Studies on Reduction of Lipolysis in Adipose Tissue on Freezing and Thawing

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Synopsis

Adrenaline-induced lipolysis in fat cells was remarkably reduced when the cells were preincubated in a dry ice-aceton bath, but their adenylcyclase and lipase activities were not reduced. In the reconstructed lipid micelles which consisted of lipase-depleted lipid micelles and lipase-containing adipose tissue extract, adrenaline, theophylline and DBCAMP-induced lipolysis was not found when lipase-depleted lipid micelles were preincubated in a dry ice-aceton bath but was found when lipase was preincubated.

It has been suggested that adrenaline increases lipolysis in adipose tissue by activating hormone-sensitive lipase via adenylcyclase and cyclic AMP-dependent protein kinase (Robinson et al., 1967; Corbin et al., 1970; Huttunen et al., 1970). However, the recent results from our group have indicated that an explanation other than this cyclic AMP theory is required for the effect of adrenaline on lipolysis (Okuda et al., 1974). Recently, we succeeded in preparing lipid micelles from fat cells by hypotonic treatment. Incubation of these lipid micelles with adrenaline resulted in marked lipolysis but no increase in protein kinase activity (Saito et al., 1974 b). These results suggest that the lipolytic action of adrenaline in these lipid micelles was not due to the increase in lipase activity. When the lipid micelles were homogenized, adrenaline no longer induced lipolysis (Okuda et al., 1974). This suggests that some structure in the lipid micelles was required for the effect of adrenaline. Mosinger and Kujalova (1964) reported that adrenaline-induced lipolysis is more unstable at 2°C. In confirmation of this, Okuda et al. (Okuda et al., 1971) reported that adrenaline-induced lipolysis was considerably inhibited by preincubation of adipose tissue slices at lower temperatures down to −10°C, although the hormone-sensitive lipase activity remained unchanged. This again suggested that adrenaline-induced lipolysis was modified, not by the change in lipase activity, but by other factors, such as the change in the physical state of endogenous fat. The present paper reports the details of the inhibitory effect of freezing and thawing on lipolysis in isolated fat cells, lipid micelles and reconstructed lipid micelles, induced by various substances, such as adrenaline, theophylline and DBCAMP. The mechanism of action of these lipolytic agents is discussed.

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Materials and Methods

Materials
Young male rats of the Wistar-King strain, weighing between 170 and 200 g, were used. They were given standard laboratory chow ad libitum. For the experiments they were sacrificed by a blow on the head and their epididymal fat pads were quickly excised.

Chemicals
Adrenaline was obtained from Sankyo Co., Ltd., dibutyryl cyclic AMP (DBcAMP) from Sigma Co., Ltd., theophylline from Wako Chemicals and Ediol (50% coconut oil) from Calbiochem. 3H-Cyclic AMP was purchased from the Japan Radioisotope Association.

Preparation of fat cells and lipid micelles
Isolated fat cells were prepared by the method of Rodbell (1964). Lipid micelles were prepared from fat cells as described previously (Okuda et al., 1974).

Estimation of lipolysis in freezing and thawing systems
Fat cells and lipid micelles were suspended separately, in Krebs-Ringer phosphate buffer (pH 7.4), and 0.5 ml of each of the suspensions, containing 1.4 and 0.6 mg of protein, respectively (equivalent to 200 mg of adipose tissue), were transferred to test tubes. The suspensions were preincubated for 30 min at 15°C, or frozen for 30 min in a dry ice-acetone bath and then the tubes were stood for 15 min at 15°C to allow the frozen fat cells or lipid micelles to thaw. Then 0.5 ml of Krebs-Ringer phosphate buffer containing various agents and 5% bovine serum albumin (BSA) was added and the mixtures were incubated at 37°C for 2 hr. Then Dole's extraction mixture was added and the free fatty acids released were estimated by the method of Dole (1956).

Preparation of lipase-depleted lipid micelles
Fat cells were suspended in 5 mM Tris-HCl buffer, pH 7.4, at a concentration equivalent to 400 mg of adipose tissue per ml. The suspension was mixed by slowly inverting the centrifuge tube several times and then centrifuged at 200×g for 3 min at room temperature. The supernatant fraction was replaced by 5 mM Tris-HCl buffer, pH 7.4, containing 0.025% triton X-100, the contents were mixed and centrifuged at 200×g for 3 min at room temperature. The fat layer was washed three times by the same procedure as that for the detergent treatment except that Triton X-100 was omitted from the buffer. Finally, the fat layer was suspended in Krebs-Ringer phosphate buffer, pH 7.4, containing 5% bovine serum albumin at a concentration equivalent to 400 mg of adipose tissue per ml. This suspension is referred to as lipase-depleted lipid micelles. It contained about 0.6 mg of protein per ml.

Estimation of lipolysis in lipase-depleted lipid micelles
Samples of 0.5 ml of lipase-depleted lipid micelle suspension (0.3 mg of protein) were mixed with 0.5 ml of Krebs-Ringer-phosphate buffer (KRP), pH 7.4, with or without adrenaline (1 μg/ml) or adipose tissue extract. The adipose tissue extract was prepared by homogenizing 2 g of adipose tissue with 8 ml of KRP by ten strokes of a Teflon homogenizer, and centrifuging the homogenate at 800×g for 30 min. The supernatant was used as the adipose tissue extract. One ml of the extract contained 6.9 mg of protein and could hydrolyze 3.5 μEq of Ediol per hour under the standard condition (Okuda et al., 1974). Reaction mixtures were incubated at 37°C for 1 hr and then Dole's extraction mixture was added and the free fatty acid released was estimated by the method of Dole (1956).

Estimation of lipase
Lipase activity was estimated as described previously (Okuda et al., 1974). For the estimation of lipase activity in the freezing and thawing system, 5 ml of suspension of fat cells or lipid micelles which is equivalent to 400 mg of adipose tissue per ml, were preincubated for 30 min at 15°C or frozen for 30 min in a dry ice-acetone bath. The frozen fat cells or lipid micelles were stood at 15°C for 15 min to thaw and homogenized by ten strokes of a Teflon homogenizer and the resultant suspensions were used as enzyme solutions. The protein concentrations of these homogenates of fat cells and lipid micelles were 2.8 and 1.2 mg per ml, respectively. Lipase activity was assayed in the presence or absence of adrenaline (1 μg/ml).

Estimation of adenylcyclase activity in the freezing and thawing system
Adenylcyclase activity was estimate by a modification of the method of Birnbaumers et al. (1969). Enzyme solution from fat cells in the freezing and thawing system was prepared by the method described under Estimation of lipase. The reaction mixture consisted of 3.2 mM ATP, 5 mM MgCl2, 10 mM theophylline, 0.1% (w/v) BSA, 25 mM Tris-HCl buffer (pH 7.4), and 10 μl of enzyme solution (about 28 μg of protein), with or without 0.1 μg of adrenaline in a final volume of 0.1 ml. The mixture was incubated for 15 min at 37°C. Then the mixture was boiled for 3 min to terminate the reaction and centrifuged. Fifty-μl aliquots of the supernatant were used to measure cAMP by the method of
Results and Discussion

Adrenaline-induced lipolysis of fat cells which were frozen in a dry ice acetone bath and thawed at 15°C was much less than that of fat cells preincubated at 15°C as shown in Fig. 1. On the other hand, adrenaline caused similar extents of activation of adenylcyclase either in the cells preincubated in a dry ice acetone bath or preincubated at 15°C. Moreover, lipase activity was not affected by the presence of adrenaline and was not reduced by freezing and thawing. These results suggest that freezing and thawing does not reduce cyclic AMP production or activation of a hormone-sensitive lipase but reduces the hydrolysis of endogenous fat in fat cells. As shown in Fig. 2, freezing and thawing greatly reduced adrenaline-induced lipolysis of lipid micelles, but not their lipase activity. Therefore, freezing and thawing probably affects a hydrolytic process in which a hormone-sensitive lipase acts on the surface of the micelles (Sarda and Desnuelle 1958; Desnuelle and Savary, 1963).

In a previous report (Okuda et al., 1974), we showed that lipolysis in the absence of adrenaline increased slightly when lipid micelles were homogenized, and we suggested that homogenization increased the basal lipolysis by increasing the rate of association of lipase with triglyceride. Since freezing and thawing did not increase the basal lipolysis, it probably did not induce a change of the fat cells or lipid micelles, which caused the increase in the rate of association between lipase and triglyceride. This suggested the more specific effect of freezing and thawing on adrenaline action than that of homogenization.

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**Fig. 1.** Effect of freezing and thawing on adrenaline-induced lipolysis, adenylcyclase activity and lipase activity in fat cells.

Fat cells were preincubated at 15°C for 30 min or frozen in a dry ice-acetone bath for 30 min and thawed. Lipolysis, adenylcyclase and lipase were estimated in the presence or absence of adrenaline as described in Materials and Methods.
Lipid micelles were preincubated at 15°C for 30 min or frozen in dry ice-acetone bath for 30 min and thawed. Lipolysis and lipase activity in the presence or absence of adrenaline as described in Materials and Methods.

Table 1. Effect of freezing and thawing on adrenaline-induced lipolysis in the lipase-depleted lipid micelle system.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Additions</th>
<th>Adrenaline (1 µg/ml)</th>
<th>FFAµEq/g/hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipase-depleted lipid micelles</td>
<td>Krebs-Ringer phosphate buffer</td>
<td>-</td>
<td>0.3</td>
</tr>
<tr>
<td>Lipase-depleted lipid micelles</td>
<td>Adipose tissue extract</td>
<td>+</td>
<td>0.2</td>
</tr>
<tr>
<td>Lipase-depleted lipid micelles preincubated in freezing and thawing</td>
<td>Adipose tissue extract</td>
<td>-</td>
<td>0.3</td>
</tr>
<tr>
<td>Lipase-depleted lipid micelles</td>
<td>Adipose tissue extract preincubated in freezing and thawing</td>
<td>+</td>
<td>0.2</td>
</tr>
</tbody>
</table>

Addipose tissue extract or lipase depleted lipid micelles were preincubated at 15°C for 30 min or frozen in dry ice acetone bath. Frozen extract or micelles were thawed at 15°C for 15 min. Adrenaline-induced lipolysis was estimated as described in the Materials and Methods.

* Lipase-depleted lipid micelles equivalent to 1 g of adipose tissue.

We found previously (Saito et al., 1976) that the treatment of lipid micelles with 0.025% Triton X–100 removed 46% of their lipase, and that this resulted in the complete loss of adrenaline-induced lipolysis. By the addition of adipose tissue extract to these lipase-depleted lipid micelles, their adrenaline-induced lipolysis was restored to almost the level in untreated lipid micelles, as shown in Table 1. When lipase-depleted lipid micelles had been frozen in a dry ice-acetone bath for 30 min, and thawed their adrenaline-induced lipolysis was not restored by the addition of lipase. On the other hand, the freezing and thawing of adipose tissue extract did not affect the recovery of adrenaline-induced lipolysis.

The lipolytic action of theophylline had been explained by the cyclic AMP theory to be due to inhibition of phospho-diesterase (Bletcher et al., 1968). However, Kuo and Renzo (1969) demonstrated that theophylline
induced lipolysis without elevating the level of cyclic AMP and we (Saito et al., 1974b) found that theophylline did not activate protein kinase in lipid micelles. Therefore, theophylline seems to exert its lipolytic action by some other mechanism. In the lipolytic system of lipase-depleted lipid micelles, DBcAMP and theophylline elicited lipolysis, as shown in Tables 2 and 3, respectively. However, their effects were not observed when the lipase-depleted lipid micelles had been frozen and thawed, although they stimulated lipolysis in the system, when the adipose tissue extract had been frozen and thawed. These results suggest that some structure of endogenous fat is required for lipolysis induced by DBcAMP, or theophylline. As previously reported (Okuda et al., 1974; Saito et al., 1974a), cAMP has been found to have no capacity to induce lipolysis in the lipid micelles, though the agent can activate protein kinase. Therefore, DBcAMP and theophylline seem to act as lipolytic agents like adrenaline without relation to cAMP.

### References


