Subcutaneous abdominal preadipocyte differentiation in vitro inversely correlates with central obesity

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Permana, Paska A., Saraswathy Nair, Yong-Ho Lee, Georgia Luczy-Bachman, Barbora Vozarova de Courten, and P. Antonio Tataranni. Subcutaneous abdominal preadipocyte differentiation in vitro inversely correlates with central obesity. *Am J Physiol Endocrinol Metab* 286: E958–E962, 2004. First published February 17, 2004; 10.1152/ajpendo.00544.2003.—Expansion of adipose tissue mass results from increased number and size of adipocyte cells. We hypothesized that subcutaneous abdominal preadipocytes in obese individuals might have an intrinsically higher propensity to differentiate into adipocytes. Thus we investigated the relationship between obesity and the level of in vitro preadipocyte differentiation in Pima Indians. Subcutaneous abdominal stromal vascular fractions containing preadipocytes were cultured from 58 nondiabetic subjects [31 M/27 F; 30 ± 6 yr, body fat 34 ± 8% by dual-energy X-ray absorptiometry (means ± SD)]. The average percentage of preadipocyte differentiation (PDIFF; cell count by microscopy) was 11 ± 11% (range 0.2–51%). PDIFF correlated negatively with percent body fat (r = −0.35, P = 0.006) and waist circumference (r = −0.45, P = 0.0004). Multiple regression analysis indicated that waist circumference (P = 0.01), sex (P = 0.01), and percent body fat (P = 0.05) were significant determinants of PDIFF. Molecular characterization of predifferentiated cultured cells was performed by real-time PCR measurements of glucocorticoid receptor-α (GRα), insulin-like growth factor I receptor (IGF-IR), peroxisome proliferator-activated receptor-γ (PPARγ), enhancer-binding protein GATA-3, CCAAT/enhancer-binding protein-α undifferentiated protein (CUP/AP-2α), and endothelial cell-specific marker 2 (ECSM2). The mRNA concentrations of GRα correlated with PDIFF (r = 0.29, P = 0.03), but the others did not (IGF-IR, r = 0.003, P = 1.0; PPARγ, r = −0.1, P = 0.5; GATA-3, r = 0.02, P = 0.9; CUP/AP-2α, r = −0.2, P = 0.1; ECSM2, r = 0.04, P = 0.7). Contrary to our hypothesis, the results may indicate a blunted in vitro differentiation potential of preadipocytes in centrally obese individuals. The lower differentiation potential of preadipocytes in the obese subjects might be due, at least partly, to decreased glucocorticoid receptor expression.

Pima Indians; gene expression; glucocorticoid receptor; adipogenesis of preadipocytes into mature adipocytes presumably occurs throughout the human life span, since preadipocytes from adult subjects can be isolated from adipose tissue, propagated, and differentiated into adipocytes in vitro (6, 7). This methodology to differentiate primary preadipocytes in culture has been widely used to dissect the highly controlled molecular process involving a complex transcriptional cascade.

As a key element of obesity development, preadipocyte differentiation may be intrinsically regulated. We hypothesized that abdominal preadipocytes in obese people would have an intrinsically elevated propensity to differentiate into adipocytes. To test our hypothesis, we investigated the relationship between the degree of obesity and the level of in vitro abdominal subcutaneous preadipocyte differentiation in subjects with a wide range of obesity. We conducted this study in Pima Indians of Arizona, a population with one of the highest prevalence rates of obesity (13).

MATERIALS AND METHODS

Subjects. Fifty-eight nondiabetic Pima or Tohono O’Odham Indians volunteered for this study. All subjects were in good health as determined by medical history and physical examination as well as routine blood and urine tests. None was taking medication at the time of the study. All subjects provided written informed consent before participation. This study was approved by the Tribal Council of the Gila River Indian Community and the Institutional Review Board of the National Institutes of Diabetes and Digestive and Kidney Diseases.

Clinical protocol. Volunteers were admitted to the Clinical Research Unit, where they were placed on a weight-maintaining diet (containing 50% of calories as carbohydrates, 30% as fat, and 20% as protein) for 2–3 days before clinical testing. Body composition was measured by dual-energy X-ray absorptiometry using a total body scanner (DPX-L; Lunar Radiation, Madison, WI) as described previously (20). Waist circumference was measured at the umbilicus with subjects in the supine position. Oral glucose tolerance tests using 75 g of glucose were performed to exclude diabetic subjects as defined by the World Health Organization criteria (1).

After an overnight fast, the subjects underwent subcutaneous abdominal fat needle biopsy under local anesthesia with 1% lidocaine. Adipose tissue was placed on a sterile nylon mesh and rinsed with sterile 0.9% NaCl solution. The tissue was then cleaned of visible connective tissue and blood vessels in Hanks’ buffered saline solution (HBSS) supplemented with 5.5 mM glucose.

Preadipocyte isolation, growth, and differentiation. Isolation of the stromal vascular fraction containing preadipocytes was performed according to a previously described method by Crandall et. al. (4),
PREADIPOCYTE DIFFERENTIATION IN OBESITY

Table 1. Real-time PCR primer and probe sequences to measure transcript levels of GRα, IGF-IR, PPARγ, GATA-3, CUP/AP-2α, and ECSM2 genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
<th>Probe</th>
</tr>
</thead>
<tbody>
<tr>
<td>GRα</td>
<td>CATTGCTCAAGGGAAAGGAGAACCTC</td>
<td>GATTTTCAAGGAGCTCTGACGATAGA</td>
<td>TTTGCTAGATTGATTAACCAGCGTCCAGTCTC</td>
</tr>
<tr>
<td>IGF-IR</td>
<td>CAAGCCTTTCTCGTTGCTGATG</td>
<td>CTCGCTTCGATGCTGCTGATG</td>
<td>CGGTCTCTCAGCAACGCAAGACATC</td>
</tr>
<tr>
<td>PPARγ</td>
<td>GGACTTTCTCAACAAGTTGAGGTTT</td>
<td>GGACTATTCTCAAGTTGAGGTTT</td>
<td>GAGAGATGGTTCTCAGTACCTAA</td>
</tr>
<tr>
<td>GATA-3</td>
<td>TTCTACGACAGTGGTTCATCGTGC</td>
<td>GCGCCATATGGGTATTTGAGGTTT</td>
<td>CTTACGAGGAACGAGAAGTAGG</td>
</tr>
<tr>
<td>CUP/AP-2α</td>
<td>CCGGAGTTAATATCCAGTCCAGATC</td>
<td>CGGAAAGGTGGTCTCAGTACCTAA</td>
<td>AATTAAAGAAAAAGGCCCTGCTGCTG</td>
</tr>
<tr>
<td>ECSM2</td>
<td>TGGAGGAGGAGGAGGCTGATT</td>
<td>CGACTGCGCTGTGGACGTTC</td>
<td>TACCGTCCGCTGCTGCTG</td>
</tr>
</tbody>
</table>

All sequences are listed from 5' to 3'. GRα, glucocorticoid receptor-α; IGF-IR, IGF-I receptor; PPARγ, peroxisome proliferator-activated receptor-γ; GATA-3, an enhancer-binding protein; CUP/AP-2α, CCAAT/ enhancer-binding protein-α undifferentiated protein; ECSM2, endothelial cell-specific marker 2.
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DISCUSSION

We utilized in vitro primary culture to investigate whether subcutaneous abdominal preadipocytes from individuals with increased obesity had a greater intrinsic potential to differentiate into adipocytes. Interestingly, our results showed that the percentage of in vitro PDIFF correlated negatively and independently with the degree of obesity and central adiposity as measured by percent body fat and waist circumference. Thus the data indicated that the stromal vascular fraction of subcutaneous abdominal fat tissue from centrally obese individuals might contain more preadipocytes with intrinsically blunted differentiation potential in vitro than tissue from lean people. This finding was consistent with data in a recent human study showing a negative correlation between body mass index and differentiation capacity in mammary preadipocytes as assessed biochemically and morphologically using a serum-free differentiation protocol (21), as well as data indicating that preadipocytes from obese rodents differentiate to a lesser extent than those from lean counterparts when subjected to a differentiation mixture similar to the one used in this study (3, 17). These findings suggest that there might be differences in the intrinsic differentiation potential of preadipocytes from lean and obese individuals, which could contribute to the development of obesity-related metabolic disorders such as insulin resistance and type 2 diabetes.

Fig. 1. Negative correlation between percent body fat and percent in vitro primary preadipocyte differentiation (PDIFF; r = −0.35, P = 0.006). ○, males; ●, females.

Table 2. Anthropometric and metabolic characteristics of Pima Indian subjects and Pearson correlations with percent PDIFF

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Male</th>
<th>Female</th>
<th>All</th>
<th>Range</th>
<th>r Value</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of subjects</td>
<td>31</td>
<td>27</td>
<td>58</td>
<td>18-44</td>
<td>−0.01</td>
<td>0.9</td>
</tr>
<tr>
<td>Age, yr</td>
<td>30±7</td>
<td>29±6</td>
<td>30±6</td>
<td>152-190</td>
<td>0.02</td>
<td>0.9</td>
</tr>
<tr>
<td>Height, cm</td>
<td>173±7</td>
<td>160±4*</td>
<td>167±9</td>
<td>46-204</td>
<td>−0.39</td>
<td>0.003</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>109±37</td>
<td>96±35</td>
<td>103±36</td>
<td>46-204</td>
<td>−0.39</td>
<td>0.003</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>36±11</td>
<td>37±13</td>
<td>37±12</td>
<td>18-70</td>
<td>−0.44</td>
<td>0.0005</td>
</tr>
<tr>
<td>Body fat, %</td>
<td>29±7</td>
<td>38±7*</td>
<td>34±8</td>
<td>12-49</td>
<td>−0.35</td>
<td>0.006</td>
</tr>
<tr>
<td>Waist circumference, cm</td>
<td>115±24</td>
<td>113±23</td>
<td>114±23</td>
<td>69-160</td>
<td>−0.45</td>
<td>0.0004</td>
</tr>
<tr>
<td>Waist-to-thigh ratio</td>
<td>1.8±0.2</td>
<td>1.8±0.2</td>
<td>1.8±0.2</td>
<td>1.4-2.4</td>
<td>−0.22</td>
<td>0.09</td>
</tr>
<tr>
<td>Fasting plasma glucose concentration, mg/dl</td>
<td>89±10</td>
<td>90±7</td>
<td>89±9</td>
<td>69-114</td>
<td>−0.28</td>
<td>0.04</td>
</tr>
<tr>
<td>Fasting plasma insulin concentration, µU/ml†</td>
<td>44±18</td>
<td>46±15</td>
<td>45±17</td>
<td>25-112</td>
<td>−0.42</td>
<td>0.002</td>
</tr>
</tbody>
</table>

Data are presented as means ± SD. PDIFF, preadipocyte differentiation. *P = 0.0001 for sex difference; †n = 55.

detector (Applied Biosystems). The final concentrations of primers and probe were 0.9 and 0.25 µM, respectively. A standard curve for each primer-probe set was generated by serial dilution of a randomly chosen cDNA sample done in triplicate. Each sample was run in duplicate, and the mean values of the duplicates were used to calculate transcript level. Real-time PCR was performed as recommended by the manufacturer as follows: 50°C, 2 min; 95°C, 10 min; 95°C, 15 s; and 60°C, 1 min for 40 cycles.

Statistical analyses were performed using the procedures of the Statistical Analysis System software (SAS Institute, Cary, NC). P values of <0.05 (unadjusted for multiple testing) were considered significant. To approximate normal distribution of percentage data, percent PDIFF was logit-transformed with the formula: logit(x) = ln (x/1-x), where 0 < x < 1. To approximate normal distribution of the gene transcript levels and plasma insulin concentration, the data were logarithmically transformed. Each gene mRNA concentration was normalized to that of cyclophilin by taking the residuals after linear regression to cyclophilin. General linear regression models were used to assess the relationships between PDIFF and obesity measures or fasting plasma insulin.

RESULTS

The anthropometric and metabolic characteristics of the subjects with a wide range of obesity (body fat 12–49%, waist circumference 69–160 cm) and their correlations with PDIFF are summarized in Table 2. The average PDIFF was 11 ± 11% (mean ± SD) with a range between 0.2 and 51%. The negative correlations of PDIFF with percent body fat and waist circumference are illustrated in Figs. 1 and 2, respectively. Table 3 shows the result of multiple regression analysis performed to identify determinants of PDIFF, with sex (P < 0.01), percent body fat (P < 0.05), and waist circumference (P = 0.01) being significant determinants. Fasting plasma insulin concentration was not a significant determinant in this analysis. Fasting plasma glucose concentration was also not a significant determinant (P = 0.8) after adjustments for sex, percent body fat, and waist circumference.

We determined the mRNA concentrations of several genes in the predifferentiated stromal vascular cells that might have contributed to the subsequent PDIFF correlations observed. The mRNA concentrations of GRs correlated with PDIFF (r = 0.29, P = 0.03; Fig. 3), but the others did not (IGF-IR, r = 0.003, P = 1.0; PPARγ, r = −0.1, P = 0.5; GATA-3, r = 0.02, P = 0.9; CUP/AP-2α, r = −0.2, P = 0.1). The expression level of ECSM2, as a measure of endothelial cell proportion in the stromal vascular cultures, was not correlated with PDIFF (r = 0.04, P = 0.7). The ECSM2 transcript was detectable in human primary culture of skin fibroblasts that could not be differentiated into adipocytes (data not shown).

The mean ± SD volume of isolated adipocytes in a subset of 14 subjects was 0.00028 ± 0.00015 mm³. PDIFF was weakly correlated with the adipocyte volume measurement (r = −0.49, P = 0.08).
results may be due to paracrine factors secreted by mature adipocytes of obese subjects that irreversibly inhibit PDIFF, such as transforming growth factor-β (22). Nevertheless, the exact mechanism requires further investigation. Additional results from our data analysis indicated that PDIFF was not correlated with the age of the young subjects in our study, which is consistent with the finding that a general decrease in the potential of PDIFF is observed mainly during middle to old age in humans (7, 12).

We reasoned that the intrinsic molecular causes of the lower differentiation potential in preadipocytes of obese individuals would be best investigated in undifferentiated stromal vascular cultures. The presumably different existing levels of several PDIFF modulators in the undifferentiated cells of the obese subjects could then negatively affect cellular adipogenesis when the cells were exposed to differentiation stimuli. These molecular modulators include receptors for insulin and hydrocortisone that comprised the differentiating mixture. We found that PDHFF correlated positively with the GR protein expression in these undifferentiated preadipocytes and GRα protein binds glucocorticoid ligand (2). The expression of GRβ is regulated by its own ligand, cortisol predominantly downregulates GRα mRNA expression and stability (2). The potential role of intracellular cortisol level in regulating GRα expression in these undifferentiated preadipocytes remains to be clarified. The lower in vitro differentiation potential of preadipocytes in the obese subjects of this study might be due, at least partly, to lower mRNA level of GRα and thus likely fewer glucocorticoid receptors in the undifferentiated preadipocytes. A recent study indicated that the ligand-binding domain of glucocorticoid receptors mediated the glucocorticoid stimulation of PDHFF through enhancement of C/EBPα expression (24). Thus the lower concentrations of glucocorticoid receptors in the undifferentiated preadipocytes of obese individuals might predispose the cells to have less effective C/EBPα activation during the PDHFF.

We also measured the mRNA concentration of IGF-IR in the undifferentiated cells, since supraphysiological concentration (10 nM) of insulin in the in vitro differentiation mixture most likely promoted differentiation through these receptors (14). The expression level of IGF-IR did not correlate with PDIFF; thus the number of these receptors before differentiation was unlikely to contribute to the variance in PDIFF. In addition, we measured the mRNA levels of the proadipogenic transcription factor PPARγ and two adipogenic inhibitors, GATA-3 (20) and CUP/AP-2α (10, 11). GATA-3 represses the transcriptional activities of key adipogenic transcription factors PPARγ and C/EBPα (20), and CUP/AP-2α inhibits the activity of C/EBPα (10, 11). Our results indicated that the mRNA levels of these transcription factors in undifferentiated stromal vascular cultures did not correlate with PDIFF. It is conceivable that the lower intrinsic differentiation potential of subcutaneous preadipocytes in the obese subjects of this study was due to the downregulation of other promoters of adipogenesis and/or upregulation of adipogenic inhibitors.

The adipocyte cell size from the available subset of 14 subjects in this study was weakly negatively correlated with PDHFF. This observation warrants further investigation in a larger number of subjects and raises the question whether a reduced differentiation potential in preadipocytes is associated with increased average size of adipocytes in people exposed to long-term excess energy intake. Enlarged subcutaneous adipocyte cell size predicts the development of type 2 diabetes (23). Thus it might be possible that blunted PDHFF in centrally obese subjects is associated with an increased risk of type 2 diabetes (5).

Table 3. Multiple regression analysis to identify determinants of PDIFF level

<table>
<thead>
<tr>
<th>Independent Variable</th>
<th>Estimate</th>
<th>Standard Error</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>0.20</td>
<td>2.06</td>
<td>0.9</td>
</tr>
<tr>
<td>Sex, male</td>
<td>1.69</td>
<td>0.67</td>
<td>0.01</td>
</tr>
<tr>
<td>Body fat, %</td>
<td>0.12</td>
<td>0.06</td>
<td>0.05</td>
</tr>
<tr>
<td>Waist circumference, cm</td>
<td>-0.13</td>
<td>0.05</td>
<td>0.01</td>
</tr>
<tr>
<td>Fasting plasma insulin, µU/ml</td>
<td>-1.17</td>
<td>1.70</td>
<td>0.5</td>
</tr>
</tbody>
</table>

As mentioned above, endothelial cells represent the major "contaminant" of cultured preadipocytes (7), and technical...
procedures during biopsy might have resulted in different proportions of endothelial cells from lean vs. obese subjects. However, this was not likely the case, because the expression level of ECFSM2, a corollary of endothelial contamination level in the cultured stromal vascular cells, did not correlate with PDIF of the same cultures. Primary fibroblast contamination of the stromal vascular fraction was also an unlikely explanation, because we found that ECFSM2 was expressed in cultured human primary fibroblasts, even though ECFSM2 appeared to be more endothelial specific than the classic endothelial marker von Willebrand factor (9).

In summary, our study shows that the level of primary preadipocyte differentiation in vitro correlated negatively with the degree of central obesity in Pima Indians. The lower differentiation potential of preadipocytes in the centrally obese subjects might be due, at least partly, to decreased expression of GRα.

ACKNOWLEDGMENTS
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DISCLOSURES
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REFERENCES