Norepinephrine-induced lipolysis in rat fat cells from visceral and subcutaneous sites: role of hormone-sensitive lipase and lipid droplets

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Abstract Norepinephrine-induced lipolysis was examined in visceral (epididymal and omental) and subcutaneous (abdominal and femoral) fat cells in rats. Hormone-induced lipolysis was considerably higher in the visceral fat cells than in the subcutaneous cells. A higher hormone-sensitive lipase (HSL) activity was present in the visceral than in the subcutaneous fat cells. Endogenous lipid droplets were prepared from rat visceral (epididymal and omental) and subcutaneous (abdominal and femoral) fat cells and norepinephrine-induced lipolysis was examined in a cell-free system consisting of the lipid droplets prepared from fat cells from each site and a fixed amount of HSL from rat epididymal adipose tissue. In this cell-free system, norepinephrine induced a higher rate of lipolysis with lipid droplets from the visceral than from the subcutaneous fat cells. These findings suggest that the difference in norpinephrine-induced lipolysis between visceral and subcutaneous fat cells may be due to the differences in HSL activity and lipid droplet character at each site.—**Morimoto, C., T. Tsujita, and H. Okuda.** Norepinephrine-induced lipolysis in rat fat cells from visceral and subcutaneous sites: role of hormone-sensitive lipase and lipid droplets. *J. Lipid Res.* 1997. **38:** 132–138.

Supplementary key words phospholipid • basal lipolysis • site-related difference

It is well known that the anatomic distribution of body fat, independent of total adiposity, is an important predictor of morbidity and mortality (1). Vague (2) originally classified obesity into visceral and femoral types, and recognized that visceral distribution of body fat was associated with an increase in the incidence of diabetes mellitus and atherosclerosis. The mechanism by which visceral distribution of fat predispose to the development of these diseases is not certain but may be related to the fact that free fatty acids (FFA) arising from lipolysis in visceral adipose tissues are drained via the hepatic portal vein directly into the liver (3).

Several mechanisms responsible for the site-specific differences in lipolysis have been proposed. These are differences in the number or type of stimulatory β-adrenergic receptors or inhibitory α_2-adrenergic receptors, differences in the sensitivity of adipocytes to adenosine-mediated suppression, as well as other factors such as site-related differences in blood flow that could modulate the flux of hormones and FFA (4–7).

It is well known that catecholamines play a central role in promoting lipolysis by white fat cells. This effect is believed to be mediated by a β-adrenergic receptor–adenylate cyclase complex, which is located in the plasma membrane of fat cells and consists of at least three distinct components, these being a β-adrenergic receptor, a nucleotide regulatory protein and adenylate cyclase (8). The receptor-controlled increase in intracellular cyclic AMP concentrations promotes activation of cyclic AMP-dependent protein kinase A, which phosphorylates a serine residue (Ser-563 for the rat and Ser-551 for the human) on HSL and promotes its activation. The resulting phosphorylated HSL is believed to stimulate lipolysis in fat cells. The theory of catecholamine-induced lipolysis is widely accepted, though we have reported experimental results that are in conflict with this theory (9).

Previously, we suggested that the phosphorylated HSL might not stimulate lipolysis in fat cells. Cyclic AMP-dependent activation of HSL certainly stimulated lipolysis of artificial lipid droplets such as [^{3}H]triolein emulsified with gum arabic, whereas it failed to stimulate lipolysis of endogenous lipid droplets from rat fat cells. The endogenous lipid droplets were prepared from the fat cells by mild procedures, including hypotonic shock, Triton X-100 treatment and brief incubation (37°C, 10 min) in a buffer. Approximately 95% of

Abbreviations: FFA, free fatty acids; HSL, hormone-sensitive lipase; TES, *N*-tris (hydroxymethyl)methyl-2-aminoethanesulfonic acid; BES, *N,N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

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the protein present in the cells was removed from the droplets which consisted of mainly triglyceride, and phosphatidylcholine (PC), phospshatidylethanolamine (PE), cholesterol, carbohydrate, and protein as minor constituents (10).

Recently, perilipin, a major adipocyte phosphoprotein, was reported to be located on the surface of endogenous lipid droplets (11). Among the minor components, PC is known to inhibit the lipolytic actions of HSL (12). These experimental results clearly indicate that lipid droplets in fat cells are quite different from artificial lipid droplets such as triolein emulsified with gum arabic. The estimation of HSL activity using artificial lipid droplets as a substrate should be performed in the presence of enzyme extracted from fat cells. In this system, lipolysis is not affected by substrate conditions (its content and character) but solely by the enzyme activity. In contrast to HSL activity, catecholamine-induced lipolysis in fat cells is not only due to HSL activity but also to the nature of the lipid droplets.

In the present investigation, we prepared lipid droplets and HSL from visceral and subcutaneous fat cells and examined the responsiveness of the droplets in relation to the levels of norepinephrine and HSL in the cells, in order to clarify the characteristic features of lipolysis in visceral fat cells.

MATERIALS AND METHODS

Animals

Young male Wistar-King rats, weighing 150 to 200 g, were given a standard laboratory diet (Oriental Yeast Co. Ltd.) and water ad libitum. They were killed by cervical dislocation and their visceral and subcutaneous adipose tissues were quickly removed.

Materials

Collagenase (type IV) was purchased from Worthington Biochemical Corp. (Freehold, NJ). Norepinephrine was obtained from Sankyo Co. Ltd. (Tokyo, Japan). TES and BES were from Wako Pure Chemical Industries (Osaka, Japan). [^{3}H]triolein (tri-[9,10-^{3}H]oleoylglycerol) was from Amersham Japan (Tokyo, Japan). Triolein was from Sigma (St. Louis, MO). Bovine serum albumin was from Wako Pure Chemical Industries and was extracted by the method of Chen (13) to remove free fatty acid.

Methods

Measurement of lipolytic activity in fat cells. Isolated fat cells were obtained from rat visceral (epididymal and omental) and subcutaneous (abdominal and femoral) adipose tissues, respectively, by the method of Rodbell (14). An aliquot of each fat cell fraction (50 µl packed volume) was incubated for 1 h at 37°C in 225 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% bovine serum albumin and 25 µl norepinephrine (0.25 µg) solution. The release of FFA was measured as described previously (15). Briefly, the incubation mixture (250 µl) was mixed with 3 ml chloroform–heptane (1:1 (v/v) containing 2% (v/v) methanol and extracted by shaking the tube horizontally for 10 min in a shaker. The mixture was centrifuged (2,000 *g*, 5 min), and the upper aqueous phase removed by suction. Copper reagent (1 ml) was then added to the lower organic phase. The tube was shaken for 10 min, the mixture was centrifuged (2,000 *g*, 10 min), and 0.5 ml of the upper organic phase, which contained the copper salts of the extracted fatty acids, was treated with 0.5 ml 0.1 % (w/v) bathocuproine in chloroform containing 0.05 % (w/v) 3(2)-tert-butyl-4-hydroxyanisole. Its absorbance was then measured at 480 nm. Lipolysis was expressed as microequivalents (µEq) of FFA released per 10^5 fat cells per h. Fat cell number was calculated by fat cell diameter and triglyceride content in each fat cell fraction.

Assay of hormone-sensitive lipase activity in fat cells. Fat cells (350 µl packed volume) were homogenized in 500 µl of buffer A in a Potter-Elvehjem homogenizer and the Teflon pestle was moved up and down three times by hand. The homogenate was centrifuged at 2,500 ***g*** for 5 min at 10°C. The resultant supernatant was used as the enzyme solution. Fifty milligrams of triolein and 220 µCi of [^{3}H]triolein were suspended in 3.75 ml of 5% (w/v) gum arabic solution and sonicated for 5 min. The assay system contained the following components in a total volume 100 µl: 50 µl of enzyme solution, 0.14 µmol of triolein, 0.45 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. The incubation was carried out for 1 h at 37°C at pH 6.8, and released [^{3}H]oleic acid was measured by the method of Belfrage and Vaughan (16). Lipase activity is expressed as nanomoles of [^{3}H]oleic acid released per 10^5 fat cells/h.

Preparation of endogenous lipid droplets. Isolated fat cells were obtained from visceral and subcutaneous adipose tissues by the method of Rodbell (10). A 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 swinging 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, followed by in-

cubation with buffer A at 37°C for 10 min, and then centrifuged at 200 *g* for 3 min. It was then washed with buffer A. Approximately 95% of protein was removed from the fat layer by these procedures. Although the fat layer was a crude preparation, it was used as endogenous lipid droplets in the experiments.

Preparation of HSL solution. Rat epididymal adipose tissue (0.7 g) was cut into small pieces with scissors and homogenized in 1 ml of buffer A in a Potter-Elvehjem homogenizer by five strokes of a Teflon pestle. The homogenate was centrifuged (2,500 *g*, 15 min) at 10°C, and the resultant supernatant was applied to a heparin-Sepharose column (5 × 20 mm), equilibrated with buffer A, to remove lipoprotein lipase. The unadsorbed fraction was used as the HSL solution. HSL activity in this fraction was not reduced by 1 M NaCl or antiserum to bovine lipoprotein lipase indicating that lipolytic activity due to lipoprotein lipase was minimal in the preparation.

Although HSL solution contained monoacylglycerol lipase as well as HSL, it alone did not affect the lipolysis in a cell-free system, because high concentration of sodium fluoride (25 mM), which inhibited HSL activity but not monoacylglycerol lipase activity (17), completely inhibited the lipolysis in the cell-free system (data not shown).

Lipolysis in a cell-free system consisting of HSL and endogenous lipid droplets. A 25 µl sample of packed endogenous lipid droplets was incubated at 37°C for 1 h with 100 µl HSL solution, 25 µl epinephrine (0.25 µg) solution, 100 µl buffer A containing 2.5% bovine serum albumin, and 25 µl buffer A. Another sample of 375 µl of packed endogenous lipid droplets was mixed with 750 µl buffer A containing 2.5% 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% bovine serum albumin for 1 h at 37°C. Lipolysis was expressed as µEq FFA per 1 ml packed lipid droplets.

Other procedures. SDS-polyacrylamide gel electrophoresis was performed in 10% gel under reducing conditions as described by Laemmli (18). The gel was stained with Coomassie Brilliant Blue R-250. The Student's *t*-test was used to determine whether the differences were statistically significant.

RESULTS

We first examined norepinephrine-induced lipolysis in rat visceral (epididymal and omental) and subcutaneous (abdominal and femoral) fat cells. As shown in **Fig. 1**, hormone-induced lipolysis was considerably higher in the visceral fat cells when compared to the subcutaneous fat cells. Basal lipolysis (lipolysis in the absence of lipolytic stimulants) was found to be 2.7 ± 0.4, 3.6 ± 1.3, 1.9 ± 0.3 and 1.3 ± 1.3 FFA µEq/10^5 cells/h in epididymal, omental, abdominal, and femoral fat cells, respectively. There was no significant difference in basal lipolysis among these fat cells. A high rate of lipolysis in rat epididymal fat cells was found at varying concentrations of norepinephrine when compared to the response of the subcutaneous (abdominal) cells (**Fig. 2**). Half-effective relative norepinephrine values (EC_{50}) were estimated to be 0.07 and 0.1 µg/ml in epididymal and abdominal fat cells, respectively.

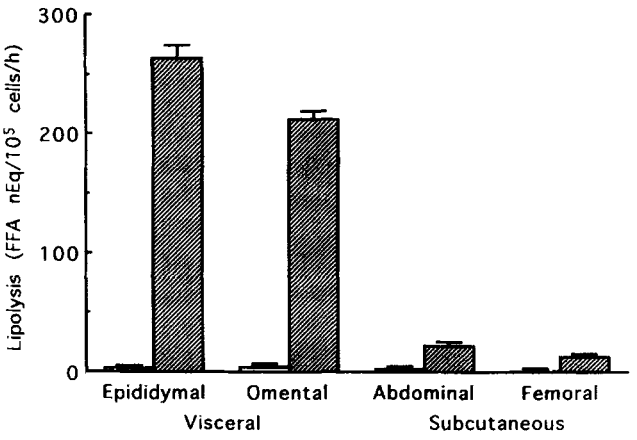

Fig. 1. Norepinephrine-induced lipolysis in rat visceral (epididymal and omental) and subcutaneous (abdominal and femoral) fat cells. Each fat cell fraction was incubated in the presence (▩) or absence (□) of norepinephrine (1 µg/ml). Values are means ± SE for four separate assays.

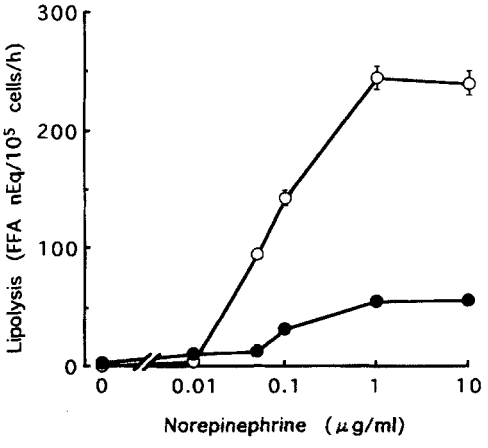

Fig. 2. Effect of norepinephrine on lipolysis in rat epididymal or abdominal fat cells. Various concentrations of norepinephrine were incubated with epididymal (○) or abdominal (●) fat cells. Each point represents the means ± SE of four separate assays.

HSL activity was estimated with artificial lipid droplets in which the lipolytic reaction was performed as a function of enzyme activity. As shown in **Fig. 3,** visceral (epididymal and omental) fat cells contained larger HSL activity than the subcutaneous (abdominal and femoral) cells.

In general, the lipolytic reaction is regulated by both the lipase content and its substrate characteristics which include substrate structure, its surface area and character. With intact lipid droplets isolated from rat epididymal fat cells, negligible amount of free fatty acids were released in the presence of HSL, whereas lipolysis was dramatically elevated with sonicated lipid droplets in the presence of the same amount of HSL (data not shown). It has been shown that this increase in lipolysis was not due to an increase in the surface area resulting from sonication but to a decrease in the phosphatidylcholine concentration on the surface of the lipid droplets (12). Therefore, it is possible that the site-related difference in lipolysis may be due to the substrate of HSL or the endogenous lipid droplets in the fat cells.

We have previously prepared a catecholamine-sensitive cell-free system consisting of the lipid droplets and HSL, and suggested that catecholamines elicited lipolysis by acting on lipid droplets (12). Extremely low level of lipolysis was found with lipid droplets both in the absence and presence of HSL. Norepinephrine-induced lipolysis was negligible in the absence of HSL but remarkable in the presence of the enzyme (data not shown). When sonicated lipid droplets were used instead of intact lipid droplets in the cell-free system, catecholamine failed to stimulate lipolysis (12).

Then, endogenous lipid droplets were prepared from rat visceral (epididymal and omental) and subcutaneous (abdominal and femoral) fat cells, and norepinephrine-induced lipolysis was examined in a cell-free system which consisted of the lipid droplets prepared from fat cells at each site, and HSL from epididymal adipose tissue. In this cell-free system, norepinephrine induced a higher rate of lipolysis with the lipid droplets from visceral fat cells than with those from the subcutaneous cells (**Fig. 4**). A higher rate of lipolysis was found in the cell-free system consisting of lipid droplets from epididymal fat cells at the various concentrations of norepinephrine when compared with the rate of lipolysis observed with lipid droplets prepared from subcutaneous (abdominal) fat cells (**Fig. 5**). As the same amount of HSL was included in these cell-free systems, the site-related difference in lipolysis may be due to the difference in the lipid droplets isolated from each site. Therefore, it follows that the difference in norepinephrine-induced lipolysis between the visceral and subcutaneous fat cells may partly be due to the difference in the lipid droplets at each site.

Table 1 shows the differences in the chemical compositions of the lipid droplets derived from rat visceral (epididymal and omental) and subcutaneous (abdominal and femoral) fat cells. There was no significant difference in cell diameter among these fat cells. The average cell diameter was found to be 64.6, 57.9, 67.7, and 74.4 μm at epididymal, omental, abdominal, and femoral lipid droplets, respectively. The lipid droplets in each site consisted mainly of triglyceride. The triglyceride and cholesterol contents were not factors that differentiated the visceral from the subcutaneous lipid droplets. On the other hand, PC and PE contents were found

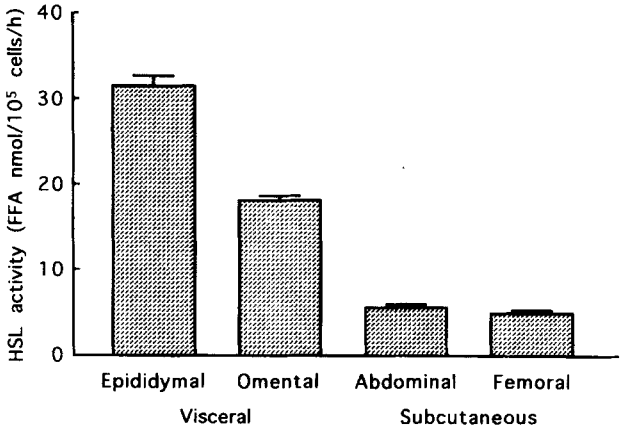

Fig. 3. Hormone-sensitive lipase activity of rat visceral (epididymal and omental) and subcutaneous (abdominal and femoral) fat cells. [^{3}H]triolein emulsion was used as a substrate as described in Materials and Methods. Values are means ± SE of four separate assays.

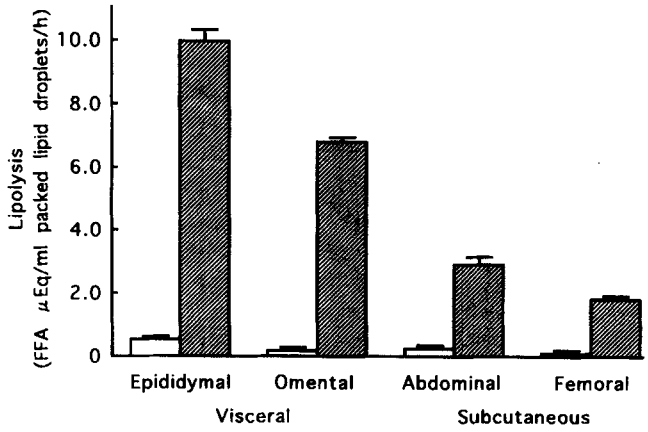

Fig. 4. Norepinephrine-induced lipolysis in a cell-free system. Endogenous lipid droplets were prepared from rat visceral (epididymal and omental) and subcutaneous (abdominal and femoral) fat cells. The lipid droplets from each site were incubated in the presence (■) or absence (□) of norepinephrine (1 μg/ml) with HSL prepared from rat epididymal adipose tissue, as described in Materials and Methods. Values are means ± SE of four separate experiments.

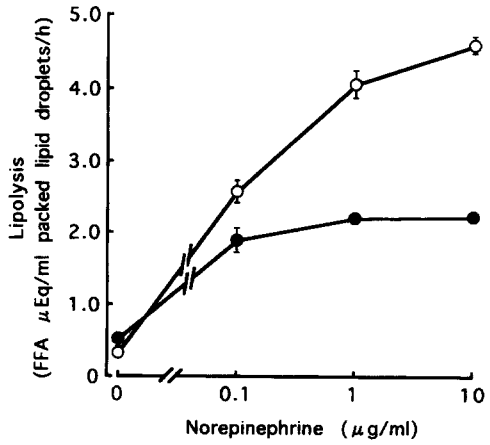

Fig. 5. Effect of norepinephrine on lipolysis in a cell-free system. The lipid droplets were prepared from epididymal and abdominal fat cells. HSL from epididymal adipose tissue was incubated with the lipid droplets from epididymal (○) or abdominal (●) fat cells, as described in Materials and Methods. Each point represents the means ± SE of four separate assays.

to be higher in the visceral lipid droplets than in the subcutaneous droplets. Protein content was also found to be higher in the visceral lipid droplets than in the subcutaneous droplets. Especially, the protein content in omental lipid droplets was about 5-fold than in the visceral droplets.

The protein fractions obtained from each droplets were analyzed by SDS-polyacrylamide gel electrophoresis. **Figure 6** shows the typical protein pattern in the lipid droplets at each site. There was no significant difference in protein patterns between abdominal and femoral lipid droplets. The protein pattern was similar in epididymal and omental lipid droplets except at peak 4 (68 kDa). Protein band at peak 4 was about 3-fold greater in the omental lipid droplets than in the epididymal droplets. There was considerable difference in protein pattern between visceral and subcutaneous lipid droplets. The protein bands at peak 1 (230 kDa), 2 (133 kDa), and 3 (115 kDa) were greater in subcutaneous lipid droplets than in visceral droplets, but peaks 6 (35.9 kDa) and 7 (31 kDa) were lower in subcutaneous lipid droplets than in visceral droplets.

DISCUSSION

Lacasa et al. (6) reported that isoproterenol-stimulated lipolytic activity was higher in the parametrial fat cells of female rats than in the femoral subcutaneous fat cells. In addition, the subcutaneous fat cells were found to have reduced cyclic AMP production and a decreased β-adrenoceptor density associated with the reduction in lipolytic activity (6). The site-related differences in lipolytic activity of the fat cells was postulated to be due to differences in cyclic AMP production and β-adrenoceptor number. This was largely based on a cyclic AMP cascade theory in which catecholamines increase cyclic AMP production by stimulating β-adrenoceptors which leads to a sequence of events including elevation of adenylyl cyclase, activation of A-kinase and phosphorylation of HSL ultimately resulting in an increase in lipolysis of fat cells.

In our previous report, however, cyclic AMP-dependent activation of the HSL stimulated lipolysis of [^{3}H]triolein emulsified with gum arabic, but not of endogenous lipid droplets prepared from rat fat cells (9). Although hydrolysis of [^{3}H]triolein emulsified with gum arabic was stimulated by HSL phosphorylated in vitro, its hydrolysis was not accelerated by HSL extracted from epinephrine-treated fat cells. Glycerol release in medium was found to be 6 ± 3 and 299 ± 5 nmol/g per 30 min in the absence and presence of epinephrine

TABLE 1. Chemical compositions of lipid droplets derived from rat visceral and subcutaneous lipid droplets

			Phospholipids		
	Triglyceride	Proteins	PC	PE	Cholesterol
Visceral	*mg*	μg	μg	μg	μg
Epididymal	302.9 ± 16.2	82.3 ± 12.0	167.0 ± 10.3	105.9 ± 8.9	162.5 ± 21.6
Omental	216.8 ± 23.5	244.0 ± 21.1	274.0 ± 14.0	170.2 ± 6.8	221.1 ± 27.8
Subcutaneous					
Abdominal	242.3 ± 13.6	54.9 ± 12.3	41.7 ± 5.0	25.1 ± 3.5	133.7 ± 16.4
Femoral	282.6 ± 20.3	47.9 ± 6.4	69.2 ± 8.7	37.7 ± 2.2	162.2 ± 16.6

The values were mean ± SE per 10^6 lipid droplets for five separate assays. Phospholipids were separated from neutral lipids by the method of Hamilton and Comai (20) and assayed by Bartlett's method (21). Cholesterol was isolated by thin-layer chromatography (Whatman K-5 silica gel plates) with hexane–diethyl ether–acetic acid 95:5:1(v/v). Lipids were located with iodine vapor and spots of cholesterol were scraped off, extracted with chloroform–methanol 2:1(v/v) and measured with a Cholesterol E-test kit (Wako Pure Chemical Industries).

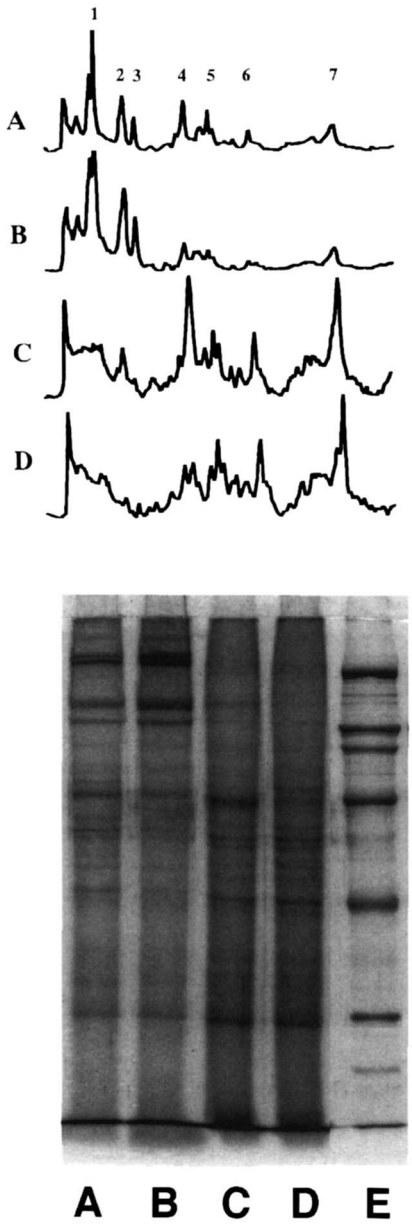

Fig. 6. SDS-polyacrylamide gel electrophoresis of proteins from femoral (A), abdominal (B), omental (C) and epididymal (D) lipid droplets. Top: Scanning densitograms on SDS-polyacrylamide gel electrophoresis. The molecular mass at each peak was estimated (from top to bottom, peak 1 (230-kda), peak 2 (133-kDa), peak 3 (115-kDa), peak 4 (68-kDa), peak 5 (43.8-kDa), peak 6 (35.9-kDa), and peak 7 (31-kDa)). Bottom: The samples (about 50 μg) were run on a 10% SDS-polyacrylamide gel under reducing conditions and the gel was stained with Coomassie Brilliant Blue. Lane E was the molecular standard (from top to bottom, myosin (200-kda), β-galactosidase (116.3-kDa), phosphorylase b (97.4-kDa), serum albumin (66.2-kDa), ovalbumin (42.7-kDa), and carbonic anhydrase (31-kDa)).

(1 μg/ml), whereas HSL activity in the extract of fat cells after 30 min incubation was 1152 ± 52 and 1223 ± 45 oleic acid nmol/g per h in the absence and presence of the hormone (10). Furthermore in a cell-free system consisting of the lipid droplets and HSL, sonication of lipid droplets caused the loss of catecholamine responsiveness (12). These results suggest that catecholamines might enhance lipolysis in fat cells through mechanisms other than through the cyclic AMP cascade.

Therefore, the precise mechanism by which catecholamines induce lipolysis is still a matter of controversy. Given this uncertainty, we have focused on HSL and lipid droplets in fat cells to clarify the factors that determine site-related differences in lipolysis. Norepinephrine-induced lipolysis was higher in visceral (epididymal and omental) fat cells than in the subcutaneous (abdominal and femoral) cells (Figs. 1 and 2). Although basal lipolysis was not significantly changed in each site, visceral fat cells contained a higher HSL activity than the subcutaneous cells (Fig. 3). Sztalryd and Kraemer (19) first reported that a lower HSL expressions including its activity and protein and mRNA amounts were found in subcutaneous rat fat cells compared to visceral cells.

Generally, HSL activity is defined as the lipolytic activity estimated under the conditions in which only HSL is a limiting factor. Therefore, HSL activity in fat cells must be differentiated from their lipolytic activity, which may be modulated by other factors such as substrate availability than HSL activity. In 1990, we found that epinephrine elicited glycerol release from rat fat cells without a concomitant increase in HSL activity (10). Previously, we established a cell-free system in which catecholamines stimulated lipolysis (12). Even in the presence of the same amount of HSL, remarkable lipolysis was found with sonicated lipid droplets as substrate in the cell-free system, whereas extremely low level of lipolysis was observed with intact lipid droplets as substrate. These results suggest that lipid droplets may also be a limiting factor for lipolysis in fat cells.

Using the cell-free system which consisted of lipid droplets and a fixed amount of HSL, norepinephrine induced a higher rate of lipolysis when the lipid droplets were derived from visceral fat cells than with those from the subcutaneous cells (Figs. 4 and 5). The result suggests that lipid droplet character may also be a factor causing difference in norepinephrine-induced lipolysis between the visceral and subcutaneous fat cells.

It is not known, however, which characteristics of the lipid droplets determine the differences in lipolysis of the fat cells derived from the different sites. Analysis of the lipid droplets revealed that PC and PE contents differentiated the visceral lipid droplets from the subcu-

taneous droplets (Table 1). There was a higher content of PC and PE in the visceral lipid droplets than in the subcutaneous droplets. Protein contents also differentiated the visceral lipid droplets from the subcutaneous droplets (Fig. 6). Protein content was greater in the visceral lipid droplets than in the subcutaneous droplets. There was considerable difference in protein composition between visceral and subcutaneous lipid droplets. In visceral lipid droplets, relative content of 230-, 133-, and 115-kDa proteins were increased, whereas 35.9-kDa and 31-kDa proteins markedly reduced. These protein might provide valuable tools for understanding the site-specific difference in lipolysis. Further experiment are needed to clarify the roles of phospholipids and proteins of the lipid droplets in norepinephrine-induced lipolysis of fat cells.

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