Basal Lipolysis in Epididymal Fat Cells from Streptozotocin-Induced Diabetic Rats

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(Received August 3, 2005)

Summary The level of free fatty acid (FFA) in plasma is increased by diabetes. The increase in plasma FFA levels accompanied the stimulation of basal lipolysis (i.e. lipolysis in the absence of lipolytic agents) in fat cells. Injection of streptozotocin with rats resulted in a significant increase in basal FFA production (5.5 fold) in fat cells. However, basal glycerol production in fat cells was increased only 1.5 fold by streptozotocin-induced diabetes, implying that FFA re-esterification in fat cells was decreased by streptozotocin-induced diabetes. The FFA re-esterification in fat cells was also decreased by 1 d of fasting. Although basal lipolysis was increased by streptozotocin-induced diabetes or 1-d fasting, neutral triacylglycerol lipase activity and the immunoreactive HSL protein content in fat cells from streptozotocin-induced diabetic rats or 1-d fasting rats were not significantly changed. Although β-blockers inhibited lipolysis induced by norepinephrine at a concentration of 10⁻⁴ M, it failed to inhibit the basal lipolysis and FFA re-esterification in fat cells from streptozotocin-induced diabetic rats. Nor did insulin or H-89, another antilipolytic agent, affect basal lipolysis or FFA re-esterification in fat cells from streptozotocin-induced diabetic rats. These results indicate that basal FFA production may be induced by a decrease of re-esterification of FFA in diabetic rats and is not affected by antilipolytic agents such as insulin, β-blockers or H-89.

Key Words basal lipolysis, FFA re-esterification, diabetes, hormone-sensitive lipase

Plasma FFA levels are affected by fat cell lipolysis, which is regulated by hormone-dependent and hormone-independent lipolytic activities. The latter is termed basal lipolysis. Basal lipolysis occurs continuously in fat cells. During lipolysis, triacylglycerol molecules are hydrolyzed by triacylglycerol lipase(s) to FFA and glycerol, which are released from adipose tissue. Because of the low level of glycerol kinase in adipose tissue, glycerol cannot be re-utilized by adipose tissue to any important extent (1). Glycerol production by adipose tissue is therefore a good index of net lipolysis. The release of FFA from adipose tissue plays an important role in the energy homeostasis of the body. Some of the FFA formed during lipolysis can be re-esterified to triacylglycerol in adipose tissue. This constitutes a futile cycle with FFA being first formed by lipolysis and then converted to triacylglycerol without the achievement of any work.

The mechanism of basal lipolysis is not well understood. Our previous study demonstrated that basal lipolysis is elevated in the enlarged fat cells of obese rats as a result of a reduction in the surface phosphatidylcholine concentration of endogenous lipid droplets (2, 3). An active hormone-sensitive lipase (HSL) is present in fat cells even in the absence of lipolytic hormones, and phosphatidylcholine on the surface of endogenous lipid droplets causes inhibition of the lipolytic action of HSL. A decrease in the surface phosphatidylcholine concentration in endogenous lipid droplets conversely causes an increase in basal lipolysis. It is well known that fasting is associated with an increase in plasma free fatty acid levels. Many investigators have reported that lipolysis in fat cells is accelerated by fasting (4, 5) and also that HSL activity or HSL protein is increased by fasting (6). However, the weight and volume of adipose tissue are readily changed a few fold by altering the nutritional conditions. Therefore, one must pay careful attention to the way of presentation of adipose tissue enzyme contents (7).

In this study, we developed microassay methods for measuring the level of glycerol and FFA, and determined the basal level of lipolysis in epididymal fat cells from streptozotocin-induced diabetic or 1-d fasting rats. We also compared the FFA re-esterification ratio and the effect of anti-lipolytic agents such as insulin or β-blocker on basal lipolysis in fat cells from diabetic or starved rats.

MATERIALS AND METHODS

Materials. Collagenase (type IV) was purchased from Worthington Biochemical Corp. (Freehold, NJ). Bovine serum albumin, soybean trypsin inhibitor, benzamidine hydrochloride, streptozotocin, insulin (human recombinant) and norepinephrine were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Albumin was extracted by the method of Chen (8) to
remove the free fatty acids.

**Animals.** Male Crj:Wistar rats weighing 170 to 200 g were given standard laboratory diets (Oriental Yeast Co., Ltd.) and water ad libitum, and maintained according to the Laboratory Animal Center guidelines at Ehime University, School of Medicine, on a 12-h (7 am to 7 pm) light-dark cycle. The rats were given a single dose of streptozotocin (65 mg/kg body weight) by femoral vein injection. Animals were killed 7 to 8 d after streptozotocin injection between 9 and 10 am, and their serum was obtained and frozen for later analysis. Epididymal adipose tissues were quickly removed and washed with Hank’s buffer for isolation of fat cells.

**Measurement of basal lipolysis in fat cells.** Glycerol and FFA were measured separately in cells and medium and the total glycerol and FFA content in each tube was determined. Fat cells (100 mL packed volume) were incubated for 2 h at 37°C in 500 mL of buffer A (25 mM TES, pH 7.4 containing 135 mM NaCl, 5 mM KCl and 1 mM MgCl₂) supplemented with 2.5% bovine serum albumin. To assay glycerol production, 100 mM of 30% perchloric acid was added, following mixing the mixture was centrifuged for 5 min at 1,000 × g. The supernatant (500 µL) was supplemented with 95 µL of 25% KOH and centrifuged for 5 min at 6,500 × g. The supernatant in the supernatant (250 µL) was assayed glycerol as described previously (9). For the assay of FFA production, 2.5 mL of Dole’s extraction mixture (isopropanol : heptane : 1 mL of H₂O) was added and the level of fatty acids released were measured with copper reagent and bathocuproine as described previously (13). The lipase activity was determined with [³H]trioleoylglycerol as a substrate (9). The lipase activity in both solutions was assayed separately, and the activities of solution I (infranatant) and II (fat layer) were combined and expressed as µmol oleic acid released per adipose tissue per hour.

**Western blotting of HSL proteins.** Adipose tissue (100 mg) was sonicated for 30 s at power 4 using a Tomy UD-200 (Tomy Co. Ltd., Tokyo, Japan) with 1 mL of Laemmli’s sample buffer containing 20% (w/v) SDS (13), and an aliquot of the suspension was subjected to SDS-polyacrylamide gel electrophoresis. The resulting proteins were transferred to a polyvinylidene difluoride membrane (BIO-RAD Laboratories, CA), which was then blocked with 5% (w/v) skim milk and incubated with anti-HSL antiserum that was produced in rabbits using a synthetic peptide as described previously (14). The immunoreactive proteins were visualized with alkaline phosphatase-conjugated goat anti-rabbit IgG and Attophos (ICN Pharmaceuticals, Inc., OH) and the enhanced chemiluminescence intensity was determined using a FluorImager, Fluorescence Imaging Analyzer (Amersham Pharmacia Biotech UK Ltd., Bucks, UK).

**General experimental procedures.** Serum free fatty acid, triacylglycerol and glucose concentrations were determined using NEFA C-Test Wako, Triglyceride E-Test Wako and Glucose C-II Test Wako test kits (Wako Pure Chemical Industries, Ltd.), respectively. Relative protein content was determined using a Bio-Rad protein assay kit (BIO-RAD Laboratories) using bovine serum albumin as a standard.

**Data and statistical analyses.** All values are expressed as means±SE. Data were analyzed by one-way ANOVA, and then differences in means among groups were analyzed using Dunnett’s test or Fisher’s Protected LSD multiple comparison test. Differences with p<0.05 were considered significant.

**RESULTS**

The lipid metabolism of streptozotocin-injected rats was compared with that of rats with similar body weight (1 wk younger than the streptozotocin-injected rats) and 1-d-fasted rats. Table 1 shows typical values of the body weight and serum concentration of triacylglycerol, free fatty acid and glucose. Streptozotocin-induced diabetes caused significant alterations in serum glucose and serum free fatty acid levels compared with those of control rats. The serum glucose concentration of the streptozotocin-injected rats was increased 2.9 fold and that of FFA increased 2.2 fold (Table 1). Serum FFA was also significantly increased by 1-d of fasting.
An increase in serum FFA levels may accompany the stimulation of basal lipolysis in fat cells (5). The basal FFA production from fat cells was significantly increased by streptozotocin-induced diabetes (5.5 fold) (Fig. 1A). However, the basal glycerol production from fat cells was increased only 1.5 fold by streptozotocin-induced diabetes (Fig. 1B). Therefore, the fractional FFA re-esterification that occurred in basal lipolysis was significantly decreased by streptozotocin-induced diabetes (Fig. 1C). The basal FFA production from fat cells was also significantly increased by 1 d of fasting (7.9 fold). However, the basal glycerol production was increased by 1 d of fasting only 1.9 fold. Therefore, the fractional FFA re-esterification that occurred in basal lipolysis was significantly decreased by 1 d of fasting.

We examined the role of endogenous lipid droplets in lipolysis in fat cells. The lipolysis in the absence of lipolytic agents (basal lipolysis) was low (Fig. 1). However, when fat cells from control rats were sonicated (homogenization of the endogenous lipid droplets), the lipolysis was markedly increased about 80 fold in the absence of lipolytic agents. However, the lipolytic activity of sonicated fat cells (both FFA and glycerol production) decreased more in streptozotocin-induced diabetic rats

**Table 1.** Body weight and serum concentration of triacylglycerol, free fatty acids and glucose in streptozotocin-induced diabetic or 1-d-fasting rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>Body weight (g)</th>
<th>Fat pad (g)</th>
<th>Triacylglycerol (mg/L)</th>
<th>FFA (μEq/L)</th>
<th>Glucose (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=8)</td>
<td>195±3.5*</td>
<td>0.66±0.03*</td>
<td>1.270±160*</td>
<td>142±26*</td>
<td>1.810±10*</td>
</tr>
<tr>
<td>Diabetic (n=10)</td>
<td>190±3.2*</td>
<td>0.47±0.02*</td>
<td>1.630±450*</td>
<td>309±30*</td>
<td>5.216±202*</td>
</tr>
<tr>
<td>Fasting (n=4)</td>
<td>168±11*</td>
<td>0.61±0.12*</td>
<td>1.040±210*</td>
<td>560±40*</td>
<td>1.190±110*</td>
</tr>
</tbody>
</table>

FFA: free fatty acid. Values not sharing a letter are significantly different, p<0.05.
than in control rats (Fig. 2). The fractional FFA re-esterification was close to zero in sonicated fat cells (data not shown).

The triacylglycerol lipase activity was determined at neutral pH (neutral triacylglycerol lipase) by the use of trioctylglycerol as a substrate. The enzyme solution from adipose tissue homogenate was prepared as described in “Materials and Methods”. The neutral triacylglycerol lipase(s) including HSL in adipose tissue might be determined in this assay system. The activity was neither inhibited by 1 M NaCl nor activated by rat serum, indicating that it included no lipoprotein lipase activity (15). The neutral triacylglycerol lipase activity was not significantly affected by streptozotocin-induced diabetes or 1 d of fasting (Fig. 3A). Similar results were observed by measuring immunoreactive HSL protein when the amount of immunoreactive HSL protein in the epididymal fat pad was determined using a rabbit polyclonal anti-HSL/synthetic peptide antibody that recognizes intact HSL. The amount of immunoreactive HSL protein was not affected by streptozotocin-induced diabetes or 1 d of fasting (Fig. 3B).

As shown in Fig. 4A, norepinephrine dependently induced lipolysis in fat cells. The pattern of dose dependency of FFA production in fat cells from streptozotocin-diabetic rats was the same as that in fat cells from control rats, although the basal lipolysis differed. It is well known that the β-blocker propranolol inhibits the induction of lipolysis by norepinephrine, whereas the α-blocker phenoxybenzamine does not inhibit such lipolysis (data not shown). Figure 4B shows the effect of β-blocker on FFA production in fat cells isolated from streptozotocin-induced diabetic rats. Propranolol at a concentration of 10⁻⁴ M completely inhibited the FFA production elicited by norepinephrine, whereas it failed to inhibit basal FFA production at a concentration of 10⁻³ M. Propranolol also failed to inhibit basal glycerol production (Fig. 5B). Therefore, the fractional FFA re-esterification in basal lipolysis in fat cells from streptozotocin-induced diabetic rats was not significantly changed by propranolol (Fig. 5C). Propranolol also failed to inhibit the stimulation of basal lipolysis induced by sonication of the cells isolated from streptozotocin-induced diabetic rats (data not shown).
Insulin failed to inhibit the basal FFA production in fat cells from streptozotocin-induced diabetic rats at a concentration of 10^{-7} M (Fig. 5A). Insulin also failed to inhibit the basal glycerol production (Fig. 5B). Therefore, the fractional FFA re-esterification in basal lipolysis in fat cells from streptozotocin-induced diabetic rats was not significantly affected by insulin (Fig. 5C). The stimulation of basal lipolysis by sonication of the cells isolated from streptozotocin-injected rats was not changed by the addition of insulin (10^{-6} M) (data not shown). H-89 (an inhibitor of protein kinase A) did not affect basal lipolysis of streptozotocin-induced diabetic rats either. Therefore the fractional FFA re-esterification in basal lipolysis was not changed by H-89 (Fig. 5C).

Adenosine also inhibited the norepinephrine induced lipolysis about 50%, whereas it failed to inhibit the basal lipolysis in the fat cells from streptozotocin-induced diabetic rats (data not shown).

**DISCUSSION**

Basal lipolysis occurs continuously in fat cells. Very little of the glycerol produced via intracellular lipolysis can be re-esterified though re-phosphorylation of glycerol by fat cells because of the low level of glycerol kinase in fat cells (1, 16). Therefore, the level of glycerol produced from fat cells is a good index of lipolysis. FFAs play an important role in the energy homeostasis of the body. Circulating FFA in plasma is derived from the breakdown of stored triacylglycerols in fat cells (17, 18). The partial hydrolysis of triacylglycerol in fat cells occurs to only a trivial extent and FFAs are not significantly oxidized in fat cells (19–21). Therefore, the release of FFA from fat cells is mainly the result of two processes occurring simultaneously in the fat cells: lipolysis and FFA re-esterification (Fig. 6). The FFA re-esterification may be assessed by determining the ratio between FFA and glycerol. Here, we measured basal lipolysis, the production of glycerol and FFA from fat cells, and compared the FFA re-esterification in fat cells from control, diabetic and fasted rats.

It is well known that diabetes is associated with an increase in plasma free fatty acid and the rate of basal lipolysis (5, 22–24). In the present study, basal FFA production was increased by diabetes (5.5 fold). Fasting is also known to be associated with an increase in plasma FFA. Basal lipolysis, measured as FFA release, is also known to be increased by fasting, with the levels of released FFA about 7.9 fold higher than control levels after 1 d of fasting (5). However, basal glycerol production was increased only 1.5 fold and 1.9 fold in diabetic and 1-d-fasting rats, respectively (Fig. 1). Therefore, FFA re-esterification was significantly decreased by diabetes and by 1 d of fasting; about 83% of FFA produced via basal lipolysis was re-esterified in control rats, but only 39% and 38% of FFA was re-esterified in the diabetic and 1-d-fasting rats, respectively (Fig. 1). Because of the low level of glycerol kinase in fat cells, glycerol-3-phosphate should be supplied from glucose for the re-esterification. Both streptozotocin-induced diabetes and starvation result in a reduced serum insulin concentration (5) and thus may cause a decrease in glucose metabolism. This may account for why the FFA re-esterification in fat cells from diabetic or fasting rats was decreased.

It has been believed that hormone-sensitive lipase (HSL) was the only enzyme to hydrolyze triacylglycerol in mammalian fat cells (25, 26). However, Zimmermann et al. reported that a second enzyme, adipose triglyceride lipase, catalyzed the initial step in triglyceride hydrolysis (27). Fortier et al. also reported, in fat cells from HSL-deficient mice, basal lipolysis as great as that of normal fat cells, proving the existence of non-HSL-mediated pathway(s) of lipolysis in fat cells (28). Therefore, we determined triacylglycerol lipase activity at neutral pH (neutral triacylglycerol lipase), which included HSL and non-HSL lipase activities. We also determined immunoreactive HSL protein content. The neutral triacylglycerol lipase activity and HSL protein were not significantly changed in streptozotocin-induced diabetic or 1-d-fasting rats (Fig. 3). However, the net basal lipolysis (glycerol production) was increased about 1.5 fold and 1.9 fold by streptozotocin-induced diabetes and 1 d of fasting, respectively (Fig. 1). This discrepancy might be explained as follows. Lipolysis in fat cells may be regulated not only by the cellular lipase activity (the catalytic activity of the enzyme), which is estimated using an artificial substrate such as trioleoylglycerol in the presence of the enzyme, which is extracted from the fat cells. In that system, the lipase activity of all samples is assayed using the same substrate conditions (artificial emulsion). Therefore, the activity is not affected by the nature of the substrate, but only by the enzyme. In contrast, lipolysis in fat cells is affected by not only the lipase (enzyme) but also by the nature of the endogenous lipid droplets (substrate) (3). Generally, interfacial reactions such as lipol-
ysis are known to be subject to regulation by the physical properties of the surface containing the substrate (29). Previously, we suggested that phosphatidylcholine on the surface of the lipid droplets may be a regulatory factor for lipolysis in fat cells (3). Egan et al. 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 to its location in unstimulated cells (13). Brassemle et al. reported that using immunofluorescence microscopy, they observed that HSL was translocated from the cytosol to the surface of lipid storage droplets in lipolytically stimulated 3T3-L1 adipocytes (30). We also investigated the translocation of HSL in fat cells in response to various lipolytic agents and reported that these agents induced HSL translocation from the cytosol to the lipid droplets in fat cells (31). These data suggest that HSL translocation may play important roles in lipolysis in fat cells. We observed the translocation of HSL from the cytosol to lipid droplets in response to fasting: the proportion of HSL protein in fat layers of fat cells was significantly increased by fasting, from 55.07 ± 1.9% of total HSL protein (mean ± SE, normally fed rats) to 64.47 ± 2.4% of total HSL protein (mean ± SE, rats that had fasted for 1 d). These data might explain the increase in the basal lipolysis (glycerol production) of adipose tissue from fasting rats. However, such translocation of HSL was not observed in the fat cells from streptozotocin-induced diabetic rats (data not shown). Thus some other mechanism might exist in the fat cells from streptozotocin-injected diabetic rats.

One of the most important metabolic actions of insulin is the inhibition of lipolytic activity in fat cells (32). Activation of cAMP phosphodiesterase by insulin is believed to be the major mechanism whereby insulin reduces cellular cAMP, which then leads to inactivation of the cAMP-dependent protein kinase, net dephosphorylation of HSL, and antilipolysis (33, 34). This mechanism of insulin-mediated antilipolysis presupposes that HSL activity is reduced. Although we found that insulin inhibited the lipolysis induced by norepinephrine in fat cells (data not shown), it did not affect the basal lipolysis induced by streptozotocin-induced diabetes or starvation in fat cells (data not shown). Propranolol, another antilipolytic agent, also failed to inhibit the basal lipolysis (Fig. 4). Previously, we measured the basal lipolysis as indicated by released FFA, and reported that the basal lipolysis was increased about 8 fold in fasting rats (5). In the present study, net basal lipolysis as indicated by glycerol production was not increased as much, only 1.9 fold by fasting (Fig. 1). The more marked increase in FFA release may have been induced by a decrease in the re-esterification of FFA under conditions of starvation. A similar mechanism may operate in diabetes.

Adipose tissue is a reservoir of FFAs that are released and used as energy substrates during periods of energy deficit. Free fatty acids are a major energy source for most tissues. Circulating FFAs in plasma are derived from the breakdown of stored triacylglycerols in adipose tissue. Physiologically, the lipolysis in the adipose tissue is controlled by hormonal balance, lipolytic hormones such as catecholamines and ACTH, and antilipolytic hormones such as insulin. Hormone-independent lipolysis is called basal lipolysis. We have considered that the contribution of basal lipolysis to the energy supply is low, because the basal level of FFA release from adipose tissue is very low under normal conditions. Under normal conditions, the basal glycerol production (net basal lipolysis) in fat cells is moderate and the ratio of FFA re-esterification is high (about 80%) (Fig. 1). Therefore, the basal FFA release from fat cells is low under normal conditions. Under conditions of diabetes or starvation, the basal glycerol production (net basal lipolysis) in fat cells is increased and the ratio of FFA re-esterification in fat cells is decreased. Therefore, the basal FFA release from fat cells is increased (Fig. 1). In conclusion, in addition to the increase of the net basal lipolysis, the decrease of FFA re-esterification in fat cells may be one of the reasons for the plasma FFA increase in diabetes and starvation.

REFERENCES


