Inhibition of cAMP-response Element-binding Protein Activity Decreases Protein Kinase B/Akt Expression in 3T3-L1 Adipocytes and Induces Apoptosis*

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White adipose tissue mass is governed by competing processes that control lipid synthesis and storage, the development of new adipocytes, and their survival. We have shown that the transcription factor cAMP-response element-binding protein (CREB) participates in adipogenesis, with constitutively active forms of CREB inducing adipocyte differentiation and dominant negative forms of CREB blocking this process. In other cell types, CREB and related factors have been shown to play important roles in survival and apoptosis. Here we demonstrate that reduction of CREB activity by ectopic expression of the dominant negative CREB, KCREB, induces apoptosis of mature 3T3-L1 adipocytes in culture. Death by apoptosis was confirmed by increased nuclear condensation, changes in membrane morphology, and increased DNA fragmentation. Gene microarray analysis indicated that KCREB expression increased expression of several pro-apoptotic genes such as Interleukin Converting Enzyme and decreased the expression of the anti-apoptotic signaling molecule, Akt/protein kinase B. Finally, introduction of constitutively active CREB, CREB-DIEOML, blocked death of mature adipocytes treated with TNF-α. The data indicate that CREB plays a central role in adipocyte survival, perhaps by regulating the expression of certain pro- and anti-apoptotic genes. These results not only extend the role of CREB in adipocyte biology but also highlight the general developmental and survival role of this factor in numerous cell and tissue types.

In addition to its primary role in energy storage, white adipose tissue plays significant roles in overall energy homeostasis and metabolic regulation and, in part, regulates satiety and insulin sensitivity (1). Diseases or dysfunction of white adipose tissue are observed with increasing frequency in clinical situations. For example, overweight and obesity and related conditions, including diabetes and cardiovascular disease, are reaching epidemic proportions worldwide (2–4). Loss or redistribution of adipose tissue associated with acquired or congenital lipodystrophies, or due to aggressive antiretroviral therapy or subcutaneous insulin injection, also constitutes a growing health concern (5–8). Although these syndromes reflect the action of numerous interacting processes, they all are characterized by changes in adipose tissue mass.

Adipose tissue mass is governed in part through competing processes that either increase or decrease the size, number, and “maturity” of fat cells (9). Adipocyte size increases via increased storage of triacylglycerol from dietary sources or generated by lipogenic pathways; new fat cells may arise via the proliferation and differentiation of pre-adipocytes or adipoblasts to mature adipocytes (10). Alternately, decreases in adipose tissue mass generally involve the loss of stored lipids by lipolytic processes. There is now evidence that decreases in adipose tissue mass may also involve the loss of mature fat cells through programmed cell death or apoptosis in certain situations (8, 11–17).

In culture, adipocytes from rodents or cell lines like 3T3-L1 undergo apoptosis upon exposure to TNF-α or HIV protease inhibitors (8, 17). Ectopic overexpression of constitutively active MKK6, an activator of p38 MAPK, also induces apoptosis and necrosis of 3T3-L1 adipocytes (11). Other studies in rodent models have demonstrated a decrease in adipose tissue DNA content due to loss of adipocytes in response to starvation or streptozotocin-induced diabetes (14). More recently, intracerebroventricular administration of leptin was also shown to decrease fat pad weight and DNA content (16). Apoptotic features, including DNA laddering and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) staining accompanied these changes. Apoptosis of adipose tissue cells has also been observed in human adipose tissue explants subjected to growth factor deprivation, elevated temperature, or TNF-α exposure as determined by changes in cell morphology and DNA laddering (12, 15). Similar results were reported for adipose tissue explants retrieved from cancer patients (13).

Previously, we have shown that the transcription factor CREB is a target for extracellular agents and intracellular signaling systems that induce adipogenesis (18, 19). Ectopic expression of a constitutively active, chimeric VP16-CREB protein was sufficient to induce adipogenesis, whereas expression...
CREB, pIND-CREB-DIEML, or pIND-LacZ plasmids were selected in zeocin, and cells stably transfected with pIND-KCREB, pIND-VP16–FCS, 1 mM glutamine, and 500 

Differentiation was initiated by addition of medium containing 10% Bt2cAMP, 1 mM glutamine, and 500 

Gels were solved on 10% polyacrylamide-SDS gels, and transferred to nitrocellulose blots were blocked with phosphate-buffered saline containing 5% dry milk and 0.1% Tween 20 and then treated with antibodies that recognize Akt, CREB, or VP16. The blots were washed and subsequently treated with goat anti-rabbit IgG conjugated to alkaline phosphatase. After the blots were washed, specific immune complexes for each target protein were visualized with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetraazolium.

**Analysis of Nuclear Condensation**—Nuclear condensation in apoptotic cells was determined by microscopic observation of cells stained with Hoechst 33342. Control cells (LacZ expressing), CREB-expressing cells, or cells treated with TNF-α were washed with PBS and fixed in PBS containing 3.7% formaldehyde for 10 min. The cells were washed in PBS and stained in PBS containing 5 μg/ml Hoechst 33342 for 15 min at ambient temperature. The monolayer was washed three times with PBS and observed by fluorescence microscopy (fluorescein isothiocyanate filter set). Images were captured on Kodak Royal Gold 200 film and scanned into a Macintosh G4 computer using an Agfa T1200 scanner.

**Flow Cytometry**—Essentially, 3T3-L1 cells were lifted from culture containers by gentle trypsinization and washed twice in PBS. The cells were resuspended in a buffer containing 100 mM Tris-HCl, pH 7.6, and 1 mM EDTA at 4°C. Immediately, an equal volume of 100% ethanol was added dropwise while vortexing, and the cells were fixed for 3 h at 4°C. After fixation, an additional volume of 100 mM Tris-HCl, pH 7.6, containing 0.1% Nonidet P-40, 20 units/ml RNase A, and 75 μg propidium iodide was added while vortexing, and the suspension was placed in a refrigerator overnight. The following day the suspensions were subjected to flow cytometry on a Becton Dickinson FACScan linked to a Macintosh G3 computer running CellQuest software.

**Atlas cDNA Array Analysis**—Total RNA was extracted from cells using the Atlas Pure Total RNA Labeling kit from CLONTECH (Palo Alto, CA). Single-strand cDNA probes were generated from total RNA using [32P]dATP. These probes were used for hybridization with separate Mouse cDNA array membranes using protocols and reagents provided by the manufacturer (CLONTECH). Arrays were subjected to autoradiography at –80°C using Kodak Lightning Plus screens. Scanned arrays were analyzed using Atlas Image software (CLONTECH), comparing relative intensities of specific cDNA “spots,” which were corrected for differences in the relative intensities of housekeeping genes between membranes prior to analysis. Three separate Array analyses were performed with probes generated from RNA resulting from two different experiments. Results for cDNAs depicted below are the mean of these three separate determinations. Data is presented as mRNA content in CREB-expressing cells relative to mRNA content in LacZ-expressing cells.

**RESULTS**

We have reported that CREB participates in the induction of adipogenesis. As part of the studies, we noted that CREB phosphorylation and activity were stimulated by conventional differentiation-inducing agents in an acute fashion, but were also transiently elevated at later times during adipogenesis. This suggested that CREB might also play a role in later stages of the differentiation process. In addition, our results in adipocyte cell lines parallel similar results in neurons wherein CREB not only participates in differentiation but also acts as a survival factor preventing apoptosis in the absence of neurotrophins. These factors led us to hypothesize that CREB may also play roles in the later stages of adipocyte differentiation or in maintaining the mature adipocyte phenotype.

As an initial test of this hypothesis, we blocked the activity of endogenous CREB in mature adipocytes through the ectopic expression of the dominant negative CREB protein, CREB–(27). CREB inhibits the action of endogenous CREB by forming heterodimers with the endogenous protein that are incapable of binding target DNA sequences. CREB–differs from CREB by the amino acid in the DNA binding domain and antibodies capable of differentiating between CREB and CREB–do not exist. However, using antibodies that recognize both proteins we were able to detect Ponasterone A-induced CREB expression in 3T3-L1 adipocytes by Western blot (Fig. 1A). Inhibition of endogenous CREB activity by CREB–was verified by demonstrating that CREB expression blocked
condensation by Hoechst staining. As shown in Fig. 2a, pre-adipocytes were transfected with an Edysone-inducible KCREB expression system as described under “Experimental Procedures.” The cells were then propagated to confluence, and adipogenesis was induced with Bt2cAMP, insulin, and dexamethasone. A, inducible expression of KCREB protein was verified by preparing nuclear extracts from mature adipocytes (day 10 in post-induction) treated with 1.0 μM Ponasterone A (Pon. A) for 24 h or untreated, control cells. 25 μg of extract protein from each extract was resolved on 10% polyacrylamide-SDS gels and transferred to nitrocellulose. After the blots were blocked, they were incubated with antibody to total CREB, which recognizes both CREB and KCREB. The panel shows a representative Western blot of induced KCREB expression. B, mature adipocytes, stably transfected with the inducible KCREB expression system, were transfected with a plasmid containing the CRE-containing somatostatin gene promoter linked to a luciferase reporter gene using Superfect amide-SDS gels and transferred to nitrocellulose. After the blots were blocked, they were incubated with antibody to total CREB, which recognizes both CREB and KCREB. The panel shows a representative Western blot of induced KCREB expression. B, mature adipocytes, stably transfected with the inducible KCREB expression system, were transfected with a plasmid containing the CRE-containing somatostatin gene promoter linked to a luciferase reporter gene using Superfect reagent. As indicated, KCREB expression was induced with 10 μM Ponasterone A (KCREB + lanes) overnight. The following day, the cells were treated with 0.3 mM Bt2cAMP for 4 h as indicated. Luciferase expression was measured in lysates as an index of transcriptional activity, and levels are shown relative to luciferase activity in cells not treated with Ponasterone A or Bt2cAMP. Levels were corrected for transfection efficiency by co-transfecting cells with a plasmid containing the Rous sarcoma virus (RSV) long terminal repeat linked to a β-galactosidase reporter. The data shown are averaged from three separate assays. C, mature adipocytes were treated with 10 μM Ponasterone A on day 0 to induce KCREB expression. The cells were refed every 3 days with complete medium containing 10 μM Ponasterone A for 9 days. Duplicate wells of cells were stained with Oil Red O and counterstained with hematoxylin on days 0 and 9. The panels show representative photomicrographs of cells on days 0 and 9 of treatment. Another hallmark of apoptotic cell death is DNA fragmentation. This parameter was measured by subjecting KCREB-expressing adipocytes to flow cytometric analysis after staining with propidium iodide. Apoptotic cells are evident on the resulting histograms as a “sub-G1/G0” peak indicative of DNA fragmentation and reduced DNA content. The number of cells in the sub-G1/G0 peak increased from day 0 through day 3 and then declined (Fig. 3); consistent with the temporal changes in nuclear condensation shown in Fig. 2A. We also noted an increase in the number of cells in the S and G2/M regions of the histograms on days 4–6. No lipid vesicles were evident during microscopic examination of cells in the S and G2/M regions (data not shown). Given the uniform expression of KCREB in the cell population (18), we speculate that these cells represent undifferentiated cells that survive KCREB expression and proliferate during and after the death of mature adipocytes. It is also possible that these cells express little of no KCREB upon
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**Fig. 3.** KCREB expression induces adipocyte apoptosis as determined by flow cytometric analysis. Mature adipocytes stably transfected with the Ecdysone-inducible KCREB expression system were treated with 10 μM Ponasterone A to induce KCREB expression on day 0. The cells were re-fed on day 2 with complete medium containing 10 μM Ponasterone A. Each day, cells grown in duplicate wells were gently trypsinized, fixed, and stained with propidium iodide as described under “Experimental Procedures.” Cell suspensions were subjected to flow cytometric analysis on a Becton Dickinson FACScan linked to a Macintosh G3 computer, and data were analyzed with CellQuest software. Each panel shows a representative histogram of fluorescence intensity (DNA content) versus cell count. The positions of the G0/G1, S, and G2/M regions are indicated in the day 0 panel, and the apoptotic sub-G0/G1 region is stippled in each panel.

exposure to Ponasterone A and, therefore, do not undergo apoptotic cell death. The specific nature of these cells is currently being investigated in our laboratory.

Given CREB’s primary function in regulating gene expression, we examined the levels of expression of several apoptotic genes in untreated adipocytes, or adipocytes inducibly expressing KCREB for 24 h. For these assays, mouse Atlas 1.2 gene arrays (CLONTECH) were employed. We found that the expression of several pro-apoptotic genes were elevated in KCREB-expressing cells, most notably Receptor Interacting Protein (RIP), and Interleukin Converting Enzyme (ICE, or caspase-1) (Fig. 4A). At the same time, expression of the anti-apoptotic signaling enzyme, protein kinase B/Akt (Fig. 4, Akt) was dramatically diminished by KCREB. Down-regulation of Akt and up-regulation of RIP and ICE by KCREB in adipocytes was confirmed by Western blot analysis (Fig. 4B). The Atlas gene arrays contain several other apoptosis-related genes, including caspases 2, 7, and 11, bcl-2, and BAX. However, either no change in the expression of these genes was observed, or no detectable signal was present on the autoradiograms even after prolonged exposure. Although a number of factors participate in apoptotic cell death, we have examined the contribution of Akt to adipocyte survival in preliminary experiments. We found that ectopic expression of dominant negative Akt (Akt(K179M)) or treatment of cells with the PI3K inhibitor, LY294002, resulted in apoptosis of adipocytes as determined by FACS analysis (Fig. 5). The percentage of apoptotic cells with either of these two treatments reached ~30% to 50% within 48 h, whereas no apoptotic cells were detected in populations expressing an LacZ protein. These preliminary data suggest that one mechanism by which inhibition of CREB induces apoptosis is through decreased Akt signaling.

The ability of KCREB to induce adipocyte apoptosis suggested that constitutively active forms of CREB might block programmed cell death. To test this concept, the Ecdysone-inducible expression system was employed to express the active CREB mutant, CREB-DIEDML, which stimulates transcription and associates with the transcriptional co-adaptor, CREB-binding protein (CBP) p300, in a phosphorylation-independent manner (28). First, we verified that the protein was expressed in response to Ponasterone A treatment by Western blot analysis (Fig. 6A). Simultaneously, we assessed the ability of CREB-DIEDML expression to drive transcription (luciferase production) from the CRE-containing, somatostatin gene promoter. We found that low doses of Ponasterone A (0.1 μM) and
DIEDML expression as indicated. The following day, TNF-α was added to the indicated levels of Ponasterone A to induce adipocyte apoptosis by preparing nuclear extracts from mature adipocytes (day 10 post-induction) treated with the indicated levels of Ponasterone A.

Verification of CREB content and phosphorylation, and a progressive decrease in transcription levels with high CREB-DIEDML expression/activity, we tested the ability of this factor to block adipocyte apoptosis induced by TNF-α. As shown in Fig. 6C, TNF-α produced a substantial increase in the number of control cells (ectopically expressing LacZ) with condensed nuclei as determined by Hoechst staining. However, in cells expressing CREB-DIEDML, fewer condensed nuclei were evident. These cell populations were subjected to FACS analysis to quantitate the number of apoptotic cells. No apoptotic nuclei were evident in untreated control cells (Fig. 6D). The percentage of apoptotic cells in the LacZ-expressing, TNF-α-treated populations averaged 30%, whereas the percentage for CREB-DIEDML-expressing, TNF-α-treated cells averaged only 5%.

**DISCUSSION**

In this paper we have shown that decreases in CREB activity generated by inducible, ectopic expression of KCREB and other dominant negative “CREBs” stimulate apoptosis of mature 3T3-L1 adipocytes in culture. Apoptosis was confirmed by chromatin condensation (Hoechst staining), changes in membrane morphology (retraction and blebbing), and cellular DNA degradation (subdiploid or sub-G0/G1 cells in FACS analysis). The ability of KCREB to induce adipocyte apoptosis appears to be due, in part, to the increased expression of certain pro-apoptotic genes, including RIP and ICE, and the decreased expression of the anti-apoptotic signaling enzyme, Akt. Although changes in the expression of most of these factors await confirmation, the decreased expression of Akt in response to KCREB was also noted by Western blot analysis. Finally, we demonstrated that ectopic expression of constitutively active forms of CREB partially inhibits the induction of adipocyte apoptosis in response to TNF-α exposure.

Other groups have shown that insulin and IGF-1 inhibit apoptosis of adipocytes and pre-adipocytes in response to TNF-α or serum deprivation (17, 29, 30). We have shown that these hormones stimulate CREB phosphorylation and transcriptional activity in several cell types, including 3T3-L1 pre-adipocytes and adipocytes through the activation of ERK and PI3K signaling systems and through the inhibition of protein phosphatase 2A (24, 25, 31, 32). Our current data implicate CREB as an important nuclear effector of the protective effects of insulin and IGF-1.

The ability of KCREB to initiate programmed cell death in adipocytes is consistent with the role of CREB in survival/apoptosis of other cells types, its regulation of survival/apoptotic genes, and its regulation by survival-associated growth factors. This is especially true of neurons. Walton et al. (33) have shown that CREB overexpression and prolonged phosphorylation protects neurons from okadaic acid-induced apoptosis, and Ginty and colleagues (26) have shown that CREB overexpression and prolonged phosphorylation protects neurons from okadaic acid-induced apoptosis. Finally, we demonstrated that KCREB partially inhibits the induction of adipocyte apoptosis in response to TNF-α exposure.
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constitutively active and dominant negative CREBs to modulate adipose tissue mass and insulin responsiveness are currently underway in our laboratories. Our data indicate that CREB plays a central role in adipocyte survival, perhaps by regulating the expression of anti-apoptotic genes such as Akt. Our results not only extend the role of CREB in adipocyte biology but also highlight the general developmental and survival role of this factor in numerous cell and tissue types.

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