Relationships between lipolysis induced by various lipolytic agents and hormone-sensitive lipase in rat fat cells

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Abstract **Norepinephrine induced lipolysis in rat fat cells, in vitro, in a time- and concentration-dependent manner, without concomitantly increasing hormone-sensitive lipase (HSL) activity. It also induced, time and concentration dependently, HSL translocation from the cytosol to the lipid droplets in fat cells. Isoproterenol, forskolin, dibutyryl cyclic AMP, and theophylline also induced lipolysis in fat cells, but did not stimulate HSL activity. These agents also induced HSL translocation from the cytosol to the lipid droplets in fat cells: about 80% to 90% of all HSL was located in lipid droplets after incubation for 1 h. These results suggest that the critical event in lipolytic activation of fat cells induced by lipolytic agents is not an increase in the catalytic activity of HSL but translocation of HSL to its substrate on the surfaces of lipid droplets in fat cells.**—Morimoto, C., K. Kameda, T. Tsujita, and H. Okuda. **Relationships between lipolysis induced by various lipolytic agents and hormone-sensitive lipase in rat fat cells.** ***J. Lipid Res.*** **2001.** 42: **120–127.**

Supplementary key words HSL acivity • HSL translocation • endogenous lipid droplet

In higher animals, stored fat is mobilized by its hydrolysis to free fatty acids (FFA) and glycerol (1). The first step in this process is regulated by a variety of hormones, such as norepinephrine, epinephrine, and adrenocorticotropic hormone (ACTH). The mechanisms of action of these lipolytic hormones are believed to be mediated by the cAMP cascade. Lipolytic hormones activate adenylate cyclase, resulting in increased synthesis of cAMP, leading to activation of cAMP-dependent protein kinase and subsequent phosphorylation and activation of hormone-sensitive lipase (HSL), resulting in the hydrolysis of stored triacylglycerol to monoacylglycerol and FFA (2).

Although the effect of the cAMP cascade on hormone-sensitive lipolysis is widely accepted, there is a discrepancy between the magnitudes of the increase in lipolysis in fat cells in response to stimulation with lipolytic hormones and the increase in HSL activity after phosphorylation by cAMP-dependent protein kinase: lipolytic hormones stimulate lipolysis in rat fat cells up to 50-fold, whereas HSL activity increases 1.5- to 2-fold after phosphorylation by cAMP-dependent protein kinase. Furthermore, we found that cAMP-dependent phosphorylation of HSL stimulated lipolysis of trioleoylglycerol emulsified with gum arabic, but not that of endogenous lipid droplets from rat fat cells (3). We analyzed their constituents and found that the physicochemical natures of the endogenous lipid droplets and artificial lipid emulsions, such as trioleoylglycerol-gum arabic emulsion, were quite different, and that the phosphatidylcholine on the lipid droplet surfaces was a regulatory factor for lipolysis in fat cells (4, 5). The lack of responsiveness of these endogenous lipid droplets to phosphorylated HSL was shown to be due to the presence of phosphatidylcholine in the droplets. These results suggest the existence of another lipolytic mechanism in fat cells.

In 1992, Egan et al. (6) demonstrated that in lipolytically stimulated fat cells, the location of HSL in cellular homogenates was shifted from the supernatant to the fat cake as compared with its location in unstimulated cells. Brasaemle et al. (7) reported that, using immunofluorescence microscopy, HSL is observed to translocate from the cytosol to the surface of lipid storage droplets in lipolytically stimulated 3T3-L1 adipocytes. Therefore, the translocation of HSL from the cytosol to its substrate on the surfaces of lipid droplets in fat cells is a candidate for another lipolytic mechanism. In this study we investigated, in detail, the translocation of HSL in fat cells in response to various lipolytic agents [norepinephrine, isoproterenol, forskolin, dibutyryl-cAMP (DBcAMP), and theophylline] and compared it with hormone-induced lipolysis and HSL activity in these cells.

Abbreviations: DBcAMP, dibutyryl cyclic AMP; FFA, free fatty acids; HSL, hormone-sensitive lipase; PVDF, polyvinylidene difluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TES, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid.

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MATERIALS AND METHODS

Animals

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

Materials

Collagenase (type IV) was purchased from Worthington Biochemical (Freehold, NJ). Norepinephrine was obtained from Sankyo (Tokyo, Japan). Trioleoylglycerol, forskolin, and DBcAMP were purchased from Sigma (St. Louis, MO). [^{3}H]Trioleoylglycerol (glycerol tri-[9,10-^{3}H]oleate) was obtained from Amersham Japan (Tokyo, Japan). [*oleate*-1-^{14}C]cholesteryloleate was obtained from NEN Life Science Products (Boston, MA). *dl*-Isoproterenol, theophylline, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), *N*-tris(hydroxymethyl)methyl-2-aminoehtanesulfonic acid (TES), and bovine serum albumin (BSA) were purchased from Wako Pure Chemical Industries (Osaka, Japan). The albumin was extracted by the method of Chen (8) to remove the FFA.

Measurement of lipolysis in fat cells by glycerol release (method 1)

Isolated fat cells were obtained from rat epididymal adipose tissues by the method of Rodbell (9). The fat cells (200-μl packed volume) were incubated at 37°C for the indicated times 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% (w/v) BSA and lipolytic agents (norepinephrine, isoproterenol, forskolin, DBcAMP, or theophylline). After incubation, the reaction mixture was centrifuged at 100 *g* at room temperature for 30 s to separate the medium and fat cells. The glycerol content of the medium was estimated by the method of Warnick (10). Briefly, the medium was heated at 70°C for 10 min, and a 50-μl aliquot was incubated with 1 ml of 100 mM HEPES buffer (pH 7.5) containing 2 mM ATP, 500 μM 4-aminoantipyrine, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.5 U of glycerol kinase, 4 U of glycerol-3-phosphate oxidase, 2 U of peroxidase, 2.7 mM *p*-chlorophenol, 0.04% (w/v) Triton X-100, and 2 mM $MgSO_4 \cdot 7H_2O$ at 37°C for 5 min. The glycerol content was then determined from the absorption at 505 nm. Lipolysis was expressed as micromoles of glycerol released per milliliter of packed fat cells per hour.

Measurement of HSL activity in fat cells (method 2)

The fat cells (200-μl packed volume) were incubated at 37°C for the indicated times in 500 μl of buffer A supplemented with 2.5% (w/v) BSA and lipolytic agents (norepinephrine, isoproterenol, forskolin, DBcAMP, or theophylline). After incubation, the reaction mixture was centrifuged at 100 *g* at room temperature for 30 s to separate the medium and fat cells. The fat cells (200-μl packed volume) were added to 450 μl of buffer B [25 mM Tris-HCl, pH 7.4, containing 254 mM sucrose, 1 mM EDTA, 100 μM benzamidine, 20 μM leupeptin, soybean trypsin inhibitor (2 mg/ml) and 1 μM okadaic acid] in a plastic tube and agitated 20 times with a handheld plastic pestle. The homogenate was centrifuged at 5,500 *g* for 10 min at 4°C and 100 μl of diethyl ether was added to the fat layer at the top of the centrifuge tube, which was shaken for 3 s and centrifuged at 1,200 *g* for 5 min at 4°C. After adding ether, the HSL activity in the fat layer dissolved readily and completely in the supernatant solution (11). As the precipitate contained no HSL activity, the supernatant, which contained HSL extracted from the fat layer, was used as the enzyme solution for assaying the HSL activity in the cells. The HSL activity in the supernatant was determined with [^{3}H]trioleoylglycerol or [1-^{14}C]cholesteryloleate as a substrate, using a previously described procedure (4) and the method of Khoo et al. (12), respectively. Cholesteryl esters are known to be more specific substrates for HSL than trioleoylglycerol (13). The amount of [^{3}H]- or [1-^{14}C]oleic acid released was measured by the method of Belfrage and Vaughan (14). Lipase activity was expressed as micromoles of oleic acid released per milliliter of packed fat cells per hour. The lipase activity was neither inhibited by 1 M NaCl nor activated by human serum, indicating that it did not contain lipoprotein lipase activity. NaF, at 25 and 100 mM, reduced the lipase activity by 50% and 70%, respectively, indicating that most of the lipase activity was due to HSL, not monoacylglycerol lipase, because the latter is not inhibited by NaF (2).

Preparation of an anti-HSL antiserum and Western blotting

An anti-HSL antiserum was raised in rabbits with a synthetic peptide, GPRLELRPRPQQAPRS, derived from rat HSL (amino acids 326–341) (15). For Western blotting, the proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA), which was blocked with 5% (w/v) skim milk and incubated with the monospecific antiserum. The immunoreactive products were visualized with alkaline phosphatase-conjugated goat anti-rabbit IgG and attophos (ICN Pharmaceuticals, Plainview, NY) and the enhanced chemifluorescence intensity was determined with a FluorImager fluorescence imaging analyzer (Amersham Pharmacia Biotech UK, Bucks, UK).

Localization of HSL in fat cells

Fat cells (200-μl packed volume) were separated from the reaction mixture, as described above, added to 450 μl of buffer B in a plastic tube and agitated 20 times with a handheld plastic pestle. After centrifugation at 5,500 *g* for 10 min at 4°C, the supernatant and fat layer were suspended in Laemmli sample buffer (16) containing 1% and 20% (w/v) SDS, respectively (6), and an aliquot of each suspension (10 to 15 μl) corresponding to 200 μl of packed fat cells was subjected to SDS-PAGE (16).

Analysis of data

The significance of differences between mean ± SE values was determined by performing Fisher's protected least significant difference test, using Super-ANOVA software (Abacus Concepts, Berkeley, CA). Differences at $P < 0.05$ were considered significant.

RESULTS

Fat cells were incubated in the presence of norepinephrine at 37°C for various times and lipolysis in fat cells was estimated by determining the amount of glycerol released into the medium as shown in **Fig. 1A**. Norepinephrine increased the amount of glycerol released after each incubation period. Estimation of the HSL protein levels of fat cells by Western blotting with the anti-HSL antiserum showed that the total HSL protein content of fat cells did not change significantly after each incubation time (data not shown). Figure 1B shows representative immunoblots of HSL in supernatant and fat layer in norepinephrine-

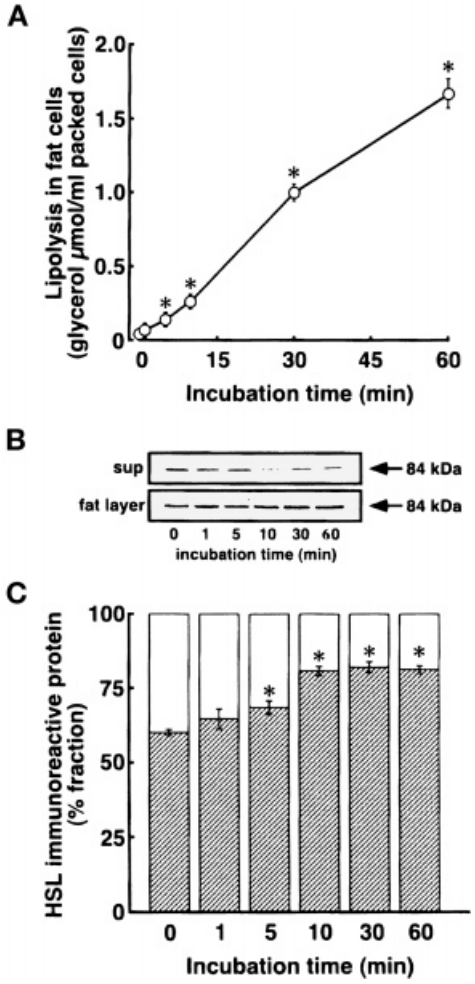

Fig. 1. Time course of norepinephrine-induced lipolysis and localization of HSL in rat fat cells. Fat cells were incubated with norepinephrine (1 μg/ml) at 37°C for various times. After incubation, each reaction mixture was centrifuged to separate the medium and fat cells, which were used to assay lipolysis and localize HSL, respectively. A: Lipolysis in fat cells was estimated by glycerol release as described in Materials and Methods (method 1). Each point represents the mean ± SE of four separate experiments; * $P < 0.01$ versus values after incubation for 0 min. B: Fat cells were homogenized and centrifuged as described in Materials and Methods and the proteins were separated by SDS-PAGE, using gels containing 8% acrylamide. Presented is a representative immunoblot showing the HSL protein levels of norepinephrine-treated cells. C: HSL immunoreactive protein levels of the supernatant (open column) and fat layer (shaded column) tabulated as a percentage fraction of the density detected by enhanced chemifluorescence. Each column represents the mean ± SE of four separate experiments; * $P < 0.01$ versus values after incubation for 0 min.

stimulated cells. Norepinephrine increased the amount of HSL protein in the fat layer in a time-dependent manner and concomitantly reduced that in the supernatant (Fig. 1C). About 60% of the total HSL protein was present in the fat layers of unstimulated cells (incubation time 0) and about 5%, 9%, and 20% of the total HSL was translocated from the supernatant to the fat layer after stimulation with norepinephrine for 1, 5, and 10 min, respectively. However, the HSL protein content of the fat layer did not increase much further after incubation for more than 10 min: 21% of the total HSL was translocated to the fat layer after incubation for 60 min.

Figure 2 shows the effect of time on the HSL activity in norepinephrine-stimulated fat cells. Fat cells were removed at the indicated times, homogenized, treated with ether, and centrifuged, and the HSL activity of each supernatant was measured with trioleoylglycerol as the substrate. The HSL activity in the fat layer was solubilized into the supernatant solution without loss of activity after this treatment (11). The HSL activity in unstimulated fat cells (incubation time 0) was high and did not increase during incubation. A similar result was obtained when cholesteryloleate was used as the substrate (data not shown).

Norepinephrine stimulated glycerol release by fat cells in a concentration-dependent manner (**Fig. 3A**). Figure 3B shows representative immunoblots of HSL in supernatant and fat layer in norepinephrine-stimulated cells. Norepinephrine increased the amount of HSL protein in the fat layer in a concentration-dependent manner and concomitantly reduced that in the supernatant (Fig. 3C). The HSL activity in the fat cells, measured with trioleoylglycerol as the substrate, was not affected by the norepinephrine concentration (data not shown). The total HSL protein content of the fat cells was also not affected significantly by the norepinephrine concentration (data not shown).

Incubation with other lipolytic agents, that is, isoproterenol, forskolin, DBcAMP, or theophylline, elicited marked glycerol release by fat cells in a time-dependent manner and the patterns of this release resembled that induced by norepinephrine (**Figs. 4A, 5A, 6A,** and **7A**, respectively). Representative immunoblots of HSL in supernatant and fat layer in these agent-stimulated cells are shown in Figs.

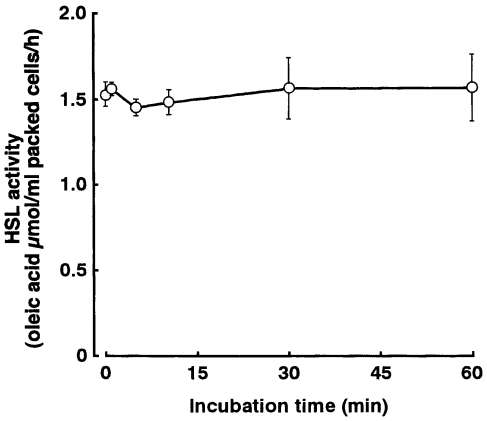

Fig. 2. Effects of norepinephrine on HSL activity in rat fat cells. Fat cells were incubated with norepinephrine (1 μg/ml) at 37°C for various times. After incubation, each reaction mixture was centrifuged to separate the medium and fat cells, and the cellular HSL activity was estimated as described in Materials and Methods (method 2). The fat cells were homogenized and centrifuged, and diethyl ether was added to the fat layer at the top of the centrifuge tube. After adding ether, the HSL activity in the fat layer dissolved readily and completely in the supernatant solution (11). The supernatant, which contained HSL extracted from the fat layer, was used as the enzyme solution for assaying the HSL activity in the cells. HSL activity was estimated using trioleoylglycerol as a substrate. Each point represents the mean ± SE of four separate experiments.

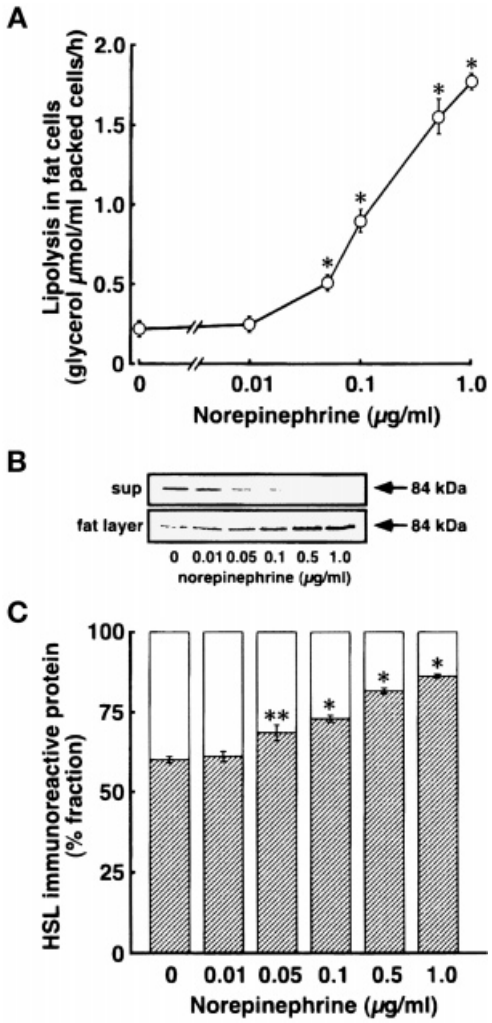

Fig. 3. Effects of norepinephrine on lipolysis and localization of HSL in rat fat cells. Fat cells were incubated with various concentrations of norepinephrine at 37°C for 1 h. A: Lipolysis in fat cells was estimated by glycerol release as described in Materials and Methods (method 1). Each point represents the mean ± SE of four separate experiments; * $P < 0.01$ versus values in the absence of norepinephrine. B: Representative immunoblot showing the HSL protein levels of norepinephrine-treated cells. C: HSL immunoreactive protein in the supernatant (open column) and fat layer (shaded column) tabulated as a percentage fraction of the density. Each column represents the mean ± SE of four separate experiments; * $P < 0.01$ and ** $P < 0.05$ versus values in the absence of norepinephrine. See legend to Fig. 1 for more detail.

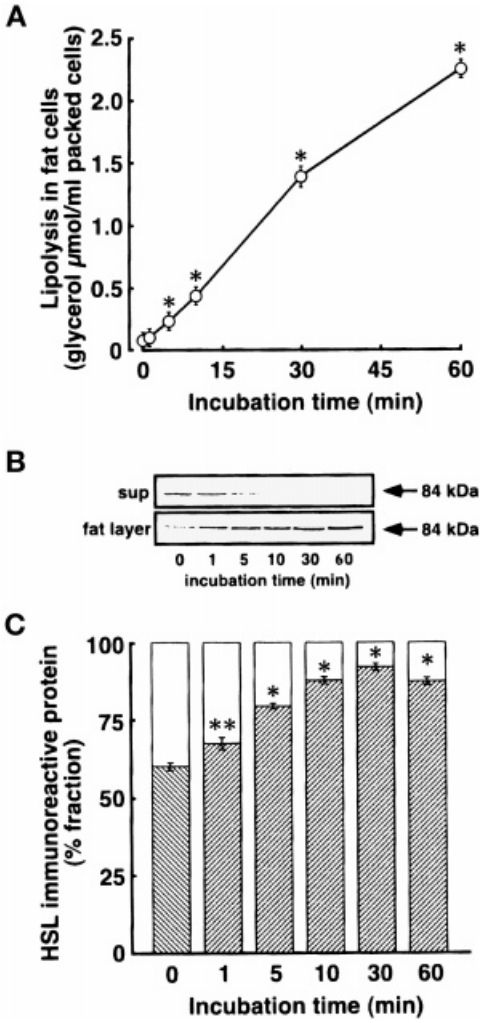

Fig. 4. Time course of isoproterenol-induced lipolysis and localization of HSL in rat fat cells. Fat cells were incubated with 1 μM isoproterenol at 37°C for various times. A: Lipolysis in fat cells was estimated by glycerol release as described in Materials and Methods (method 1). Each point represents the mean ± SE of four separate experiments; * $P < 0.01$ versus values after incubation for 0 min. B: Representative immunoblot showing the HSL protein levels of isoproterenol-treated cells. C: HSL immunoreactive protein levels of the supernatant (open column) and fat layer (shaded column) tabulated as a percentage fraction of the density. Each column represents the mean ± SE of four separate experiments; * $P < 0.01$ and ** $P < 0.05$ versus values after incubation for 0 min. See legend to Fig. 1 for more detail.

4B, 5B, 6B, and 7B, respectively. These agents also induced translocation of HSL from the supernatant to the fat layer in a time-dependent manner (Figs. 4C, 5C, 6C, and 7C, respectively). However, their HSL translocation patterns differed. The isoproterenol-stimulated and norepinephrine-stimulated HSL translocation patterns were similar: HSL translocation reached saturation after incubation for 10 min and the HSL protein levels of the fat layers did not increase with further incubation (Fig. 4C). Forskolin did not stimulate translocation significantly after incubation for 5 min, but did so thereafter for up to 30 min (Fig. 5C). DBcAMP and theophylline stimulated translocation linearly up to 60 min (Figs. 6C and 7C). When HSL activity in fat cells was estimated with trioleoylglycerol or cholesteryloleate as the substrate after incubation with various lipolytic agents for 60 min, appreciable levels of HSL activity were present in fat cells in the absence of lipolytic agents and did not change significantly during lipolytic agent-mediated stimulation of lipolysis in fat cells (**Table 1**).

DISCUSSION

It is difficult to determine the exact HSL activity in fat cells because the fat layers formed after centrifugation of fat cell homogenates contain large amounts of HSL.

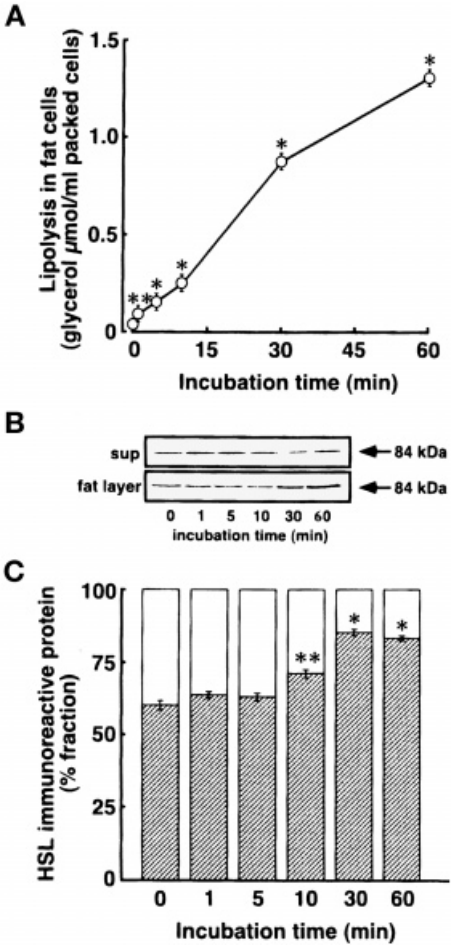

Fig. 5. Time course of forskolin-induced lipolysis and localization of HSL in rat fat cells. Fat cells were incubated with 10 μM forskolin at 37°C for various times. A: Lipolysis in fat cells was estimated by glycerol release as described in Materials and Methods (method 1). Each point represents the mean ± SE of four separate experiments; * $P < 0.01$ versus values after incubation for 0 min. B: Representative immunoblot showing the HSL protein levels of forskolin-treated cells. C: HSL immunoreactive protein levels of the supernatant (open column) and fat layer (shaded column) tabulated as a percentage fraction of the density. Each column represents the mean ± SE of four separate experiments; * $P < 0.01$ and ** $P < 0.05$ versus values after incubation for 0 min. See legend to Fig. 1 for more detail.

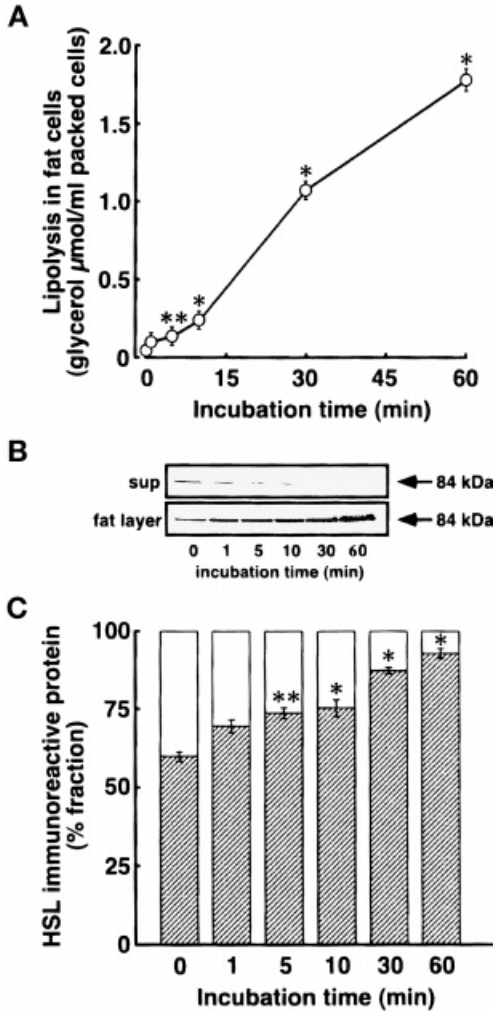

Fig. 6. Time course of DBcAMP-induced lipolysis and localization of HSL in rat fat cells. Fat cells were incubated with 1 mM DBcAMP at 37°C for various times. A: Lipolysis in fat cells was estimated by glycerol release as described in Materials and Methods (method 1). Each point represents the mean ± SE of four separate experiments; * $P < 0.01$ and ** $P < 0.05$ versus values after incubation for 0 min. B: Representative immunoblot showing the HSL protein levels of DBcAMP-treated cells. C: HSL immunoreactive protein levels of the supernatant (open column) and fat layer (shaded column) tabulated as a percentage fraction of the density. Each column represents the mean ± SE of four separate experiments; * $P < 0.01$ and ** $P < 0.05$ versus values after incubation for 0 min. See legend to Fig. 1 for more detail.

Therefore, we first tried to solubilize fat-associated HSL from the fat layer, using ether treatment, as described by Strand, Vaughan, and Steinberg (17), and found that all the lipolytic activity in the fat layer was recovered in the buffer solution after this treatment (11). As there was no HSL activity in the precipitate, the supernatant, which contained HSL extracted from the fat layer, was used as the HSL solution for the assay. Although norepinephrine elicited marked glycerol release from rat fat cells, the HSL activity did not change significantly during norepinephrine-mediated stimulation of lipolysis in fat cells (Figs. 1 and 2). Furthermore, norepinephrine did not affect the HSL protein content of the fat cells. Similar results were observed with four other lipolytic agents: isoproterenol, forskolin, DBcAMP, and theophylline (Table 1). These results provide evidence that lipolytic agents induce lipolysis in fat cells without activating the catalytic activity of HSL, and conflict with the cAMP cascade theory.

Some investigators reported that lipolytic hormone stimulated the HSL activity (18, 19). However, they confused "lipolysis in fat cells" (glycerol or FFA release from fat cells) with "HSL activity" (HSL catalytic activity). The lipolysis in fat cells is estimated with FFA or glycerol released by incubating intact fat cells with lipolytic agents such as norepinephrine. Therefore, lipolysis in fat cells reflects not only their HSL catalytic activity but also other factors such as the translocation of HSL to the endogenous lipid droplets. On the other hand, the HSL activity is

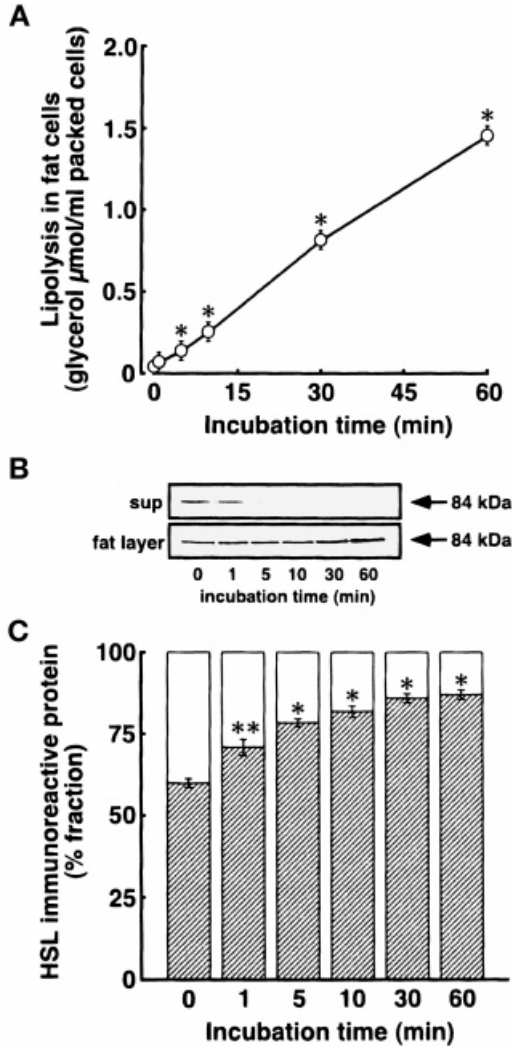

Fig. 7. Time course of theophylline-induced lipolysis and localization of HSL in rat fat cells. Fat cells were incubated with 1 mM theophylline at 37°C for various times. A: Lipolysis in fat cells was estimated by glycerol release as described in Materials and Methods (method 1). Each point represents the mean ± SE of four separate experiments; * $P < 0.01$ versus values after incubation for 0 min. B: Representative immunoblot showing the HSL protein levels of theophylline-treated cells. C: HSL immunoreactive protein levels of the supernatant (open column) and fat layer (shaded column) tabulated as a percentage fraction of the density. Each column represents the mean ± SE of four separate experiments; * $P < 0.01$ and ** $P < 0.05$ versus values after incubation for 0 min. See legend to Fig. 1 for more detail.

estimated with an artificial substrate such as trioleoylglycerol emulsified with gum arabic in the presence of the enzyme (HSL) extracted from fat cells. In this system, the activity is determined under similar substrate conditions and may be affected solely by the catalytic power of the enzyme. Although Berger and Barnard (18) and Slavin, Ong, and Kern (19) suggested that catecholamines stimulated HSL activity in fat cells, they did not estimate the catalytic activity of HSL but determined catecholamine-stimulated lipolysis in fat cells by measuring glycerol release from fat cells. In this article, various lipolytic agents such as norepinephrine, isoproterenol, forskolin, DBcAMP, and theophylline stimulated lipolysis in fat cells (Figs. 3, 4, 5, 6, and 7, respectively), but did not stimulate HSL activity (Table 1).

TABLE 1. Effects of various lipolytic agents on HSL activity in rat fat cells

Lipolytic Agents	HSL Activity	
	[^{3}H]Trioleoylglycerol	[1-^{14}C]Cholesteryloleate
	oleic acid μmol/ml packed cells/h	
Basal (no addition)	1.50 ± 0.04	0.75 ± 0.04
Norepinephrine (1 μM)	1.49 ± 0.02	0.78 ± 0.06
Isoproterenol (1 μM)	1.43 ± 0.05	0.73 ± 0.08
Forskolin (10 μM)	1.50 ± 0.10	0.73 ± 0.03
DBcAMP (1 mM)	1.48 ± 0.04	0.68 ± 0.04
Theophylline (1 mM)	1.48 ± 0.06	0.77 ± 0.08

Fat cells were incubated with various lipolytic agents at 37°C for 1 h. After incubation, each reaction mixture was centrifuged to separate the medium and fat cells, and the fat cells were homogenized. The homogenate was centrifuged and diethyl ether was added to the fat layer at the top of the centrifuge tube. After adding ether, the HSL activity in the fat layer dissolved readily and completely in the supernatant solution (11). The supernatant, which contained HSL extracted from the fat layer, was used as the enzyme solution for assaying the HSL activity in the cells. HSL activity was estimated by using either trioleoylglycerol or cholesteryloleate as the substrate as described in Measurement of HSL Activity in Fat Cells (Method 2). Basal HSL activity was estimated with fat cells incubated without lipolytic agent. Values represent mean ± SE of four separate experiments.

Previously, we reported that HSL activity was stimulated by the addition of cAMP and cAMP-dependent protein kinase, using trioleoylglycerol emulsified with gum arabic as a substrate (3). Therefore, our assay system of HSL activity can detect the activation of HSL by its phosphorylation. It seems likely that the lipolytic agents in the present investigation fail to stimulate HSL activity because of the reduced rate of phosphorylation as compared with the previous experiments (3). We reported that isoproterenol was found to stimulate lipolysis in the cell-free system without an increase in the phosphorylation of HSL, suggesting that isoproterenol-induced lipolysis may not be mediated through the phosphorylation and activation of HSL (20). Further experiments are needed to clarify this possibility.

Egan et al. (6) found that during incubation at 37°C for 5 min, HSL shifted quantitatively from the supernatant of control cells to the fat layer of lipolytically stimulated cells: nearly all the HSL was present in the supernatant fraction of centrifuged homogenates of unstimulated fat cells, whereas nearly all the HSL was associated with the fat layer of homogenates of isoproterenol-treated cells. However, we could not reproduce these data (data not shown). In our study, we found large amounts of HSL protein (60% of the total) in the fat layers of centrifuged homogenates of unstimulated cells (Figs. 1, 3, 4, 5, 6, and 7), but in spite of these large amounts of HSL, lipolysis was not observed in these fat cells. There are three possible explanations for this phenomenon. One is experimental artifact: HSL protein may have moved from the supernatant (cytosol) to the fat layer during homogenization and centrifugation of the fat cells. The second is the purity of the isolated fat cake: membranes of endoplasmic reticulum and other organelles intimately associated with the cellular lipid droplets might contaminate the lipid fat cake.

The third is that two types of HSL binding, catalytic and noncatalytic, to the lipid surfaces occurred. Tsujita, Muderkwa, and Brockman (21) prepared films of mixtures of dioleoylglycerol and phosphatidylcholine, exposed them to various lipases, and found that the extent of dioleoylglycerol hydrolysis was less than 5% when the molar fraction of dioleoylglycerol was 0.5, and increased abruptly to 95% when the molar fraction was 0.6. When the molar fraction was 0.5, a considerable amount of catalytically active enzyme was adsorbed by the substrate film, but dioleoylglycerol hydrolysis did not occur. These workers showed that this "switching" phenomenon was related not to the enzyme concentration, but to the ratio of dioleoylglycerol to phosphatidylcholine in the mixed lipid film. In previous studies, we demonstrated that the phosphatidylcholine of the endogenous lipid droplets played an important role in the lipolytic process (4, 5). Clifford et al. (22) reported that there was no HSL translocation but a movement of perilipin away from the lipid droplets on lipolytic stimulation in fat cells from mature rats. These data suggest that the catalytically active HSL that binds to the lipid droplets does not always hydrolyze triacylglycerols.

Norepinephrine rapidly induced HSL translocation from the cytosol to the lipid droplets in fat cells. This translocation was observed after stimulation with norepinephrine for 1 min and almost reached saturation within 10 min (Fig. 1), whereas lipolysis in fat cells increased slightly to about 15% of that after 60 min, within 10 min of stimulation with this hormone (Fig. 1A). Similar results were observed when isoproterenol was used as a lipolytic hormone (Fig. 5). These results suggest that HSL translocation may occur before lipolysis starts in catecholamine-stimulated fat cells, but does not occur after the lipolytic products, such as fatty acids and glycerol, have accumulated.

The patterns of HSL translocation induced by forskolin, DBcAMP, and theophylline differed from those of the catecholamines, norepinephrine and isoproterenol. Forskolin did not significantly stimulate translocation after incubation for 5 min (Fig. 5C), whereas DBcAMP and theophylline stimulated translocation linearly for up to 30 min (Figs. 6C and 7C). The HSL activities did not change significantly during stimulation of lipolysis in fat cells by these five agents (Table 1). Although the HSL translocation patterns differed, our results suggest that the lipolytic agents we tested did not induce lipolysis in fat cells through activation of the catalytic activity of HSL.

It is widely accepted that lipolytic hormones such as catecholamine, ACTH, and glucagon cause an activation of cAMP-dependent protein kinase that phosphorylates and activates HSL (23, 24). The lipolytic action of various lipolytic agents such as forskolin, DBcAMP, and theophylline, is also believed to be mediated by the cAMP cascade (25–27). However, in a previous study, we demonstrated that the mechanism(s) of the lipolytic action of these agents (forskolin, DBcAMP, and theophylline) may be different from that of the cAMP-mediated pathway and, therefore, another lipolytic mechanism may exist. We reported that the cAMP content of fat cells and a cell-free system consisting of HSL and endogenous lipid droplets did not correlate with forskolin-induced lipolysis (28). Okuda et al. (29) reported that when fat cells were incubated with [^{3}H]cAMP and [^{3}H]DBcAMP, these agents accumulated in the cells in approximately equal concentrations and DBcAMP was not hydrolyzed to cAMP in the cells. However, lipolysis in fat cells was increased by DBcAMP but not by cAMP. Miyoshi et al. (30) reported that theophylline-induced lipolysis in fat cells did not accompany activation of HSL activity. Our results in the present study suggest that these lipolytic agents elicit lipolysis in fat cells by stimulating HSL translocation from the cytosol to the lipid droplets in fat cells.

This raises questions concerning the mechanism responsible for HSL translocation induced by these lipolytic agents. Holm et al. (31) postulated that HSL may undergo a conformational change as a result of cAMP-protein kinase phosphorylation and that this change may play an important role in HSL translocation. Londos et al. (32) hypothesized that modifications of a lipid droplet surface protein (perilipin) were importantly involved in the lipolytic process. Clifford et al. (22, 33) reported that lipolysis in fat cells was closely related to phosphorylation of perilipin, which was tightly associated with the surfaces of lipid droplets in fat cells, and suggested that nonphosphorylated perilipin provided a barrier against hydrolysis by HSL, and that this barrier was removed when perilipin was phosphorylated by cAMP-dependent protein kinase. Shen et al. (34) suggested an additional mechanism for the control of HSL activity in the fat cells via oligomerization. Syu and Saltiel (35) reported that lipotransin, which is an HSL-interacting protein, might direct the hormonally regulated redistribution of HSL. Furthermore, Osuga et al. (36) reported the possibility of another hormone-regulated lipase in fat cells, using HSL knockout mice. These data suggest that the mechanisms regulating lipolysis in fat cells cannot explain only the activation of HSL catalytic activity by the phosphorylation.

In conclusion, we have demonstrated clearly that various lipolytic agents do not increase the catalytic activity (i.e., turnover rate) of HSL when lipolytic activation in fat cells occurs. Our data also suggest that HSL protein translocation from the cytosol to the lipid droplets and changes in the physicochemical character of the endogenous lipid droplet surfaces may play important roles in the lipolysis in fat cells induced by these lipolytic agents. Experiments are now in progress to elucidate the mechanism(s) responsible for the translocation of HSL from the cytosol to the lipid droplets in fat cells mediated by these lipolytic agents.

This work was supported by research grants from Fuji-Bio Co. Ltd. (Shizuoka, Japan).

Manuscript received 28 October 1999, in revised form 11 February 2000, in re-revised form 5 June 2000, and in re-re-revised form 25 September 2000.

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