Effect of substrates on the cyclic AMP-dependent lipolytic reaction of hormone-sensitive lipase

Hiromichi Okuda,1 Chic Morimoto, and Takahiro Tsujita

2nd Department of Medical Biochemistry, School of Medicine, Ehime University, Shigenobu-cho, Onsen-gun, Ehime 791-02 Japan

Abstract Cyclic AMP-dependent activation of hormone-sensitive lipase (HSL) stimulated lipolysis of [3H]triolein emulsified with gum arabic, but not that of endogenous lipid droplets from rat fat cells. The absence of responsiveness of the lipid droplets to activation of HSL was found to be caused by some factor other than their surface area. The activated HSL showed a higher rate of lipolysis than nonactivated HSL on lipid droplets sonicated with gum arabic. Addition of phosphatidylcholine, which is one of the minor components of intact lipid droplets, to triolein emulsified with gum arabic, but not that of endogenous lipid droplets sonicated with gum arabic, showed a higher rate of lipolysis than nonactivated HSL on lipid droplets. These results suggest that loss of responsiveness of the endogenous lipid droplets in fat cells to activated HSL may be due to phosphatidylcholine in the lipid droplets. The absence of responsiveness of the lipid droplets containing phosphatidylcholine with phospholipase C restored the responsiveness. These results suggest that loss of responsiveness of the endogenous lipid droplets in fat cells to activated HSL may be due to phosphatidylcholine in the lipid droplets. Okuda, H., C. Morimoto, and T. Tsujita. Effect of substrates on the cyclic AMP-dependent lipolytic reaction of hormone-sensitive lipase. J. Lipid Res. 1994; 35: 1267–1273.

Supplementary key words activated HSL • lipid droplets • sonicated lipid droplets • phosphatidylcholine

The breakdown of stored triacylglycerols in adipose tissue to free fatty acids is controlled by various hormones such as catecholamines and insulin. The control is believed to be mediated through reversible phosphorylation of HSL, the enzyme catalyzing the rate-limiting step in lipolysis (1). A cDNA from a rat library encoding the HSL polypeptide has recently been cloned and sequenced, and the primary structure of HSL of 757 amino acids has been deduced (2). The activity of rat HSL is believed to be controlled by cyclic AMP-dependent phosphorylation of the single serine residue (Ser-563) in the regulatory phosphorylation site (2, 3). HSL is activated by its phosphorylation by cyclic AMP-dependent protein kinase, and is inactivated by its dephosphorylation by protein phosphatases (3). In addition to the regulatory site, Ser-565 is reported to be a “basal” site, which is a target for both calcium/calmodulin-dependent and AMP-activated protein kinases. Interestingly, phosphorylation at these two sites is mutually exclusive. Therefore, dephosphorylation at Ser-563 is a prerequisite for phosphorylation at Ser-565 (3).

In most studies on HSL, artificial lipid emulsions, such as trioien emulsified with gum arabic, have been used as substrates, although the physiological substrate of HSL is endogenous lipid droplets in fat cells. In general, lipase activity is markedly influenced by the physical properties of the surface of the substrate (4). Tsujita, Muderkwa, and Brockman (4) prepared films of mixtures of 1,3-dioleoyl-sn-glycerol and 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine at an argon–buffer interface and exposed these lipid films to various lipases, such as pancreatic carboxylester lipase and milk carboxylester lipase. They found that the extent of hydrolysis of 1,3-dioleoylglycerol was less than 5% up to a mole fraction of 0.5 and increased abruptly to 95% at a mole fraction of 0.6. They showed that this “switch” was not related to the surface pressure, reaction time, or enzyme concentration, but was due to the ratio of 1,3-dioleoylglycerol to phosphatidylcholine in the mixed lipid films (4). There is no evidence that the surface properties of artificial lipid droplets, such as trioien emulsified with gum arabic, are the same as those of the endogenous lipid droplets in fat cells.

Previously, we isolated endogenous lipid droplets from rat fat cells and established a cell-free system consisting of the droplets and HSL, in which epinephrine and forskolin stimulated lipolysis of the droplets by the lipase (5, 6). In the present study, we prepared membrane-bound HSL from chicken adipose tissue and studied the effect of phospholipid on hydrolysis of triglyceride by activated or nonactivated HSL as a model of lipolysis in fat cells. The HSL from chicken adipose tissue is activated to a greater extent than HSL from rats by cyclic AMP-protein kinase system, making it more useful in studies of the activation process (7).

Abbreviations: HSL, hormone-sensitive lipase; TES, N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid; BES, N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; cyclic AMP, cyclic adenosine monophosphate; EDTA, ethylenediaminetetraacetic acid; DTT, dithiothreitol; FFA, free fatty acids.

1To whom correspondence should be addressed.
The purpose of the present investigation was to examine whether cyclic AMP-dependent activation of chicken adipose tissue HSL increases the hydrolysis of endogenous lipid droplets from rat fat cells.

MATERIALS AND METHODS

Animals
Young male Wistar-King rats, weighing 150-200 g, were given standard laboratory diet and water ad libitum. 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). Triolein, cyclic AMP, bovine heart protein kinase (3',5'-cyclic AMP-dependent), and egg phosphatidylcholine were from Sigma (St. Louis, MO). TES and BES were from Wako Pure Chemical Industries (Osaka, Japan). Leupeptin and pepstatin were from the Peptide Institute (Osaka, Japan). Phospholipase C (Uppsala, Sweden). Leupeptin and pepstatin were from Pure Chemical Industries (Osaka, Japan). Heparin-Sepharose was from Pharmacia, LKB Biotechnology (Uppsala, Sweden). Leupeptin and pepstatin were from the Peptide Institute (Osaka, Japan). Phospholipase C (type I, from Clostridium perfringens) was from Sigma and was purified further by affinity chromatography on agarose-linked egg yolk lipoprotein, followed by gel filtration on Sephadex G-100 (8) before use. The purified enzyme appeared homogeneous on polyacrylamide gel electrophoresis and its specific activity was 210 U/mg protein per min. One enzyme unit (U) was defined as the amount required to hydrolyze 1 μmol of lecithin/min. Bovine serum albumin was from Wako Pure Chemical Industries and was extracted by the method of Chen (9) to remove free fatty acid.

Methods
Preparation of endogenous lipid droplets. Isolated fat cells were obtained by the method of Rodbell (10), and 1 ml packed volume of cells was suspended in 4 ml of 5 mM Tris-HCl buffer (pH 7.4). The suspension was mixed by slowly inverting the centrifuge tube three times and then centrifuged at 200 g for 3 min at room temperature. The fat layer was mixed with 4 ml of 5 mM Tris-HCl buffer (pH 7.4) containing 0.025% Triton X-100 by slowly swirling the tube three times and the mixture was centrifuged at 200 g for 3 min at room temperature. The fat layer was washed once with buffer A (25 mM TES, pH 7.4, containing 135 mM NaCl, 5 mM KCl and 1 mM MgCl₂), incubated with buffer A at 37°C for 10 min, centrifuged at 200 g for 3 min, and washed again with buffer A. Lipase activity was removed from the fat layer by these procedures. Addition of epinephrine to the fat layer failed to stimulate lipolysis in the absence of added lipase (5).

One gram (dry weight) of the fat layer consisted of 870 μmol triglyceride, 0.71 μmol phospholipid, 0.52 μmol cholesterol, 342 μg carbohydrate, and 63 μg protein. Of the phospholipid fraction, 75% was phosphatidylcholine and 25% was phosphatidylethanolamine. As measured with radioactive Triton X-100, about 16% of added Triton X-100 was included in the fat layer but did not affect the rate of activation of HSL due to ATP, cyclic AMP and cyclic AMP-dependent protein kinase. Although the fat layer was a crude preparation, it was used as endogenous lipid droplets in this study.

Preparation of chicken HSL. Laying hens (White Leghorns) were killed by decapitation, and adipose tissue was excised from their abdomen and from around the gizzard. The tissue was minced and homogenized for 60 sec at 10-15°C in a Waring Blender with 2 vol of buffer solution consisting of 0.25 M sucrose, 1 mM EDTA, 0.1% mercaptoethanol, 0.1 mM benzanilide, leupeptin (1 mg/l), pepstatin A (1 mg/l) and 25 mM Tris- HCl, pH 7.4. The homogenate was centrifuged at 10,000 g for 20 min and the bulk of the floating fat cake was removed. The infranatant fluid was centrifuged at 100,000 g for 60 min. Residual floating fat was removed by suction, and the infranatant fluid was filtered through glass wool. The solution was carefully adjusted to pH 5.2 with 0.1 M acetic acid in an ice bath with constant stirring, and after 30 min, insoluble material was collected by centrifugation at 10,000 g for 15 min. This material was suspended in 20 mM Tris-HCl, pH 7.4, containing 20% glycerol, 1 mM EDTA, 0.1 mM EDTA, 0.1 mM benzanilide, leupeptin (1 mg/l), and pepstatin (1 mg/l) and stored at -80°C.

Just before experiments, the stored material was thawed at 4°C and applied to a heparin-Sepharose column (5 × 20 cm) equilibrated with buffer A to remove lipoprotein lipase. The unadsorbed fraction was called the 5.2P fraction and used as chicken HSL. HSL activity in this fraction was not reduced by 1 M NaCl, indicating that lipolytic activity due to lipoprotein lipase was minimal in the preparation.

Measurements of the lipolytic reaction with activated and nonactivated HSL. For activation of HSL, the 5.2P fraction was incubated at 25°C for 10 min with an equal volume of activation mixture consisting of 2 mM EDTA, 2 mM DTT, 10 mM MgCl₂, 1 mM ATP, 20 μM cyclic AMP, cyclic AMP-dependent protein kinase (0.625 mg/ml), and 100 mM Tris-HCl, pH 7.0. ATP, cyclic AMP, and the protein kinase were omitted from the control mixture containing the nonactivated HSL. Enzyme activity was determined by measuring the rate of release of free fatty acids using endogenous lipid droplets with or without sonication as substrate. The assay system contained the following components in a total volume of 250 μl: 22.5 μl of packed lipid droplets or 67.5 μl of sonicated lipid droplets, which corresponded to 18.8 μmol equivalents to triolein, 125 μl of enzyme solution, 3.58 mg of bovine serum albumin, 20...
μmol of KCl, 5 μmol of NaCl, and 11.25 μmol of BES. Sonicated lipid droplets were prepared by sonication of a mixture of 22.5 μl of packed lipid droplets, 10.17 mg of gum arabic, and 45 μl of water. The reaction mixture was incubated for 1 h at 37°C at pH 6.8, and the reaction was stopped by adding 3 ml of a 1:1 (v/v) mixture of chloroform and heptane containing 2% (v/v) methanol. The amount of fatty acid released was then measured (11).

The lipase activity was also determined by a radiochemical method in which the release of [3H]oleic acid from [3H]triolein was measured. A suspension of 50 mg of triolein and 220 μCi of [3H]triolein in 3.75 ml of 5% (w/v) gum arabic solution was sonicated for 5 min. The assay system contained the following components in a total volume of 100 μl: 50 μl of enzyme solution, 0.136 μmol of triolein, 0.452 mg of gum arabic, 1.43 mg of bovine serum albumin, 8 μmol of KCl, 2 μmol of NaCl, and 4.5 μmol of BES. Incubation was carried out for 1 h at 37°C at pH 6.8 and released [3H]oleic acid was measured by the method of Belfrage and Vaughan (12).

RESULTS

The lipolytic activities of activated and nonactivated HSL with [3H]triolein emulsified with gum arabic as substrate were compared as functions of the enzyme concentration. The activated HSL showed higher lipolytic activity than nonactivated HSL (Fig. 1). Omission of ATP from the activation mixture or single addition of ATP, cyclic AMP, and/or the protein kinase instead of the mixture of these components failed to stimulate the lipase activity. Furthermore, addition of skeletal muscle protein kinase inhibitor inhibited the activation, indicating that it was mediated by phosphorylation of HSL (data not shown).

We then compared the lipolytic activities of the activated and nonactivated HSL with endogenous lipid droplets from rat fat cells as substrate. In contrast to the case with

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**Fig. 1.** Effect of HSL concentration on hydrolysis of triolein emulsion. Hydrolysis of [3H]triolein emulsion with activated (●) or nonactivated (○) HSL was measured. Values in this and other figures are means ± SE of four separate assays.

**Fig. 2.** Effect of HSL concentration on hydrolysis of endogenous lipid droplets from rat fat cells. Hydrolysis of endogenous lipid droplets with activated (●) or nonactivated (○) HSL was measured.

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**Fig. 3.** Effect of surface areas of substrates on lipolysis in the presence of activated or nonactivated HSL. Lipolysis by the activated (●) or nonactivated (○) HSL was measured at fixed surface areas of [3H]triolein emulsified with gum arabic (A) or endogenous lipid droplets from rat fat cells (B). The lipid droplets and triolein emulsified with gum arabic were mixed with buffer A containing 2% osmium chloride. The mixture was kept for 3 h at 4°C and then subjected to scanning electron micrography (Hitachi H-500). The average diameter (2r) of each substrate was estimated from the electron micrographs with an image analyzer. The surface area was calculated as 4πr².
[3H]triolein emulsified with gum arabic, lipolysis of the lipid droplets was not enhanced by activation of HSL at any concentration tested (Fig. 2).

In 1958, Sarda and Desnuelle (13) proposed that the surface area of the substrate is one factor that regulates lipase activity. Therefore, it seemed possible that the loss of responsiveness of the lipid droplets to the activation of HSL might be caused by their surface area. However, this was not the case, because activation of HSL increased its hydrolysis of [3H]triolein emulsion but not of lipid droplets with the same range of surface areas of 25 to 400 cm²/ml reaction mixture (Fig. 3).

Although lipolysis of intact lipid droplets was not enhanced by activation of HSL, the rate of lipolysis of sonicated lipid droplets by activated HSL was higher than that by nonactivated HSL (Fig. 4). The average diameter of intact lipid droplets was 56.6 ± 0.7 μm and that of sonicated droplets was 4.6 ± 0.2 μm. Therefore, the average surface area of sonicated droplets was about 12 times that of intact droplets. However, from the results in Fig. 3, it seems unlikely that increase of the surface area by sonication of the droplets caused their responsiveness to activation of HSL.

As described in Materials and Methods, the intact lipid droplets contained several amphiphilic components such as phosphatidylcholine, phosphatidylethanolamine, cholesterol, and protein, which might be localized at the surface of the droplets. The average diameters of the lipid droplets were decreased about 12-fold by sonication, with an increase in the droplet number. Thus, the increase in the surface area of the droplets was proportional to the ratio of the average diameter of the intact lipid droplets to that of the sonicated ones. Therefore, the concentrations of these amphiphilic components at the surface might be reduced about 12-fold by sonication, and this reduction of their concentrations at the surface might induce responsiveness to activation of HSL.

To examine this possibility, we emulsified [3H]triolein with gum arabic and each of these amphiphilic components at about 100-fold their concentration in intact lipid droplets. Then we sonicated the mixtures for 5 min and incubated the resulting triolein emulsions in the presence of activated or nonactivated HSL. Of these amphiphilic components, phosphatidylcholine was found to inhibit more of both absolute and relative rates of enhancement on lipolysis due to activation of HSL than other components (Table 1). Phosphatidylethanolamine and cholesterol also inhibited the enhancing effect of activation of HSL, but less than phosphatidylcholine.

Next we emulsified [3H]triolein with gum arabic and various amounts of phosphatidylcholine, sonicated the mixtures for 5 min, and incubated them with activated and nonactivated HSL. As shown in Fig. 5, addition of phosphatidylcholine to the triolein emulsion strongly inhibited the rates of lipolysis by both activated and nonactivated HSL, and reduced the enhancing effect of activation of HSL. In addition to phosphatidylcholine, phosphatidic acid inhibited the enhancing effect of activation of HSL (data not shown).

As the enhancing effect was nearly zero at a phosphatidylcholine concentration of 0.025 μg/ml, [3H]triolein was measured with activated or nonactivated HSL. Each point represents the mean ± SE of four separate assays.

### Table 1. Effect of amphiphilic constituents in the lipid droplets on hydrolysis of triolein emulsion

<table>
<thead>
<tr>
<th>Addition (w/w to triolein)</th>
<th>Lipase Activity</th>
<th>Absolute Rate of Enhancement</th>
<th>Relative Rate of Enhancement</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nonactivated</td>
<td>Activated</td>
<td>FFA μmol/mg protein/h</td>
</tr>
<tr>
<td>None</td>
<td>1.99 ± 0.07</td>
<td>3.72 ± 0.17</td>
<td>1.73</td>
</tr>
<tr>
<td>Phosphatidylcholine (0.05)</td>
<td>0.29 ± 0.01</td>
<td>0.57 ± 0.03</td>
<td>0.08</td>
</tr>
<tr>
<td>Phosphatidylethanolamine (0.0175)</td>
<td>2.01 ± 0.06</td>
<td>2.96 ± 0.12</td>
<td>0.95</td>
</tr>
<tr>
<td>Cholesterol (0.025)</td>
<td>1.29 ± 0.04</td>
<td>1.87 ± 0.08</td>
<td>0.58</td>
</tr>
<tr>
<td>Protein (0.01)</td>
<td>1.49 ± 0.05</td>
<td>2.72 ± 0.15</td>
<td>1.23</td>
</tr>
</tbody>
</table>

Hydrolysis of [3H]triolein was measured with activated or nonactivated HSL. Each point represents the mean ± SE of four separate assays.
Effect of phosphatidylcholine on hydrolysis of triolein emulsion by activated and nonactivated HSL. Hydrolysis of \(^{14}C\)triolein emulsion containing various amounts of phosphatidylcholine with activated (●) or nonactivated (○) HSL was measured.

The inhibitory action of phosphatidylcholine was also observed with sonicated lipid droplets (Fig. 7). The intact lipid droplets were sonicated with gum arabic and various amounts of phosphatidylcholine and then incubated with activated or nonactivated HSL. In these sonicated lipid droplets phosphatidylcholine inhibited the rate of lipolysis by both activated and nonactivated HSL at lower concentrations than those causing inhibition in the triolein emulsion. Furthermore, the enhancing effect of activated HSL was completely inhibited by phosphatidylcholine at a ratio phosphatidylcholine to triglyceride of 0.01 (w/w), which was 10-fold lower than that with the triolein emulsion.

Finally, phosphatidylcholine was added to intact lipid droplets in a ratio of 0.01 (w/w). The mixture was subjected to sonication with gum arabic and incubated with activated or nonactivated HSL in the presence of various amounts of phospholipase C. As shown in Fig. 8, phospholipase C enhanced the effect of activated HSL on lipolysis of the sonicated lipid droplets. Heat-treated (100°C, 10 min) phospholipase C had no enhancing effect. These results also suggest that phosphatidylcholine, especially its phosphate group, in the sonicated lipid droplets inhibits the increase in rate of lipolysis due to activation of HSL.

DISCUSSION

A number of substrates and emulsifiers have been used for assay of HSL. Chicken adipose tissue HSL was known to hydrolyze long chain tri-, di-, and 1(3)-monoaecylglycerol at relative maximal rates of 1:13:10 (7). On the other hand, the percentage activations of lipolysis due to activation of HSL were 213 with triolein, 16 with diolein, and...
emulsifier, and surface area of the substrate emulsion. The present experiments suggest that loss of responsiveness of intact lipid droplets to activation of HSL is not due to the HSL content, substrate structure, or surface area of the droplets but to the emulsifier, phosphatidylcholine (Figs. 1, 2, and 3). Addition of phosphatidylcholine to triolein or the sonicated lipid droplet emulsion induced loss of responsiveness to activation of HSL and treatment of these substrates containing phosphatidylcholine with phospholipase C restored the responsiveness (Table 1, Figs. 5, 6, 7, and 8). In these experiments, gum arabic was used as an emulsifier of triolein or the sonicated lipid droplets to stabilize the emulsions. Therefore, addition of phosphatidylcholine did not change the size of droplets in these emulsions (data not shown). The diameters of triolein and sonicated lipid droplets emulsified with gum arabic were found to be 1.39 ± 0.03 μm and 4.56 ± 0.17 μm, respectively, by scanning electron micrography (Hitachi H-500). Thus, the surface area per triglyceride of triolein emulsion was about 3 times that of the sonicated emulsion. This might be one reason why a higher concentration of phosphatidylcholine was needed to inhibit hydrolysis by HSL and the enhancing effect of activation of HSL with triolein emulsion than with sonicated lipid droplets.

Although the intact lipid droplets failed to respond to activation of HSL, the sonicated lipid droplets emulsified with gum arabic responded to its activation (Fig. 4). This finding was in contrast to the report of Wise and Jungas (16) that activation of HSL did not stimulate the hydrolysis of endogenous triglyceride in an adipose tissue homogenate. The difference might have been due to different states of the endogenous substrates. In our experiment, endogenous lipid droplets were sonicated with gum arabic and the resultant sonicated lipid droplets with an average diameter of 4.6 μm were stable during the period of incubation with HSL. On the other hand, average diameter of homogenized lipid droplets was 33.9 ± 0.9 μm (average diameter of intact lipid droplets: 56.6 ± 0.7 μm). Thus, homogenization of the intact lipid droplets caused only about 1.7-fold increase in the average surface area and so 1.7-fold reduction of the phosphatidylcholine content at the surface. Moreover, homogenized lipid droplets were unstable, possibly due to the absence of gum arabic, and readily fused into an oil layer during the period of incubation with HSL. Therefore, it seems likely that loss of responsiveness of triglyceride in the adipose tissue homogenate to activation of HSL in the study by Wise and Jungas (16) may have been due to the small reduction of the phosphatidylcholine content on the surface of homogenized lipid droplets containing triglyceride and the instability of these droplets during the incubation period.

If loss of responsiveness of intact lipid droplets to activation of HSL is due to the presence of phosphatidylcholine on their surface, phospholipase C treatment of these droplets should restore the responsiveness. However, we failed to demonstrate this, because phospholipase C-treated intact droplets were unstable, possibly because of the absence of gum arabic during the incubation period with HSL. Therefore, further experiments are needed to
determine whether loss of responsiveness of endogenous
lipid droplets in fat cells to activation of HSL is actually
due to phosphatidylcholine on their surface. However,
as activation of HSL by ATP, cyclic AMP, and protein
kinase does not stimulate lipolysis of intact lipid droplets
in fat cells, it seems possible that there may be another
mechanism for stimulation of lipolysis.  

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