Age-Related Adipose Tissue mRNA Expression of ADD1/SREBP1, PPARγ, Lipoprotein Lipase, and GLUT4 Glucose Transporter in Rhesus Monkeys

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Aging has been shown to have an effect on the capacity to differentiate preadipocytes and on the expression of some genes expressed in adipose tissue. The mRNA levels of adipocyte differentiation-related genes were examined in rhesus monkeys (Macaca mulatta) ranging in age from 7 to 30 years. The effect of aging on the expression of peroxisome proliferator-activated receptor γ (PPARγ), adipocyte determination- and differentiation-dependent factor 1/stereol regulatory element binding protein 1 (ADD1/SREBP1), CCAAT/enhancer binding protein α (CEBPα), lipoprotein lipase (LPL), GLUT4 glucose transporter, and adipin were examined by slot blot analysis. Significant inverse correlations were observed between age and the mRNA levels of PPARγ, ADD1/SREBP1, LPL, and GLUT4. The coordinate downregulation of these genes may be linked to the declining fat mass of senescent animals. There was no correlation between age and the mRNA levels of adipin. The mRNA levels of these genes were not correlated to body weight or fasting plasma insulin. These findings indicate that aging may have an effect on the adipocyte differentiation program and this effect appears to be gene specific.

Both fat mass and adipocyte function change during aging (1,2). Fat mass has been shown to increase until middle age or early old age, followed by declining fat mass in aging humans (1) and rats (3,4). The increase in fat mass is a result of an increase in both fat cell number and size in humans, rats, and monkeys (3,5,6). The decrease in fat mass in senescent rats is a result of decreased fat cell size, not a result of decreased fat cell number (2). Fat cell number continues to increase (epididymal fat depot) or remains stable (perirenal fat depot) in the senescent rats in which fat mass decreases (4).

Preadipocytes are progenitor cells and can differentiate into fat cells which store the lipid droplet. These preadipocytes exist throughout the life span (2,7,8). The number of preadipocytes increases or remains stable with age (9). However, the intrinsic preadipocyte replication potential and the capacity of preadipocytes to differentiate were observed to decline with age in both rats and humans (7,8,10–13). Fat cells in senescent animals can develop from preadipocytes even though the capacity to fully differentiate is reduced. Such fat cells may have a reduced lipogenic capacity, resulting in decreased cell size. Thus, the decline in preadipocyte capacity for fully differentiating may contribute to the decrease in fat mass with aging.

During differentiation, coordinated transcriptional activation of many genes, such as adipocyte determination- and differentiation-dependent factor 1/stereol regulatory element binding protein 1 (ADD1/SREBP1), peroxisome proliferator-activated receptor γ (PPARγ), CCAAT/enhancer binding protein α (CEBPα), lipoprotein lipase (LPL), GLUT4 glucose transporter, and adipin occurs. These gene products are believed to be important for the acquisition and maintenance of the adipocyte phenotype (14,15). The mRNA levels of LPL, glycerol-3-phosphate dehydrogenase (G3PD), adipocyte fatty acid binding protein (apo2), and adipin were decreased with age in the differentiated adipocytes of rats (2,8,16).

Thus, aging affects the expression of adipocyte differentiation-related genes, which may lead to the decreased capacity for full differentiation. However, the effects of aging upon the expression levels of the transcription factors, PPARγ, C/EBPα and ADD1/SREBP1, which play important roles in coordinated transcriptional regulation during adipocyte differentiation (14,15,17–21), have not been previously investigated. Thus, we have examined the mRNA levels of these genes, as well as other adipocyte differentiation-related genes, including LPL, GLUT4, and adipin, in the adipose tissue of rhesus monkeys to elucidate the effect of aging on genes involved in the adipocyte differentiation program.

Methods

Animals

Twenty adult rhesus monkeys (Macaca mulatta) (7 to 30 years) were used in this study. Either Ensure (Ross Laboratories, Columbus, OH), a nutritionally complete liquid diet, or monkey chow (Purina Mills Inc., St. Louis, MO) and fresh water were provided for 8 hours per day ad libitum to individually housed monkeys. Their body weights ranged widely from 7.2 to 22.6 kg. Monkeys with type 2 diabetes were not included in this study; thus, all monkeys had normal plasma glucose levels (~0.0 mM). Some monkeys were obese (body fat >22%) and had hyperinsulinemia; therefore fasting plasma insulin varied widely from 174 pM to 1494 pM. The monkeys were maintained according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Experimental Procedures

Plasma samples were obtained following a 16-hour fast from monkeys which were under light anesthesia (ketamine hydrochloride 10 mg/kg body weight). The plasma samples were
kept frozen for later assays. The adipose tissue samples were taken under fentanyl or ketamine hydrochloride anesthesia from the subcutaneous abdomen just below and lateral to the navel. Tissues were flash frozen and stored at −80°C.

Northern and Slot Blot Analyses
Total RNA was extracted from tissues using TRIzol reagent (Life Technologies, Bethesda, MD). The concentration of total RNA was determined by measurements of absorbance at 260 nm. For Northern blot analysis, 5 μg total RNA was separated on 0.8% agarose containing 2.2 M formaldehyde. The RNA was transferred to Nylon membranes (Hybond-N, Amersham Corp., Amersham, UK). To measure the relative mRNA levels of PPARγ, ADD1/SREBP1, C/EBPα, GLUT4, LPL, or adipin, and heat shock protein 83 (HSP83), slot blot analysis was performed. We used HSP83 as an internal control (22). Three micrograms of total RNA was applied on Nitrocellulose (Bio-Rad Laboratories, Hercules, CA), an amount which was within the binding capacity of the membrane. The filters were hybridized as described previously (23). The relative amounts of mRNAs were quantitated by densitometry (Molecular Dynamics, Sunnyvale, CA).

DNA Probes
The cDNAs utilized as probes in Northern and slot blot analyses were prepared as follows. Primers were synthesized using sequences previously published for human PPARγ2 (24), C/EBPα (25), LPL (26), GLUT4 (27), ADD1/SREBP1 (28), and adipin (29). To facilitate cloning, each primer contained additional 5’ sequences encoding the EcoRI, Xhol, or Xbal restriction site.

ADD1/SREBP1:
5'-CGCGCTCTAGACCGCAACXfACfGGAAACCCXf-3’;
5'-CGCGGCTCGAGTGGTGCGTGCCGGTTGCAGGT-3’.
PPARγ:
5'-CGCGTCTAGACCGCAACXfACfGGAAACCCXf-3’ and
5'-CGCGCTCGAGGCCACGGTGCCXfACAGAGAAA-3’.
C/EBPα:
5'-CGCGGAATTCAAGGACCCfGAAGACACAGCf-3’;
5'-CGCGCTCGAGAGTTGCCCXfGGCCTTGACCAA-3’;
5'-CGCGTCTAGAACCfGGCAGAGTGTGCAGGGGXf-3’ and
5'-CGCGCTCGAGTCTCACAGATGGTCAGGCTGGCT-3’.
LPL:
5'-GCGCGGCTCGAGGCCACGGTGCCXfACAGAGAAA-3’;
5'-CGGCGTAATTCGCCGAGAGCTGGTGCCGAA-3’;.
GLUT4:
5'-GAAAGAAGCCAACACAGGCACAGCT-3’
5'-GGATCTACGAGCTTCCACCATCAGGACAGAA-3’.

Reverse-transcription reaction was carried at 25°C for 10 minutes and 42°C for 50 minutes in a final concentration of 20 mM Tris-Cl, pH 8.4; 50 mM KCl; 2.5 mM MgCl₂; 10 mM dithiothreitol (DTT); 500 μM of each dNTP; 10 U/μL of Superscript II reverse transcriptase (Life Technologies, Bethesda, MD), and 7.5 ng/μL of random hexamers, using 5 μg of total RNA purified from monkey adipose tissue. The partial sequences of PPARγ, ADD1/SREBP1, C/EBPα, GLUT4, LPL, and adipin were amplified from 2 μL of the reverse-transcription reaction mixture by polymerase chain reaction (PCR) in a final volume of 100 μL containing 1× PCR buffer (10 mM Tris-Cl, pH 8.3; 50 mM KCl; 150 μM MgCl₂; 0.001% w/v gelatin), 100 μM of each dNTP, 2.5 U/μL of Taq DNA polymerase (Perkin-Elmer Co., Norwalk, CT), 0.55 μg/tube TaqStart antibody (Clontech Laboratories, Inc., Palo Alto, CA), and 0.1 μM of each primer. PCR was performed using 35 cycles of denaturation (1 minute at 94°C), annealing (1 minute at 50°C), and extension (1 minute at 72°C). The fragments were cloned into pBluescript II KS (+) (Stratagene, La Jolla, CA). Each clone was sequenced to confirm the sequences of the monkey homologues of PPARγ, ADD1/SREBP1, C/EBPα, GLUT4, LPL, or adipin. The DNA fragments of PPARγ, ADD1/SREBP1, C/EBPα, GLUT4, LPL, or adipin were digested at restriction endonuclease sites which had been inserted in the primer sequences, and were used as probes. The probes were labeled with α-[32P]dCTP (3,000 Ci/mmol, Amersham), using Random Primers DNA Labeling System (Life Technologies). The probe for HSP83 was prepared as described before (23).

Analysis of Splicing Alternatives of Monkey PPARγ
The ratio of PPARγ2 mRNA levels to total PPARγ mRNA levels (sum of PPARγ1 and PPARγ2) was measured as described (30,31). The following PCR primers were synthesized according to monkey PPARγ1 and PPARγ2 sequences (31). 5’-GGATCTGTTCTTGTGAATGGA-3’ and 5’-GGAGTCAACGAGACAT-3’ (specific to the PPARγ1 sequence) and 5’-CCTGCACAACATATCACA-3’ and 5’-CAGACCTACCATCCACACAT-3’ (specific to the PPARγ2 sequence). Reverse transcription was performed using the same conditions described above. PCR was performed using three primers in the same reaction concentration as described above except adding 1 μCi/tube of α-[32P]dCTP (3,000 Ci/mmol, Amersham). Twenty cycles of PCR, which was within the logarithmic phase of amplification, were performed. The length of 254 bp of PPARγ1 and 205 bp of PPARγ2 PCR products were radiolabeled, which were separated on an 8% polyacrylamide gel. Autoradiography was performed after the gel was dried. The relative amounts of PPARγ1 and PPARγ2 were quantitated by densitometry (Molecular Dynamics) and corrected for GC content and the lengths of each PCR product. To determine if these two mRNA splicing variants were being amplified in an unbiased manner, a validation experiment was performed. In vitro synthesized monkey PPARγ1 and PPARγ2 mRNAs (using T7 RNA polymerase, Life Technologies) were mixed in varying ratios and were measured as described above. The observed ratio of PPARγ2 mRNA to total PPARγ mRNA (%PPARγ2) was highly correlated to the expected ratio (r = .990). A cycle titration revealed that the logs of the relative amounts of the PCR products (both PPARγ1 and PPARγ2) were highly correlated to the number of cycles (in the range 19 to 25 cycles, r = .997), indicating that 20 cycles was within the logarithmic phase of amplification. These data confirmed that the two splicing mRNA variants were being amplified in an unbiased manner and provided validation of this method.

Other Assays
Plasma glucose concentrations were determined using a Beckman glucose autoanalyzer II (Beckman Instruments, Fullerton, CA). Plasma insulin levels were measured by radioimmunooassay (32). Body fat was measured by the tritiated water dilution method (33).

Statistical Analysis
Linear relationships between key variables were tested using
RESULTS

The body weights of these adult monkeys varied widely (mean ± SE: 14.1 ± 1.1 kg, range: 7.2–22.6 kg) and did not show a significant correlation with age, whereas percent body fat and fat weight increased with age (Table 1). Fasting plasma glucose levels (3.7 ± 0.1 mM, 2.7 to 5.0 mM) were within normal range, as diabetic monkeys were excluded from this sample; however, glucose levels increased slightly with age. Fasting plasma insulin levels (695 ± 111 pM, 174 to 1459 pM) in these monkeys also were not significantly correlated with age.

Northern blot analysis revealed that each probe hybridized to one specific band, verifying that these probes could be used for slot blot analysis (Figure 1A). We next examined the mRNA levels of these genes by slot blot analysis. The mRNA levels of ADD1/SREBP1, PPARγ, LPL, and GLUT4 were significantly inversely correlated with age (Table 1, Figures 1B, 2), but were not correlated with either body weight or fasting plasma insulin levels (Table 1). The mRNA levels of C/EBPα decreased slightly, although not significantly, with aging. The mRNA levels of adipin, however, were not related to age (Table 1, Figures 1B, 2). The mRNA levels of HSP83, which we used as an internal control, did not show any changes with age (Table 1, Figure 2). The addition of other factors, that is, body weight, body fat, fasting plasma glucose, and fasting plasma insulin, did not improve the fit of the linear model of the mRNA levels of PPARγ and LPL and age. The percent body fat improved the linear model of the mRNA levels of ADD1/SREBP1 and GLUT4 and age. Longitudinal studies have revealed that many rhesus monkeys spontaneously develop obesity at middle age. Many of these obese monkeys go on to develop type 2 diabetes. When aging rhesus monkeys lose weight, lean body mass declines (34), whereas plasma glucose, and percent body fat increase with aging. Thus, the inverse correlation between percent body fat, and the mRNA level of ADD1/SREBP1 and GLUT4 may be the result of an age-related decrease of mRNA expression and age-related increase of plasma glucose and body fat (Table 1).

Table 1. Correlations Between Age and Parameters and mRNA Levels in 20 Rhesus Monkeys

<table>
<thead>
<tr>
<th>Age</th>
<th>Body Weight</th>
<th>% Body Fat</th>
<th>Fat Weight</th>
<th>Plasma Glucose</th>
<th>Plasma Insulin</th>
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<tbody>
<tr>
<td>Body weight</td>
<td>.23</td>
<td>.71***</td>
<td>.55*</td>
<td>.51*</td>
<td>.68***</td>
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<tr>
<td>% Body fat</td>
<td>.61***</td>
<td>.66**</td>
<td>.59**</td>
<td>.70***</td>
<td>.64**</td>
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<tr>
<td>Fat weight</td>
<td>.48**</td>
<td>.66**</td>
<td>.59**</td>
<td>.70***</td>
<td>.65**</td>
</tr>
<tr>
<td>Plasma glucose</td>
<td>.09</td>
<td>.73***</td>
<td>.44</td>
<td>.64**</td>
<td>.65**</td>
</tr>
<tr>
<td>ADD1/SREBP1</td>
<td>-.58**</td>
<td>-.21</td>
<td>-.70***</td>
<td>-.46*</td>
<td>-.41</td>
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<td>-.30</td>
<td>-.42</td>
<td>-.36</td>
<td>-.47*</td>
</tr>
<tr>
<td>C/EBPα</td>
<td>-.32</td>
<td>-.43</td>
<td>-.52*</td>
<td>-.44</td>
<td>-.23</td>
</tr>
<tr>
<td>LPL</td>
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<td>-.25</td>
<td>-.32</td>
<td>-.25</td>
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<tr>
<td>GLUT4</td>
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<td>-.44</td>
<td>-.68***</td>
<td>-.57*</td>
<td>-.46*</td>
</tr>
<tr>
<td>Adipin</td>
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<td>-.49*</td>
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<td>-.42</td>
<td>-.15</td>
</tr>
<tr>
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<td>.04</td>
<td>-.04</td>
<td>-.09</td>
<td>-.03</td>
<td>.20</td>
</tr>
</tbody>
</table>

The % PPARγ mRNA levels to total PPARγ (PPARγ1 and PPARγ2) mRNA levels.

*p < .05; **p < .01; ***p < .001.
Figure 2. Relationship between age and the mRNA levels of ADD1/SREBP1, PPARγ, C/EBPα, LPL, GLUT4, and adipsin in the adipose tissue of 20 rhesus monkeys. The mRNA levels of each gene were measured by densitometry of slot blot hybridization as described in Methods. The ratio of PPARγ2 to total PPARγ (PPARγ1 and PPARγ2) mRNA (%PPARγ2) was determined as described in Methods.

When percent body fat was excluded, the addition of other factors (body weight, fasting plasma glucose, and fasting plasma insulin) did not improve the linear model of the mRNA levels (ADD1/SREBP1 and GLUT4) and age. Adjustment of each mRNA level for HSP83 produced similar correlations with age to those shown in Figure 2 (unadjusted for HSP83). HSP83-adjusted significant correlations with age were: ADD1/SREBP1: $r = -0.50, p < 0.05$; PPARγ: $r = -0.65, p < 0.001$; GLUT4: $r = -0.68, p < 0.001$; and LPL: $r = -0.68, p < 0.001$. The mRNA levels of ADD1/SREBP1 ($r = 0.49, p < 0.05$), C/EBPα ($r = 0.65, p < 0.01$), GLUT4 ($r = 0.71, p < 0.001$), and LPL ($r = 0.82, p < 0.001$) were significantly correlated to the mRNA levels of PPARγ as we have demonstrated previously (31). Thus, the mRNA levels of PPARγ, ADD1/SREBP1, GLUT4, and LPL decreased with age coordinately in the adipose tissue of monkeys.

The ratio of PPARγ2 to total PPARγ mRNA was significantly correlated with body weight and fasting plasma insulin, but not with age (Table 1).

**DISCUSSION**

During adipocyte differentiation, coordinated gene expression of transcription factors, that is, PPARγ, ADD1/SREBP1, and C/EBPα, occurs. PPARγ and C/EBPα are important for maintaining the fully differentiated state. ADD1/SREBP1 also
plays an important role in adipocyte differentiation through increasing the activity of PPARγ (14,15). We have shown that the mRNA levels of PPARγ, C/EBPα, and ADD1/SREBP1 decrease with age in rhesus monkeys. This reduction in mRNA levels may result from failure of adipocytes in aged animals to be fully differentiated compared to younger animals. The down-regulation of PPARγ, C/EBPα, and ADD1/SREBP1 with aging in the adipose tissue of monkeys could also be due to the decreased capacity of preadipocytes to differentiate, which was reported in humans and rats (7,8,10,12,13).

PPARγ, C/EBPα, and ADD1/SREBP1 regulate many fat-specific genes including LPL and GLUT4, as well as trigger the program of adipocyte differentiation. Thus, the decreased mRNA expression of LPL and GLUT4 would be the result of the decreased expression of PPARγ, C/EBPα, and ADD1/SREBP1. LPL and GLUT4 play important roles in lipogenesis, and lipogenesis has also been reported to decrease with age (2). The decreased mRNA expression of LPL and GLUT4 may therefore be related to reduced lipogenesis with age. Thus, the adipocytes of senescent animals may not store as much lipid as that observed in younger animals because of decreased expression of lipid metabolism-related genes, which in turn may be linked to reduced fat mass in senescent animals.

The age-related decreases in the mRNA levels of PPARγ, C/EBPα, ADD1/SREBP1, GLUT4, and LPL were coordinated. Gene transcription of many transcriptional factors occurs as a cascade during adipocyte differentiation. The regulation of this cascade may be by PPARγ itself or by ADD1/SREBP1 itself (14,15). These transcriptional factors also include C/EBPβ and C/EBPδ, which are involved in the transcriptional control of PPARγ (35). Thus, PPARγ itself or ADD1/SREBP1 itself, or
C/EBPβ, C/EBPδ, or other unknown factors may coordinately regulate the expression of PPARγ, C/EBPα, ADD1/SREBP1, GLUT4, and LPL, and this regulation may decrease with age, and appears to precede the loss of fat mass with senescence.

The mRNA levels of adipin were not correlated with age in the monkey, thus, the effect of aging on the mRNA levels of adipocyte differentiation-related genes is not uniform. The mRNA levels of adipin were previously reported to decrease with age in the rat (16). Thus, there may be species differences in the effect of aging on these adipocyte mRNA expression levels. The present data suggest the possibility that aging affects the regulation of the adipocyte differentiation program, and that this effect is gene specific.

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REFERENCES

17. Lin E-T, Lane MD. CAAT/enhancer binding protein α is sufficient to initiate the 3T3-L1 adipocyte differentiation program. Proc Natl Acad Sci USA. 1994;91:8757–8761.

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