Increased CUG Triplet Repeat-binding Protein-1 Predisposes to Impaired Adipogenesis with Aging*

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Preadipocyte differentiation capacity declines between middle and old age. Expression of the adipogenic transcription factors, CCAAT/enhancer-binding protein (C/EBP) α and peroxisome proliferator-activated receptor γ (PPARγ), is lower in differentiating preadipocytes from old than young animals, although no age-related changes occur in C/EBPβ mRNA, which is upstream of C/EBPα and PPARγ. C/EBPβ-liver-enriched inhibitory protein (C/EBPβ-LIP), a truncated C/EBPβ isoform that is a dominant inhibitor of differentiation, increases with aging in rat fat tissue and preadipocytes. CUG triplet repeat-binding protein-1 (CUGBP1) binds to C/EBPβ mRNA, increasing C/EBPβ-LIP translation. Abundance and nucleotide binding activity of CUGBP1 increased with aging in preadipocytes. CUGBP1 overexpression in preadipocytes from young animals increased C/EBPβ-LIP and impaired adipogenesis. Decreasing CUGBP1 in preadipocytes from old rats by RNA interference reduced C/EBPβ-LIP abundance and promoted adipogenesis. Tumor necrosis factor-α, levels of which are elevated in fat tissue with aging, increased CUGBP1 protein, CUGBP1 binding activity, and C/EBPβ-LIP in preadipocytes from young rats. Thus, CUGBP1 contributes to regulation of adipogenesis in primary preadipocytes and is responsive to tumor necrosis factor-α. With aging, preadipocyte CUGBP1 abundance and activity increases, resulting in enhanced translation of the C/EBPβ-LIP isoform, thereby blocking effects of adipogenic transcription factors, predisposing preadipocytes from old animals to resist adipogenesis. Altered translational processing, possibly related to changes in cytokine milieu and activation of stress responses, may contribute to changes in progenitor differentiation and tissue function with aging.

Through middle age fat depot sizes increase. However, in advanced old age, fat depot sizes decrease, and fat becomes redistributed to tissues outside fat depots (1–3). The decline in fat depot mass is because of a decrease in fat cell size, as fat cell numbers are constant or increase in different depots with increasing age (1, 2). Because capacity for lipolysis decreases in fat cells with aging (4, 5), declines in capacity for lipid accumulation could be particularly important in contributing to reduced adipocyte size. New fat cells arise from preadipocytes throughout the life span, because new fat cells continue to appear from preadipocytes during adulthood, and preadipocytes are present in fat depots at all ages (1, 6–10). These findings are compatible with the possibility that reduced fat depot size with aging may be related to decreased capacity of preadipocytes to differentiate and of fat cells arising from them to accumulate lipid. Indeed, preadipocytes isolated from old animals accumulate lipid less extensively than cells from young animals, even after weeks in culture under identical conditions (11, 12). Thus, in addition to the influence of possible age-related changes in diet, activity, hormones, circulation, or other factors extrinsic to adipose cells, altered preadipocyte intracellular characteristics with aging may contribute to changes in fat depot size and function.

Reduced expression of the key adipogenic transcription factors, CCAAT/enhancer-binding protein α (C/EBPα)2 and peroxisome proliferator-activated receptor γ (PPARγ), contribute to decreasing capacity of preadipocytes to differentiate with aging (3, 13). Overexpression of C/EBPα in preadipocytes from old animals restores adipogenesis, consistent with a contribution of mechanisms upstream of adipogenic transcription factor expression to the age-related decline in differentiation. C/EBPβ expression increases early during adipogenesis, contributing to enhanced PPARγ and C/EBPα expression (14, 15). The early differentiation-related increase in C/EBPβ mRNA is similar across age groups (13), implicating events between the increase in C/EBPβ mRNA and subsequent increases in PPARγ and C/EBPα expression. Increases in anti-adipogenic regulators that act at this point, including the liver-enriched inhibitory protein (LIP) isoform of C/EBPβ and C/EBP homologous protein (CHOP), occur with aging (3, 13). Abundance of the alternatively translated C/EBPβ-LIP, which lacks the full transactivation domain of longer C/EBPβ isoforms, is substantially higher in fat tissue from old than young rats and

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‡ The abbreviations used are: C/EBP, CCAAT/enhancer-binding-protein; PPARγ, peroxisome proliferator-activated receptor γ; TNFα, tumor necrosis factor-α; ANOVA, analysis of variance; siRNA, small interfering RNA; PBS, phosphate-buffered saline; LIP, liver-enriched inhibitory protein; LAP, liver-enriched activating protein; CHOP, C/EBP homologous protein; GFP, green fluorescent protein.
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Increases within hours after induction of adipogenesis (13). This prompted us to investigate potential upstream regulators of C/EBPβ mRNA translation that could contribute to decreased adipogenesis with aging.

(CUG)_n triplet repeat RNA-binding protein (CUGBP) was identified through its ability to bind specifically to (CUG)_n oligonucleotides in vitro (16, 17). CUGBP was postulated to play an important role in the pathogenesis of myotonic dystrophy, because the 3’-untranslated region of the myotonin protein kinase gene contains a (CTG)_n repeat expansion in patients with this disease. There are two CUGBP isoforms, CUGBP1 and CUGBP2, the cellular localization of which is regulated by phosphorylation by as yet unknown kinases (18). By binding to target mRNAs, CUGBP1 can lead to alternative isoform production, both through affecting the translation apparatus and alternative splicing of pre-mRNA molecules (19–21).

C/EBPβ mRNA is among the transcripts to which CUGBP1 can bind, leading to production of low molecular weight isoforms of C/EBPβ in vitro translation and partial hepatectomy experiments (19). Two CUGBP1-binding sites have been identified side by side between the first and second AUG codons in the 5’ region of C/EBPβ mRNA, one in frame and the second out of frame. The latter is located in a small open reading frame that has been shown to regulate initiation of translation from different AUG codons of the C/EBPβ mRNA.

Because C/EBPβ is an important regulator of adipogenesis and the ratio of its activating to inhibitory isoforms is subject to a significant decline with aging, we speculated that CUGBP1 has a role in preadipocyte differentiation and declining adipogenesis in advanced old age. Additionally, we examined whether TNFα affects CUGBP1 expression and activity for the following reasons. In liver, CUGBP1 activity is increased by lipopolysaccharide, suggesting that CUGBP1 is a stress-responsive protein (22). Furthermore, C/EBPβ-LIP generation in response to lipopolysaccharide in the liver becomes exaggerated with aging. TNFα abundance is greater in fat tissue from old than young rats (23). TNFα is anti-adipogenic and down-regulates expression of PPARγ (24). Hence, we speculated that decreased adipogenesis with aging could result, at least partially, from effects of TNFα on CUGBP1.

MATERIALS AND METHODS

Preadipocyte Isolation and Culture—Preadipocytes were isolated from the epididymal or caudal portion (to exclude brown fat (25)) of the perirenal depots of 3-, 17-, and 24–30-month-old male, barrier-reared, specific pathogen-free Brown Norway rats (median survival 32 months; maximum survival 43 months (26, 27)) from the National Institute on Aging colony maintained by Harlan Sprague-Dawley (Indianapolis, IN). The protocol was approved by the Boston University Institutional Animal Care and Use Committee. Separate groups of animals were used for each experiment. All experiments were conducted in parallel, and animals were autopsied to exclude gross pathology. Fat depots were minced, digested in a collagenase solution (1 mg of collagenase/ml Hanks’ balanced salt solution; 3 ml/g tissue), filtered, and centrifuged at 1200 rpm for 10 min (25). The pellet was resuspended in α-minimal essential medium (αMEM) containing 10% fetal bovine serum, and the cells were plated for 20 h. The cells were washed, trypsinized until 90% of the cells had lifted, and replated at 4 × 10^4 cells/cm^2. This method results in 70–90% pure preadipocyte populations (determined by counting colonies derived from single cells that are able to accumulate more lipid than similarly treated skin or lung fibroblasts), irrespective of age or fat depot origin (12).

Furthermore, preadipocyte recoveries, determined by adding various numbers of preadipocytes to fat tissue aliquots before processing, were similar across depots and age groups. To induce differentiation, confluent preadipocyte cultures were exposed to a serum-free differentiation-inducing medium (DM) containing 5 μg/ml insulin, 10 μg/ml transferrin, and 200 μM triiodothyronine, in 1:1 Dulbecco’s modified Eagle’s medium MEM/Ham’s F12 (28). Media were changed every 2 days.

Determination of Preadipocyte Differentiation Stage—Extent of lipid accumulation was assessed by phase contrast microscopy by observers who were not aware of the treatments cells had received, using methods described previously (12, 29, 30). Cells were considered to be in morphological stage 0 when no intracellular granules were visible by phase contrast light microscopy, stage 1 when small dark inclusions were visible, and stage 2 when they contained a few small inclusions that were doubly refractile at medium power (20× objective). Cells were considered to be in at stage 3 when inclusions were larger and more numerous. When the inclusions were large enough to be doubly refractile at low power (10× objective) and filled most of the cytoplasm, they were classified as stage 4. They were considered to be stage 5 when they contained 2–5 large droplets and stage 6 when they contained a single large inclusion that laterally displaced the nucleus. The lipid nature of these inclusions was confirmed by staining with oil red O (31, 32). In previous experiments, we demonstrated that inclusions of the type in stage 4 to 6 cells only appear in differentiating preadipocytes and not other cell types (e.g. lung fibroblasts) in the differentiation medium we used, and that the proportion of such cells is highly correlated with glycerol-3-phosphate dehydrogenase activity (12, 31, 32).

RNA Analysis—Total RNA was extracted from preadipocytes using the guanidinium thiocyanate/phenol method (33). 1 μg of total RNA was reverse-transcribed with Moloney murine leukemia virus-reverse transcriptase (Invitrogen) and random oligonucleotides (Amersham Biosciences). PCRs were performed using an ABI 7500 real time PCR system and software (Applied Biosystems, Foster City, CA). Amplification was performed in a final volume of 20 μl, containing 5 μl of cDNA from the reverse-transcribed reaction, primer mixture (1 μM of each primer), and 10 μl of 2× SYBR Green Master Mix (Applied Biosystems). Analysis of CUGBP1 mRNA expression was carried out during the exponential phase of amplification using the following primers: sense, 5’-CCAGACACCAGATCTT-GATGCT-3’, and antisense, 5’-AGGGTGTACATCTGTATAAGG-TGTAGT-3’ (34). CUGBP1 was expressed as a function of hypoxanthine guanine phosphoribosyltransferase, which was used as an internal control. The expression of hypoxanthine guanine phosphoribosyltransferase was first evaluated using serially diluted reverse-transcribed cDNA. Primers used for hypoxanthine guanine phosphoribosyltransferase amplifica-
tion were sense, 5′-CTTGCCTGAGATGTCATGAAG-3′, and antisense, 5′-GTTTGCATTGTTTACCAGTG-3′.

**Protein Analysis**—For protein analysis, cells were harvested and blotted as described previously (35). Membranes were probed with monoclonal antibody to human CUGBP1 (which has 98% sequence homology with rat CUGBP1 (16)) and goat polyclonal glycerol-3-phosphate dehydrogenase (Santa Cruz Biotechnology catalog sc-20357, Santa Cruz, CA). Visualization of binding of the horseradish peroxidase-conjugated secondary antibody was performed by chemiluminescence. Total protein contents (pg/cell) were 305 ± 32 and 310 ± 38 in undifferentiated and differentiated preadipocytes from 3-month-old rats, respectively, and 335 ± 39 and 333 ± 42 in undifferentiated and differentiated preadipocytes from 24-month-old rats (n = 16 in each group). Equal amounts of protein from undifferentiated or differentiated preadipocytes from young or old rats were loaded in parallel on the same gels. Scanning densitometry was performed using a Hewlett-Packard 3970 scanner (Hewlett-Packard, Palo Alto, CA) and Quantscan software (Biosoft, Ferguson, MT). Densitometric results were expressed as a percentage of total optical density within each gel and were normalized to reflect differences in cellular protein content.

**CUGBP1 UV Cross-link Immunoprecipitation Activity Assay**—RNA oligomers (CUG)$_n$ were labeled with [γ-32P]ATP and T4 kinase. Equal amounts of the radioactive RNA probe (50–200,000 cpm) were then incubated with the total nuclear and cytosolic protein extracts isolated from preadipocytes of each group (preadipocytes isolated from young or old animals, from young animals with or without TNFα treatment) for 30 min at room temperature. Subsequently, the reactions were subjected to UV treatment for 5 min at 125 mJ and separated by SDS-PAGE, as described previously (16). CUGBP1 was precipitated from the reaction with an anti–CUGBP1 antibody linked to protein A-agarose beads. The immunoprecipitates were then washed four times with PBS and run on a 12% polyacrylamide gel under non-denaturing conditions (36). After transfer to a nitrocellulose membrane, x-ray film was exposed to the filter for 3–4 days.

The specificity of the interaction was examined by the addition to the reaction mix of 100 ng of unlabeled RNA competitor prior to the addition of protein. To verify the concentration of proteins used for the assay, the filters were stained with Coomassie Blue after the UV cross-link analysis.

**Generation of Recombinant Adenoviruses**—An ~1.4-kb fragment of CUGBP1 cDNA was isolated with the use of AflII and HindIII restriction endonucleases from the pOPI3CAT CUGBP expression vector (Stratagene, La Jolla, CA) with an Rous sarcoma virus promoter. Following blunting of its ends, the CUGBP1 cDNA fragment was ligated into the EcoRV site of the cytomegalovirus adenovirus shuttle vector, pAd Track-CMV (37). The pAd-Easy system for production of recombinant adenoviruses was used (Invitrogen). The isolated plasmid DNA was subjected to digestion with the appropriate restriction endonucleases and run on a 0.7% agarose gel to check for the proper orientation of the insert. Colonies containing the correct plasmid were then grown into 500-ml LB cultures, and the pAd Track-CMV/GoI plasmid DNA (containing the CUGBP1 sequence) was isolated by the CsCl gradient ultracentrifugation method (38). The recombinant vector along with the pAd Easy-1 helper vector (which represents the genome of the serotype 5 human adenovirus) were used to electroporate BJ 5183 Escherichia coli cells (39), which produce the deleted adenoviral E1 gene in trans. pAd Easy-1 contains the viral genome along with the adenoviral long terminal repeats that allow the formation, through homologous recombination, of the recombinant adenovirus containing the CUGBP1 gene (37). The viral genome was then linearized with PacI and used to transfect 911 packaging cells (40). The cells were lysed, and the lysate was subjected to CsCl gradient ultracentrifugation (41). The band was loaded on a cassette and dialyzed to remove residual CsCl. A plaque assay was used for titrating the virus (41).

**Adenoviral Infection**—Confluent preadipocytes were washed twice with PBS, and growth medium was replaced with α-minimum Eagle’s medium containing 1% fetal bovine serum. 4 × 10⁹ adenoviral particles were diluted in 100 μl of PBS containing 0.4 mM polyethyleneimine and incubated for 20 min at room temperature. After incubation, the viral polyethyleneimine complex was added to preadipocytes for 18 h. After infection, cells were washed twice with PBS, and growth or differentiation medium was added. Infection efficiency was assessed by determining the percentage of cells expressing green fluorescent protein (GFP) by fluorescence microscopy. CUGBP1 expression was verified by Western blot analysis 48–72 h after infection.

**Short Interference RNA-mediated CUGBP1 Knockdown**—CUGBP1 siRNA (AACCGATCTTGTAGCTATCA) was synthesized (Qiagen, Valencia, CA). CUGBP1 siRNA was labeled with a Cy3 labeling kit (Mirus, Madison, WI). The labeled siRNA was transfected into epididymal rat preadipocytes at 75% confluence with the RNAiFfect transfection reagent (Qiagen, Valencia, CA). As a control, transfection with nonsilencing, fluorescein-labeled siRNA was carried out under the same conditions. After 6 h, the cells were washed with PBS, and α-minimum Eagle’s medium containing 10% fetal bovine serum was added. Differentiation was initiated 12 h after the siRNA transfection for up to 8 days. Medium was changed every 2nd day.

**Statistical Analysis**—Results are presented as means ± 1 S.E. Each n represents cultures from separate groups of animals. Significance was determined by t tests or single factor ANOVA, as appropriate (42, 43). Appropriate post hoc tests were used to determine differences among groups. In transfection experiments, comparisons of numbers of transfectants containing lipid inclusions to mock-transfected cells were made by logistic regression analysis with p values determined from log likelihood ratios. In infection experiments, repeated measures of ANOVA with treatment as the nest factor were used in order to determine whether there are differences in lipid accumulation between cells infected with CUGBP1-expressing or control adenovirus. Variation among subjects, due to effect of treatment and due to random effects of subjects within treatment, as well as variation within each subject, was tested. p < 0.05 was considered significant.
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CUGBP1 Protein Abundance Increases Progressively with Aging—Significantly higher CUGBP1 protein abundance was found in epididymal preadipocytes isolated from middle-aged than young rats (Fig. 1; n = 6; p < 0.01; ANOVA and Duncan’s multiple range test). CUGBP1 levels were higher again in old than middle-aged animals (17 versus 30 months; p < 0.01; Duncan’s multiple range test). CUGBP1 mRNA levels changed in a similar way with aging (27 ± 3 arbitrary units by real time PCR in young (mean ± 1 S.E.) and 44 ± 3 in old animals; p < 0.01; n = 4; t test). Thus, CUGBP1 protein abundance increases throughout the life span and involves increasing transcript abundance.

CUGBP1 Nucleotide Binding Activity Increases with Aging—CUGBP1 activity was determined by measuring binding to (CUG)₈ triplets in an UV cross-link immunoprecipitation assay. Cytosolic extracts of preadipocytes isolated from old rats exhibited higher binding activity than extracts from young rat preadipocytes (Fig. 2; p < 0.005; n = 7; t test). CUGBP1 activity was absent in nuclear extracts from the same cells (data not shown). Thus, increased abundance of CUGBP1 protein with aging is associated with increased binding activity. The activity is present in the cytosolic fraction of the preadipocyte, where the translational machinery is located. Activity of the cytosolic isoform is enhanced when hyperphosphorylated by as yet unknown kinases (18). Indeed, CUGBP1 phosphorylation appears to be required for LIP isoform production (44). Thus, the greater increase in CUGBP1 activity than protein levels with aging (depicted in Figs. 1 and 2) may be due to its regulation via phosphorylation.

CUGBP1 Overexpression Decreases the Ability of Preadipocytes to Accumulate Lipid—When perirenal preadipocytes from young rats, which are capable of extensive lipid accumulation (12), were infected with adenovirus expressing the CUGBP1 gene (Fig. 3A), they exhibited decreased capacity for lipid accumulation and C/EBPα expression compared with controls infected with a GFP-only expressing adenovirus. Seven days after infection, the extent of lipid accumulation was assessed by light microscopy in individual infected cells identified by fluorescence microscopy by observers who were not aware of which treatment the cells had received (Fig. 3A). Fewer preadipocytes transduced with CUGBP1 could accumulate lipid extensively compared with cells treated with the control virus (Fig. 3B; p < 0.0001; n = 4; 300 cells/experiment; repeated measurements ANOVA with treatment as the nest factor). Lipid accumulation also decreased in 3T3-L1 preadipocytes infected with the CUGBP1 adenovirus (data not shown). The C/EBPβ-LIP isoform was increased in preadipocytes infected with the Ad-GFP/CUGBP1 adenovirus and exposed to differentiation medium for 4 h compared with controls infected with Ad-GFP (Fig. 4; p < 0.01; n = 3; t test; C/EBPβ-LIP/LAP ratios were 1.22 ± 0.05 in the Ad-GFP/CUGBP1 adenovirus-expressing cells and 0.79 ± 0.04 in the control cells). The Ad-GFP/CUGBP1 adenovirus had little effect on C/EBPβ-LAP abundance. The 14-kDa C/EBPβ isoform associated with proteolytic degradation of C/EBPβ (45) could not be detected in the Ad-GFP/CUGBP1 adenovirus-expressing cells.

CUGBP1 Inhibition Decreases C/EBPβ-LIP in Preadipocytes from Old Animals—To test if reducing CUGBP1 expression reduces abundance of the C/EBPβ-LIP isoform, 12 h before induction of differentiation, epididymal preadipocytes from old animals were transfected with a CUGBP1 siRNA construct that reduced CUGBP1 to 30% that in cells transfected with a scrambled nucleotide control construct (Fig. 5). Six hours following induction of differentiation, the CUGBP1 siRNA treatment had reduced C/EBPβ-LIP to 32 ± 8% that in cells transfected with the control construct (p < 0.05; n = 3; t test). C/EBPβ-LAP abundance in the CUGBP1 siRNA-treated cells was 95 ± 3% that in control cells (C/EBPβ-LIP/LAP ratios were 0.49 ± 0.09 in the CUGBP1 siRNA-treated cells and 1.47 ± 0.08 in the control.
cells; \( p < 0.005; t \) test; note that the cells in this experiment were from old animals, although those in the above overexpression experiment were from young animals, potentially explaining the higher \( C/EBP \beta\)-LIP/LAP ratios in control cells in this experiment). Thus, CUGBP1 siRNA treatment resulted in reduced \( C/EBP \beta\)-LIP upon induction of differentiation.

CUGBP1 Inhibition Enhances Primary Preadipocyte Lipid Accumulation—Epididymal preadipocytes from 30-month-old rats, which are resistant to adipogenesis, were transfected with the CUGBP1 siRNA or control, nonsilencing scrambled oligonucleotides. More preadipocytes transfected with the CUGBP1 siRNA were able to accumulate lipid extensively following treatment with differentiation-inducing medium than control cells (Fig. 6; \( p < 0.0005; n = 4; 400 \) cells/experiment; repeated measurements ANOVA with treatment as the nest factor). Consistent with previous findings that lipid accumulation by
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differentiating preadipocytes decreases with aging, control epididymal preadipocytes from 3-month-old rats accumulated lipid to a greater extent than control cells from the old animals \((p < 0.0001; n = 4\) young and 4 old rats). Treatment of preadipocytes from the young rats with CUGBP1 siRNA also enhanced lipid accumulation \((p < 0.0001; n = 4; 300\) cells/experiment). The relative increase in lipid accumulation following CUGBP1 siRNA exposure was greater in the cells from the old animals (age-CUGBP1 siRNA interaction \(p = 0.005\)) and approached that of the control cells from young animals.

**TNFα Increases Preadipocyte CUGBP1**—Preadipocytes were exposed to TNFα for 24 h to test if the chronically elevated levels of TNFα in fat tissue of old animals (23) might contribute to increased CUGBP1. TNFα treatment for 24 h increased CUGBP1 protein in epididymal preadipocytes isolated from young rats in a dose-dependent manner (Fig. 7A; \(p < 0.05\) that 1 nm TNFα increased CUGBP1, Fig. 7B, \(n = 5\); t test). Lipopolysaccharide \((10\) mg/ml) also increased preadipocyte CUGBP1 protein abundance (data not shown), as has been shown in cultured hepatocytes (22). TNFα treatment increased preadipocyte cytosolic binding activity to \((CUG)_2\) oligomer repeats (Fig. 7C).

Epididymal preadipocytes from 3-month-old rats were treated with 500 pm TNFα for 24 h and then differentiation medium for 4 h. C/EBPβ-LIP protein was higher in lysates from TNFα-treated than control preadipocytes \((p < 0.0005; n = 4, t\) test), although C/EBPβ-LAP was similar in the treated and control cultures (Fig. 7D; C/EBPβ-LIP/LAP ratios were 0.83 ± 0.11 in the cells treated with TNFα and 0.45 ± 0.03 in the control cells), indicating that the effect of TNFα on C/EBPβ-LIP is specific. The 14-kDa C/EBPβ proteolytic degradation product (45) was not detected in cells treated with TNFα despite use of antibody that binds to this isoform. Thus, TNFα increases preadipocyte CUGBP1 abundance and activity.

LIP forms heterodimers with C/EBPβ-LAP, C/EBPβ, and C/EBPα, the adipogenic potential of these regulators is abrogated (49–52). C/EBPβ-LIP homodimers also block adipogenesis, because C/EBPβ-LIP DNA binding affinity is higher than that of C/EBPβ-LAP (49). C/EBPβ-LIP can be generated through alternative translation of C/EBPβ mRNA (mediated by CUGBP1 (19, 22)) or through degradation of larger C/EBPβ isoforms by caspases (45). We previously found that abundance of the 14-kDa major caspase degradation product of C/EBPβ was not increased as C/EBPβ-LIP increased with aging (13), implying that protein degradation is unlikely to be the main cause of increased fat tissue C/EBPβ-LIP with aging. Furthermore, despite constant C/EBPβ mRNA levels, C/EBPβ-LAP did not decrease with aging, as would be expected if proteolysis of C/EBPβ translational products principally accounted for increased C/EBPβ-LIP. In this study, no increase in the 14-kDa isoform could be detected in preadipocytes infected with the CUGBP-expressing adenovirus or after TNFα treatment, conditions that caused an increase in the 19-kDa C/EBPβ-LIP. Studies using cycloheximide in breast epithelial cells have indicated that increases in C/EBPβ-LIP associated with increased CUGBP1 activity occur independently from changes in protein turnover (44). Thus, it appears unlikely that increases in C/EBPβ-LIP due to aging, TNFα exposure, or enhanced CUGBP1 expression result from C/EBPβ proteolysis, although there remains a possibility that selective changes in C/EBPβ isoform degradation could contribute.

A cancer treatment drug, oltipraz, increases C/EBPβ-LIP in hepatocyte-derived cells (53). In 3T3-L1 cells, oltipraz increased cytoplasmic localization and binding activity of CUGBP1 (54) and was associated with increased C/EBPβ-LIP and reduced differentiation. Oltipraz also increased C/EBPβ-LIP and reduced differentiation of rat epididymal preadipo-

**DISCUSSION**

We demonstrated that CUGBP1 is implicated in differentiation of primary preadipocytes through enhancing C/EBPβ-LIP expression. CUGBP1 overexpression in undifferentiated preadipocytes caused increased C/EBPβ-LIP and reduction in lipid accumulation and C/EBPα expression. Increased CUGBP1 abundance and activity with aging predisposed preadipocytes to resist adipogenesis. CUGBP1 RNA interference decreased C/EBPβ-LIP and promoted lipid accumulation, even in preadipocytes cultured from old animals. TNFα, which increases in fat tissue with aging, enhanced CUGBP1 expression and activity.

C/EBPβ-LAP homodimers and C/EBPβ-LAP/C/EBPβ heterodimers promote PPARγ and C/EBPα expression, driving adipogenesis (14, 46–48). However, when C/EBPβ-LIP forms heterodimers with C/EBPβ-LAP, C/EBPβ, and C/EBPα, the adipogenic potential of these regulators is abrogated (49–52). C/EBPβ-LIP homodimers also block adipogenesis, because C/EBPβ-LIP DNA binding affinity is higher than that of C/EBPβ-LAP (49). C/EBPβ-LIP can be generated through alternative translation of C/EBPβ mRNA (mediated by CUGBP1 (19, 22)) or through degradation of larger C/EBPβ isoforms by caspases (45). We previously found that abundance of the 14-kDa major caspase degradation product of C/EBPβ was not increased as C/EBPβ-LIP increased with aging (13), implying that protein degradation is unlikely to be the main cause of increased fat tissue C/EBPβ-LIP with aging. Furthermore, despite constant C/EBPβ mRNA levels, C/EBPβ-LAP did not decrease with aging, as would be expected if proteolysis of C/EBPβ translational products principally accounted for increased C/EBPβ-LIP. In this study, no increase in the 14-kDa isoform could be detected in preadipocytes infected with the CUGBP-expressing adenovirus or after TNFα treatment, conditions that caused an increase in the 19-kDa C/EBPβ-LIP. Studies using cycloheximide in breast epithelial cells have indicated that increases in C/EBPβ-LIP associated with increased CUGBP1 activity occur independently from changes in protein turnover (44). Thus, it appears unlikely that increases in C/EBPβ-LIP due to aging, TNFα exposure, or enhanced CUGBP1 expression result from C/EBPβ proteolysis, although there remains a possibility that selective changes in C/EBPβ isoform degradation could contribute.

A cancer treatment drug, oltipraz, increases C/EBPβ-LIP in hepatocyte-derived cells (53). In 3T3-L1 cells, oltipraz increased cytoplasmic localization and binding activity of CUGBP1 (54) and was associated with increased C/EBPβ-LIP and reduced differentiation. Oltipraz also increased C/EBPβ-LIP and reduced differentiation of rat epididymal preadipo-

**FIGURE 6. Lipid accumulation is enhanced by inhibiting CUGBP1 in preadipocytes from old animals.** A, epididymal preadipocytes from 30-month-old rats were transfected with Cy3-labeled CUGBP1 siRNA, a fluorescent-labeled nonsilencing, scrambled oligonucleotide control, or were not transfected. Phase contrast (left) and fluorescence photomicrographs representative of four experiments are shown. B, higher proportions of epididymal preadipocytes from young (3M) rats accumulated lipid extensively than from old (30M) animals. Lipid accumulation in preadipocytes from both 3- and 30-month-old rats transfected with CUGBP1 siRNA (solid bars) was more extensive than in cells transfected with the control construct (open bars). \(n = 4\) separate experiments.
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FIGURE 7. TNFα increases CUGBP1 abundance and activity. A, TNFα increases CUGBP1 protein abundance in a dose-dependent fashion in lysates of epididymal preadipocytes isolated from young rats. B, epididymal preadipocytes from young rats were exposed to 1 nM TNFα for 24 h. A representative Western immunoblot analysis is shown in the top panel, and mean optical densities in arbitrary units are shown in the bottom panel (n = 5). C, TNFα increases CUGBP1 binding activity. UV cross-link immunoprecipitation analysis showed increased CUGBP1 binding activity to (CUG)₈ oligomer repeats in cytosolic extracts of young rat epididymal preadipocytes treated with 1 nM TNFα for 24 h compared with control cells. Coomassie Blue staining is shown to the right. D, TNFα increases C/EBPβ-LIP abundance in epididymal preadipocytes from young rats. Representative Western immunoblots of lysates of cells treated with 500 pM TNFα for 4 h then differentiation medium for 24 h are shown in the top panel and mean optical densities (arbitrary units; n = 4) in the bottom panel.

cytos. These findings imply an association between increased CUGBP1 activity and impaired differentiation. In this study, we demonstrated that increasing CUGBP1 causes decreased adipogenesis, and reducing CUGBP1 enhances adipogenesis in primary preadipocytes. In preliminary studies, we found this also to be true in human preadipocytes (data not shown). Thus, CUGBP1 activity and adipogenesis appear to be causally linked. In addition to adipogenesis, CUGBP1 is involved in muscle (55) and cardiac (56) development. Because TNFα, lipopolysaccharide, epidermal growth factor (44), and oltipraz (54) increase CUGBP1 abundance or activity in 3T3-L1 cells, euploid preadipocytes, or other cell types, CUGBP1 activity is subject to regulation, offering a potential avenue for influencing fat tissue development and function.

We found that CUGBP1 overexpression increased C/EBPβ-LIP, inhibited C/EBPα, and decreased lipid accumulation in differentiating primary preadipocytes. In 3T3-L1 cells, others reported that CUGBP1 overexpression did not increase C/EBPβ-LIP (54). In primary preadipocytes, after considerable trial and error we found that CUGBP1 had to be expressed or knocked down shortly before initiation of adipogenesis, which is followed by a brief peak in C/EBPβ mRNA levels, to demonstrate effects. In addition to protein abundance, a number of other factors likely influence CUGBP1 activity, including phosphorylation. CUGBP1 affects processing or translation of other transcripts, including p21 and the insulin receptor, which are involved in regulating adipogenesis (57, 58). Calreticulin and possibly other proteins interact with CUGBP1 or its targets, affecting its activity (57). Thus, the extent and timing of increases or decreases in CUGBP1 abundance and activity, together with cellular context, would be anticipated to modulate its effects on adipogenesis, which likely occur through multiple pathways.

Despite the fact that perirenal preadipocytes isolated from young rats differentiate readily to a great extent, among the cells that were infected with the CUGBP1-expressing adenovirus, few showed signs of lipid accumulation and even fewer accumulated large lipid droplets compared with cells treated with the control adenovirus. Although CUGBP1 overexpression significantly reduced the capacity of these cells to accumulate lipid, this was not completely abolished. One reason might be that the measurements of lipid accumulation were made 15 days after infection, when levels of the protein expressed by adenoviruses are decreased, potentially allowing some cells to accumulate lipid. Another reason is that, as reflected in Figs. 3B and 6B, considerable variation in capacity for adipogenesis occurs among cells in both primary and 3T3-L1 clonal preadipocyte populations for as yet unknown reasons (59, 60). Other pathways are involved in regulating adipogenesis that act independently from the pathway we investigated. For example ADD-1/SREBP-1 acts in a C/EBPβ- and C/EBPα-independent manner by binding to the PPARγ promoter, inducing its expression, or by regulating pathways that in turn regulate production of PPARγ ligands (61, 62). Such mechanisms could account, at least in part, for the ability of some of the CUGBP1-expressing preadipocytes to overcome its inhibition of adipogenesis.

We reported previously that C/EBPα and PPARγ are decreased and C/EBPβ-LIP is increased in cultured preadipocytes and freshly isolated whole fat tissue from old compared with young rats (13). In this study, we found increased CUGBP1 abundance and activity in undifferentiated preadipocytes with aging. It appears that, by favoring the production of C/EBPβ-LIP instead of C/EBPβ-LAP, increased CUGBP1 with aging impedes preadipocyte differentiation. The preadipocytes from animals of different ages had been maintained in culture under
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identical conditions in parallel, reducing effects of such potential in vivo differences as hormones, food intake, and activity among age groups. Cells were isolated using conditions designed to exclude other cell types, such as macrophages, from the cultures. Thus, increased CUGBP1 and its contribution to impaired adipogenesis may reflect effects of inherent aging processes that predispose undifferentiated preadipocytes from old animals to resist adipogenesis.

We found that CUGBP1 is up-regulated by TNFα, a cytokine that activates cellular stress responses. Furthermore, TNFα has been shown to induce LIP expression (44). Thus, it appears the CUGBP1-C/EBPβ-LIP pathway is at least one mechanism through which cytokines can influence the capacity for initiation of adipogenesis, in addition to effects of TNFα on insulin signaling and other pathways. Together with the observations that lipopolysaccharide up-regulates liver CUGBP1 activity (22) and that preadipocyte CUGBP1 increases with aging, increased CUGBP1 in response to TNFα is consistent with a link between activation of cellular stress responses and increased CUGBP1 activity. Fat tissue TNFα increases with aging (23). TNFα is produced by macrophages (63) and possibly fat cells (64) or other cell types in fat tissue. Thus, the preadipocytes resident in the fat tissue of aging animals may be subject not only to increased CUGBP1 activity because of inherent, age-related processes within preadipocytes but also because of high local TNFα concentrations. The possible pathways involved in effects of TNFα on CUGBP1 expression remain to be defined (e.g. NF-κB).

Other mechanisms likely contribute to reduced adipogenesis with aging. CHOP regulates C/EBPβ nuclear translocation and, like C/EBPβ-LIP, can form heterodimers with other C/EBP family members, reducing their adipogenic potential (65). We have noted that expression of CHOP increases with aging (3). We found that C/EBPβ protein abundance decreases with aging (13). C/EBPβ, as a heterodimer with C/EBPβ, is particularly effective in initiating adipogenesis (14). Furthermore, TNFα, which increases in adipose tissue with aging, impairs adipogenesis through multiple pathways in addition to enhancing CUGBP1 expression (29, 66–68). Thus, redundant mechanisms may contribute to decreased capacity for initiation of adipogenesis with aging.

Fat depot size increases through middle age in rodents and humans before decreasing in old age (3). Decreased preadipocyte differentiation capacity may serve to limit even greater increases in fat mass up to middle age that might occur otherwise. In advanced old age, this potentially protective mechanism might ultimately contribute to dysfunction, particularly because fat cell turnover is relatively slow, with preadipocytes in young animals exhibiting qualities that do not become apparent in fat cells until later in life (3). Adipose tissue, with its potentially high concentrations of cytotoxic free fatty acids, may be a particularly harsh microenvironment. Coupled with high numbers of macrophages and pro-inflammatory cytokines, this environment could inflict damage to preadipocytes. Increasing numbers of damaged cells over time with cellular stress response activation may underlie increases in CUGBP1 activity and C/EBPβ-LIP abundance, contributing to impaired adipogenesis. Thus, decreasing adipogenesis with aging may protect the organism from development of dysfunctional fat tissue arising from defective progenitors. After middle age or early old age, reduced adipogenesis may contribute to decreased fat depot size and thus to deposition of lipids outside fat tissue, leading to dysfunction in other tissues through lipotoxicity (69). Decreasing adipogenesis with aging may therefore be an example of antagonistic pleiotropy: adaptive responses that are advantageous in younger individuals that have an adverse impact in advanced old age (70).

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