

Mechanism of the stimulatory action of okadaic acid on lipolysis in rat fat cells

Chie Morimoto,^{*,§} Atsuko Kiyama,^{**} Kenji Kameda,[†] Hiroyuki Ninomiya,^{*} Takahiro Tsujita,[†] and Hiromichi Okuda^{1,*}

2nd Department of Medical Biochemistry* and Central Research Laboratory,[†] School of Medicine, Ehime University, Shigenobu-cho, Onsen-gun, Ehime 791-0295; Department of Chemistry,[§] Faculty of Science, Ehime University, Matsuyama, Ehime 790-8577; and Department of Human and Natural Environment Sciences,^{**} Faculty of Integrated Arts and Sciences, Tokushima University, Minamijousanjima-cho, Tokushima 770-8502, Japan

Abstract Okadaic acid was found to induce concentration- and time-dependent lipolysis in rat fat cells in the absence of lipolytic hormones, but it did not significantly increase the total hormone-sensitive lipase (HSL) activity in these fat cells, the activity of HSL extracted from fat layer and that of HSL in the supernatant of homogenized fat cells. Western blotting of fat cell homogenate fractions with an antiserum raised against synthetic peptide derived from rat HSL showed that HSL protein shifted from the supernatant to the fat layer in response to okadaic acid, which increased the HSL protein content on the fat layer and concomitantly reduced that of the supernatant, concentration- and time-dependently. Sonication of the fat cells abolished their responsiveness to okadaic acid. The lipolytic action of okadaic acid was examined and its site was identified using a cell-free system comprising lipid droplets isolated from rat fat cells and HSL. Okadaic acid induced lipolysis in this cell-free system and sonication of the lipid droplets caused disappearance of lipolytic action of okadaic acid. Okadaic acid failed to stimulate lipolysis in a cell-free system comprising HSL and artificial lipid droplets (trioleoylglycerol emulsified with gum arabic) instead of lipid droplets isolated from rat fat cells. These results suggest that okadaic acid does not increase the catalytic activity of HSL but induces translocation of HSL to the lipid droplets isolated from rat fat cells. The site of the lipolytic action of okadaic acid in relation to the interaction between HSL and lipid droplet is discussed.—Morimoto, C., A. Kiyama, K. Kameda, H. Ninomiya, T. Tsujita, and H. Okuda. **Mechanism of the stimulatory action of okadaic acid on lipolysis in rat fat cells.** *J. Lipid Res.* 2000. 41: 199–204.

Supplementary key words okadaic acid • lipolysis • HSL activity • fat cell • endogenous lipid droplet

Okadaic acid, a polyether fatty acid isolated from the black sponge *Halichondria okadai*, is a potent inhibitor of both type-1 and type-2 phosphatases (1, 2). It has been a useful tool for studying the biological significance of serine/threonine protein phosphorylation due to its ability to activate S_6 kinase and the mitogen-activated protein kinases (3–

6) and to inhibit phosphatidylinositol 3'-kinase-mediated tyrosine kinase-dependent signal transduction. (7, 8).

In 1997, Sekar et al. (9) were the first to report that okadaic acid activated lipolysis in rat fat cells in the absence of lipolytic hormones. Generally, the mechanisms of action of lipolytic hormones such as catecholamines and ACTH are believed to be mediated by the cAMP cascade: lipolytic hormones activate adenylate cyclase, thereby increasing cAMP formation (10, 11). Then cAMP promotes lipolytic activity by activating cAMP-dependent protein kinase, which phosphorylates HSL (12), resulting in the hydrolysis of stored triacylglycerol by HSL to fatty acids and monoacylglycerol and subsequent hydrolysis of monoacylglycerol by monoacylglycerol lipase to FFA and glycerol.

In a previous study, we isolated endogenous lipid droplets from rat fat cells and analyzed their constituents. We found that the physicochemical nature of the endogenous lipid droplets was quite different from that of artificial lipid droplets such as trioyleoylglycerol–gum arabic emulsion and demonstrated that the phosphatidylcholine on the surfaces of the lipid droplets was a factor that regulated lipolysis in fat cells (13). In this study, we focused on the substrates, endogenous lipid droplets, and investigated the mechanism of the lipolytic action of okadaic acid using rat fat cells and a cell-free system comprising lipid droplets from these fat cells and HSL.

MATERIALS AND METHODS

Animals

Young male Crj:Wistar rats, weighing between 150 to 200 g, were given a standard laboratory diet (Oriental Yeast Co. Ltd) and

Abbreviations: FFA, free fatty acids; HSL, hormone-sensitive lipase; TES, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; PSL, photo-stimulated luminescence.

¹ To whom correspondence should be addressed.

water ad libitum, and cared for in the Laboratory Animal Center at Ehime University, School of Medicine. They were killed by cervical dislocation to minimize endogenous catecholamine secretion and their epididymal adipose tissues were quickly removed.

Materials

Collagenase (type IV) was purchased from Worthington Biochemical Corp. (Freehold, NJ). Trioleoylglycerol was purchased from Sigma (St. Louis, MO). [³H]trioleoylglycerol (glycerol tri-[9, 10-³H]oleate) was obtained from Amersham Japan (Tokyo, Japan). [³²P]orthophosphoric acid was obtained from New England Nuclear Life Science Products (Boston, MA). Okadaic acid, TES, and bovine serum albumin were purchased from Wako Pure Chemical Industries (Osaka, Japan). The albumin was extracted by the method of Chen (14) to remove FFA.

Measurement of lipolysis in fat cells by glycerol release

Isolated fat cells were obtained from rat epididymal adipose tissue by the method of Rodbell (15). The fat cells (200 μ l packed volume) were incubated at 37°C in 450 μ l of buffer A (25 mM TES, pH 7.4, containing 135 mM NaCl, 5 mM KCl, and 1 mM MgCl₂) supplemented with 2.5% (w/v) bovine serum albumin and 50 μ l okadaic acid solution. The final concentration of okadaic acid was 10.0, 1.0, or 0.1 μ M. After incubation, the reaction mixture was centrifuged at 100 *g* for 30 sec to separate the medium from the fat cells. The medium and fat cells were used to assay lipolysis and lipase activity, respectively. The glycerol content of the medium was estimated by the method of Warnick (16). Briefly, the medium was heated at 70°C for 10 min, then a 50- μ l aliquot was incubated at 37°C for 5 min with 1 ml 100 mM HEPES buffer (pH 7.5) containing 2 mM ATP, 500 μ M 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% (w/v) Triton X-100, and 2 mM MgSO₄·7H₂O. The glycerol content was then determined from the absorption at 505 nm. Lipolysis was expressed as nmol glycerol released per ml packed fat cells per h.

Measurement of lipolysis in fat cells by FFA release

The fat cell fraction was divided into two portions. One portion (50 μ l packed volume) was incubated for 1 h at 37°C in 225 μ l buffer A supplemented with 2.5% (w/v) bovine serum albumin and 25 μ l of okadaic acid solution to produce a final okadaic acid concentration of 10.0, 1.0, or 0.1 μ M. The other portion (50 μ l packed volume) was suspended in 125 μ l buffer A supplemented with 2.5% (w/v) bovine serum albumin and 2.26 mg gum arabic. After sonication of the mixture for 5 min, 100 μ l of buffer A containing 2.5% (w/v) albumin was added. The mixture was incubated for 1 h at 37°C with 25 μ l of okadaic acid solution to produce a final concentration as described above. The amount of FFA released was measured as described previously (17) and lipolysis was expressed as microequivalents (μ Eq) of FFA per ml packed fat cells per h.

Measurement of HSL activity in fat cells

Fat cells (200 μ l packed volume) were separated from the reaction mixture, as described above, added to 450 μ l buffer B (50 mM Tris-HCl, pH 7.0, containing 250 mM sucrose, 1 mM EDTA, 2 μ g leupeptin, and various concentrations of okadaic acid equivalent to those used during the incubation period) in a plastic tube and agitated 20 times using a hand-held plastic pestle. The homogenate was centrifuged at 5,500 *g* for 10 min at 4°C, 100 μ l diethyl ether was added to the fat layer at the top of the centrifuge tube, which was shaken for 3 sec and centrifuged at 1,200 *g* for 5 min at 4°C. The HSL activity in the fat layer dissolved readily and completely in the buffer solution after ether was added to the fat layer (18). As the precipitate contained no HSL activity, the supernatant, which

contained HSL extracted from the fat layer, was used as an enzyme solution to assay the HSL activity in the cells. The HSL activity in the supernatant was determined using [³H]trioleoylglycerol as a substrate, as described elsewhere (19) and the amount of [³H]oleic acid released was measured by the method of Belfrage and Vaughan (20). Lipase activity was expressed as μ mol oleic acid released per ml packed fat cells per 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 at 25 and 100 mM reduced the lipase activity to 50% and to 30%, respectively, indicating that most of the lipase activity was due to HSL, not monoacylglycerol lipase, because the latter is not inhibited by NaF (21).

Measurement of [³²P]HSL in fat cells

The amount of phosphorylated HSL in fat cells was measured by the method of Nilsson et al. (22). Briefly, fat cells (50 μ l packed volume) were incubated with 190 μ l buffer A supplemented with 3.5% (w/v) bovine serum albumin and 5 μ l carrier-free [³²P]orthophosphoric acid (4.63 MBq/ml final concentration) at 37°C for 40 min; 5 μ l okadaic acid solution was added and incubation was continued for 5 min. The final concentration of okadaic acid was 10.0, 1.0, or 0.1 μ M. The fat cells were separated from the incubation medium by centrifugation at 10,000 *g* for 5 sec with dinonylphthalate and then placed in a tube containing a 70- μ l aliquot of solution comprising 3% (w/v) SDS, 100 mM 2-mercaptoethanol, 50 mM NaPi, 10 mM EDTA, 10 mM NaF, and 10% (v/v) ethanol, pH 8.5, in which they dissolved in <15 sec. The cell proteins (100 to 150 μ g) were precipitated in ice-cold acetone, extracted with organic solvents, and dissolved in 100 μ l Laemmli sample solution (23) by repeated heating to 96°C for 1.5 min, sonication for 10 min, and vortex mixing for 30 sec.

SDS-PAGE with 8% acrylamide gel was performed (23) as described previously, each gel was dried and the amount of radiolabeled proteins were determined directly using a BAS-3000, Bio-Imaging Analyzer (Fuji Film, Tokyo, Japan). The intensity of the light is represented as photo-stimulated luminescence (PSL).

Preparation of an anti-HSL antiserum and Western blotting

An anti-HSL antiserum was raised in rabbits using a synthetic peptide, GPRLELRPRPQQAPRS, derived from rat HSL (amino acid sequences from 326 to 341) (24). For Western blotting, the proteins were separated by SDS-PAGE, transferred to a PVDF membrane (Bio-Rad Laboratories, CA), which was blocked with 5% (w/v) skim milk and incubated with the mono-specific antiserum. Immunoreactivity was visualized with alkaline phosphatase-conjugated goat anti-rabbit IgG and attophos (ICN Pharmaceuticals, Inc., OH), and the enhanced chemifluorescence intensity was determined using a FluorImager, Fluorescence Imaging Analyzer (Amersham Pharmacia Biotech UK Ltd, Bucks, U.K.).

Localization of HSL in fat cells

Fat cells (200 μ l packed volume) were separated from the reaction mixture, as described above, added to 450 μ l buffer C (25 mM Tris-HCl, pH 7.4, containing 254 mM sucrose, 1 mM EDTA, 100 μ M benzamidine, 20 μ M leupeptin, 2 mg/ml soybean trypsin inhibitor, and various concentrations of okadaic acid equivalent to those used during the incubation period) in a plastic tube and agitated 20 times using a hand-held 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 containing 1 and 20% (w/v) SDS, respectively (25), and an aliquot of each suspension (10 to 15 μ l) corresponding to 200 μ l packed fat cells was subjected to SDS-PAGE (23).

Measurement of lipolysis in a cell-free system comprising HSL and endogenous lipid droplets

Endogenous lipid droplets were prepared from rat fat cells and HSL solution was obtained from rat epididymal adipose

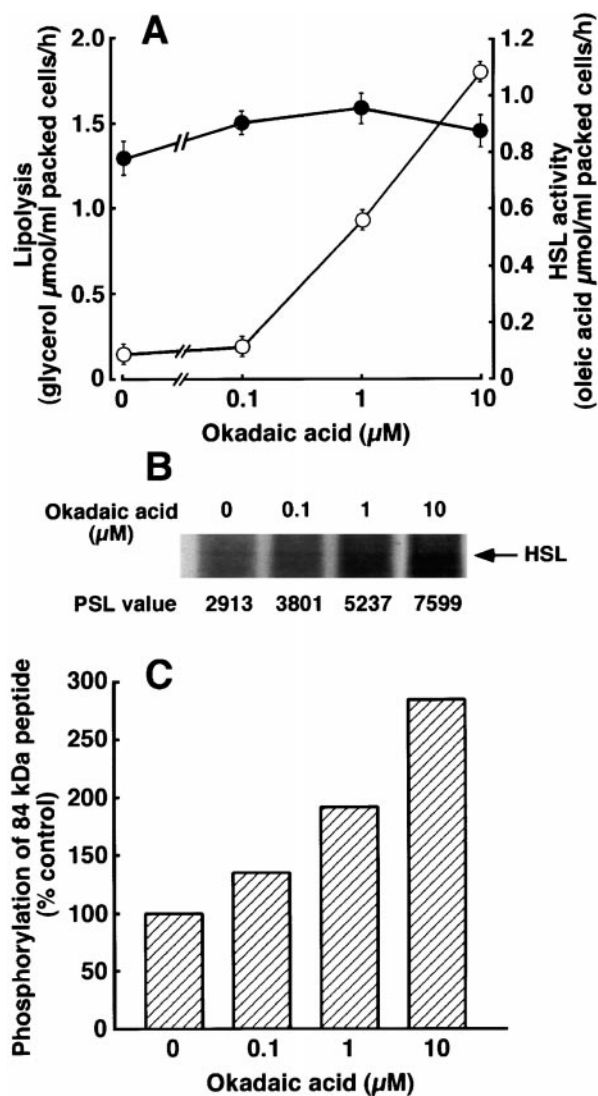


Fig. 1. Effects of okadaic acid on lipolysis, HSL activity, and HSL phosphorylation in rat fat cells. **A:** Fat cells were incubated with various concentrations of okadaic acid at 37°C for 1 h. After incubation, each reaction mixture was centrifuged to separate the medium from the fat cells. Lipolysis (\circ) and HSL activity (\bullet) were estimated as described in Materials and Methods. Each point represents the mean \pm SE of four separate experiments. **B:** The autoradiograph of the 84,000-Da [^{32}P]phosphopeptide band and PSL value determined as described in Materials and Methods. **C:** HSL phosphorylation was tabulated as a percentage relative to the density in unstimulated fat cell.

tissues as described previously (13). A 25- μl sample of packed endogenous lipid droplets was incubated at 37°C for 1 h with 100 μl HSL solution, 25 μl okadaic acid solution, 100 μl buffer A containing 2.5% (w/v) bovine serum albumin and 25 μl buffer A. Another sample (375 μl) was mixed with 750 μl buffer A containing 2.5% (w/v) bovine serum albumin and 16.95 mg gum arabic, the mixture was sonicated for 5 min, and the sonicate (75 μl) was incubated with 100 μl HSL solution and 100 μl buffer A containing 2.5% (w/v) bovine serum albumin for 1 h at 37°C . The amount of FFA released by each cell sample was measured, as described above, and lipolysis was expressed as μEq of FFA per ml of packed lipid droplets per h.

Analysis of data

Statistical analysis was performed by the Fisher's Protected LSD test to determine the significance of differences using Super ANOVA Software.

RESULTS

Okadaic acid-induced lipolysis and the HSL activity in rat fat cells were determined (**Fig. 1A**). Fat cells were incubated with various concentrations of okadaic acid and lipolysis was measured by determining the amount of glycerol released into the medium. Fat cells (200 μl packed volume) added to 450 μl of buffer B, were homogenized, centrifuged, and separated into fat layer (40 μl), supernatant (500 μl), and precipitate (trace amounts) fractions. Fat-associated HSL was solubilized from fat layer in the buffer solution by ether treatment without loss of activity (18). As 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

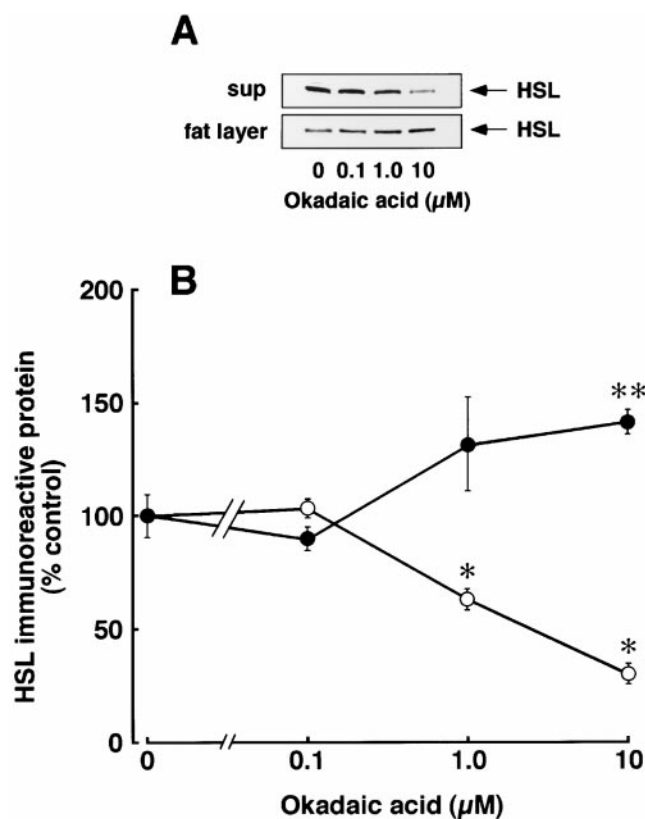


Fig. 2. Effect of okadaic acid on localization of HSL in rat fat cells. Fat cells were incubated with various concentrations of okadaic acid at 37°C for 1 h. After incubation, the fat cells were homogenized and centrifuged as described in Materials and Methods, and the proteins were separated by SDS-PAGE using gels containing 8% acrylamide. **(A):** A representative immunoblot that compares HSL protein in okadaic acid-treated and control cells. **(B):** HSL immunoreactive protein in supernatant (\circ) and fat layer (\bullet) tabulated as a percentage relative to the density detected by enhanced chemifluorescence in unstimulated fat cells. Each point represents the mean \pm SE of four separate experiments; * $P < 0.01$ and ** $P < 0.05$ vs. values in the absence of okadaic acid.

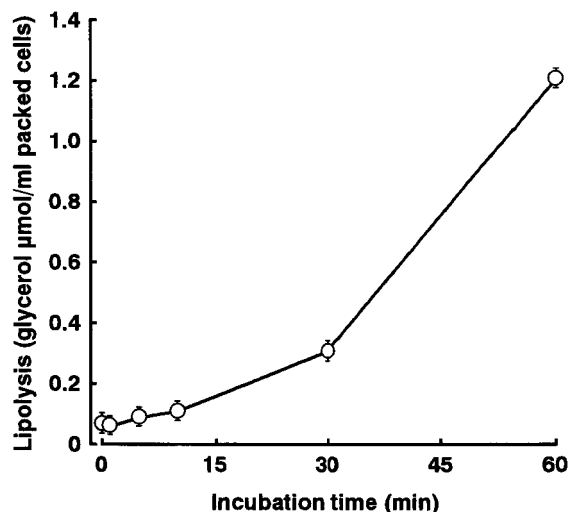


Fig. 3. Time course of okadaic acid-induced lipolysis in rat fat cells. Fat cells were incubated with $1 \mu\text{M}$ of okadaic acid at 37°C for various times. After incubation, each reaction mixture was centrifuged to separate the medium from the fat cells and lipolysis was estimated as described in Materials and Methods. Each point represents the mean \pm SE of four separate experiments.

as an HSL solution in assays. Okadaic acid ($1 \mu\text{M}$) increased lipolysis in fat cells in the absence of lipolytic hormones 6- to 8-fold. The HSL activity increased slightly in response to the addition of $1 \mu\text{M}$ okadaic acid, but this increase was not significant and extremely small in comparison with the increase in glycerol release induced by okadaic acid. The incorporation of [^{32}P]phosphate into HSL protein in fat cells was analyzed using SDS-PAGE. Okadaic acid increased the density of the 84,000-Da [^{32}P]phosphopeptide band in a concentration-dependent manner (Fig. 1B and C). The HSL protein level was examined by Western blotting with an anti-HSL antiserum, and okadaic acid treatment for 1 h did not significantly affect the total HSL protein content of fat cells (data not shown). However, okadaic acid increased the HSL protein level of the fat layer and reduced that of the supernatant concomitantly (Fig. 2).

The addition of $1 \mu\text{M}$ okadaic acid increased glycerol release from fat cells in a time-dependent manner (Fig. 3), but it did not significantly increase the total HSL activity in fat cells (data not shown), indicating that okadaic acid induced lipolysis in the fat cells without increasing the HSL activity. Treatment with $1 \mu\text{M}$ okadaic acid increased the HSL protein level of the fat layer and reduced that of the supernatant concomitantly in a time-dependent manner (Fig. 4). Little lipolysis in fat cells occurred in the absence of okadaic acid, but sonication of the fat cells elicited marked lipolysis, which was not affected by the addition of okadaic acid (Fig. 5).

Next, the lipolytic action of okadaic acid was examined in a cell-free system comprising endogenous lipid droplets and HSL in order to identify the site of the lipolytic action of okadaic acid. Okadaic acid stimulated lipolysis in the cell-free system in a concentration-dependent manner (Fig. 6), whereas little lipolysis in the cell-free system oc-

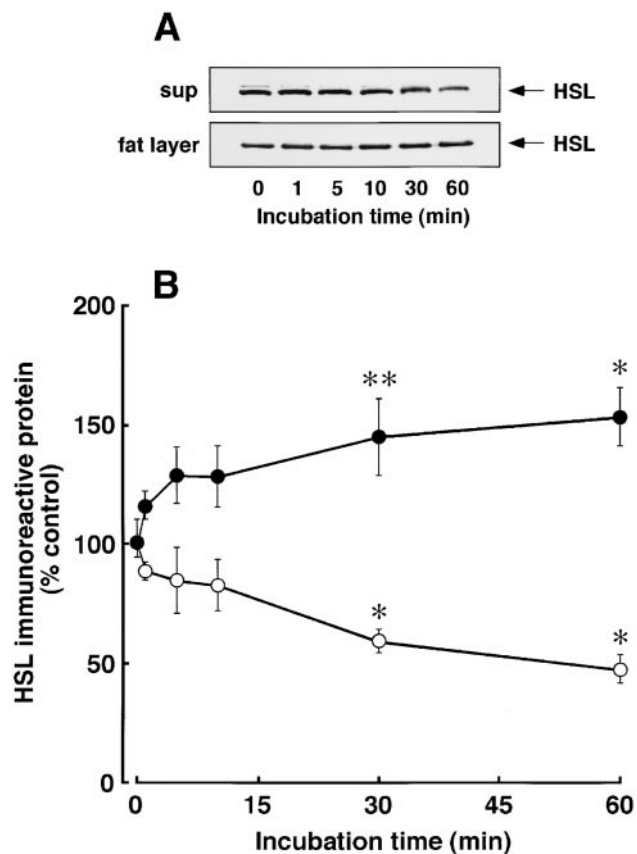


Fig. 4. Localization of HSL in rat fat cells. Fat cells were incubated with $1 \mu\text{M}$ of okadaic acid at 37°C for various times. After incubation, the fat cells were homogenized and centrifuged as described in Materials and Methods, and the proteins were separated by SDS-PAGE using gels containing 8% acrylamide. A: A representative immunoblot showing HSL protein in okadaic acid-treated and control cells. B: HSL immunoreactive protein in supernatant (○) and fat layer (●) tabulated as a percentage relative to the density detected by enhanced chemifluorescence in unstimulated fat cells. Each point represents the mean \pm SE of four separate experiments; * $P < 0.01$ and ** $P < 0.05$ vs. values after incubation for 0 min.

curred in its absence. However, sonication of the endogenous lipid droplets elicited marked lipolysis, which was not affected by the addition of okadaic acid (Fig. 6). When trioleoylglycerol emulsified with gum arabic was used in the cell-free system instead of endogenous lipid droplets, lipolysis was also observed and it was not stimulated by okadaic acid. In the presence of HSL, the rate of lipolysis with sonicated lipid droplets was 19.80 ± 0.24 nEq of FFA per mg triacylglycerol per h, and that with trioleoylglycerol emulsified with gum arabic was 3.56 ± 0.22 nmol of oleic acid per mg trioleoylglycerol per h.

DISCUSSION

In the absence of lipolytic hormones, okadaic acid stimulated lipolysis and increased the phosphorylation of HSL in rat fat cells in a concentration-dependent manner (Fig. 1). As lipolysis is believed to be mediated by the cAMP signaling pathway, and okadaic acid inhibits both type-1 and

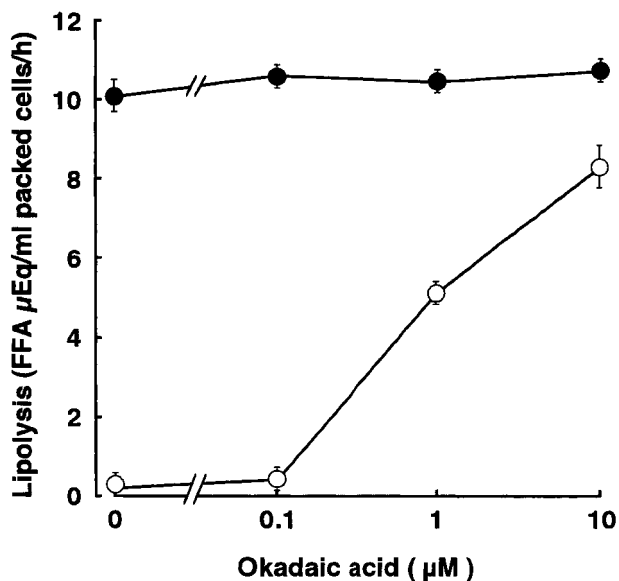


Fig. 5. Effect of okadaic acid on lipolysis in rat fat cells. The experimental procedure is described in Materials and Methods; intact fat cells (○) and sonicated fat cells (●). Each point represents the mean \pm SE of four separate experiments.

type-2 phosphatases (1, 2), it is likely that the effects of okadaic acid on lipolysis are mediated by the cAMP cascade. The treatment of fat cells with okadaic acid results in the increased phosphorylation of HSL, and the stimulation of lipolysis. Before the addition of okadaic acid, the level of HSL activity in rat fat cells was appreciable and it did not change significantly during okadaic acid-mediated stimulation of lipolysis (Fig. 1). Furthermore, okadaic acid did not affect the HSL protein level which was estimated by Western blotting with an anti-HSL antiserum. These

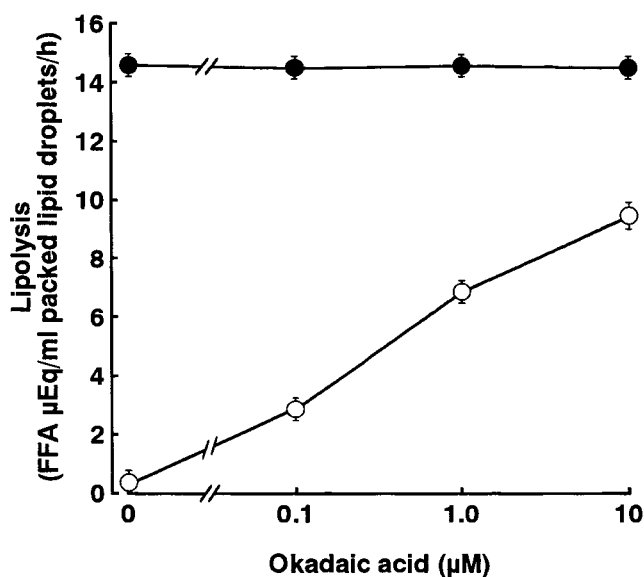


Fig. 6. Effect of okadaic acid on lipolysis in the cell-free system comprising lipid droplets and HSL. The experimental procedure is described in Materials and Methods; lipid droplets isolated from rat fat cells (○) and sonicated lipid droplets (●). Each point represents the mean \pm SE of four separate experiments.

results provide evidence that okadaic acid induces lipolysis without activating the catalytic activity of HSL.

Egan et al. (25) found that HSL shifted quantitatively from the supernatant of control cells to the fat layer of lipolytically stimulated cells; nearly all the HSL was found 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, in our study, HSL protein (55 to 65% of the total HSL protein) was found in the fat layers of centrifuged homogenates of unstimulated cells (Figs. 2 and 4). In spite of the considerable amount of HSL present in the fat layer, lipolysis was not observed in the unstimulated fat cells. Similar results were observed with the cell-free system comprising lipid droplets isolated from rat fat cells and HSL; in the absence of lipolytic agents, a considerable amount of HSL was adsorbed by the endogenous lipid droplets and the amount of adsorbed HSL was not increased significantly by the addition of lipolytic agents (data not shown). Although a considerable amount of HSL was adsorbed by the lipid droplets, lipolysis was not observed in the absence of lipolytic agents in the cell-free system (Fig. 6). These results suggest that even if the HSL adsorbed by the endogenous lipid droplets is catalytically active, it does not always evoke lipolysis in fat cells.

Although okadaic acid stimulated lipolysis in intact fat cells, it failed to elicit lipolysis in sonicated fat cells (Fig. 5), indicating that some intracellular factor that was destroyed by sonication plays a role in okadaic acid-induced lipolysis. Therefore the lipolytic action of okadaic acid was examined in a cell-free system comprising lipid droplets from rat fat cells and HSL to identify the site of lipolytic action of okadaic acid. Okadaic acid induced lipolysis in this cell-free system and the destruction of the lipid droplets by sonication resulted in loss of responsiveness to okadaic acid (Fig. 6). Substituting trioleoylglycerol emulsified with gum arabic for the lipid droplets in the cell-free system also abolished the lipolytic action of okadaic acid. These results suggest that the site of the lipolytic action of okadaic acid is not HSL but the lipid droplets in rat fat cells and the structure of the lipid droplets may be closely related to this lipolytic action.

In general, lipase activity is influenced markedly by the physical properties of the surface of the substrate. Tsujita, Muderkwa, and Brockman (26) prepared films of mixtures of 1,3-dioleoylglycerol and 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine at an argon-buffer interface and exposed them to various lipases, such as pancreatic carboxylester lipase. They found that the extent of hydrolysis of 1,3-dioleoylglycerol was less than 5% at a molar fraction of 0.5 and increased abruptly to 95% at a molar fraction of 0.6. However, the amounts of lipases adsorbed by the lipid films at molar fractions of 0.5 and 0.6 did not differ much (27). At the molar fraction of 0.5, a considerable amount of catalytically active enzyme adsorbed to the substrate film, but hydrolysis of 1,3-dioleoylglycerol did not occur. These workers showed that this "switching" phenomenon was not related to the enzyme concentration, but was related to the ratio of 1,3-dioleoylglycerol to phos-

phatidylcholine in the mixed lipid films. Considering based on the experimental results of Tsujita et al., it seems likely that okadaic acid stimulates lipolysis through changing the physical properties of the surface of the lipid droplets.

In conclusion, this study has yielded the novel information that okadaic acid evoked glycerol release from rat fat cells in the absence of lipolytic hormones, but did not activate HSL catalytic activity. Similar results were obtained by Egan et al. (25), who found that upon lipolytic activation of adipocytes induced by isoproterenol, the critical event was not an increase in catalytic activity (i.e., turnover number) but translocation of the lipase to its substrate on the surfaces of the lipid storage droplets. Okadaic acid also promoted the translocation of HSL from the cytosol to the endogenous lipid droplets in a concentration- and time-dependent manner (Figs. 2 and 4). However, the stimulation of lipolysis by okadaic acid cannot be explained only by translocation of HSL. In previous papers, we suggested that the physicochemical character of the endogenous lipid droplets plays an important role in the lipolytic process; phosphatidylcholine and some proteins in the lipid droplets may be involved in the mechanism of the stimulatory effect of lipolytic hormones (13, 19). Wise and Jungas (28) proposed that the cellular lipolytic response to epinephrine involves "substrate activation" of HSL. Souza et al. (29) hypothesized that when perilipins located on the surfaces of the endogenous lipid droplets in fat cells were phosphorylated by protein kinase, their barrier function of perilipins is attenuated, giving HSL access to the endogenous lipid droplets. Furthermore, Clifford et al. (30) discussed the possibility that insulin might mediate some of its anti-lipolytic effect via dephosphorylation of perilipin. These data suggest that the translocation of HSL protein from the cytosol to the lipid droplets and change of the surface physicochemical character of the endogenous lipid droplets play important roles in the lipolysis in fat cells induced by okadaic acid and other lipolytic hormones. ■

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