Adipocyte size and cellular expression of caveolar proteins analyzed by confocal microscopy

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Hulstrøm V, Prats C, Vinten J. Adipocyte size and cellular expression of caveolar proteins analyzed by confocal microscopy. Am J Physiol Cell Physiol 304: C1168–C1175, 2013. First published April 10, 2013; doi:10.1152/ajpcell.00273.2012.—Caveolae are abundant in adipocytes and are involved in the regulation of lipid accumulation, which is the main volume determinant of these cells. We have developed and applied a confocal microscopic technique for measuring individual cellular expression of the caveolar proteins cavin-1 and caveolin-1 along with the size of individual adipocytes. The technique was applied on collagenase isolated adipocytes from ad libitum fed Sprague-Dawley rats of different age (4–26 wk) and weight (103–629 g). We found that cellular expression of caveolar proteins was variable (SD of log expression in the range from 0.25 to 0.65). Regression analysis of protein expression on adipocyte size revealed that the expression of the caveolar proteins cavin-1 and caveolin-1 on adipocytes from individual rats was tightly related to adipocyte cell surface area (mean coefficient of regression was 0.83 for cavin and 0.77 for caveolin), indicating that caveolar density was the same in membranes from all cells within a biopsy. This intrinsic relation remained unchanged with animal age, but adipocytes from animals with increasing age showed a decrease in mean expression of caveolar proteins per unit cell surface. The different relation between adipocyte size and cellular expression levels of caveolar proteins within and between individuals of different age shows that caveolar density is an age-sensitive characteristic of adipocytes.

Adipocyte size; caveolae; cavin-1; caveolin-1

MEAN ADIPOCYTE SIZE AND ADIPOSE tissue cellularity increases with age in sedentary inbred rodents. Furthermore, it is well documented that obesity is associated with decreased insulin sensitivity of fat cells (6, 40). Many research groups have studied the relationship between a number of metabolic parameters and adipocyte size (8, 11, 15, 35). Early studies of possible functional impacts of adipocyte size were carried out on pools of cells from animals of different age or weight and consequently different mean cell size (29, 30, 36). Interpretation of data from such studies is difficult since it is not possible to dissociate an influence of cell size per se from an effect of interindividual factors correlated to cell size during aging. To analyze whether the functions of the fat cell vary with adipocyte size per se, it is necessary to separate fat cells of different sizes in the same biopsy. Techniques from the early 1970s took advantage of differences in flotation rates of small and large adipocytes (1) or fractionation of adipocytes by filtering the adipocyte suspension through nylon mesh screens of different pore size (13) subdividing isolated fat cells in populations with different mean diameters. Those studies showed that both basal and insulin-stimulated glucose metabolism increase with cell size so that the insulin fold response and insulin sensitivity were the same for all cells in a biopsy (1, 9, 13).

In a recent study of fatty acid uptake in explants of adipose tissue from various depots, Varlamov et al. (41) quantified fatty acid uptake of individual adipocytes, reporting a decreased insulin fold response related to increased cell size or age in middle and lower body fat depots.

Resolving how expression of functional proteins relates to cell size is important in understanding the implications of holding adipocytes of different sizes. It is well documented that adipocyte size varies considerably not only between but also within the same depot in individuals. The cause of the within depot adipocyte size heterogeneity is unknown. One underlying factor could be that adipocytes in a depot have different ages (37) or that adipocytes interact differently with stromal cells during maturation (21). Increases in the size of adipocytes depend on lipid accumulation regulated by insulin action on GLUT4 and hormone-sensitive lipase. It is unknown, however, whether a change in fat deposition induces adipocyte protein expression or if protein expression governs fat deposition and thus adipocyte size. In different nonfat cells, caveolae have been implicated in mechanosensing where caveolae may act as a membrane reserve and modulate volume-regulated ion channel activation by sensing the increase in membrane tension during cellular swelling (10, 16) and/or uniaxial stretching (34).

Caveolae are small (50–100 nm) invaginations of the cell surface membrane that are highly enriched in cholesterol, sphingolipids, and the specific proteins cavin and caveolin (27, 44). Caveolae constitute as much as 30–50% of the adipocyte surface area (7, 22, 39) and have, in addition to their volume regulatory role, been implicated in a range of cellular processes such as signal transduction and transmembrane transport of lipids (18–20, 26, 33). Recently, it was demonstrated that expression of caveolar proteins and cholesterol is critical for insulin signaling in adipocytes (4). In adipocytes, caveolae could, therefore, well play a role in a locally acting homeostatic mechanism adjusting the size of growing adipocytes to their number of caveolae.

Here, we developed a fluorescent imaging assay that can quantify specific protein expression and cell size in individual adipocytes from the same biopsy. Analysis of expression of the caveolar proteins cavin-1 and caveolin-1 (referred to as cavin and caveolin in the following) as a function of adipocyte size revealed that the expression of cavin and caveolin both are positively related to adipocyte surface in biopsies from rats of different age. Surprisingly, the age-related increases in mean adipocyte size decreased the mean density of caveolar proteins expressed per adipocyte surface area.
METHODS

Animal model. Fat cells were isolated from epididymal adipose tissue of Sprague-Dawley rats of different weights (103–629 g) and different ages (4–26 wk), and they were fed a normal diet (13 kcal %fat, Altromin 1319; Brogaarden, Lyngby, Denmark) ad libitum. All animal studies were performed according to the Guide for the Care and Use of Laboratory Animals, published by the US National Institutes of Health (NIH publication No. 85–23, revised 1996) and approved by the Animal Experiments Inspectorate of the Danish Ministry of Justice.

Collagenase isolation and fixation. Adipocytes were isolated by collagenase digestion for 45 min. The procedures for isolating fat cells have been described previously (42). The adipocytes were freely dispersed in the medium; there was rarely clumping of the cells. The cell suspension was fixed with 4% formalin, mixed with melted agarose (Agarose A6877 Type II, Medium EEO; Sigma-Aldrich), and spread on a slide in a 200-μm-thick layer. Upon cooling at 4°C for 10 min, a gel slab, adherent to the slide, formed. This gel slab was immersed in acetone for 30 min. Upon a brief wash in distilled water specimens were air dried.

Antibodies. The primary antibodies used were mouse anti-ZF11, a protein-G purified monoclonal antibody against cavin, prepared as described previously (44) and rabbit anti-caveolin (no. 610060; BD Transduction Laboratories). Secondary antibodies used were Alexa Flour 568 goat anti-mouse (no. A11031) or Alexa Flour 488 goat anti-rabbit (no. A11034) from Molecular Probes. Control slides were incubated with secondary antibodies alone or with mouse IgG1 (Dako). None of the controls showed any labeling.

Immunofluorescence staining. The slides were washed in TBS (0.05 M Tris, pH 7.6, and 0.15 M NaCl, pH 7.4). Antigen retrieval was performed for 1 h at 70°C by using TEG buffer, pH 9. Upon heat treatment, the slides rested for 30 min at room temperature and were then washed in TBS. Sections were blocked with 10% goat serum for 30 min to reduce nonspecific protein binding and incubated overnight at 4°C with primary antibodies diluted in the preincubation medium. All sections were incubated overnight at 4°C with primary antibodies against caveolin and cavin diluted in TBS with 0.1% Triton X-100. Slides were then rinsed in TBS and incubated with secondary antibodies for 2 h. The incubation and washing solutions contained 0.1% Triton X-100. Temperatures >80°C caused the tissue to wash off the slide. Microwave or water bath heat generated bubbles, which lifted the tissue off the slide.

Phase contrast microscopy. The preservation of cell size through the present preparatory procedure was analyzed by measuring projected cell areas of formalin-fixed fat cells embedded in the slab of agarose cast on the slide, and, following acetone extraction drying and rehydration, the same area of extracted cells were measured. More than 200 cells from 4 different animals covering the cell diameter range between 11 and 177 μm were measured before and after acetone extraction using a Zeiss Axiosplan 2 microscope equipped with phase contrast optics using the same image analysis settings. Area measurements were analyzed as described in Image processing and analysis.

Confocal microscopy image acquisition. Ten to fifteen confocal sections spaced 0.93 μm apart in the z-plane, were collected with a Zeiss LSM 710 through a ×20/0.8 NA objective. The 488 and 568 nm Ar/Kr laser lines were employed, and settings were optimized to minimize fluorescence bleedthrough. Optical slice thickness was 0.93 μm and pinhole size 38 airy disc. No labeling was detectable in the absence of primary antibody or in the presence of “nonsense” primary antibody. From each animal between 27 and 102 adipocytes were imaged.

Image processing and analysis. Confocal images were analyzed with ImageJ (by W. S. Rasband; National Institutes of Health, Bethesda, MA; http://rsb.info.nih.gov/ij/) as follows: the confocal z-stacks were projected into a single image (SUM z-projection) resulting from the sum of each grey scale value of a pixel at the different z-planes. Cell area and total and mean fluorescence intensity in regions of interest were quantified. Background fluorescence was measured in a random field outside of each cell and subtracted from the intensity cell measurement.

The sampled values were as follows: 1) cell area corresponding to cell boundaries (area of the selection in square micrometers); 2) integrated density (the product of area and mean gray value = total intracellular fluorescence); the integrated density was used as a relative measure of the amount of protein labeled by the specific antibody; and 3) a “wafer” selected as a circular sample of the cell disc (representing the collapsed cell) with an area <10% of the total disc area away from the edge in an area with apparently uniform labeling intensity was quantified. The mean gray value of each protein in the wafer was sampled from each cell (average gray value within the selection; this is the sum of the gray values of all pixels in the selection divided by the number of pixels representing mean intracellular fluorescence). In the following, all comparisons of labeling are relative and therefore independent of absolute labeling efficiency.

Calculation of cell diameter. The cell diameter was calculated from measurements of the projected area on the assumption that cells were spherical.

Statistical analysis. Multiple linear regression analysis on log-transformed data with dummy variables was used to test the hypothesis that a common exponent of one could describe the relation between cellular expression of cavin and caveolin with cell area. The sum of squares due to the hypothesis was used to calculate the appropriate variance ratio, which was referred to the F-distribution for calculation of P.

One-way ANOVA of log transformed labeling densities was used to test homogeneity between samples from rats of different age. Multiple linear regression analysis on log transformed data with dummy variables was also used to test the hypothesis of homogeneity of the relations between expression densities of cavin and caveolin in cells from rats of different ages. All statistical analyses were done using SAS Enterprise 5.1.

RESULTS

Relation between cell area before and after acetone water/triglyceride extraction. Water and triglyceride removal left the adipocytes as “collapsed ghosts,” approximately circular discs with a thickened perimeter in a 20-fold flattened gel slab, still adherent to the slide (Fig. 1). The cytoplasmic rim (i.e., the plasma membrane and the underlying cytoplasm) of the adipocytes appeared rather unaffected in the central part of the disc, whereas the thickened perimeter displayed circular folds (Fig. 1). In a rehydrated specimen, the circular discs of cellular remains (the extracted cell) was ~8-μm thick (7–12 μm) for cells in a diameter range of 11 to 177 μm before extraction. The relation between the projected area of >200 adipocytes before extraction and the area of the collapsed disc remaining after acetone extraction in four cell samples from different animals covering the cell diameter range between 11 and 177 μm is displayed in Fig. 1. The points are well fitted by a straight line through the origo, i.e., the relation is not different from proportionality. A proportionality factor of 1.25 is calculated for cell areas, which is constant over the entire range of cell sizes.

Colocalization of cavin and caveolin. Cavin and caveolin were colocalized. The colocalization of the two proteins was very prominent in the folded perimeter of the cell where parts of the membrane stands perpendicular to the line of sight (Figs. 3 and 4).
Relation between cell size and protein expression. The protein expression data showed variation in the cellular cavin and caveolin levels with SD of log cavin in the range from 0.25 to 0.65 and SD of log caveolin in the range from 0.26 to 0.62 in the biopsies from rats of different age.

The model

\[ y = k(\pi d^2)^\beta \]

\[ \log_e(y) = \beta \log_e(\pi d^2) + \log_e(k) \]

where \( y \) is protein expression level per cell and \( \pi d^2 \) is cell surface area, was fitted to the data by linear regression separately in all animals. From the model \( y = k(\pi d^2)^\beta \), it is seen that a \( \beta \)-estimate of one would indicate that protein expression is proportional to cell surface area, \( \beta = 0.5 \) to cell diameter and \( \beta = 1.5 \) to cell volume.

In addition to the statistical advantage in fitting a linear model, the distribution of raw data and residuals approached normal distributions better by the log transformation. It should be noted that the estimate of a slope in a regression with error also in the independent variable will result in a systematic underestimation. This bias was corrected for by means of the error estimate from the size measurement validation procedure described above, using the formula of Carroll et al. (2). The individual estimates of \( \beta \) from 21 different rats are shown in Table 1. To test whether the data could be fitted with a common slope, a linear regression with individual intercepts and a slope common to all animals was calculated. An ANOVA test showed that this simplification was permissible for the two caveolar proteins (\( P = 0.31 \) for cavin and \( P = 0.42 \) for caveolin) and that the common slopes were 0.98 ± 0.12 for cavin and 1.01 ± 0.029 for caveolin. Mean coefficients of determination showed that in the average animal 66% of the variation in cellular cavin expression and 60% in caveolin expression could be explained by variation in cell area. This simple relation between cell surface area and expression level of caveolar proteins predicts that mean labeling intensity per unit area of the fat cell membrane should be independent of cell.
size. To test this prediction, central circular parts of the extracted cells (wafers), which appeared flat and evenly labeled, were analyzed. Regression analysis showed that mean gray values of the wafers were independent of the area of the cell ($P = 0.18$; see Fig. 8); thus this mean intensity might estimate expression in two layers of plasma membrane and cytoplasmic rim. If this were the case, the true surface area of a (spherical) cell before extraction ($A_{sphere}$) could be estimated by dividing total labeling intensity of a protein with even distribution in the cell membrane and cytoplasmic rim in an extracted and collapsed cell with half the mean intensity per unit area of the wafer. From $A_{sphere}$, a true projected cell area could be calculated, and this quantity could be compared with the directly measured area of the cell after acetone extraction.

The double logarithmic plot of these two areas from 1,334 cells showed that they could be proportional (slope not different from one, $P = 0.3$) and the estimate of the proportionality constant was 1.31 (Fig. 5), which is not significantly different from the value of 1.25 found by the direct approach of measuring projected areas before and after extraction. If the estimate of mean wafer intensity was biased, a deviation from 1.25 would occur. The agreement between the two methods of estimating the degree of cellular deformation during extraction shows that an unbiased measure of labeling density of membrane and cytoplasmic rim proteins can be sampled in the planar part of the extracted cell. The agreement does, however, not imply that cell surface can be replaced by the indirect estimate without loss of accuracy, since the error variance of this indirect estimate of the total surface area (0.093) is more than fivefold larger than the error variance of the area of the collapsed cell (0.017).

The cellular expression of caveolar proteins was also examined in preparations of epididymal fat cells from ad libitum fed rats of different age and, consequently, different mean cell size (Table 2). In these rats, total cellular expression is positively correlated to cell area within each cell sample from an indi-

### Table 1. Regression analysis for cellular cavin and caveolin expression for animals prepared on different days

<table>
<thead>
<tr>
<th>Age, wk</th>
<th>Animal Weight, g</th>
<th>Number of Cells</th>
<th>Mean Area (log$_k$)</th>
<th>Regression Coefficient (Cavin)</th>
<th>$r^2$</th>
<th>Regression Coefficient (Caveolin)</th>
<th>$r^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>104</td>
<td>70</td>
<td>7.46 ± 0.51</td>
<td>0.96 ± 0.08</td>
<td>0.68</td>
<td>0.97 ± 0.09</td>
<td>0.62</td>
</tr>
<tr>
<td>4</td>
<td>103</td>
<td>95</td>
<td>7.52 ± 0.48</td>
<td>0.97 ± 0.07</td>
<td>0.71</td>
<td>1.02 ± 0.08</td>
<td>0.63</td>
</tr>
<tr>
<td>4</td>
<td>113</td>
<td>102</td>
<td>7.57 ± 0.52</td>
<td>1.08 ± 0.07</td>
<td>0.72</td>
<td>1.07 ± 0.06</td>
<td>0.77</td>
</tr>
<tr>
<td>4</td>
<td>113</td>
<td>55</td>
<td>7.83 ± 0.37</td>
<td>0.94 ± 0.08</td>
<td>0.74</td>
<td>0.92 ± 0.07</td>
<td>0.78</td>
</tr>
<tr>
<td>6</td>
<td>226</td>
<td>98</td>
<td>8.04 ± 0.41</td>
<td>0.90 ± 0.07</td>
<td>0.63</td>
<td>0.81 ± 0.09</td>
<td>0.45</td>
</tr>
<tr>
<td>6</td>
<td>222</td>
<td>69</td>
<td>8.11 ± 0.40</td>
<td>0.99 ± 0.05</td>
<td>0.69</td>
<td>1.03 ± 0.06</td>
<td>0.76</td>
</tr>
<tr>
<td>6</td>
<td>229</td>
<td>73</td>
<td>7.91 ± 0.50</td>
<td>0.91 ± 0.05</td>
<td>0.84</td>
<td>0.92 ± 0.05</td>
<td>0.85</td>
</tr>
<tr>
<td>6</td>
<td>227</td>
<td>50</td>
<td>8.10 ± 0.44</td>
<td>0.92 ± 0.06</td>
<td>0.81</td>
<td>0.90 ± 0.06</td>
<td>0.81</td>
</tr>
<tr>
<td>8</td>
<td>278</td>
<td>99</td>
<td>8.30 ± 0.42</td>
<td>0.84 ± 0.08</td>
<td>0.53</td>
<td>0.86 ± 0.08</td>
<td>0.57</td>
</tr>
<tr>
<td>8</td>
<td>293</td>
<td>27</td>
<td>8.31 ± 0.42</td>
<td>1.05 ± 0.10</td>
<td>0.83</td>
<td>1.02 ± 0.14</td>
<td>0.67</td>
</tr>
<tr>
<td>8</td>
<td>344</td>
<td>33</td>
<td>8.61 ± 0.25</td>
<td>0.94 ± 0.11</td>
<td>0.68</td>
<td>0.95 ± 0.15</td>
<td>0.57</td>
</tr>
<tr>
<td>10</td>
<td>381</td>
<td>27</td>
<td>8.71 ± 0.27</td>
<td>1.00 ± 0.20</td>
<td>0.51</td>
<td>0.96 ± 0.22</td>
<td>0.42</td>
</tr>
<tr>
<td>10</td>
<td>371</td>
<td>50</td>
<td>8.55 ± 0.24</td>
<td>1.10 ± 0.17</td>
<td>0.47</td>
<td>0.96 ± 0.14</td>
<td>0.49</td>
</tr>
<tr>
<td>10</td>
<td>446</td>
<td>34</td>
<td>8.45 ± 0.24</td>
<td>1.05 ± 0.14</td>
<td>0.64</td>
<td>1.06 ± 0.17</td>
<td>0.55</td>
</tr>
<tr>
<td>10</td>
<td>468</td>
<td>86</td>
<td>8.97 ± 0.26</td>
<td>0.93 ± 0.11</td>
<td>0.45</td>
<td>0.94 ± 0.18</td>
<td>0.25</td>
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<tr>
<td>16</td>
<td>480</td>
<td>87</td>
<td>9.50 ± 0.40</td>
<td>0.95 ± 0.09</td>
<td>0.55</td>
<td>0.84 ± 0.07</td>
<td>0.64</td>
</tr>
<tr>
<td>16</td>
<td>460</td>
<td>55</td>
<td>8.93 ± 0.26</td>
<td>1.15 ± 0.17</td>
<td>0.46</td>
<td>1.04 ± 0.06</td>
<td>0.28</td>
</tr>
<tr>
<td>16</td>
<td>509</td>
<td>43</td>
<td>9.00 ± 0.25</td>
<td>1.18 ± 0.13</td>
<td>0.68</td>
<td>1.12 ± 0.16</td>
<td>0.56</td>
</tr>
<tr>
<td>26</td>
<td>591</td>
<td>55</td>
<td>9.41 ± 0.24</td>
<td>1.04 ± 0.10</td>
<td>0.68</td>
<td>1.17 ± 0.12</td>
<td>0.57</td>
</tr>
<tr>
<td>26</td>
<td>629</td>
<td>55</td>
<td>9.38 ± 0.31</td>
<td>0.90 ± 0.08</td>
<td>0.89</td>
<td>1.04 ± 0.09</td>
<td>0.72</td>
</tr>
<tr>
<td>26</td>
<td>537</td>
<td>41</td>
<td>9.55 ± 0.25</td>
<td>0.97 ± 0.09</td>
<td>0.75</td>
<td>1.00 ± 0.14</td>
<td>0.55</td>
</tr>
</tbody>
</table>

Estimates are followed by ± SE. Test of homogeneity of a logarithmic relation between cellular surface area and cavin expression in isolated epididymal adipocytes prepared from different rats on different days. Table gives the individual parameter estimates for the equation: expression = $k$ (surface area)$^P$, which is transformed into $\log_e$ (expression) = $\beta \log_e$ (surface area) + $\log_e (k)$ corresponding to a straight line with slope $\beta$ and intercept $\log_e (k)$. Estimates of $\beta$ were corrected for the effect of error in the size measurement using the formula of (Ref. 2). ANOVA was done by analyzing the increase in residual variance which results from fitting the data by a common $\beta$ and individual intercepts. Test showed that calculation of a common $\beta$ was permissible ($P = 0.53$ for cavein; $P = 0.42$ for cavin) and that the common estimate of $\beta$ could be equal to 1 ($P < 0.001$). Mean coefficient of determination ($r^2$) was 0.66 for cavin and 0.60 for caveolin. In other words, a proportionality with adipocyte cell surface could explain 66% of the variation in cellular cavin expression and 60% of the variation of cellular caveolin expression in individual rats.

**Fig. 4.** Indirect immunofluorescence labeling of an acetone extracted adipocyte for caveolin and cavin from a 26-wk-old animal. Nucleus stained with DAPI (A), cavin (B), caveolin (C), and a merged image (D) of A–C, with yellow color demonstrating colocalization, very prominent in the folded perimeter of the cell where parts of the membrane stands perpendicular to the line of sight.
to cell size and animal age (Figs. 6 and 7). Moreover, removing
sion showed that these caveolar proteins had similar relations
Analysis of covariation of cellular cavin and caveolin expres-

ted; this could indicate that the proteins were present in
caveolae in a fixed proportion and that the density of caveolae
in the fat cell membrane is subject to some variation also in
individual, cellular expression of the caveolar proteins cavin
DISCUSSION

We have developed and applied a technique for measuring
individual, cellular expression of the caveolar proteins cavin
and caveolin along with cell size in collagenase-isolated adi-
pocytes.

Upon embedding aldehyde-fixed adipocytes in agarose on a
slide, acetone extraction and rehydration leave the nonlipid cell
constituents as a discoid body that has a diameter proportional
to that of the original cell. Furthermore, the specimen can be
immunostained and the middle part of the discoid cell constit-
ients are representative of an en face view of two layers of
plasma membrane with underlying cytoplasm, both by their

Fig. 5. Relation between calculated area and measured area, equation: $y = x + 0.27$, $r^2 = 0.86$. The calculated surface area of a (spherical) cell before extraction was estimated by dividing total labeling of an extracted and collapsed cell with half the mean intensity per unit area of central circular parts of the extracted cells, which appeared flat and evenly labeled. This calculated area is compared with the measured area after extraction. Linear regression analysis of the logarithm of these 2 areas from 1,334 cells shows that they could be proportional (slope not different from one, $P = 0.3$). A proportionality constant of 1.31 was calculated from the mean logarithmic difference of $0.27 \pm 0.008$, which is not significantly different from the value of $0.222 \pm 0.009$ found by the direct approach of measuring projected areas before and after extraction ($P = 0.4$). The agreement shows that an unbiased measure of labeling density of membrane and cytoplasmic rim proteins can be sampled in the planar part of the extracted cell.

Fig. 6. Relation between cellular expression of cavin and cell surface. Isolated epididymal adipocytes from 5 animals of different age are shown in a double logarithmic plot. Linear regression lines for each animal are also shown. ANOVA showed that the regression lines could all have the same slope of 1 ($P = 0.20$). In this experiment variation in cell surface area explained 72% of the variation in cellular cavin expression. $\bullet$: 4 wk; $\circ$: 6 wk; $\triangledown$: 10 wk; $\triangle$: 16 wk; $\blacksquare$: 26 wk (same rate as shown in Table 2).

Table 2. Regression equations for cavin and caveolin expression for five animals (same settings)

<table>
<thead>
<tr>
<th>Animal Age, wk</th>
<th>Weight, g</th>
<th>Number of Cells</th>
<th>Mean Area (log.)</th>
<th>Regression Line (Cavin)</th>
<th>$r^2$</th>
<th>Regression Line (Caveolin)</th>
<th>$r^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>113</td>
<td>44</td>
<td>8.11 $\pm$ 0.30</td>
<td>$y = (0.91 \pm 0.10)x + 8.09$</td>
<td>0.65</td>
<td>$y = (0.8 \pm 0.10)x + 8.10$</td>
<td>0.62</td>
</tr>
<tr>
<td>6</td>
<td>227</td>
<td>50</td>
<td>8.10 $\pm$ 0.44</td>
<td>$y = (0.92 \pm 0.06)x + 8.11$</td>
<td>0.82</td>
<td>$y = (0.90 \pm 0.06)x + 8.27$</td>
<td>0.81</td>
</tr>
<tr>
<td>8</td>
<td>344</td>
<td>33</td>
<td>8.61 $\pm$ 0.25</td>
<td>$y = (0.94 \pm 0.11)x + 7.84$</td>
<td>0.68</td>
<td>$y = (0.95 \pm 0.15)x + 7.71$</td>
<td>0.57</td>
</tr>
<tr>
<td>16</td>
<td>509</td>
<td>43</td>
<td>9.00 $\pm$ 0.24</td>
<td>$y = (1.18 \pm 0.13)x + 5.74$</td>
<td>0.68</td>
<td>$y = (1.12 \pm 0.16)x + 5.49$</td>
<td>0.56</td>
</tr>
<tr>
<td>26</td>
<td>591</td>
<td>41</td>
<td>9.55 $\pm$ 0.25</td>
<td>$y = (0.97 \pm 0.09)x + 6.68$</td>
<td>0.75</td>
<td>$y = (1.00 \pm 0.14)x + 6.06$</td>
<td>0.55</td>
</tr>
</tbody>
</table>

Estimates are followed by $\pm$ SE. Table gives the individual parameter estimates for the equation: expression $= k$ (surface area)$^{\beta}$ which is transformed into log$_{e}$ (expression) $= \beta$ log$_{e}$ (surface area) + log$_{e}$ (k) corresponding to a straight line with slope $\beta$ and intercept log$_{e}$ (k). ANOVA testing similar to one described in Table 1 showed that lines could have a common slope of 1 ($P = 0.50$ for cavin; $P = 0.75$ for caveolin) and that intercepts were different ($P < 0.001$ for cavin; $P < 0.001$ for caveolin).
cellular caveolin expression. Experiment variation in cell surface area explained 62% of the variation in (same rats as shown in Table 2 and Fig. 6).

The tiny caveolae, which are abundant in the adipocyte plasma membrane, cannot be studied directly with the present method due to their minute size; uneven labeling of the planar parts of the cells showed the same pattern for caveolin and cavin (colocalization) and is interpreted as resulting from variations in the lateral density of caveolae in the plasma membrane of adipocytes. Colocalization of high intensity labeling of both caveolar proteins also occurred in the peripheral edge of the discoid cell, which appeared with artifacts in the form of circular wrinkles where the membrane and cytoplasmic rim was parallel with the line of sight. All observations on the localization of the two proteins in the present preparation were in concert with their caveolar association previously established by ultrastructural methods (43). Analysis of the relation between adipocyte size and cellular expression of caveolar proteins in isolated epididymal adipocytes from rats of various ages showed that in individual rats more than half of the variance in cellular expression was explained by the variation in surface area of the adipocytes. This simple relation was valid for cavin as well as for caveolin, both of which appeared equally dependent on cell diameter. The simple relation implies that the density of caveolar proteins in the plasma membrane should be constant and independent of cell size; this also held true when tested on sample images of the planar part of the lipid extracted cells. The finding of a significant correlation between the individual densities in samples of the planar part of individual cells. The latter finding suggests that the variation in cellular expression, not explained by surface variation, is not entirely caused by measurement error, i.e., that a true variation in caveolar density between individual adipocytes is present.

In addition to involvement in lipid metabolism, caveolae have been implicated in lipid transport and metabolism (25), the present finding of a constant density of caveolar proteins in the plasma membrane and cytoplasmic rim is consistent with a recent report on uptake of fluorescent fatty acids, which was also constant when expressed per cellular area and thus increased with cell size (41). It could be noted in this connection that earlier published data from our laboratory on cellular lipogenesis from glucose in single adipocytes have likewise showed near proportionality between lipogenesis and cell surface (9).

The mutual correlation of cellular expressions of the caveolar proteins was even higher than their correlation with cell size, also evidenced by the finding of a significant positive correlation between their individual densities in samples of the planar part of individual cells. The latter finding suggests that the variation in cellular expression, not explained by surface variation, is not entirely caused by measurement error, i.e., that a true variation in caveolar density between individual adipocytes is present.

for one of the caveolar proteins. Considering that caveolae have been implicated in lipid transport and metabolism (25), the present finding of a constant density of caveolar proteins in the plasma membrane and cytoplasmic rim is consistent with a recent report on uptake of fluorescent fatty acids, which was also constant when expressed per cellular area and thus increased with cell size (41). It could be noted in this connection that earlier published data from our laboratory on cellular lipogenesis from glucose in single adipocytes have likewise showed near proportionality between lipogenesis and cell surface (9).

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In addition to involvement in lipid metabolism, caveolae have been implicated in a number of cellular regulatory mechanisms (24, 25). To explain the presently observed surface dependence of expression of caveolar proteins, two caveolar functions might be particularly important in adipocytes: Firstly, Sens and Turner (31) proposed a theoretical model in which invaginations in the plasma membrane (e.g., caveolae) could serve as a membrane reservoir able to buffer variation in membrane tension. This prediction was corroborated by experimental studies in which cell stretching or osmotic swelling resulted in a reversible caveolar flattening (16, 23, 34). An early recognized feature of caveolae is their dependence on cholesterol, loss of which results in collapse of caveolae (12, 28). A decrease in membrane cholesterol with increasing fat cell size has been reported (17), suggesting that a relation between membrane cholesterol/caveolae density and cell size exists. Flattening of caveolae could constitute the sensor in a

Fig. 7. Relation between cellular expression of caveolin and cell surface. Isolated epididymal adipocytes from 5 animals of different age are shown in a double logarithmic plot. Linear regression lines for each animal are also shown. ANOVA by multiple linear regression with dummy variables showed that the regression lines could have the same slope of 1 ($P = 0.20$). In this experiment variation in cell surface area explained 62% of the variation in cellular caveolin expression. $\bullet$: 4 wk; $\square$: 6 wk; $\blacktriangledown$: 10 wk; $\triangle$: 16 wk; $\blacksquare$: 26 wk (same rats as shown in Table 2 and Fig. 6).

Fig. 8. Relation between expression of cavin per unit area of wafer against cell size. ANOVA by multiple linear regression with dummy variables showed independence of within rat cell size variation ($P = 0.2$) and a significant ($P < 0.001$) heterogeneity (decrease) of mean expression per unit area with the increase in mean cell area which occurred during aging. $\bullet$: 4 wk; $\square$: 6 wk; $\blacktriangledown$: 10 wk; $\triangle$: 16 wk; $\blacksquare$: 26 wk (same rats as shown in Table 2 and Fig. 6).
mechanism, which tends to keep membrane tension constant, the effector being the sensitivity to insulin as regulated by insulin receptor expression, which has been shown to be markedly decreased by accelerated insulin receptor degradation in adipocytes lacking caveolae due to knockout of the caveolin or cavin genes (5, 14, 18, 26). In view of the paramount importance of insulin signaling for lipid storage in adipocytes, such negative feedback regulation of lipid accumulation in individual cells would tend to bring about proportionality between expression of caveolar proteins and cell surface area. While a mechanism adjusting cellular lipid storage so that caveolar density is kept constant is consistent with the present results on individual rats, comparisons between rats of different age shows another phenomenon, namely that that the expression levels of caveolar proteins per cell surface area decreased with age in a way which kept the cellular expression approximately constant. Several studies have reported that increase in average adipocyte size that occurs during aging does not produce a surface proportional increase in adipocyte protein expression or functionality (3, 32, 38, 45). Caveolae have been reported to constitute 30–50% of the adipocyte surface area in young rats (7, 22, 39). The present results indicate that this percentage decreases with age.

To accommodate the present finding that the average density of caveolar proteins in the plasma membrane and cytoplasmic rim decreased with the increase in mean adipocyte size that occurs with increasing age, the above-suggested mechanism, which would tend to keep the cell surface proportional to the number of caveolae per cell and which might operate on a short time scale, needs to be extended. One remedy would be to suggest that increasing age, on a longer time scale, promotes the advent of caveola-free membrane, the existence of which is already suggested by the high residual correlation between the cellular densities of caveolar proteins noted above.

Expression of caveolar proteins occurs early in adipocyte differentiation and the marked variation in cellular level of this expression might be of importance for the size variation between adipocytes. Factors involved in this variation are not known but are likely to include interactions with the extracellular matrix of adipose tissue, notably the adipocyte plasma membrane strain brought about by contact with fibrous elements of the tissue. Such forces might contribute to the remarkable size variation among adipocytes, which, in turn, could be considered useful in their tight packing in adipose tissue.

The present results show that it is possible to measure adipocyte size along with expression levels of caveolar proteins by immunolabeling and confocal microscopy. The different relations between cell surface and expression levels within and between individuals of different ages imply the existence of at least two distinct mechanisms regulating fat storage in individual adipocytes, of which one mechanism involves age sensitivity of the density of caveolae in the adipocyte plasma membrane.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

Author contributions: V.H., C.P., and J.V. conception and design of research; V.H. performed experiments; V.H. and J.V. analyzed data; V.H. and J.V. interpreted results of experiments; V.H. prepared figures; V.H. drafted manuscript; V.H., C.P., and J.V. edited and revised manuscript; V.H., C.P., and J.V. approved final version of manuscript.

REFERENCES


