Single-cell analysis of insulin-regulated fatty acid uptake in adipocytes

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Varlamov O, Somwar R, Cornea A, Kievit P, Grove KL, Roberts CT Jr. Single-cell analysis of insulin-regulated fatty acid uptake in adipocytes. Am J Physiol Endocrinol Metab 299: E486–E496, 2010. First published June 22, 2010; doi:10.1152/ajpendo.00330.2010.—Increased body fat correlates with the enlargement of average fat cell size and reduced adipose tissue insulin sensitivity. It is currently unclear whether adipocytes, as they accumulate more triglycerides and grow in size, gradually become less insulin sensitive or whether obesity-related factors independently cause both the enlargement of adipocyte size and reduced adipose tissue insulin sensitivity. In the first instance, large and small adipocytes in the same tissue would exhibit differences in insulin sensitivity, whereas, in the second instance, adipocyte size per se would not necessarily correlate with insulin response. To analyze the effect of adipocyte size on insulin sensitivity, we employed a new single-cell imaging assay that resolves fatty acid uptake and insulin response in single adipocytes in subcutaneous adipose tissue explants. Here, we report that subcutaneous adipocytes are heterogeneous in size and intrinsic insulin sensitivity. Whereas smaller adipocytes respond to insulin by increasing lipid uptake, adipocytes with cell diameters larger than 80–100 μm are insulin resistant. We propose that, when cell size approaches a critical boundary, adipocytes lose insulin-dependent fatty acid transport. This negative feedback mechanism may protect adipocytes from lipid overload and restrict further expansion of adipose tissue, which leads to obesity and metabolic complications.

ADIPOSE TISSUE PLAYS A CRITICAL ROLE in energy homeostasis by providing fatty acids (FA) as an oxidative fuel for other tissues. It also secretes biologically active, hormone-like adipokines that target various peripheral tissues and the brain (3). The transport of FA into adipocytes is mediated by FA transport protein-1 (FATP1) and the scavenger receptor FAT/CD36 (11). Insulin increases FA uptake in adipocytes by stimulating the translocation of FATP1 from intracellular vesicles to the plasma membrane (40). Alterations of adipose tissue lipid metabolism and the loss of insulin regulation of FA and glucose uptake and lipolysis contribute to the development of obesity, metabolic syndrome, and peripheral and systemic insulin resistance (4, 9, 20, 31, 32).

The distribution of adipose tissue plays an important role in the development of insulin resistance and type 2 diabetes. Visceral fat is morphologically and biochemically different from subcutaneous fat (15). Visceral adipocytes are more sensitive to β-adrenergic agonists and as a result exhibit higher lipolysis rates than subcutaneous adipocytes (10, 17). Visceral adipocytes are also more insulin sensitive than subcutaneous adipocytes (24), although this is affected by sex difference (28), and secrete higher levels of proinflammatory adipokines that are involved in the development of insulin resistance and metabolic syndrome (48). Recent studies by Tran et al. (45) have shown that subcutaneous fat transplanted into the visceral cavity of mice exerts a metabolically beneficial effect by improving insulin sensitivity and increasing whole body glucose uptake. However, the transplantation of epididymal fat pads intraperitoneally also improves glucose tolerance and insulin sensitivity in mice (21). It is currently unclear whether subcutaneous fat derived from different anatomic locations in the body has a differential effect on whole body insulin sensitivity and how it is involved in FA uptake and utilization. It has been suggested that different subcutaneous depots may have different developmental origins based upon the regional adipose phenotype of knockout mice lacking various differentiation factors (reviewed in Ref. 16) as well as the fact that some human lipodystrophies result in distinct segmental redistribution of adipose tissue (reviewed in Ref. 2). For instance, Dunnigan-type familial partial lipodystrophy is characterized by a preferential loss of adipose tissue in the extremities and trunk but not in the neck and face.

Early studies showed that, compared with small adipocytes, large adipocytes exhibit decreased rates of glucose oxidation and reduced rates of insulin-stimulated glucose uptake and are less sensitive to the antilipolytic action of insulin (19, 33, 36, 38). Obese individuals with increased subcutaneous adipose tissue have larger adipocytes and are more hyperinsulinemic and glucose intolerant than lean individuals with smaller adipocytes (12, 14, 22, 35, 37, 41, 44), and the presence of larger subcutaneous adipocytes may predict the development of type 2 diabetes (49). More recent studies have shown that the peroxisome proliferator-activated receptor-γ agonist pioglitazone or weight loss increases the number of smaller adipocytes in subcutaneous fat depots and improves insulin sensitivity, indicating that insulin resistance can be reversed by decreasing adipocyte size (30, 34). Although these studies suggest an inverse correlation between fat cell size and whole body insulin sensitivity, it remains to be determined whether the enlarged cell size itself is predictive of impaired insulin regulation of lipid uptake. Here, we employed a single-cell assay in ex vivo adipose explant cultures from lean adult rhesus macaques to quantify FA uptake and insulin sensitivity of individual adipocytes in different subcutaneous fat depots.

MATERIALS AND METHODS

Animals and adipose tissue extraction. All animal procedures were in accordance with the guidelines of the Oregon Health and Science University Institutional Animal Care and Use Committee. Four lean nondiabetic rhesus macaque females utilized in the present work were subjects of unrelated studies, and the adipose tissue used in this study...
was obtained as excess tissue at necropsy. These animals were fed standard monkey chow that contained sufficient vitamin, mineral, and protein content for normal growth. The weight of the animals, 6.0 ± 0.67 kg (n = 4), matches the average weight of female rhesus macaques in the Oregon National Primate Research Center colony. The night prior to necropsy, all animals were deprived of food and water. At necropsy, fat (typically 0.1–0.5 g) was dissected from different anatomic locations. Retropertoneal fat, subcutaneous upper body fat (from lower axial armpit areas), middle body fat (from abdominal area), and lower body fat (from the outer hip area) were collected in 50-ml tubes filled with 20 ml of medium M199 (Invitrogen, Carlsbad, CA) at room temperature, and hormonal treatment was started within 30 min of necropsy.

Fluorescent labeling and hormonal treatment of fat explants. One- to two-millimeter portions of adipose tissue (explants) were dissected using sharp surgical scissors. Explants were immediately placed at the bottom of plastic eight-well chambers (Lab-Tek II chambered no. 1.5, German coverglass system; Nunc), covered with squares of light stainless steel mesh (0.4 mm, TWP) to prevent floating and resultant adipocyte rupture, and layered with 0.4 ml of 37°C M199 supplemented with 0.1% FA-free BSA (Sigma-Aldrich, St. Louis, MO) alone or together with 10 nM human insulin (Sigma). Explants were incubated for 2 h in an atmosphere of 5% CO2 at 37°C, and 100 μl of 10 μM green fluorescent Bodipy-500/510 C1C12 (Bodipy-C12; Invitrogen) solution in medium M199 containing 0.1% FA free BSA was added to the chamber. The medium was mixed by repeated pipetting, and the chambers were incubated for an additional 10 min at 37°C. Reactions were stopped by placing chambers on ice and washing explants four to five times with ice-cold 0.1% FA-free BSA in PBS. Explants were then fixed at room temperature with 4% paraformaldehyde in PBS for 30 min, washed four times with PBS, and stored in the dark in PBS at 4°C for ≥2 days before analysis. To identify dead cells, 30 min prior to addition of Bodipy-C12, 2 μl of ethidium homodimer (LIVE/DEAD Viability/Cytotoxicity Kit; Invitrogen) was added to 400 μl of insulin-containing M199 medium. Dead cells exhibit red nuclear staining. Germ agglutinin (WGA-Alexa633, 1:50 dilution; Invitrogen) was added to fixed, Bodipy-stained adipose tissue and incubated for 5–10 min prior to imaging. To colabel adipocytes with Bodipy-C12 and NBD-2-deoxyglucose (Invitrogen), explants were incubated for 1 h in glucose-free DMEM (Invitrogen) containing 10 mM insulin, washed twice with PBS, and then incubated for additional 10 min with 200 μM NBD-2-deoxyglucose in 200 μl of PBS at 37°C. Tissue was washed with PBS and overlaid with 200 μl of M199 medium, and NBD fluorescence was collected as described below. Following NBD imaging, 200 μl of prewarmed QBT Fatty Acid Uptake Kit (Molecular Devices, Sunnyvale, CA) was carefully added to the same well. Green fluorescent images were collected over 10 min of incubation.

Confocal microscopy. Image recording was conducted using an inverted Leica SP5 AOBS spectral confocal system equipped with a motorized, temperature-controlled stage and HC PL FLUOTAR 10.0 × 0.30 and ×20 PL APO NA 0.70 dry objectives. Bodipy-C12 (excitation peak, 488 nm) was excited with an Argon laser, and images were recorded at emission bandwidth of 500–550 nm. For QBT/NBD-2-deoxyglucose double-labeling experiments, NBD-2-deoxyglucose-labeled tissue was illuminated with an excitation wavelength of 488 nm (16% power), and fluorescence was collected at emission bandwidth of 498–606 nm. Tissue was labeled with QBT (green Bodipy-C12) and illuminated with excitation wavelength of 488 nm (6% power), and fluorescence was collected at emission bandwidth of 500–524 nm. Because NBD fluorescence appears weak compared with Bodipy fluorescence, it is possible to perform sequential double-labeling experiments by lowering excitation power and narrowing the emission bandwidth of the second (Bodipy) channel without a substantial bleed-through from the NBD channel. WGA-Alexa fluor 633 was excited at 633 nm. In Bodipy/ethidium homodimer colabeling experiments, Bodipy was excited at 488 nm and fluorescence collected at 500–550 nm, and ethidium was excited at 561 nm and fluorescence collected at 570–650 nm.

Image processing and analysis. The image analysis algorithm is illustrated in Supplemental Fig. S4 (Supplemental Material for this article can be found on the AJP-Endocrinology and Metabolism website). Typically, 20–30 z-sections, 5 μm apart, were collected at 400 Hz, resulting in individual image dimensions of 1,550 × 1,550 μm. For high-resolution imaging, images were collected at 1-μm intervals. Leica “lift” stacks of images were opened with the LOCI plug-in data browser and analyzed in Image J as follows. The stack of images was projected onto a single flat image (z-project, sum slices) containing integrated pixel intensities of the z-stack (Supplemental Fig. S4A). The regions of interest (ROI), corresponding to cell boundaries, were drawn manually (ROI manager). Cells with poorly defined boundaries were excluded from analysis (Supplemental Fig. S1A). Mean intracellular fluorescence and the equatorial cell area of the ROI were determined using z-projections. We define the efficiency of Bodipy-C12 transport across the plasma membrane as the amount of total intracellular fluorescence accumulated in 10 min, normalized to the surface area of the cell. Equation 4 in Supplemental Fig. S4 shows the linear relationship between the efficiency of Bodipy-C12 transport and mean intracellular fluorescence. The equatorial cell diameter was estimated on the basis of the circumference of the ROI (cell shape frequently deviates from strictly round). Because the level of fluorescence in dead cells was typically 10–15% of that in live cells, the former appear as very faint and were excluded from analysis (Supplemental Fig. S2).

Real-time microscopy of FA uptake in adipose tissue explants. Fat explants were pretreated with 10 nM insulin as described above and labeled for 10 min with 2.5 μM of red fluorescent Bodipy 558/568 C12 (Invitrogen) to establish a focal plane for real-time imaging of FA uptake. Real-time FA uptake, shown in Fig. 4A, was performed using a QBT Fatty Acid Uptake Kit. Briefly, the eight-well chamber containing insulin-pretreated explants and 100 μl of M199 medium was placed on a stage of the confocal microscope and equilibrated to 37°C. One hundred microliters of prewarmed QBT reagent was added at time “0”, and images were collected in a single plane every 10 s.

Statistical analysis. GraphPad Prism and Excel were used to analyze statistical differences between insulin sensitivity of adipocytes in different subcutaneous adipose depots of rhesus macaques

Table 1. Average adipocyte size in different subcutaneous adipose depots of rhesus macaques

<table>
<thead>
<tr>
<th>Animal ID</th>
<th>Weight, kg</th>
<th>Sex</th>
<th>Glucose, mg/dl</th>
<th>Triglycerides, mg/dl</th>
<th>Insulin, μU/ml</th>
<th>Upper Body Cell Size, μm²</th>
<th>Middle Body Cell Size, μm²</th>
<th>Lower Body Cell Size, μm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (24720)</td>
<td>6.45 F</td>
<td></td>
<td>30 (53)</td>
<td>38</td>
<td>2.7</td>
<td>1,316 ± 47</td>
<td>970 ± 51</td>
<td>1,828 ± 64</td>
</tr>
<tr>
<td>2 (22340)</td>
<td>4.25 F</td>
<td></td>
<td>35 (57)</td>
<td>45</td>
<td>8.6</td>
<td>1,217 ± 66</td>
<td>950 ± 50</td>
<td>1,251 ± 91</td>
</tr>
<tr>
<td>3 (21870)</td>
<td>6.7 F</td>
<td></td>
<td>63</td>
<td>52</td>
<td>11.3</td>
<td>9,087 ± 642</td>
<td>15,616 ± 715</td>
<td>11,377 ± 558</td>
</tr>
<tr>
<td>4 (22934)</td>
<td>6.6 F</td>
<td></td>
<td>42 (47)</td>
<td>17</td>
<td>6.5</td>
<td>7,127 ± 282</td>
<td>9,578 ± 338</td>
<td>8,436 ± 354</td>
</tr>
</tbody>
</table>

Values are means ± SE (n = 50–80). F, female. Cell size was determined as described in MATERIALS AND METHODS. No significant differences between the average sizes of adipocytes from 3 anatomic locations were detected (repeated-measures ANOVA test). Blood samples were collected from live anesthetized animals immediately prior to euthanasia and analyzed for glucose, triglyceride, and insulin levels, as described in MATERIALS AND METHODS. Glucose levels shown in parentheses represent blood samples collected several weeks prior to necropsy.
cytes of different sizes. For every animal, adipocytes were divided into cell size groups, and the mean intracellular fluorescence of individual groups was determined under basal or insulin-stimulated conditions. For each size group, the average fluorescence of insulin-treated cells was normalized to the average fluorescence of untreated cells. Insulin-stimulated/basal fluorescence ratios, represented by at least two animals per size group, were compared using the one-way Tukey multiple comparison ANOVA test.

**Blood tests.** Blood samples of animals obtained immediately prior to euthanasia by the attending pathologist using approved procedures were analyzed as described below. In an age-matched cohort, average glucose level is 58.3 ± 1.4 mg/dl (range 46–71 mg/dl; n = 19), average insulin level is 20.7 ± 3 µU/ml (range 5.6–56.5 µU/ml; n = 19), and homeostasis model assessment of insulin resistance (HOMA-IR) values ranged from 0.77 to 8.23 (n = 19). Because some serum samples collected at necropsy were close to hypoglycemic values (Table 1), we also included glucose levels of serum samples that had been collected, analyzed, and archived several weeks prior to necropsy (Table 1; glucose levels shown in parentheses). Thus, the glucose levels and HOMA-IR values of experimental animals were within the normal range of the colony. Serum concentrations of insulin were determined by the Endocrine Technology and Support Core at the Oregon National Primate Research Center using an Immulite 2000, a chemiluminescence-based automatic clinical platform (Siemens Healthcare Diagnostics, Deerfield, IL). The Endocrine Technology and Support Core has validated the usage of Immulite

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**Fig. 1.** The use of Bodipy-C12 for quantifying fatty acid (FA) uptake in adipose tissue explants. A: adipose tissue explants were immobilized at the bottom of the imaging chamber with 0.4-mm stainless steel mesh, and M199 medium was added alone or in the presence of 10 nM insulin. Following a 2-h insulin treatment, explants were labeled for 10 min with 2.5 µM green Bodipy-C12, fixed, and analyzed by confocal microscopy. For each explant, 3 independent sectors of Bodipy-labeled fat cells (quadrant 8, red squares) were analyzed. Each experimental condition (basal or insulin treatment) was duplicated, and the data collected from 6 sectors were pulled together for further analysis. B: Co-uptake of Bodipy-C12 (green) and NBD-2-deoxyglucose (red). Top images were taken using a ×10 objective; bottom images represent higher-resolution images taken with a ×20 objective. Bars = 100 µm. C: confocal images of the Bodipy-C12-labeled fat explant. Fat explants pretreated with insulin were labeled with red Bodipy-C12 for 2 h (red; middle), washed, and pulsed for 10 min with green Bodipy-C12 (green; left). Longer exposure to red Bodipy-C12 resulted in the labeling of the majority of the cells in the explant, although the efficiency of labeling varied. Right: bright-field image showing the fat explant and a part of metal mesh. Images represent the sum of z-slices. Bars = 100 µm. D: Bodipy-C12 transport into insulin-sensitive adipose tissue requires cell integrity. To identify live and dead adipocytes, insulin-pretreated fat explants were labeled with ethidium homodimer, as described in MATERIALS AND METHODS, and then with Bodipy-C12. The nuclei of dead cells are dye permeable and stained in red. Note that areas of adipose tissue exhibiting red staining are devoid of green fluorescence. Bars = 100 µm. E: insulin-resistant adipose tissue does not accumulate significant amounts of Bodipy-C12. Adipose tissue comprised of large adipocytes was treated with insulin, ethidium homodimer, and Bodipy-C12, as described above. Left: normal contrast; right: enhanced contrast images. Bars = 100 µm.
2000 for monkey serum hormones, including insulin. The validation includes a direct comparison of monkey serum samples analyzed coordinately by an Immulite 2000 and a Roche Elecsys 2010 analyzer (also a chemiluminescence-based clinical platform from F. Hoffmann-La Roche, Basel, Switzerland). The sensitivity for the insulin assay is 2 mIU/ml, with a range between 2 and 300 μU/ml. The intra- and interassay variation with the Immulite 2000 is <10%. Serum glucose and triglyceride levels were determined by Rhein Consulting Laboratories (Portland, OR), using a Cobas Mira Plus chemistry analyzer (Roche Diagnostic Systems, Indianapolis, IN).

RESULTS

The use of fluorescence in biological assays has a great advantage over radioisotopes in that fluorescence adds a spatial dimension and improves the temporal resolution of an assay. For example, fluorescent lipids have previously been used in combination with radioisotopically labeled FA for the measurement of FA uptake in single cells (such as 3T3-L1-derived and primary adipocytes) and white and brown adipose tissue and have been shown to efficiently integrate into intracellular triacylglycerides (23, 25, 27, 51, 52). In this study, we adopted a fluorescent assay to determine whether tissue adipocytes of different sizes exhibit differences in insulin sensitivity and FA uptake. Microscopic fragments (explants) of retroperitoneal and subcutaneous adipose tissue, isolated from lean nondiabetic rhesus macaques (see MATERIALS AND METHODS and Table 1 for details), were immobilized at the bottom of an imaging chamber, treated for 2 h with 10 nM insulin, and labeled with a green fluorescent derivative of lauric acid, Bodipy-C12. The mean intracellular fluorescence was then quantified using confocal microscopy (Fig. 1A).

Adipose tissue comprised of adipocytes with diameters <80–100 μm (cell area 5,000–7,000 μm²) displayed robust insulin-dependent FA uptake; a 10-min pulse of Bodipy-C12 resulted in fluorescent labeling of the peripheral cell layer, whereas longer exposure to Bodipy-C12 resulted in the labeling of the entire explant (Fig. 1C). The interior of explants was not labeled with fluorescence after a short exposure to Bodipy-C12, likely due to its hydrophobic nature and slow diffusion in the interior of the explant (Fig. 3). In contrast, a 10-min exposure to hydrophilic water-soluble NBD-2-deoxyglucose resulted in labeling of the entire population of fat cells in the explant (Fig. 1B). Dead cells did not accumulate a significant amount of Bodipy-C12, indicating that FA uptake requires membrane integrity (Figs. 1D and 5). Adipose tissue comprised of adipocytes with diameters >80–100 μm displayed a very different pattern of fluorescence; Bodipy-C12 staining was weak and diffuse, and dead and live fat cells accumulated similar amounts of fluorescence regardless of the presence of insulin (Fig. 1E).

Imaging through adipose tissue potentially imposes significant technical problems in that large lipid droplets scatter fluorescent light and create uneven illumination and light collection. Additionally, uneven diffusion of fluorescent dye in fat tissue may contribute to variability of results. To address the light-scattering problem, we analyzed three-dimensional images of the first layer of large insulin-nonresponsive adipocytes that showed a diffuse Bodipy-C12 pattern (Figs. 1E and 2A). Vertical sections through the stacks showed that images were not severely distorted, the shape of adipocytes appeared principally circular, and fluorescence intensity did not vary with distance from the coverslip (Fig. 2, A and B). These experiments indicate that, under the imaging conditions used, spherical aberrations and scattering of fluorescence light from the outer cell layer of fat explants are negligible.

Figure 3 illustrates the diffusion of Bodipy-C12 fluorescence in an explant containing large adipocytes. There was an apparent delay in the accumulation of fluorescence, possibly because Bodipy-C12 must partition in and cross the lipid bilayer and subsequently accumulate in sufficient quantities to give a measurable intracellular signal. At 600 s, Bodipy-C12 preferentially accumulated in the outer layer of the fat explant, with little labeling detected in deeper cell layers (Fig. 3, A and C). Within each cell layer, there was very little cell-to-cell variability in accumulated fluorescence intensities (Fig. 3, B, C, and D), suggesting homogeneous Bodipy-C12 diffusion through the explant. As expected for unfacilitated transport, within the outer cell layer, small and large fat cells accumulated similar amounts of fluorescence (Fig. 3B). This analysis confirms that Bodipy-C12 diffusion into the outer layer of the explant is unrestricted and that the cell-to-cell variations in fluorescence intensities described below represent physiologically relevant differences.

Insulin-responsive adipose tissue is composed of relatively small adipocytes ~40–70 μm in diameter. We explored further the details of Bodipy-C12 movement through the cell using live-cell microscopy. The time course of FA uptake in insulin-treated retroperitoneal fat explants is shown in Fig. 4A. A focal plane for real-time imaging was established by prela-
Fig. 3. Diffusion of Bodipy-C12 in adipose tissue. A: a sample of adipose tissue that was insulin resistant and transported Bodipy-C12 by diffusion was incubated with Bodipy-C12 and the fluorescent quencher, as described in MATERIALS AND METHODS. Time-lapse microscopy shows dye penetration in the explant. Shown in red, the explant was prelabeled with red Bodipy-C12. The regions of interest used to quantify mean intracellular fluorescence are shown as white (outer cell layer), yellow (intermediate cell layers), and green circles (deep cell layers). Bar = 100 μm. B: correlation between cell size and mean intracellular fluorescence. Adipocytes were binned according to sizes, and their average intracellular fluorescence was plotted against the average cell area. Three independent explants were analyzed; each size group contained 8–15 cells. C: an example of fluorescent traces. The outer layer of cells in the explant (white circle) is easily accessible to Bodipy-C12, whereas inner layers of cells (yellow and green circles) receive dye after a substantial delay. D: at 600 s, Bodipy-C12 preferentially accumulates in the outer layer of the explant, with little labeling detected in deeper cell layers. Black bar represents background fluorescence.

buling explants with red fluorescent Bodipy-C12 (Fig. 4A, top left, shown in red). When green Bodipy-C12 and the membrane-impermeable fluorescence quencher (see MATERIALS AND METHODS for details) were added to the medium, fluorescence accumulated at the cell periphery, which likely represents cytoplasmic structures, and in the interior of lipid droplets. There was a 200-s time delay between the addition of Bodipy-C12 and the appearance of fluorescence in the cytoplasm and lipid droplets of the cells. This delay is possibly due to the binding of Bodipy-C12 to FA transporter proteins at the cell surface and FA translocation across the membrane. After an initial time delay, there was a rapid increase in fluorescence intensity in the cell cytoplasm coupled with a slower increase in fluorescence in the interior of lipid droplets (Fig. 4B). Because the kinetics of fluorescence accumulation in the cell cytoplasm was approximately linear during the 3- to 10-min time interval, and intradroplet fluorescence contributed only a small fraction of the total cellular fluorescence (Fig. 4B), we used a 10-min time point as readout of FA transport into the cell. Figure 4, C–E, represents high-resolution, three-dimensional images of adipocytes labeled with Bodipy-C12 fluorescence. The label appears as punctuate vesicles or clusters of fluorescence directly adjacent to the surface of lipid droplets. Very little label was detected at the plasma membrane of fat cells costained with wheat germ agglutinin (Fig. 4D), indicating that Bodipy-C12 clusters are intracellular. In addition to unilocular adipocytes, adipose tissue also contains small, 25- to 30-μm-diameter, multilocular cells (Fig. 4E), possibly representing an early differentiation stage of adipocytes, in which large lipid droplets form by homotypic fusion of small lipid droplets. It has previously been reported that FA uptake in adipocytes occurs by a dual mechanism comprising a saturable transport component and a nonsaturable passive flip-flop and/or diffu-
tion component (42). Whereas in 3T3-L1 preadipocytes the
nonsaturable component is the main mode of FA transport,
during adipocyte differentiation the rates of saturable transport
increase progressively, parallel to the induction of FA trans-
porters (47, 56). It has been demonstrated that the saturable
mechanism is the main mode of FA transport in isolated rat
adipocytes (42). We confirmed that, in in vitro-differentiated
3T3-L1 adipocytes, passive Bodipy-C12 transport constitutes
~10% of the total influx of FA across the plasma membrane
and that active transport can be stimulated by insulin (data not
shown). We next tested whether Bodipy-C12 uptake in insulin-
sensitive tissue adipocytes also occurs by a dual mechanism.
Whereas live cells accumulated bright punctuate fluorescence,
dead cells were weakly fluorescent (Fig. 5, A and B, arrow-
heads and asterisks, respectively). An excess of unlabeled FA
added during Bodipy-C12 uptake blocked the appearance of a
population of fat cells containing bright cytoplasmic and in-
tradroplet fluorescence but had little effect on the fluorescent of
dead cells (Fig. 5, C–E). In the presence of lipid block, the
levels of fluorescence in live cells were 10-fold lower than that
in the absence of lipid block (Fig. 5E). These experiments
demonstrate the competitive uptake of FA uptake in adipose
tissue.

Adipose tissue harbors populations of adipocytes of different
sizes (17, 29, 49). It is possible that smaller and large adipocytes
residing in the same adipose tissue differ in their meta-
bolic rates and the efficiency of signal transduction. Because
free FA are precursors for triglycerides that constitute a sig-
nificant fraction of the adipocyte volume, and FA transport is
regulated by insulin, we tested whether large and small adipocytes
derived from three subcutaneous fat depots (upper body
fat from the lower armpit area, middle abdominal body fat, and
lower body outer hip fat) differ in size and insulin sensitivity.

All animals used in this study were lean and had normal serum
insulin, glucose, and triglyceride levels but displayed signifi-
cant individual variation in their average fat cell sizes (Table.
1 and Fig. 7, A, C, and E). Out of four animals, two had
small/intermediate-sized adipocytes with an average cell area

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**Fig. 4.** Real-time imaging of FA uptake in living adipose tissue. A: explants from insulin-sensitive adipose tissue were pretreated with 10 nM insulin and labeled with red Bodipy-C12 and placed on a confocal stage equilibrated to 37°C, and a focal plane was captured using the red fluorescent image (top left). The QBT reagent was added at time “0”, and single-plane images were recorded every 10 s. Because of the presence of a membrane-impermeable Bodipy quencher in the medium, background fluorescence remains low. Bar = 100 μm. B: time course of Bodipy-C12 uptake. Compartmental fluorescence was calculated as mean fluorescence of the regions of interest containing portions of cell cytoplasm (inset) and lipid droplets. Error bars represent SE; n = 20. C and D: high-resolution 3D images of retroperitoneal adipocytes labeled with Bodipy-C12 for 10 min. In D, cells were costained with Bodipy-C12 (green) and wheat germ agglutinin (red) that outlines the plasma membrane. Bar = 10 μm. E: example of very small adipocytes containing multiple lipid droplets. Bar = 10 μm.
of 1,000–3,000 μm² (35–60 μm in diameter, animals 1 and 2), and two had very large adipocytes with an average cell area of 6,000–20,000 μm² (87–160 μm in diameter, animals 3 and 4). The Kolmogorov-Smirnov test was employed to show the normal distribution of cell sizes within each fat depot of individual animals. No significant differences between the average cell sizes from three anatomic locations were detected (repeated-measures ANOVA test; Table 1). This analysis demonstrates the heterogeneity of cell sizes within a population of adipocytes derived from lean animals.

To test whether large and small adipocytes differed in their insulin response, we incubated explants with or without insulin, labeled with Bodipy-C12, and quantified the mean intracellular fluorescence of individual adipocytes. Insulin stimulated the appearance of bright fluorescence in adipocytes from animals with small cell sizes (Fig. 6, A and C, and Supplemental Fig. S3, A, D, and G) but had no effect on fluorescence of adipocytes from animals with large cells (Fig. 6, B and D, and Supplemental Fig. S3, B, E, and H). The latter showed a weak diffuse fluorescent pattern independent of the presence of insulin and cell sizes (Fig. 6E, ○ and gray diamonds). Areas of adipose tissue containing dead cells appear very dim (Supplemental Fig. S2) and were excluded from the analysis. In a group of animals with small adipocytes there was a gradient of fluorescent intensities, and smaller cells tended to appear brighter than their larger neighbors (Fig. 6, A and E, gray circles). Thus, there is size-dependent intradepot heterogeneity in the levels of insulin-stimulated FA uptake in subcutaneous adipose tissue.

Individual insulin-responsive depots show a relatively narrow dynamic range of cell size distributions (Fig. 7, A, C, and E, animals 1 and 2). To determine how insulin sensitivity changes over a broad range of cell sizes, we compared a variation in insulin response between adipocytes from four experimental animals. This approach dramatically increases the dynamic range of cell sizes. Abdominal subcutaneous
adipocytes with cell areas <7,000 μm² (100 μm in diameter) showed a significantly higher insulin sensitivity than larger adipocytes. The later showed similar rates of the basal and insulin-stimulated FA uptake (Fig. 7D). A qualitatively similar relationship between insulin sensitivity and cell size was observed for lower body adipocytes, although statistical significance was not achieved (Fig. 7F). In contrast, upper body adipocytes were equally sensitive to insulin regardless of cell size (Fig. 7B). In general, adipocytes >5,000–7,000 μm² in size accumulated similar levels of fluorescence whether insulin was present or not, suggesting a relatively insulin-resistant state of large adipocytes. Taken together, these data indicate that, as cell size approaches a critical boundary, lipid uptake in adipocytes becomes insulin resistant.

**DISCUSSION**

An impaired response of skeletal muscle, adipose tissue, and liver to insulin is a common condition in obesity and a precursor to the onset of type 2 diabetes, resulting in the loss of insulin regulation of glucose uptake (3, 18). Insulin-sensitive glucose uptake, glucose oxidation, and the antilipolytic action of insulin decrease proportionally with an increase in average fat cell size (14, 19, 22, 33, 35, 37, 41, 49). Considering that the main biological function of adipose tissue is to manage lipid storage and metabolism, it is also important to understand how adipocyte size affects FA uptake and its regulation by insulin. Here, we applied a single-cell microscopy assay to determine how FA uptake and insulin sensitivity correlated with the size of individual adipocytes in subcutaneous adipose tissue.

Traditional radioactive techniques are based on the measurement of glucose and FA uptake in fragments of adipose tissue, representing the average response of a potentially heterogeneous population of adipocytes. The method developed in the present study offers a significant advantage over the standard approach for the measurement of FA uptake using radioactive FA analogs (5). This microscopy-based approach allows both a
single-cell and a population response to be quantified in microscopic quantities of adipose tissue obtained from microbiopsies or laparoscopic surgeries. Furthermore, the metabolic state, cell size, hormonal responsiveness, and potentially other biochemical and spatiotemporal parameters such as gene expression, protein levels, and the rates of metabolic reactions can be measured simultaneously at a single-cell level. Because organotypic cultures of adipose tissue retain morphological and biochemical properties of adipose tissue (39, 43), and the time delay between tissue extraction and hormonal treatments is minimal, it is likely that the fat explants used in the present study more closely resemble the true state of adipose tissue in vivo. In contrast, collagenase-treated dissociated cultures of primary adipocytes may lose their native properties due to a loss of cell-cell contacts, cell lysis, a proper three-dimensional substrate, and long postextraction manipulations.

The main findings of the present study are that small adipocytes are insulin sensitive, whereas large adipocytes are typically less responsive, and that adipose tissue can contain a heterogeneous population of adipocytes that differ in size and insulin sensitivity within the same anatomic location. In all three subcutaneous depots evaluated in this study, adipocytes with an average cell size larger than 80–100 µm were insulin insensitive. Previous studies in humans have reported that subcutaneous fat contains two populations of cells with sizes of 20–50 and 100–120 µm in diameter, and only small differences in size were found between insulin-sensitive and insulin-resistant individuals (29). Unfortunately, the technical limitations of those studies preclude the direct estimation of the insulin sensitivity of individual adipocytes present in adipose tissue. It is still possible that, within the same animal and anatomic location, small adipocytes are more insulin sensitive than large adipocytes. To the best of our knowledge, the present study is the first attempt to directly measure insulin response in individual adipocytes present in nonhuman primate (or human) adipose tissue. Although all animals used in the present study had normal insulin and glucose levels, indicating possibly normal glucose disposal in liver, muscle, and visceral fat, two out of four contained large adipocytes that were insulin resistant and showed no correlation between cell sizes and FA uptake (Fig. 7 and Table 1). Recent studies have argued against differential insulin sensitivity of adipocytes from high-fat diet...
and insulin-resistant mice, since small and large cells had similar insulin sensitivity (53). We hypothesize that, under physiological conditions, insulin-sensitive lipid uptake (the present study) and the antilipolytic action of insulin in adipose tissue (19, 38) are negatively regulated by cell size. This mechanism may protect adipose tissue from lipid overload and the development of complications associated with the enlargement of adipocyte size, such as local hypoxia, inflammation, elevated basal lipolysis, and systemic insulin resistance (46, 50, 53, 55). Consistent with this hypothesis, adipose tissue insulin receptor knockout (FIRKO) mice are protected against obesity and obesity-related glucose intolerance (6). These effects may be mediated by factors other than the impaired glucose transport in adipocytes, because mice with local inactivation of the GLUT4 transporter in adipose tissue develop muscle and liver insulin resistance and glucose intolerance (1). Similar to FIRKO mice, FATP1-knockout mice are also protected against diet-induced obesity and systemic insulin resistance, and insulin-stimulated FA uptake in adipocytes is completely abolished (52). These and earlier findings support our study and the hypothesis that, as the lipid storage capacity of adipocytes reaches threshold values, a negative feedback mechanism begins to inhibit insulin-dependent lipogenic processes, restricting further lipid accumulation and increased cell size.

This cell size-sensing mechanism is likely to control multiple aspects of adipocyte function, including lipid metabolism and insulin signaling, and is likely activated by changes in biophysical properties or lipid chemistry of fat cells (13, 26, 54). Extensive proteomic and gene expression analyses have demonstrated a remarkable heterogeneity related to adipocyte size. For example, small adipocytes appear to express significantly lower levels of the adipogenic transcription factor C/EBPα than large adipocytes. Many other classes of molecules involved in secretion, signaling, trafficking, and cytoskeleton function, as well as mitochondrial and lipid metabolism enzymes, were found to be differentially expressed in small and large adipocytes (7, 8). Thus, adipocyte growth triggers a gene expression program that downregulates insulin-regulated lipid accumulation in the cell. Identification of key molecules that control adipocyte growth may provide new therapeutic targets for obesity and diabetes.

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DISCLOSURES

The authors have nothing to disclose.

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