MORPHOLOGICAL CHANGES OF THE ADIPOSE CELL PLASMA MEMBRANE DURING LIPOLYSIS

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ABSTRACT

Morphological changes of the plasma membrane in the white adipose cell associated with lipid mobilization were assessed qualitatively and quantitatively on freeze-fracture replicas of epididymal adipose tissue from fasted and from streptozotocin-diabetic rats. The number of plasma membrane invaginations and intramembranous particles were evaluated per square micrometer of membrane and per entire adipocyte. These two determinations show that the number per square micrometer (local concentration) of both structural features progressively increases with the duration of diabetes and fasting, while that at the same time their number per entire cell (total content) remains unchanged. These data thus show: (a) a reorganization of the adipose cell plasma membrane during lipolysis; and (b) that this reorganization can be detected only by determining the concentration and the total content of the structural features of the membrane involved.

MATERIALS AND METHODS

Animals

Young male Wistar rats (200-290 g) were used throughout.

Fasting

20 animals were placed in individual cages containing water but no food while 6 others used as controls were fed normally with Purina Chow. Six rats of the fasting group were killed after 3 days, six after 6 days, and four after 9 days (four died at the 8th day of fasting).

Diabetes

18 rats were injected in the tail vein with streptozotocin (75 mg/kg) kindly supplied by Dr. W. Dulin (The
Preparation of Tissue

Immediately after the killing of the animals in each experimental group, the tip of the epididymal fat pads was excised on both sides and quickly minced in a 4% glutaraldehyde solution buffered with 0.1 M phosphate buffer, pH 7.4, at room temperature. After 2 h of fixation, small pieces of tissue were immersed in 30% glycerol buffered with 0.1 M phosphate buffer, pH 7.4, for at least 30 min, rapidly frozen in Freon 22 cooled in liquid nitrogen, then fractured and shadowed in a Balzers BAF 301 apparatus (Balzers High Vacuum Corp., Balzers, Liechtenstein) according to the technique of Moor and Mühletaler (11). The freeze-fracture replicas were cleaned in a sodium hypochlorite solution for 2 h and then soaked twice for 20 min each in a freshly prepared mixture of 20 ml chloroform and 10 ml methanol, and finally three times, each time for 20 min, in dimethyl formamide (Merck A.G., Darmstadt, West Germany). As described in our previous paper (3), this step is of the utmost importance for obtaining clean, fat-free replicas. The replicas were then rinsed in distilled water, mounted on copper grids, and examined in a Philips EM 300 electron microscope. Magnifications were calibrated with a reference grid (Ernest F. Fullam Inc., Schenectady, N. Y., 2,160 lines/mm). In addition, glutaraldehyde-fixed fragments were postfixed in 2% phosphate-buffered OsO4, dehydrated in graded ethanols, and embedded in Epon for examination by light and conventional electron microscopy. Semithin sections were examined in a phase-contrast microscope (Carl Zeiss, Oberkochen, West Germany) while thin sections stained with uranyl acetate and lead citrate were examined in a Philips EM 300 electron microscope.

Quantitative Evaluation

For each animal, 12 membrane faces present in a minimum of three different replicas were used. The membrane faces were carefully selected for flatness. In each membrane face, four pictures were taken at a fixed magnification of 35,000. As previously described (3), the parameters used in the study of the adipocyte membrane were the number and size of invaginations ("phagocytosis") and the number and size of intramembrane particles on both faces of the membrane. These structural features of the membrane stand out clearly in freeze-fracture. Quantitative evaluation of membrane invaginations and membrane particles was carried out on 3×-enlarged positive prints by a person who did not know the experimental conditions. All diameter measurements were performed with the aid of an 8× magnifier containing a reticle calibrated in tenths of a millimeter. The number of membrane invaginations and intramembranous particles (the latter corrected so as to subtract the surface of the membrane occupied by invaginations) was expressed per unit area (1 μm²) of membrane, and per entire adipose cell. For this purpose, we estimated the surface area of adipocytes under the different experimental conditions. In a first step, cell diameter was measured on light micrographs of adipocytes in semithin sections. For each animal, about 150 cell diameters from three different pieces of tissue were measured at a magnification of 192. Mean diameter obtained for each animal was corrected according to Weibel (21). However, since during lipolysis and lipid droplet reduction the adipocytes form numerous cytoplasmic processes, the surface area of lipolytic adipocytes as calculated with the equation \( S = \pi D^2 \) was grossly underestimated. We tried, therefore, to obtain a corrective factor for these shrunken adipocytes. For each animal, 25 electron micrographs of thin-section preparations (magnification 16,000) were taken and on each micrograph the length of the adipose cell plasma membrane was determined with a curvimeter. The lengths were pooled and the sum was divided by the sum of the perimeters of the same adipocytes as drawn without indentations (see Fig. 3). A ratio was thus obtained, and for each animal the mean perimeter of the adipocytes (evaluated as \( 2\pi R \), where \( R \) is derived from light-microscope measurements) was multiplied by this ratio. A corrected diameter \( (D) \) of the shrunken adipocyte was then derived from the corrected perimeter and a corrected surface \( (S) \) obtained with the equation \( S = \pi D^2 \).

RESULTS

Biology

As expected, a progressive loss of weight reaching 35% of the initial weight was observed during fasting (Table I). Weight loss in streptozotocin-
TABLE I

Loss of Weight, the Plasma Glucose, Plasm Immunoreactive Insulin (IRI), and Immunoreactive Glucagon (IRG) in Control and Experimental Animals

<table>
<thead>
<tr>
<th>Loss of weight</th>
<th>Glucose</th>
<th>IRI</th>
<th>IRG</th>
</tr>
</thead>
<tbody>
<tr>
<td>% initial weight</td>
<td>mg%</td>
<td>ng/ml</td>
<td>ng/ml</td>
</tr>
<tr>
<td>Fasting</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control, n = 6</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 days, n = 6</td>
<td>21.3 ± 0.8*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 days, n = 6</td>
<td>27.5 ± 1.4*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9 days, n = 4</td>
<td>39.0 ± 2.2*</td>
<td>65.5 ± 13.7</td>
<td>0.59 ± 0.08</td>
</tr>
<tr>
<td>Diabetes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control, n = 6</td>
<td>0</td>
<td>102.3 ± 24.6</td>
<td>2.82 ± 0.75</td>
</tr>
<tr>
<td>3 days, n = 6</td>
<td>3.6 ± 0.5*</td>
<td>496.2 ± 18.8*</td>
<td>1.68 ± 0.37</td>
</tr>
<tr>
<td>6 days, n = 6</td>
<td>7.7 ± 0.9*</td>
<td>475.0 ± 32.1*</td>
<td>1.89 ± 0.38</td>
</tr>
<tr>
<td>9 days, n = 6</td>
<td>10.4 ± 3.2*</td>
<td>393.4 ± 27.4*</td>
<td>0.56 ± 0.14‡</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SEM; n = number of animals. Statistical significances determined with the Student's t-test vs. the control group are expressed as follows: (*): P < 0.005. (‡): P < 0.05.

Figure 1a-c: Phase-contrast micrograph of semi-thin section of epididymal adipose tissue from control (a), 3-day (b), and 6-day diabetic (c) rats taken at the same magnification. Note the striking decrease in size of the adipocytes in diabetic rats as compared to controls. ×192.
treated animals seemed to be related to the severity of diabetes as judged by immunoreactive insulin (IRI), immunoreactive glucagon (IRG), and blood glucose values (Table I): while some diabetic animals lost as much as 19% of their initial weight in 3 days, the weight of others was maintained constant, or even increased in some animals.

Morphology

LIGHT MICROSCOPY: The striking decrease in the size of adipocytes from either fasting or diabetic animals as compared to the adipocytes from control animals is shown in Fig. 1 a-c.

CONVENTIONAL ELECTRON MICROSCOPY: The electron microscopic appearance of control fat cells corresponds fully to that reported previously (1, 4, 5, 12, 17, 20, 23). In the resting cell, the cytoplasm is reduced to a thin rim surrounding the central lipid droplet (Fig. 2). The plasma membrane shows numerous microvesicular invaginations, ~45 nm in diameter, and is surrounded by a continuous layer of granular and filamentous material ("basal lamina"). Collagen fibers are scattered outside the basal lamina.

The changes in the morphology of the adipocyte induced by lipolysis are also well documented (13, 14, 16, 19, 22, 23): they consist mainly of a decrease of the central lipid droplet accompanied by a folding of the plasma membrane which is thrown into numerous processes (Fig. 3); the latter become progressively more prominent and complex with the duration of lipolysis, whether through fasting or through acute diabetes.

**FIGURE 2** Thin section of a portion of a control fat cell. The cytoplasm is reduced to a thin rim surrounding the central lipid droplet (L). It contains a small lipid droplet (I) and dense black spots representing glycogen particles. The arrows point to flask-like invaginations of the plasma membrane. BL, basal lamina; CF, collagen fibers. × 28,000.

**FIGURE 3** Portion of a lipid-depleted fat cell from a 6-day diabetic rat. The cytoplasm forms variegated processes bounded by the plasma membrane at the level of which one can see numerous microvesicular invaginations (arrows). The dotted line outlines the actual perimeter of the adipocyte without indentations (see Materials and methods). L, lipid droplet; CF, collagen fibers; BL, basal lamina; m, mitochondria. × 23,000.
FREEZE-FRACTURE: On the A face (or P face, as recently suggested by Branton et al. [2]) of the adipocyte plasma membrane (Fig. 4), the necks of membrane invaginations appear as small circular depressions, while on the B face (or E face in the newly proposed terminology [2]) (Fig. 5) they look like raised craters. Their number, disposition, and size are similar on both faces. In areas free of invaginations, the fracture faces of the adipocyte plasma membrane show small globular elevations representing the intramembranous particles. Their number is higher on A than on B faces of the membrane (insets, Figs. 4 and 5). These basic features are altered during lipolysis: instead of being disposed at random on the fracture face (Fig. 6), the membrane invaginations become progressively clustered (Figs. 7-9). The degree of clustering increases with the duration of lipolysis due to either fasting or diabetes, so that in heavily shrunken adipocytes the membrane invaginations seem to be restricted to depressed areas at the bottom of membrane folds (Figs. 7-9). On the contrary, the overall disposition of intramembranous particles does not seem to be affected by the experimental conditions.

QUANTITATIVE EVALUATION: As shown in Fig. 10, the diameter of adipose cells as well as their surface (corrected as described in Materials and Methods) decreases during lipolysis due to a loss of mass from the lipid droplet. However, while the reduction in the size of adipocytes was comparable in each group of fasting animals, the decrease in size was subject to large variations in each experimental group of streptozotocin-treated rats. This is probably due to the fact that the severity of diabetes (see Table I), thus the degree of lipolysis and weight loss, was very different from one animal to another. The changes produced in the number of membrane invaginations by lipolysis are summarized in Fig. 11. When evaluated per square micrometer of membrane, the number of membrane invaginations increases significantly during lipolysis induced by either fasting or diabetes. However, when the results are expressed per adipose cell rather than by square micrometer (this is carried out by multiplying in each animal the number of invaginations per square micrometer by the mean surface of the adipocyte), no statistically significant differences are noted. As shown in Fig. 12, no change in the mean size or in the size distribution of membrane invaginations occurs as the result of lipolysis.

Fig. 13 shows the modifications induced by each lipolytic condition in the number of intramembranous particles, as evaluated per square micrometer of membrane or per entire adipose cell. Both lipolytic conditions induced a significant and progressive increase of intramembranous particles per square micrometer of the cytoplasmic (A) face of the membrane. On B faces, an increase of intramembranous particles was also often found. When the results are expressed per entire cell rather than per square micrometer of membrane, no significant difference in the number of particles was noted, except at 9 days' diabetes. As shown in Fig. 14, the mean size and the size distribution of intramembranous particles do not appear to be affected by lipolysis, whether due to fasting or to streptozotocin diabetes.

DISCUSSION
Previous studies by conventional thin-section electron microscopy have shown, although not quantitatively, an apparent increase of membrane invaginations or pinocytosis in the plasma membrane of adipocytes during lipolysis (13, 14, 16, 19, 22, 23). Our evaluation of freeze-fracture replicas allows, for the first time, a quantitative study of the membrane changes occurring during lipolysis.

As far as the number of membrane invaginations per square micrometer of adipocyte plasma membrane is concerned, our data correlate well with previous conventional microscope analysis (13, 14, 16, 19, 22, 23). However, when the

Figure 4 Freeze-fracture replica showing the A face (inner leaflet) of the membrane, the cross-fractured cytoplasm (Cy), and the central lipid droplet of a control adipose cell. On the A face, the necks of membrane invaginations appear as small circular depressions (i). Intramembrane particles (inset) are numerous and randomly distributed. CF, collagen fibers; BL, basal lamina. × 42,000. Inset, × 65,000.

Figure 5 Freeze-fracture replica of an area of an adipose cell comparable to that in Fig. 4, but revealing the B face (outer leaflet) of the plasma membrane. In this case, the necks of the invaginations are seen as elevated craters (i); the entire membrane of several invaginations (arrows) can be seen bulging from the cross-fractured cytoplasm (Cy). Intramembrane particles (inset) are less numerous than on the A face. CF, collagen fibers. × 33,000. Inset, × 65,000.
Figure 6 Overall view at low magnification of an A face of a control adipocyte. It shows that membrane invaginations (i) evenly pit the membrane surface. × 12,000.
Figure 7 A face of an adipose cell plasma membrane from a 6-day diabetic rat. The surface of the cell is irregular and slightly indented. Membrane invaginations (i) appear concentrated at the bottom of indented areas. BL, basal lamina; CF, collagen fibers; Cy, cytoplasm. × 33,000.
FIGURE 8 Freeze-fracture replica of a portion of an adipocyte after 9-day fasting. The cell surface (A face) is deeply indented. Invaginations (i) are present in discrete clusters at the bottom of membrane folds. Cy, cytoplasm; CF, collagen fibers. × 27,000.
FIGURE 9  B face of an adipose cell plasma membrane of a 9-day fasting rat. Clusters of invaginations (i) are seen on protruding areas of the outer leaflet of the membrane. CF, collagen fibers. × 21,000.
FIGURE 10 Diameter (a) and corrected surface (b) of adipocytes in control (□ and ■), diabetic (○—○), and fasting (●—●) rats. Results are expressed as mean ± SEM; the statistical significance of the difference between the control and the experimental animal is evaluated with the Student's t-test; number of animals (n) = 6; P values are expressed as follows: (*) P < 0.05; (**) P < 0.02; (***) P < 0.005. After 9-days fasting, determination of the diameter of adipocytes in two of the four animals was impossible due to the drastic shrinkage of the cells. This experimental point was thus withdrawn.

FIGURE 11 Number of membrane invaginations per square micrometer of adipocyte plasma membrane (a) and per total adipose cell (b) in control (□ and ■), diabetic (○—○), and fasting rats (●—●). Results are expressed as mean ± SEM. In (a), n represents the number of membrane faces studied = 60 (except in 9-day fasting, n = 48); in (b), n represents the number of animals studied = 6. Statistical significance is determined vs. the control group and P values are expressed as in Fig. 10: (***) P < 0.005.
invaginations per square micrometer, which represents the local concentration of these structural features, and their number per entire cell, which represents the total content of membrane invaginations in a given adipocyte. The determination of these two parameters, rendered possible only by a quantitative approach, indicates that the dramatically shrunken adipocyte maintains its total content of membrane invaginations. This is done by increasing the local concentration of invaginations, the latter phenomenon seen qualitatively as a progressive clustering of the invaginations during lipolysis. The consequences of such a redistribution of the membrane invaginations remain unknown at present; however, the fact that the total number of invaginations remains constant during lipolysis seems to bring further support to the conclusions of Jarett and Smith (9), who see the invaginations as finger-like processes permanently open to the extracellular space. The same arguments used for interpreting the variations in membrane invaginations can be applied to the changes observed in the number of intramembranous particles. Indeed, the local concentration of particles per unit area of membrane was increased during lipolysis but not the total content of particles in a given adipose cell membrane. As for invaginations, the functional implication of this redistribution of intramembranous particles in the adipose cell membrane is linked to the elucidation of the function of individual particles. Since particles represent protein-containing structures intercalated in the lipid bilayer (6, 10, 15, 18), our results imply that during lipolysis the rapid changes in cell size (and surface) are achieved primarily by changing the lipid component of the membrane, but do not affect the total protein content of this membrane. This in turn is likely to modify the protein:lipid ratio in the membrane, as well as the respective topographical relationships between proteins and lipids.

We thank Dr. W. A. Muller for performing glucose and hormone determinations in blood samples and Dr M. Amherdt for help with quantitative evaluation of the morphological data. We are indebted to M. Sidler-Ansermet, O. Jerotic, I. Bernard, and P. Fruleux for skilled technical assistance.

A preliminary report of this work was presented at the 8th Annual Meeting of the Union of Swiss Societies for Experimental Biology (April 1976), Fribourg, Switzerland, and at the 10th Annual Meeting of the European Society for Clinical Investigation (April 1976), Rotterdam, The Netherlands.
This investigation was supported by grant no 3.553.75, from the Fonds National Suisse de la Recherche Scientifique.

Received for publication 19 May 1976, and in revised form 13 September 1976.

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