Chronological changes in metabolism and functions of cultured adipocytes: a hypothesis for cell aging in mature adipocytes

Yi-Hao Yu and Huaijie Zhu
Department of Medicine, Columbia University College of Physicians and Surgeons, New York, New York 10032
Submitted 3 June 2003; accepted in final form 13 November 2003

Yu, Yi-Hao, and Huaijie Zhu. Chronological changes in metabolism and functions of cultured adipocytes: a hypothesis for cell aging in mature adipocytes. Am J Physiol Endocrinol Metab 286: E402–E410, 2004. First published November 18, 2003; 10.1152/ajpendo.00247.2003.—The growth and aging of 3T3-L1 adipocytes were investigated in a synchronized tissue-culture system. We systematically characterized several major aspects of adipocyte metabolism and functions as variables of cell age. We found that terminal differentiation of 3T3-L1 cells is followed by a near-linear hypertrophic growth (increase in triglyceride content) of the cultured adipocytes throughout a 20-day study period. However, three metabolically and functionally distinct stages are recognized. The first stage overlaps with differentiation and is represented by small immature adipocytes. The second stage is characterized by fully mature adipocytes that show peaked overall metabolic activities. The third stage is marked by cell aging, with deterioration in every major aspect of the cell’s functionality except for the function of net energy storage, which is preserved even in aged adipocytes. Compared with young mature adipocytes, older cells are increasingly insulin resistant, have decreased glucose uptake and fuel consumption, and show impaired glycerokinase-mediated fatty acid reesterification. Moreover, aged adipocytes show reduced gene expression for adiponectin and leptin, each of which is important in systemic regulation of energy metabolism. The characterization of these cell age-dependent changes in adipocyte functionality provides a model for understanding dynamic changes at the tissue level and suggests that adipose tissue is modifiable via adipocyte aging.

triglyceride; adiponectin; leptin; glycerokinase and futile cycle; insulin sensitivity

ADIPOSE TISSUE IS ESSENTIAL in maintaining systemic energy metabolism and homeostasis (26, 42). Although anatomic location, neuronal innervation, and hormonal stimulation may all affect the gross functional status and, possibly, the fine architectures of adipose tissue, individual adipocytes represent primary functional units of adipose tissue. Therefore, they are the basis of tissue functionality.

Although it was observed more than two decades ago that adult adipose tissue contained a relatively constant number of adipocytes (17, 18), adipocyte de novo production was also documented in adult adipose tissue (22, 25, 37). It is now believed that adipocyte cellularity (cell number) changes in adult animals. This notion is supported not only by the evidence of adipocyte proliferation but also by adipocyte apoptosis, which has been widely documented (10, 12, 32–34, 36). It seems likely that individual adipocytes turn over routinely in mature adipose tissue, and that constant adipocyte turnover may be an important mechanism underlying the heterogeneity of adipocyte size and function observed in both normal and abnormal adipose tissues (4, 30, 31, 46). At steady state, with a relatively constant adipocyte cellularity in most adult animals, changes in adipocyte life span and turnover rate, or alteration of the cell-aging process, would be expected to result in changes in the overall adipocyte composition of the tissue. Specifically, the average age of the cell population, or the proportion of young vs. old adipocytes in a given tissue, would change if the de novo production and/or the cell-aging process were altered.

An important question is whether changes in adipocyte age are linked to changes in adipocyte function and, therefore, in tissue function. In contrast to the early adipocyte differentiation process, which has been extensively studied (see Refs. 23 and 38), there is a lack of published data related to the aging of mature adipocytes. In the present study, we systematically investigated adipocyte aging in a synchronized tissue culture system. We are able to show that specific changes in adipocyte metabolism and functions occur during adipocyte growth and aging, supporting a cell-aging hypothesis that may account for differences in the final makeup of adipose tissues under various conditions.

MATERIALS AND METHODS

Materials. [2-3H]glycerol (200 mCi/mmol), D-[1-14C(U)]glucose (5 mCi/mmol), 2-deoxy-D- D-[1-14C]glucose (50 mCi/mmol), and [α- 32P]dCTP (800 Ci/mmol) were purchased from PerkinElmer Life Sciences. Thin-layer chromatography (TLC) plates were obtained from Merck. Culture media, supplements, and antibiotics were all purchased from Life Technologies. Taq polymerase was purchased from Invitrogen. Triglyceride assay kits (Trig/GB) were from Roche Diagnostics. Most other chemicals and reagents were purchased from Sigma, with the highest purity available.

Cell culture. 3T3-L1 preadipocytes were maintained in high-glucose DMEM with 10% calf serum. Medium was changed every other day, and cells were always split and passed before they reached full confluence. To induce differentiation, 3T3-L1 cells were plated at a density of 1 × 10⁵ cells/35-mm dish (6-well plates). Forty-eight hours after reaching confluence, cells were incubated in an induction medium of high-glucose DMEM containing 10% fetal bovine serum, 2 μM dexamethasone, 500 μM IBMX, and 5 μg/ml insulin. At postinduction day (PID) 3, the medium was changed to a growth medium containing high-glucose DMEM and 10% fetal bovine serum. The culture medium was routinely changed every other day. Biochemical measurements were conducted at different time points after a 2-day induction, as indicated by PID, to designate the
ages of differentiated adipocytes. PID 0 refers to preadipocytes just before induction. Assays were conducted either at basal state (in an assay medium devoid of insulin) or after a defined period of short-term insulin stimulation, as indicated in each assay. When assays were carried out on cells at PID 3, the induction medium was first withdrawn. Cells were washed and then incubated in a regular growth medium for 6 h before assays (with or without insulin stimulation) were conducted.

**Oil-red-O staining for intracellular triglycerides.** Oil-red-O (0.4%) in isopropanol solution was freshly made. Fine particles were allowed to precipitate out after standing at 23°C for 10 min. After a brief spin, clear supernatant was transferred to a new tube and mixed with one-half volume of H2O. After 10 min at 23°C, the dye was spun again, and the clear supernatant was used for staining immediately. To stain for triglycerides (TGs), cells in the monolayer were first washed with phosphate-buffered saline (PBS) three times and then fixed in 37% formaldehyde solution for 30–60 min without shaking. Next, formaldehyde was washed off with six washes in PBS buffer. Fixed cells devoid of formaldehyde were stained with the freshly prepared Oil-red-O solution for 10 min at 23°C, followed by extensive washes with H2O (6 times).

**Total TG mass.** Cellular lipids were extracted using hexane-isopropanol (3:2 by volume). One milliliter of the organic solvent was freshly made. Aliquots of the extracts were dried under a nitrogen stream, and lipids were redissolved in hexane. TG mass was determined enzymatically with colorimetric kits (Trig/GB from Roche Diagnostics). TG standards were used for quantification.

**Quantitative RT-PCR.** RNA was prepared from cultured cells with TRIzol reagent by following the protocol provided by the manufacturer (Life Technologies). Reverse transcription was carried out with reverse transcriptase and random hexamer primers. An aliquot of the reverse transcriptase and random hexamer primers. An aliquot of the reaction mixture containing reverse transcripts was used for subsequent quantitative PCR reaction, as previously described (49). Pairs of sequence-specific primers (Table 1) were designed to amplify a small DNA fragment (~150 bp) of specific cDNAs. When information was available, pairs of primers that span an intron sequence were chosen. Amplification was pretitrated using [32P]dCTP labeling, and quantification was made within a linear range of amplification (usually within the first 25 cycles). Simultaneous amplification of β-actin mRNA was used as an internal control, and the relative expression levels of a specific gene were determined by the ratio of the mRNA level of the specific gene in question to the level of β-actin.

**Northern blot.** Fifteen to fifty micrograms of RNA were applied to each lane in a 1% agarose gel with formaldehyde, and Northern analysis was performed as previously described (49). The probes used for hybridization were generated by RT-PCR labeling with [32P]dCTP by use of RNAs isolated from mouse adipose tissue as initial templates.

**TG turnover.** To study TG turnover, cells were labeled with a larger amount of [3H]glycerol (30 μCi in 2 ml medium) for 2 h. At the end of labeling, cells were washed three times with PBS before change to a chase medium (high-glucose DMEM with 10% fetal bovine serum in the absence of radioactive tracers). Lipids were extracted by hexane-isopropanol at the following time points during chase: 0, 24, 48, and 72 h, and 3H-labeled TG from each sample was quantified by scintillation counting after TLC separation.

**CO2 production rates.** Measurements of CO2 production rates were based on the method described by D’Girofalo (9) but modified as follows. Briefly, preadipocytes (PID 0) or adipocytes (at various PIDs) in 6-well plates were first washed and trypsinized (0.25 mg/ml) in 0.3 ml of trypsin-EDTA solution. After cells were completely detached, 0.2 ml of 1× trypsin inhibitor solution (Sigma, T7659) was added to neutralize trypsin activity. Next, 2 ml of Krebs-Ringer bicarbonate buffer containing 4% BSA and 6 mM glucose was added, and the cell suspension was transferred to a 15-ml culture tube. Then, 0.5 ml of KRB-4% BSA-6 mM glucose solution containing 1 μCi of [3H(U)]glucose, with or without 4.5 μM insulin, was added. The suspension was immediately gassed with 95% O2-5% CO2 for 15 s, and the culture tube was sealed with a rubber stopper that was attached to a central well containing a piece of filter paper to collect CO2 at the end of assay. Cells were incubated at 37°C for 60 min with constant rotation (80 cycles/min). At the end of 60-min incubation, 0.5 ml of 2.5 N H2SO4 was injected into the culture tube through the rubber stopper to destroy the cells and stop the metabolic incubation. To trap CO2 gas produced during the 60-min culture period, 0.2 ml of benzenthionium hydroxide was injected into the filter paper inside the suspended central well. Collection of CO2 was completed after another 20-min incubation at 80 cycles/min. After disassembling of the culture tube, the central well containing trapped [3H]CO2 was transferred into a scintillation vial, and the labeled CO2 was quantified.

**Cellular ATP levels.** A luciferase method was used to determine intracellular ATP levels. 3T3-L1 cells in 6-well plates were washed three times with ice-cold PBS and lysed in 1 ml of solution containing 0.1 N NaOH and 0.5 mM EDTA. The lysates were incubated at 60°C for 20 min and stored at ~80°C before assay. To measure ATP levels, samples were diluted 200-fold, and an aliquot of 20 μl was assayed in a reaction mixture containing 100 μl of luciferase-luciferin reagent (Sigma). A 15-s light emission was measured in a luminometer (Monolight 3010, Pharmingen). ATP concentrations were determined and calibrated with a standard ATP curve generated under the same assay conditions.

**Trypan blue staining.** Before staining, the adipocyte monolayer was washed three times with PBS. Cells were stained in situ at 0.04% trypan blue in PBS for 5 min at room temperature before microscopic examination. To quantify the percentage of permeable cells, monolayer cells were first released by trypsinization (0.25 mg/ml trypsin). By the end of digestion, a culture medium containing 20% calf serum was gently added back to the cell suspension. Cells were stained with trypan blue (0.04% final concentration) for 2 min and were then immediately examined under a microscope. Stained and unstained

Table 1. Sequence-specific primer pairs amplifying a small DNA fragment of specific cDNAs

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bax</td>
<td>5'–gcaatagggaggctgcagagga–3'</td>
<td>5'–gccttgagcaccaggttgca–3'</td>
</tr>
<tr>
<td>LITAF</td>
<td>5'–tcccctcctgtcctcaggtc–3'</td>
<td>5'–ctgtagggcgcagagggtgc–3'</td>
</tr>
<tr>
<td>HSL</td>
<td>5'–cgctgacaccaatcacccgac–3'</td>
<td>5'–cgatggagagaagtctgcag–3'</td>
</tr>
<tr>
<td>aP2</td>
<td>5'–gacgacagggagtggagggc–3'</td>
<td>5'–gcctttcataacacattccacc–3'</td>
</tr>
<tr>
<td>Gyk</td>
<td>5'–ggggacacccgctgtaaggct–3'</td>
<td>5'–gttccactgtcctccaccaagt–3'</td>
</tr>
<tr>
<td>Adiponectin</td>
<td>5'–tgcagccaccaaacctccctcta–3'</td>
<td>5'–gacccagaggataagtcctaa–3'</td>
</tr>
<tr>
<td>adipocyte lipid-binding protein-2; Gyk, glycerokinase.</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Bax, a member of the Bel-2 family (a proapoptotic polypeptide); LITAF, lipopolysaccharide-induced TNF-α factor; HSL, hormone-sensitive lipase; aP2, adipocyte lipid-binding protein-2; Gyk, glycerokinase.
cells were counted. The percentage of stained cells at each time point was obtained after four independently stained cell populations had been examined. At least 500 individual cells were counted for each specimen.

**Free glycerol release.** Before assay, 3T3-L1 adipocytes in 6-well plates were washed three times with PBS. Cells were then incubated with 1 ml of regular DMEM culture medium containing 10% fetal bovine serum. After 1 h of incubation, medium was collected, and an aliquot of the medium was taken out to measure free glycerol concentration. Free glycerol levels were determined in an enzymatic reaction mixture in which lipase was omitted, and the levels were determined by colorimetric analysis (Trig/GB from Roche Diagnostics). Glycerol standards were used for calibration.

**[^H]glycerol incorporation assay.** To measure rates of glycerol incorporation into TG, adipocytes in culture plates were first washed three times with PBS before the assay. Cells were then incubated with[^H]glycerol in high-glucose DMEM culture medium (10 μCi in 2 ml medium) for 1 h. After the wash, lipids were extracted from the monolayer cells with organic solvents made of hexane and isopropanol in 3:2 ratio. The extraction was carried out either at 23°C for 2 h or at 4°C overnight. To determine glycerol incorporation into TG, an aliquot of the lipid extract was taken out and dried under a liquid nitrogen stream. The lipids were redissolved in 20 μl of hexane and then analyzed by TLC. ^3H-labeled TG was quantified by scintillation counting.

**2-Deoxyglucose uptake.** Before the hexose measurements, cells in 6-well plates were washed three times with PBS, followed by incubation in a serum-free low-glucose DMEM medium for 1 h. Cells were washed three times with, and then incubated in, KRP-HEPES buffer (10 mM HEPES, pH 7.4, 131.2 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO4, 2.5 mM CaCl2, 2.5 mM NaH2PO4) in the presence or absence of 0.75 μM insulin for 20 min. The uptake was initiated by addition of 2-deoxy-D-[1-^14C]glucose to a final concentration of 0.02 mM (1 μCi). The uptake was allowed for 10 min. At the end of 10 min, the medium was immediately removed, and cells were washed three times with ice-cold PBS. Cells were then dissolved in 1 ml of 0.1 M NaOH-0.1% SDS, and an aliquot was used to quantify 2-deoxy-D-[1-^14C]glucose in a scintillation counter. As indicated in Fig. 6, 2-deoxyglucose uptake was also measured at a physiological concentration of glucose. In these experiments, glucose, at the final concentration of 4.5 mM was included in the incubation media before and during 2-deoxy-D-[1-^14C]glucose uptake.

**Cell number determination.** Cell numbers were determined by quantifying DNA content with fluorescent dye Hoechst 33342. Cells cultured in 6-well plates were first washed with PBS, and then 1 ml of 0.1% Triton X-100 in 3:2 ratio. The extraction was carried out at 23°C for 2 h or at 4°C overnight. To determine glycerol incorporation into TG, an aliquot of the lipid extract was taken out and dried under a liquid nitrogen stream. The lipids were redissolved in 20 μl of hexane and then analyzed by TLC. ^3H-labeled TG was quantified by scintillation counting.

**Data treatment and statistical analysis.** Most experiments were done in triplicate, and some with repeated triplicates. Values obtained from each experiment were normalized either by β-actin levels (Northern or quantitative RT-PCR) or by cell numbers (all other assays), as indicated in Figs. 1–7. Data were expressed as means ± SE. Differences between means were analyzed by repeated-measures ANOVA with Statistica V6.0. P values of <0.05 and <0.001 were used to indicate statistical significance.

**RESULTS**

The growth of 3T3-L1 adipocytes is accompanied by a linear increase in TG accumulation, and cell aging commences with an elevation in aging markers. We used 3T3-L1 adipocytes for this study, because ages of the cultured mature adipocytes can be relatively synchronized from the start of terminal differentiation (the cell ages are designated by postinduction days). 3T3-L1 cells began to differentiate after induction with dexamethasone, insulin, and IBMX. After a lag of ~3 days postinduction, during which time terminal adipocyte markers rose rapidly (data not shown), cells became competent to store TG. Between PID 5 and PID 10, adipocytes became fully mature and overtly lipid laden, with a readily recognizable morphology under a microscope (Fig. 1A). Mature adipocytes continued to store TG at a constant rate (Fig. 1B), but after PID 10, cellular aging markers began to increase (Fig. 1C). To monitor the aging process, we quantified the gene expression of Bax and lipopolysaccharide-induced TNF-α factor (LITAF). Bax is a member of the Bcl-2 family and a proapoptotic polypeptide that is elevated in dying adipocytes (15, 20). LITAF is an inducible gene by proapoptotic protein p53 (27, 29). It also functions as a transcription factor of TNF-α expression (27, 29). As shown in Fig. 1C, expression levels of both Bax and...
LITAF were relatively stable initially but became increasingly elevated between PID 10 and PID 20, correlating with the aging process during this period. We also measured the gene expression of TNF-α, which is a multifunctional cytokine related to several deteriorative metabolic changes and apoptosis of adipocytes (see Ref. 40). However, due to the extremely low level of signals detected in our cultured adipocytes, quantification was not possible (data not shown).

Adipocyte aging is marked by a decrease in fuel uptake and TG turnover and overall cellular catabolism. Despite the continuous hypertrophic growth (increase in TG content), even at the time when aging markers were increasing (Fig. 1), the uptake of glucose (the only significant energy source present in the culture medium) declined during aging. The 2-deoxyglucose uptake at PID 20 was only 40% of that at PID 10 (Fig. 2A), whereas net TG mass increased more than twofold during the same period of time (Fig. 2A). To understand how TG mass increased, we examined TG turnover rates during this aging period. We conducted a 72-h TG turnover study on PID 15. In this study, intracellular TG was pulse-labeled with [3H]glycerol for 1 h, and the labeled TG was isolated and quantified immediately after pulse labeling and at different time points during subsequent chasing. As shown in Fig. 2B, a rapid decline in TG turnover was demonstrated over time. Whereas ∼50% of labeled TG turned over during the 1st day, only 28 and 12% of the labeled TG from the previous days turned over on the 2nd and the 3rd days, respectively. The semi-log plot is shown in the inset in Fig. 2B. The nonlinearity is consistent with changes in turnover rate.

We next examined changes in overall catabolic activities of the cells. The cellular respiration rate, as measured by the production rate of CO2 that derives from terminal catabolism, was investigated during adipocyte growth and aging. This respiration rate displayed a complex change over time. It increased initially as cells underwent differentiation and maturation, peaked at about PID 6–8, and then began to decline quickly as cells aged. Such changes occurred both in the basal state and, more profoundly, when measured during insulin stimulation (Fig. 2C). Consistent with these changes, cellular levels of ATP, the product of oxidative phosphorylation generated during catabolism, showed a similar pattern of chronological changes. In particular, a continuous and significant decline from the peak (~PID 6) was observed during subsequent aging (Fig. 2D). To ensure that the decline of intracellular ATP levels over time was not due to progressive losses of membrane integrity in older cells, we assessed the cell membrane’s permeability by trypan blue exclusion staining. Only nonviable cells trapped in the adipocyte monolayer took up the dye and stained dark blue (Fig. 3A). To further quantify the percentage of permeable cells, we stained individual adipocytes after they were released from the culture plate by trypsinization (Fig. 3B). In this way, the stained and unstained individual cells could be easily distinguished and accurately counted. By use of this method, 10–17% were stained positive at each time point during adipocyte aging (Fig. 3C). However, no trend of increase in cell membrane permeability was observed over cell aging, and the differences between different time points were not statistically significant. The variations in percentage of trypan blue staining were in part introduced by various extents of additional cell damage rendered during the trypsinization procedure, because the number of nonviable cells observed in these experiments was considerably higher than that estimated from in situ staining (data not shown).

Taken together, the above metabolic studies showed that adipocyte aging is associated with a decrease in metabolic (mainly catabolic) activities and an increase in energy inefficiency, reflected by continuous TG accumulation despite decreasing levels of glucose uptake and metabolism.

Adipocyte aging is accompanied by a reduction in the glycerokinase-mediated “futile cycle” of fatty acid reesterification. In addition to the decrease in TG catabolism as a primary cause of the slowed TG turnover during cell aging, we examined TG lipolysis. We first quantified the gene expression...
of hormone-sensitive lipase (HSL), a key enzyme for lipolysis (see Ref. 24), and adipocyte lipid-binding protein-2 (aP2), a member of the intracellular fatty acid-binding protein family, which also appears to participate in lipolysis; adipocytes deficient in aP2 are consistently impaired in lipolysis (7, 39). Whereas the expression of HSL was relatively constant over time, with only a modest decline at PID 20 compared with that at PID 10, the expression level of aP2 underwent a more significant change, with a steady decline from PID 10 to PID 20 (Fig. 4A), suggesting that a decrease in lipolysis was associated with adipocyte aging. To quantify actual fatty acid release from stored TG, we measured release of free glycerol. Despite reduced gene expression of HSL and aP2, suggesting a decline in lipolysis rate during aging, the net TG hydrolysis rate was ~10% higher at PID 20 than that at PID 10, although the difference did not reach the statistical significance of \( P < 0.5 \) (Fig. 4B).

To explain the discrepancy between changes in lipolytic enzyme expression and in actual glycerol release, we investigated the futile cycle reesterification activities. To measure the reesterification rate, we incubated cells with \(^{3}H\)glycerol and quantified the rate of incorporation of the labeled glycerol into TG. As shown in Fig. 5A, the rate of incorporation of \(^{3}H\)glycerol into TG by cultured adipocytes was at the highest level at the beginning of our measurement (PID 5) and steadily declined as cells aged (down to ~10% of the initial level by PID 20). This reduction in reesterification activity is in agreement with the change in the gene expression levels of glycerokinase, a key enzyme in this process. Compared with the peak level at PID 5, the expression of glycerokinase decreased to only about one-third by PID 20 (Fig. 5B). The initial increase in glycerokinase gene expression (Fig. 5B) was expected, and it coincided with adipocyte differentiation and maturation. These data indicate that younger (but mature) adipocytes are more capable of reesterification of free fatty acids (FFAs) and glycerol, and therefore more capable of controlling FFA release compared with older cells.
Adipocyte aging is associated with a decline in insulin sensitivity and a reduction in gene expression of the secretory proteins adiponectin and leptin. We further assessed effects of aging on two important functional aspects of adipocytes, i.e., insulin sensitivity and gene expression of leptin and adiponectin. To assess adipocyte insulin sensitivity, we first measured basal and insulin-stimulated 2-deoxyglucose uptakes in the absence of cold glucose in the medium, a condition conventionally used in uptake assays. Compared with young adipocytes (PID 10), aged cells (PID 22) had a considerably lower uptake both at the basal state (50% reduction) and under insulin stimulation (73% reduction) (Fig. 6A). Next, we examined the insulin sensitivity at a more physiological glucose concentration in the medium. In these experiments, we included 4.5 mM glucose in the incubation medium before and during the uptake assay (see MATERIALS AND METHODS). Figure 6B shows the results of such experiments conducted with adipocytes of different age groups as indicated. As expected, the net insulin-stimulated 2-deoxyglucose uptake increased initially as cells underwent differentiation and maturation. This insulin responsiveness was relatively stable at the peak level between PID 5 and PID 10 and then decreased as cells aged. The insulin responsiveness at PID 20 was less than one-half that of the peak value (Fig. 6B).

We compared mRNA levels of adiponectin and leptin between the young mature (PID 10) and the aged (PID 22) adipocytes. Northern blot analysis showed a marked decrease in mRNA levels of both leptin (~70% reduction) and adiponectin (~85%) from PID 10 to PID 22 (Fig. 7A). Figure 7B shows chronological changes in the mRNA levels of the two proteins from PID 3 to PID 20 by quantitative RT-PCR. The expression of both proteins quickly rose during the first few days, which coincided with the initial stage of adipocyte differentiation and development, as cells became filled with lipids. After expression peaked at maturity, the mRNA levels of leptin and adiponectin each followed their own courses of decline as cells went through an aging process. The decline started earlier in the gene expression for adiponectin than for leptin, and leptin appeared to sustain a high level of gene expression until after PID 15, when a decrease became evident (Fig. 7B).

**DISCUSSION**

The cell culture data showed that the chronology of 3T3-L1 adipocytes paralleled a continuous near-linear accumulation of TGs but was characterized by three functionally and metabolically distinct stages that marked development, maturation, and...
aging. Cells first underwent terminal differentiation and maturation. Full maturity was reached and sustained between PID 5 and 10, as marked by peaked metabolic activity coinciding with high levels of several other functions. Then, cellular aging became increasingly apparent, and it was accompanied with increasing levels of gene expression of cellular “aging markers,” Bax and LITAF. During aging, cells became both more metabolically inert and more energy efficient (i.e., more TG storage, but less fuel uptake and consumption). However, as a result of this acquired metabolic inactivity, older cells metabolized less glucose at the basal level and became less responsive to insulin stimulation. In our experiments, adipocytes were followed only up to PID 20, primarily because there were considerable degrees of cell proliferation (undifferentiated cells) observed after PID 20, likely due to available growth spaces vacated by the death and detachment of some mature adipocytes at this time. This new cell growth and subsequent differentiation made the entire cell population sufficiently unsynchronized in age after PID 20 and was not desirable for further biochemical and metabolic assays. However, the average half-life of the cultured 3T3-L1 adipocytes was estimated to be 40–60 days under these culture conditions (data not shown).

Although gene expression of HSL and aP2 at PID 20 was ~25 and ~43% less, respectively, than at PID 10, suggesting a decrease in lipolysis rate, the net free glycerol release was not decreased in older cells (Fig. 4B). This latter observation actually agrees with the previous finding that larger adipocytes have higher lipolytic activity (6, 35). This apparent discrepancy between aP2 expression and free glycerol release may be explained if one assumes that aP2 is usually present in large abundance. Thus, although a decrease of aP2 expression in older cells was statistically significant, the magnitude of the decrease was physiologically too small to affect the lipolysis rate. We also sought alternative explanations. Because the net release of FFAs or glycerol from stored TG is a result of a combined action of lipolysis and FFA glycerol reesterification, we investigated the reesterification process during adipocyte aging. FFA reesterification is also termed the futile cycle, and glycerokinase is a key enzyme in this process, converting free glycerol to glycerol 3-P, which is necessary for subsequent esterification with acyl-CoA. This futile cycle was initially described in brown adipose tissue for its contribution to heat production (see Ref. 3). It is likely that this reesterification process also occurs in white adipose tissue in controlling FFA release (14, 35). High levels of glycerokinase activity are present in white adipose tissue, especially under conditions such as thiazolidenedione treatment (14). We found that both free glycerol incorporation into TG and the gene expression of glycerokinase decreased markedly from PID 5 to PID 20 (Fig. 5), making older cells less capable of controlling FFA release. Uninhibited FFA release from adipocytes is believed to be an important cause of systemic lipotoxicity and insulin resistance (see Refs. 5, 43, and 44).

To assess the cell’s capacity to make adipocyte-derived hormones, we quantified the gene expression levels of leptin and adiponectin, two well-documented hormones with “antidiabetic” properties. Although adiponectin has been shown to enhance insulin sensitivity in peripheral tissues in normal and insulin-resistant mice (2, 8, 47, 48), leptin exerts its antidiabetic effects both peripherally and centrally via central nervous system pathways (11, 21, 28, 41, 45). We found that process parallel, but opposite, to the increasing levels of Bax and LITAF gene expression. mRNA levels of adiponectin and leptin decreased during aging, although each followed its own course of decline. The continuous decrease in adiponectin mRNA level observed after PID 10 is in agreement with previously reported findings that the expression of this protein is inversely related to fat mass (1, 19). Our older adipocytes had higher TG content than younger ones (Fig. 1). However, cell aging may also contribute to the course of the change. The aging effect was clearly evident when the change in leptin gene expression was analyzed. Leptin production usually correlates positively with fat mass and adipocyte volume (13, 50). This correlation may be partially reflected in our experiments between PID 5 and PID 15 (Fig. 7B), because leptin mRNA levels increased during this period of time, but the increase was only marginal (Fig. 7B) as opposed to the linear increase in adiposity (Fig. 1). After PID 15, however, a decrease in leptin mRNA level was eventually observed, which was unexpected because cells continued to enlarge their TG mass even at these advanced ages. Therefore, we conclude that the reduction of leptin gene expression in these fatter but aged cells is due to a previously unrecognized cell-aging effect. It is possible that aging plays a negative role in leptin gene expression and that this negative effect becomes dominant and overrides the positive effect of fat mass on leptin gene expression in the “very old” adipocytes.

Taken together, the present study showed that, although TG-storing function was maintained during adipocyte aging (i.e., between PID 10 and PID 20), deterioration occurred in every other aspect of adipocyte functionality examined. Compared with young (but fully mature) adipocytes (e.g., PID 10), aged adipocytes (e.g., PID 20) were much more metabolically inert and insulin resistant. In addition, aged cells became significantly impaired in functions related to systemic regulation of energy metabolism secondary to increasingly limited gene expression of adiponectin and leptin. Furthermore, due to the decreased capacity in glycerokinase-mediated glycerol-FFA reesterification, FFA release from stored TG may become uninhibited, which is a potential cause for systemic lipotoxicity.

Adipocytes are functional building blocks of adipose tissue. Therefore, changes in adipocyte functions are expected to affect tissue functionality. Differences in cell size and functions among individual adipocytes in adipose tissues have long been observed, and the degree of the heterogeneity varies widely under different conditions (4, 30, 31, 46). The relationship between adipocyte size and functions (e.g., esterification, lipolysis, and insulin sensitivity) has been investigated in some earlier studies (4, 6, 35). However, it was not clear how cell size correlated with actual cell age. Adipocyte size may be greatly influenced by the physiological status of individual animals (e.g., obese vs. lean, acutely on high-fat diet vs. chronically on low-fat diet). Even within a single adipose depot, size heterogeneity is present and may be a result of differential paracrine/autocrine (or neuronal innervation) effects on individual adipocytes, because there may be distinctive differences in cell position within the fine architecture of the tissue.

Our present study tracked chronological changes in adipocyte metabolism and functions during prolonged cell culture.
The increasing expression of Bax and LITAF over time in these cultured adipocytes suggested that an aging process was underlying these metabolic and functional changes. Similar increased expressions of Bax and LITAF were also observed in long-term cultured, confluent preadipocytes (data not shown), suggesting that the expression of these aging markers was not unique to adipocyte aging. Because the expansion of TG mass in the cultured adipocytes paralleled adipocyte age, it is possible that the metabolic and functional deterioration observed was actually secondary to the increase in fat mass, rather than increase in cell age. However, the fact that the metabolic and functional changes did not always correlate inversely with cellular TG mass argues against cell size as a primary underlying cause for the observed changes. In fact, the metabolic and functional changes were characterized by an initial increase (roughly from PID 0 to PID 5), then a peak (roughly between PID 5 and PID 10), and then a course of eventual decline (roughly from PID 0 to PID 5), then a peak (roughly between PID 5 and PID 10), and then a course of eventual decline (roughly from PID 0 to PID 5), then a peak (roughly between PID 5 and PID 10), and then a course of eventual decline (roughly from PID 0 to PID 5), then a peak (roughly between PID 5 and PID 10), and then a course of eventual decline (roughly from PID 0 to PID 5), then a peak (roughly between PID 5 and PID 10), and then a course of eventual decline (roughly from PID 0 to PID 5), then a peak (roughly between PID 5 and PID 10), and then a course of eventual decline (roughly from PID 0 to PID 5), then a peak (roughly between PID 5 and PID 10), and then a course of eventual decline (roughly from PID 0 to PID 5), then a peak (roughly between PID 5 and PID 10), and then a course of eventual decline (roughly from PID 0 to PID 5), then a peak (roughly between PID 5 and PID 10), and then a course of eventual decline (roughly from PID 0 to PID 5), then a peak (roughly between PID 5 and PID 10), and then a course of eventual decline (roughly from PID 0 to PID 5), then a peak (roughly between PID 5 and PID 10), and then a course of eventual decline (roughly from PID 0 to PID 5), then a peak (roughly between PID 5 and PID 10), and then a course of eventual decline.

Finally, it is possible that the observed aging process was primarily a phenomenon in cell culture, because the half-life of adipocytes may be considerably longer in vivo than in culture, although because of the difficulty of tracking the ages of individual adipocytes in vivo, the former remains unknown at this time. Obviously, data from our tissue culture cannot be simply extrapolated to composite adipocytes in the tissue, and we cannot be certain at present that cell age-related metabolic and functional changes occur in vivo. If verified in vivo, however, cell aging would help to explain adipocyte heterogeneity (both in size and functions) observed in adipose tissue; it would suggest that unsynchronized cell turnover and aging result in differences in size and functions of composite adipocytes, and that the relative proportion of “young” to “old” adipocytes has a significant impact on the overall metabolic profile of the tissue. We hypothesize that the functional status of adipose tissue is modifiable through aging of individual adipocytes. Changes in the ratio of young to old adipocytes may result from aberrant cell aging or turnover process, which may occur under certain physiological and pathological conditions. Even a brief period of disruption of or discordance between adipocyte production and apoptosis is expected to lead to shifts of the average age of adipocyte population during the ensuing period of time in an animal’s life. Imbalanced acceleration in cellular aging may also occur. In this regard, obesity or animals started on a high-fat diet may offer examples of accelerated adipocyte hypertrophy and aging, leading to faster turnover (22, 25, 37). We speculate that premature deterioration in adipocyte functions under these conditions may result in a seemingly young population of adipocytes with the metabolic profile of old cells. Moreover, it is possible that, during a prolonged course of overdemand, adipocyte de novo production may eventually fail to keep up with the speed necessary to compensate for accelerated cell aging. If this occurs, a truly defective adipose tissue composed of only aged adipocytes may result. Finally, there is some evidence that reagents that are capable of modifying adipocyte aging or turnover process can cause improvement in compositional and functional profiles of adipose tissue. In this regard, both leptin and the thiazolidinediones improve adipose function by promoting apoptosis of aged adipocytes (15, 33) and by stimulating de novo production of young adipocytes (16, 30), respectively. Future investigations would require careful monitoring and characterization of the adipocyte aging process and changes in turnover rates under these and various other conditions. Technical advances in isolation and separation of adipocytes by age would greatly facilitate these studies.

ACKNOWLEDGMENTS

We thank Drs. Henry Ginsberg, Ira Goldberg, and Yiying Zhang for reading the manuscript and for their helpful comments and suggestions.

GRANTS

This work was supported by Pfizer Grant ARAP CU-51736901.

REFERENCES


null