter corresponded to HSL activity. These results suggest that the HSL activity in the fat layer was

solubilized into the buffer solution without loss of activity by the ether treatment. Since there was no HSL activity in the precipitate fraction, the aqueous extracts from the ether-treated fat layer and the supernatant were combined for assay of HSL.

As shown in Fig. 1, fat cells were incubated at 37°C for various times in the presence or absence of NE, and lipolysis was estimated by determining the amount of glycerol released into the medium. Fat cells were removed at the indicated times and their HSL activities were measured. Triolein or cholesteryloleate was used as a substrate for the HSL assay. NE induced an increase in glycerol release at each incubation time. The active lipase was found in fat cells before incubation and did not increase during incubation as estimated with either triolein or cholesteryloleate as substrate. Western blot analysis with antiserum against HSL clarified that NE did not affect the HSL protein content in fat cells (Fig. 2). These results clearly indicate that NE induced an increase in glycerol release (*i.e.* lipolysis) in fat cells without a concomitant elevation of HSL activity.

Propranolol is known to inhibit catecholamine-induced lipolysis (*17*). NE-induced glycerol release was markedly inhibited by 10^{-4} M propranolol, as shown in Fig. 3, whereas HSL activity measured with triolein as the substrate did not decrease at any incubation time. Cholesteryloleate-hydrolyzing activity also did not decrease at any incubation time (data not shown). This indicates that propranolol inhibited NE-induced lipolysis in fat cells without a concomitant reduction of HSL activity.

One of the most important metabolic actions of insulin is the inhibition of lipolytic activity in fat cells (*18*). Insulin inhibited NE-induced glycerol release in fat cells as shown in Fig. 4. However, it failed to reduce HSL activity measured with triolein as the substrate except after 1 h of incubation. A similar result was observed when cholesteryloleate was used as the substrate (data not shown). The insulin-induced reduction in HSL activity was, however, extremely low when compared with the decrease in hormone-induced glycerol release. These results suggest that insulin might exert its antilipolytic action on NE-induced lipolysis through a mechanism other than inhibition of HSL activity.

Table II shows the effect of ACTH on lipolysis and lipase activity. ACTH (0.1 μg/ml) was incubated with fat cells at 37°C for 1 h, and the glycerol released during incubation was estimated. ACTH-induced glycerol release was inhibited by insulin. ACTH did not activate HSL activity when triolein was used as the substrate. Although the HSL activity in the presence of ACTH was found to be slightly

TABLE I. **Triacylglycerol-hydrolyzing activities of the fat layer before and after ether treatment.**

Condition	Triacylglycerol-hydrolyzing activity (μmol/ml packed cells/h)
Homogenized fat layer	0.17±0.01
Ether-treated fat layer+triolein	0.18±0.02

The procedures are described in "MATERIALS AND METHODS." Values are means±SE of six separate experiments.

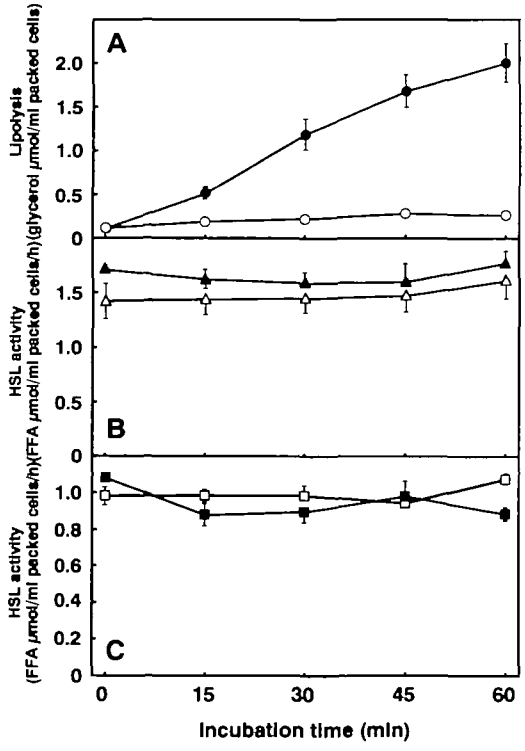

Fig. 1. **Relationship between norepinephrine-induced lipolysis and HSL activity in incubated fat cells.** The cells were incubated in the presence (●, ▲, ■) or absence (○, △, □) of NE (0.1 μg/ml). Lipolysis in the cells was estimated by determining the amount of glycerol released into the medium (A). HSL activity in fat cells was estimated using either triolein (B) or cholesteryloleate (C) as the substrate. Each point represents the mean±SE of six separate experiments.

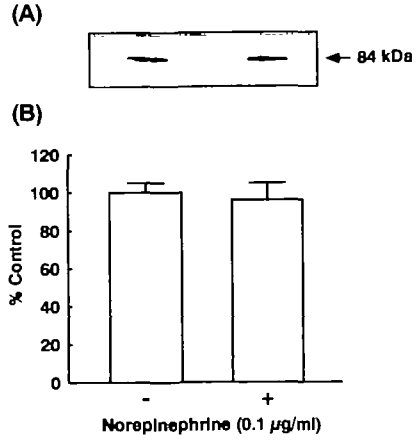

Fig. 2. **Western blotting of the aqueous extracts from fat cell homogenate with antiserum against HSL.** Fat cells were incubated for 1 h at 37°C in the presence or absence of NE (0.1 μg/ml). After incubation, the fat cells were subjected to homogenization and centrifugation as described in "MATERIALS AND METHODS." Then diethyl ether was placed onto the fat layer in the centrifuge tube. After aspirating the upper ether layer, an aliquot of the supernatant was used for Western blotting. The supernatant proteins were separated by SDS-PAGE in gels containing 8% acrylamide, a system that effectively separates polypeptide in the 84-kDa range. (A): Western blot of HSL protein in NE-treated cells and unstimulated cells. (B): HSL protein tabulated as a percentage relative to the density detected by enhanced chemiluminescence in unstimulated fat cells.

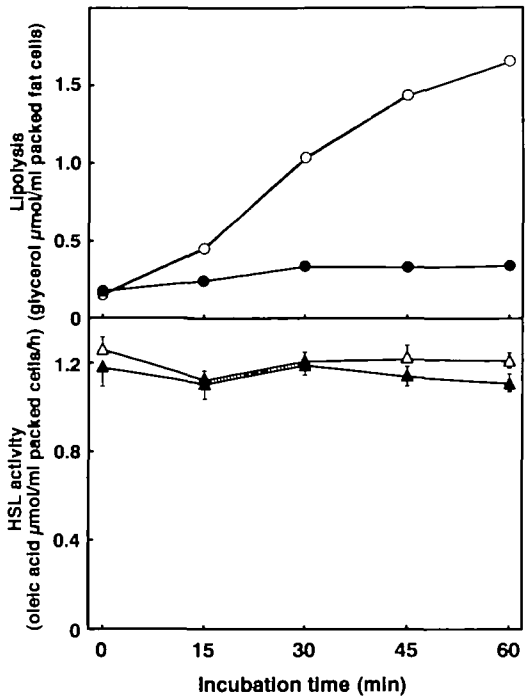

Fig. 3. **Relationship between norepinephrine-sensitive lipolysis and HSL activity: Effect of propranolol.** The lipolysis and lipase activity were examined in the presence of NE (0.1 μg/ml), and each fat cell fraction was incubated in the presence (●, ▲) or absence (○, △) of propranolol (10^{-4} M). Lipolysis was estimated by determination of glycerol release (○, ●), while HSL activity was estimated using triolein (△, ▲) as the substrate. Each point represents the mean ± SE of six separate experiments.

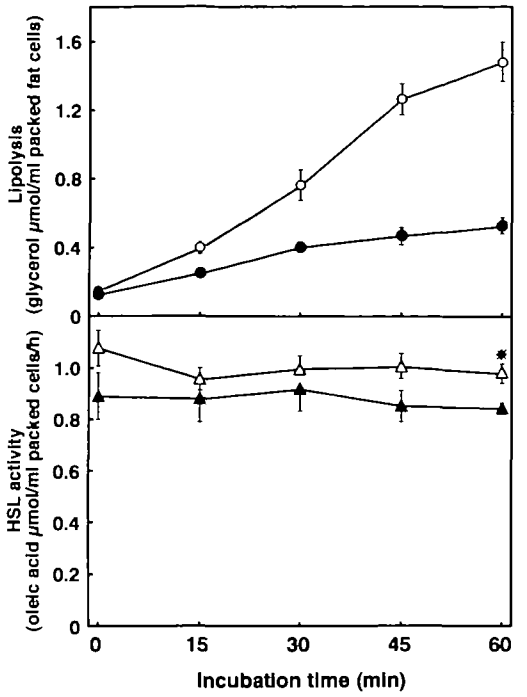

Fig. 4. **Relationship between norepinephrine-sensitive lipolysis and HSL activity: Effect of insulin.** The lipolysis and lipase activity were examined in the presence of NE (0.1 μg/ml), and each fat cell fraction was incubated in the presence (●, ▲) or absence (○, △) of insulin (10^{-8} M). Lipolysis was estimated by determination of glycerol release (○, ●), while HSL activity was estimated using triolein (△, ▲) as the substrate. Each point represents the mean ± SE of six separate experiments. *$p < 0.05$ *vs.* corresponding value with insulin.

reduced by insulin, the reduction in enzyme activity produced by the antilipolytic agent was very low when compared with its effect on glycerol release. When HSL activity was estimated with cholesteryloleate as the substrate, ACTH slightly increased the activity and insulin did not affect the HSL activity. The fluctuation in HSL activity did not, however, correspond to the lipolysis induced by ACTH or insulin. These experimental results suggest that ACTH-induced lipolysis and its inhibition by insulin might not be mediated by changes in HSL activity.

DISCUSSION

In 1964, Strand *et al.* first reported that ether treatment was highly effective in solubilizing the HSL activity associated with endogenous fat in adipose tissue homogenates (*19*).

In the present investigation, we used this technique to solubilize HSL activity from the fat layer formed after centrifugation of rat fat cell homogenate, and found that all the lipolytic activity of the fat layer was recovered in the buffer solution after ether treatment (Table I). Since there was no HSL activity in the precipitate, the combined supernatant obtained after centrifugation of the ether-treated fat layer and the supernatant were used as an HSL solution in the assay, with triolein or cholesteryloleate as the substrate. Although NE elicited marked glycerol release from rat fat cells, no rise in HSL activity could be found when estimated with either triolein or cholesteryloleate as the substrate (Fig. 1). Western blot analysis with antiserum against HSL proved that NE did not affect the HSL protein content in the fat cells (Fig. 2).

Vaughan *et al.* showed that the lipolytic activity of the whole homogenate of rat epididymal adipose tissue could be increased by prior incubation of the intact tissue with epinephrine or ACTH (*6*). They called this lipolytic activity hormone-sensitive lipase, because FFA production was a linear function of the amount of homogenate and did not depend on the exogenous substrate. Generally, lipolysis is affected by both lipase activity and substrate conditions such as substrate content, surface area, and surface character (*20*).

In 1992, Egan *et al.* found that HSL shifted quantitatively from the supernatant of control fat cells to the floating "fat cake" of lipolytically stimulated cells (*5*). They proposed that the endogenous lipid droplets in fat cells might be more than a passive participant in the lipolytic process.

Recently, we suggested that the surface physicochemical character of the endogenous lipid droplets plays an important role in the lipolytic process in fat cells, since sonication of endogenous lipid droplets from fat cells induced an increase in lipolysis in the presence of HSL (*8*). This increase in lipolysis was due not to an increase in the surface area resulting from sonication, but to a decrease in the phosphatidylcholine concentration on the surface of the lipid droplets. Presumably, such a change in the surface character promotes association of HSL with the lipid

droplets and causes an increase in lipolysis.

Therefore, at least two mechanisms can be postulated to explain the increase of lipolysis in adipose tissue homogenate produced by prior incubation of the intact tissue with epinephrine or ACTH, reported by Vaughan *et al.* (*6*). The first is direct activation of HSL by these hormones; and the second is a hormone-mediated change in the surface character of the endogenous lipid droplets, followed by increased association between HSL and the droplets. Since there is a possibility that both HSL and the endogenous lipid droplets may have participated in the lipolysis of the homogenate in the experiments of Vaughan *et al.* (*6*), this lipolytic activity cannot strictly be termed hormone-sensitive lipase activity. HSL activity is defined as lipolytic activity estimated under conditions in which only HSL is the limiting factor.

In the present investigation, we estimated the HSL activity of NE-treated and nontreated fat cells with exogenous substrates, and found that these were not limiting factors in the enzyme assay. We clarified that NE elicited glycerol release from fat cells without a concomitant elevation of HSL activity. Propranolol, a β-blocker, inhibited NE-induced lipolysis, but did not reduce HSL activity (Fig. 3). These results indicate that neither NE-induced lipolysis nor its inhibition by propranolol were mediated by HSL activity. As with propranolol, insulin inhibited glycerol release from fat cells without a concomitant decrease in HSL activity over a 45-min incubation period (Fig. 4).

Although a statistically significant reduction in HSL activity was caused by insulin after 60 min of incubation, a considerable discrepancy was found between the insulin-induced reductions in lipolysis and lipase activity. Insulin inhibited 70% of the lipolytic activity but only 13.7% of the HSL activity. Thus, the insulin-induced reduction in lipolytic activity could not be explained by a decrease in HSL activity. These results suggest that NE-induced lipolysis and its inhibition by propranolol or insulin might involve mechanisms other than a change in HSL activity. ACTH caused a 680% increase in glycerol release from fat cells, whereas HSL activity did not change when triolein was used as the substrate, and increased by only 14% when cholesteryloleate was used as the substrate (Table II).

Insulin inhibited 79% of the lipolytic activity in the presence of ACTH, whereas HSL activity did not change when cholesteryloleate was used as the substrate and decreased by only 14% with triolein as the substrate (Table II). From these results, it was concluded that neither ACTH-induced lipolysis nor its inhibition by insulin was accompanied by changes in HSL activity.

TABLE II. **Relationship between ACTH-sensitive lipolysis and lipase activity.**

Additions	Lipolysis (glycerol μmol/ml packed cells/h)	HSL activity	
		Triolein hydrolyzing (oleic acid μmol/ml packed cells/h)	Cholesteryloleate hydrolyzing
None	0.20 ± 0.01	1.24 ± 0.08	0.79 ± 0.03
ACTH	1.56 ± 0.06 *	1.38 ± 0.05 **	0.90 ± 0.02 **
ACTH+insulin (10^{-9} M)	0.32 ± 0.01 *	1.18 ± 0.06 **	0.92 ± 0.02

Lipolysis and lipase activity were examined in the presence of 1 μg/ml ACTH. HSL activity was estimated with either triolein or cholesteryloleate as the substrate. Values are means$\pm$SE of four separate experiments. *$p<0.01$, **$p<0.05$.

ACTH is well known to increase cAMP production in fat cells, whereas insulin decreases the levels of cyclic nucleotides (*21-23*). Increased cAMP levels in fat cells cause phosphorylation of HSL at its regulatory site through the action of cAMP-dependent protein kinase (*3*). Phosphorylation of HSL has been shown to stimulate the lipolysis of artificial substrate droplets which did not contain phosphatidylcholine, but not that of fat cell lipid droplets which were surrounded by a phosphatidylcholine monolayer (*4*). Therefore, it seems likely that the small degrees of activation or inhibition induced by ACTH or insulin may be due to the extent of HSL phosphorylation, because phosphatidylcholine-depleted artificial lipid droplets were used as substrates in the present investigation.

If fluctuations in lipolysis in fat cells are not due to HSL, they must be controlled by some other factor. In connection with this, we established a catecholamine-sensitive cell-free system consisting of lipid droplets and HSL isolated from fat cells, and found that the lipid droplets, especially their structure, may be closely related to the action of catecholamines (*8*). Further experiments are needed to confirm this hypothesis.

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