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Alteration by 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin of CCAAT/Enhancer Binding Protein Correlates with Suppression of Adipocyte Differentiation in 3T3-L1 Cells

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SUMMARY

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and related compounds elicit multiple effects on the function of adipose tissue and adipogenic cell lines, including the suppression of adipocyte differentiation. We began to examine the mechanism by which TCDD inhibits differentiation of the established preadipocyte cell line 3T3-L1. Examination of the expression of several early marker genes of preadipocyte differentiation through Northern blot analysis and of differentiation-dependent mitosis showed that TCDD did not interfere with the earliest known responses of preadipocytes to inducers of differentiation. Analysis of mRNA for three isoforms of the CCAAT/enhancer binding protein (C/EBP) revealed that TCDD (5 nM) selectively inhibited the induction of C/EBPα mRNA but did not block the induction of C/EBPβ and C/EBPδ in response to differentiation inducers. The differentiation-dependent induction of PPARγ mRNA was also blocked by TCDD. Immunoblot analysis with specific antibodies to each C/EBP isoform demonstrated that the levels of C/EBPδ and C/EBPβ protein were rapidly induced (by day 1) and then abrogated by day 4 and 8, respectively, in solvent-treated (control) cells. In TCDD-treated cells, however, the levels of C/EBPβ and C/EBPδ protein persisted at these time points. In contrast, C/EBPα protein was markedly suppressed by TCDD in concordance with its level of RNA. Both translational products of C/EBPα, p30 and p42, were dose-dependently decreased by TCDD. Gel shift analysis of nuclear extract binding to an oligonucleotide containing a C/EBP DNA recognition sequence revealed no difference between extracts from control and TCDD-treated cells in the binding pattern at day 2 of differentiation. At days 4 and 8, the band corresponding to the C/EBPα/DNA complex (as determined with supershift assays) was dramatically decreased in the treated extracts in comparison to control extracts. In contrast, a band corresponding to a C/EBPβ/DNA complex was found to be enriched in the treated samples. These data indicate that suppression of differentiation in the 3T3-L1 preadipocyte cell line by TCDD occurs at a short but defined period, during the differentiation program, and involves altered regulation of C/EBP, including the inhibition of C/EBPα.

TCDD is the most toxic member of a large family of halogenated polyaromatic hydrocarbons that includes dioxins, furans, biphenyls, and naphthalenes. These compounds bind to a cytosolic, high affinity receptor known as the AhR, which mediates pleiotrophic responses in many target tissues and cells, leading to changes in gene expression, cellular metabolism, differentiation, and proliferation (for reviews, see Refs. 1 and 2).

One characteristic effect of TCDD poisoning is the perturbation of energy storage and utilization. Although specific alterations in metabolism vary by species and strain, commonly observed symptoms include a reduction of adipose tissue mass, increase in serum triglycerides, redistribution of fatty acids (3–5), alteration of lipid and carbohydrate metabolism (6), and the onset of diabetic-like symptoms (5). Both the liver and adipose tissue, which are the primary sites of carbohydrate and lipid metabolism, respectively, show impaired synthesis and storage of energy after exposure to TCDD. For example, gluconeogenesis is significantly decreased in the livers of TCDD-treated animals (7, 8). In adipose tissue, glucose transport (9, 10), lipoprotein lipase activity (5), and fatty acid synthesis (11) are inhibited. Furthermore, the levels of mRNA for several adipogenic genes, including the *GLUT4*, *LPL*, and *FABP* (or *aP2*), are selectively reduced compared with pair-fed control animals (10). These changes indicate an overall shift in adipose function

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ABBREVIATIONS: TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; AhR, aryl hydrocarbon receptor; C/EBP, CCAAT/enhancer binding protein; GLUT4, insulin-responsive glucose transporter; FABP, fatty acid binding protein; LPL, lipoprotein lipase; DMEM, Dulbecco's modified Eagle's medium; DEX, dexamethasone; IBMX, isobutylmethylxanthine; LAP, liver activator protein; LIP, liver inhibitory protein; PPAR, peroxisome proliferator activated receptor; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DTT, dithiothreitol; DMSO, dimethylsulfoxide; SDS, sodium dodecyl sulfate.

from a lipogenic to a lipolytic or an antilipogenic mode. The biochemical and molecular bases for these changes in energy homeostasis are not well understood, in part because TCDD affects numerous cell and tissue types and elicits complex changes in endocrine function.

To understand more thoroughly the direct effects of TCDD on adipocyte function, we used the established preadipocyte cell line 3T3-L1. These preadipocytes are fibroblast-like cells that, on quiescence, can be induced to differentiate into cells that morphologically and biochemically resemble adipocytes (12, 13). The adipocyte differentiation program involves a limited number of mitoses followed by a cessation of proliferation, change in cell morphology, and coordinate transcriptional activation of a group of genes encoding adipogenic proteins, which allows the cells to synthesis and accumulate lipid. Regulation of this program is believed to be controlled by at least two major families of transcription factors: the C/EBP and the PPAR (for a review, see Ref. 14).

The C/EBP family is composed of several distinct members that share a common basic-leucine zipper motif. The first member to be cloned was C/EBPα (15). Several lines of evidence suggested that C/EBPα was obligatory for the conversion to and maintenance of differentiated adipocytes. First, C/EBPα expression was dramatically increased during adipocyte differentiation, and its expression preceded the appearance of mRNA for a number of marker genes of terminal adipocyte differentiation, including *FABP* and *GLUT4* (16). Analysis of the promoters of the *FABP* and *GLUT4* genes revealed C/EBP binding sites, and C/EBPα has been shown to *trans*-activate the promoters of both of these genes (16, 17). Treatment of 3T3-L1 preadipocytes with antisense RNA that blocked C/EBPα expression also suppressed terminal differentiation and triglyceride accumulation (18, 19). Finally, forced expression of C/EBPα in preadipocytes and nonadipogenic fibroblasts triggered differentiation without the need for exogenous hormonal stimulants (20).

Genes encoding several other C/EBPs have been cloned, including C/EBPβ (21), also called NF-IL6 (22), LAP (23), AGP/EGP (24) or IL-6DBP (25), and C/EBPδ (21), which are expressed in differentiating adipocytes. The roles of C/EBPβ and C/EBPδ in the differentiation program are less defined than for C/EBPα; however, it has been demonstrated that they respond to inducers of differentiation (C/EBPβ to isobutylmethylxanthine and C/EBPδ to dexamethasone), indicating a possible role in the signal transduction of these agents (26). Interestingly, both C/EBPβ and C/EBPδ are transcriptionally activated before C/EBPα, suggesting that one or both may participate in the activation of C/EBPα. Precocious expression of either C/EBPβ or C/EBPδ enhanced differentiation, and ectopic expression of C/EBPβ in NIH 3T3 cells stimulated their conversion to adipocytes, further documenting a role for these factors in the program (26).

The PPAR nuclear protein family is also composed of several members, one of which, PPARγ, was shown to be expressed selectively and at high levels in adipocytes (27). PPARγ, like C/EBPα, is believed to regulate the adipocyte differentiation program, based on results of studies showing that it is induced early in the program and is capable of *trans*-activating genes containing an adipocyte-responsive element (27, 28). Overexpression of PPARγ in NIH 3T3 fibroblasts resulted in adipocyte conversion of these cells in a PPAR ligand-dependent mechanism; however, cotransfection of C/EBPα and PPARγ greatly stimulated differentiation, even in the absence of inducers (29). Thus, PPARγ seems to cooperate with C/EBPα to promote adipogenesis optimally.

Recent experiments in our laboratory have demonstrated that TCDD treatment of 3T3-L1 preadipocytes suppressed their conversion into fat cells in a dose- and AhR-dependent manner (30). Inhibition of cellular differentiation occurred at an early stage in the program and did not seem to be reversible. In view of the role of C/EBP and PPARγ proteins as putative regulators of adipocyte differentiation, we hypothesized that the effect of TCDD on 3T3-L1 differentiation could be mediated through its actions on one or more of these factors. We concentrated our initial efforts on C/EBP proteins because they have been proposed to be important regulators of energy homeostasis (31), which is severely disrupted in TCDD-treated animals. We began to investigate the mechanism by which TCDD suppresses adipocyte differentiation. The results reported here demonstrate that TCDD does differentially alter the expression and DNA binding of C/EBPα, C/EBPβ, and C/EBPδ. We further show that differentiation-dependent induction of PPARγ is blocked by TCDD treatment as well.

Materials and Methods

Cell culture and treatment. 3T3-L1 preadipocytes (obtained from American Type Culture Collection) were grown and induced to differentiate as described previously (30). TCDD was dissolved in DMSO and added as a 1:2000 dilution (5 nM final concentration). For studies on the ability of TCDD to suppress differentiation, TCDD or solvent alone (control) was added to the medium (DMEM supplemented with 10% fetal bovine serum) with the inducers of differentiation (0.25 μM DEX, 0.5 mM IBMX, and 10 μg/ml insulin). The day on which the differentiation protocol was initiated was referred to as day 0. On day 2, the cells were fed with fresh medium containing 10 μg/ml insulin. Thereafter, cells were fed at 2-day intervals. Insulin supplementation of the medium was stopped on day 6.

Plates of differentiated cells were stained for triglyceride accumulation with oil red O. The stain was quantified spectrophotometrically as we have described previously (30).

RNA isolation and Northern blot analysis. Total RNA was isolated from 3T3-L1 cells using the acid phenol/guanidinium isothiocyanate method (32). The RNA samples were subjected to Northern blot analysis as described previously (10). Membranes were stripped in 10 mM Tris-Cl, pH 7.5, 1 mM EDTA, and 0.2% SDS at 70° with shaking and reprobed. cDNA probes for murine c-*jun*, c-*fos*, c-*myc*, α2 chain of collagen IV, PPARγ, and LPL were cloned through polymerase chain reaction amplification with primers based on the published sequences. Plasmids containing full-length cDNAs for C/EBPα, C/EBPβ, and C/EBPδ were generously provided by Dr. Steven McKnight (Tularik, South San Francisco, CA). A cDNA for FABP was a gift from Dr. Howard Green (Harvard Medical School, Charlestown, MA). A 24-base oligonucleotide (5′-ACGGTATCTGATCGTCTTCGAACC-3′) (33) corresponding to the mouse 18*S* ribosomal RNA sequence was used for normalization of loading and transfer.

Preparation of cell lysates and immunoblot analysis. Nuclei from plates of 3T3-L1 cells were isolated according to the method of Dignam *et al.* (34). The packed pellet of nuclei was resuspended in lysis buffer (60 mM Tris-Cl, pH 6.8, 1% SDS) and boiled for 5 min with occasional mixing by vortex. Insoluble debris was removed through centrifugation at 15,000 rpm for 5 min at 4°. The amount of soluble protein was measured according to the method of Bradford (35) with bovine serum albumin standards. Protein electrophoresis and immunoblotting were performed as described previously (10). Protein/antibody complexes were detected with an anti-rabbit horse-

radish peroxidase-linked secondary antibody and the enhanced chemiluminescence detection system (Amersham). Polyclonal anti-C/EBPα, C/EBPβ, and C/EBPδ antibodies were purchased from Santa Cruz Biotechnology.

Preparation of nuclear extracts and electrophoretic mobility shift assays. Nuclei were isolated from 3T3-L1 cells according to the method of Dignam *et al.* (34). Nuclear extracts were prepared from these nuclei according to a slightly modified procedure of Lavery and Schibler (36). Briefly, pelleted nuclei were resuspended in a minimal volume of MDKEH (3 mM $MgCl_2$, 1 mM DTT, 15 mM KCl, 0.1 mM EDTA, and 10 mM HEPES, pH 7.9). Extraction buffer was added to give a final concentration of 350 mM NaCl, 0.5 M urea, 1% Nonidet P-40, 1 mM DTT, and 25 mM HEPES, pH 7.9. The samples were mixed vigorously by vortex and incubated on ice for 20 min. The extracts were clarified through centrifugation at 15,000 rpm for 20 min at 4° in a microcentrifuge. The supernatant was adjusted to 10% (v/v) glycerol, snap-frozen, and stored at −80° until use. The protein concentrations of the extracts were in the range of 2–10 mg/ml.

A double-stranded oligonucleotide corresponding to the C/EBP binding site in the C/EBPα promoter (37) was synthesized, annealed, and end-labeled with [γ-^{32}P]ATP (Amersham) and T4 kinase (Promega) according to standard methods. The sequence of this probe was 5′-tcgaGCGTTGCGCCACGATCTCT-3′. The nuclear extracts (10 μg) were incubated in a buffer containing 25 mM HEPES, pH 7.9, 10% glycerol, 0.5 mM DTT, 5 μg of acetylated bovine serum albumin, 2 μg of poly(dI-dC) (Boehringer Mannheim), and 60 mM NaCl at room temperature for 15 min. Specific or nonspecific competitor DNAs were added at the end of this period; then, 50,000 cpm of oligonucleotide (~0.3 ng of DNA) was added, and the samples were incubated for an additional 15 min. For supershift assays, antiserum was added after the second incubation period, mixed gently, and incubated for an additional 20 min at room temperature. The samples were resolved on a 4% nondenaturing polyacrylamide gel in 0.5× Tris/borate/EDTA buffer (1× = 45 mM Tris-borate and 1 mM EDTA). After being dried, the gel was exposed to X-ray film in the presence of an intensifying screen at −80°.

Results

TCDD effects on the earliest responses of preadipocytes to inducers of differentiation. Because we observed previously that TCDD was unable to suppress preadipocyte conversion when added 2 days after the initiation of differentiation (30), we investigated several early events in the differentiation process. The earliest known steps involve the activation of a number of genes encoding transcription factors (14). As shown in Fig. 1, a number of these genes were transiently activated in response to the differentiation medium, which is supplemented with fetal bovine serum, DEX, IBMX, and insulin. The levels of mRNA for c-*fos*, c-*myc*, *jun*-B, and c-*jun* (the latter is not shown) increased rapidly (≤1 hr) and then decreased by 8 hr. Cotreatment of the cells with TCDD (5 nM) and these inducers of differentiation showed little difference between control and treated cells in the inducibility or persistence of these genes. At several hours after the cells were exposed to the differentiation inducers, they begin to round up. Expression of mRNA encoding the α2 chain of collagen IV is induced during this period (38) and is not inhibited by treatment with TCDD (Fig. 1). Thus, we did not detect any differences between control and TCDD-treated cells in the inducibility of several important genes during the earliest stages of 3T3-L1 differentiation.

TCDD effects on cell growth and morphology. Differentiating preadipocytes undergo several rounds of clonal cell division after exposure to the inducers of differentiation and

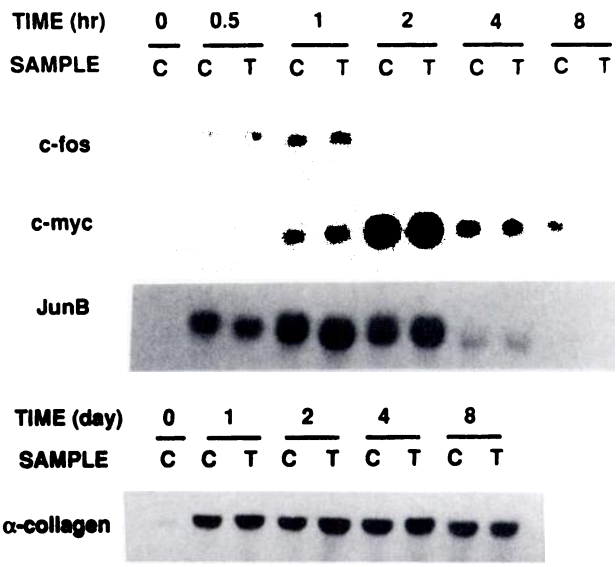

Fig. 1. Effect of TCDD on the expression of rapidly induced genes in 3T3-L1 cells stimulated with inducers of differentiation. 3T3-L1 cells were induced to differentiate as described in Materials and Methods in the presence of 5 nM TCDD or solvent alone (0.05% v/v DMSO) and harvested at the times shown. Total RNA was isolated, electrophoresed (12 μg/lane), and subjected to Northern blot analysis with radiolabeled cDNA probes for the mRNAs indicated. *α-Collagen*, α2 chain of collagen IV.

before the accumulation of lipid (14). The cell number increased nearly 3-fold from day 1 to day 6 (Fig. 2A), but the final number of cells per plate was not significantly different between control ($8.91 \times 10^6 \pm 0.37 \times 10^6$) and TCDD-treated groups ($8.20 \times 10^6 \pm 1.01 \times 10^6$). The preadipocytes underwent a change in appearance from the characteristic spindle-like shape of fibroblasts (Fig. 2B) to a rounded form (Fig. 2C) during the first 2 days of exposure to the inducers of differentiation. Control and TCDD-treated cells were virtually indistinguishable at this time, but by day 4, triglyceride droplets were evident in the control cells (Fig. 2D), whereas TCDD-treated cells showed little lipid accumulation (Fig. 2E). Furthermore, control cells remained rounded, whereas TCDD-treated cells reverted to a fibroblast-like shape. These data indicated that both control and treated 3T3-L1 preadipocytes were capable of completing differentiation-dependent mitosis and that the effect of TCDD on differentiation was not manifested until the cells had begun to synthesize and store lipid, suggesting that the target may be related to a factor or factors involved in the initiation or coordination of lipid accumulation.

Differential effects of TCDD on the levels of C/EBP mRNA during differentiation. To determine whether TCDD altered the induction of key adipogenic genes in response to the inducers of differentiation, we followed the expression of three C/EBP genes during the differentiation period. In agreement with previous findings (21, 26), C/EBPβ and C/EBPδ were induced rapidly (Fig. 3, *day 1*) before the induction of C/EBPα mRNA, which was visible only after day 3. TCDD had little effect on the levels of C/EBPβ and C/EBPδ mRNA, but it markedly suppressed the induction of C/EBPα mRNA. Expression of PPARγ, another regulator of adipogenesis, was similarly suppressed in TCDD-treated cells. Cells that accumulated little C/EBPα and PPARγ mRNA also showed reduced levels of mRNA for a number of other adi-

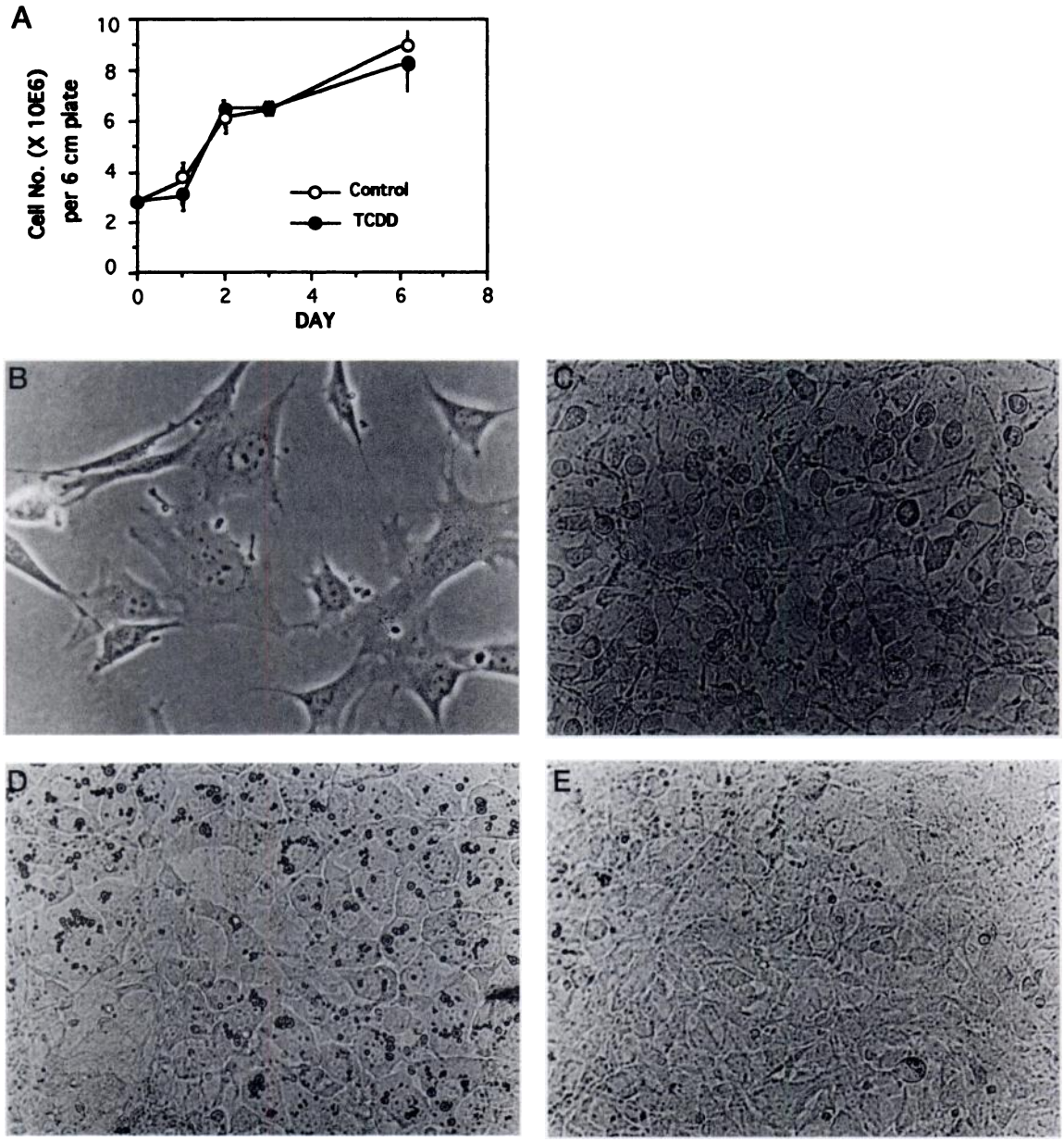

Fig. 2. Growth curve and photomicrographs of 3T3-L1 cells during different stages of differentiation. A, Changes in cell number after induction of preadipocyte differentiation. *Day 0*, day on which the cells were induced to differentiate as described in Materials and Methods. Each point represents the mean and standard deviation of four plates. B, Proliferating 3T3-L1 preadipocytes. C, DMSO-treated control cells on day 2. D, Control cells on day 4; most of the cells are rounded and have begun to accumulate droplets of triglyceride. E, TCDD-treated cells on day 4. Few cells have lipid droplets; most have reverted to a flat, fibroblast-like shape.

pogenic genes, including *LPL*, *FABP*, *GLUT4*, hormone-sensitive lipase, and fatty acid transporter, as expected (Fig. 3 and data not shown).

TCDD prolongs the presence of C/EBPβ and C/EBPδ proteins but suppresses accumulation of C/EBPα. To determine whether the differential effect of TCDD on C/EBP mRNA levels also affected cellular protein levels of these genes, we conducted immunoblotting on nuclear extracts of 3T3-L1 cells. Levels of C/EBPβ protein (Fig. 4A) in general mirrored the mRNA levels in the control (solvent-treated) cells, showing a rapid, transient increase. Two translational products, LAP (36 kDa) and LIP (24 kDa) (39) were strongly induced during the first 4 days of differentiation. The induction of LAP seemed to be slightly accelerated in the TCDD-treated cells on day 1 but showed little difference from the control samples on days 2 and 4. The appearance of LIP, however, was inhibited by TCDD on days 1 and 2. On day 8, both forms of C/EBPβ declined in control samples, whereas in the treated cells LAP remained elevated. A similar trend was observed with C/EBPδ (Fig. 4B); in the TCDD-treated cells, there was no difference in the levels of protein on days 1 and 2 compared with the control samples, but on day 4, levels of C/EBPδ remained elevated in TCDD-treated samples, even though the protein was virtually absent in the control group. In contrast to the stabilization by TCDD of C/EBPδ and C/EBPβ protein levels, a distinct suppression of C/EBPα protein was evident in cells exposed to TCDD in comparison to vehicle controls (Fig. 4C). Levels of both of the transla-

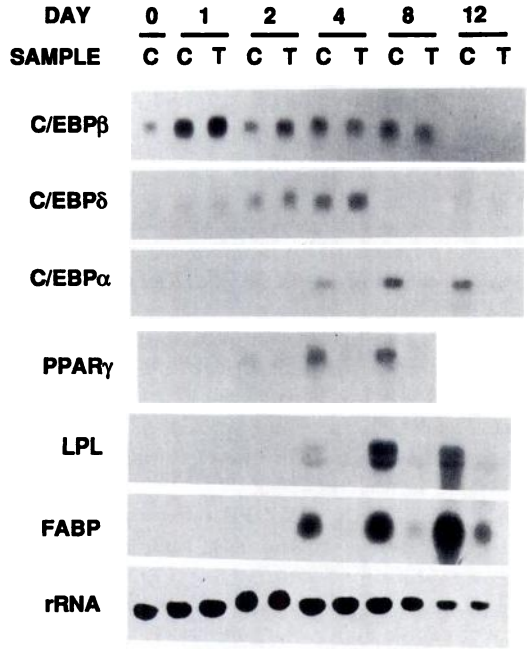

Fig. 3. TCDD treatment of differentiating 3T3-L1 cells differentially affects expression of C/EBPα, C/EBPβ, and C/EBPδ mRNAs. Total RNA (15 μg) from 3T3-L1 cells were analyzed through Northern blotting with cDNA probes specific for each form of C/EBP. Blots were stripped and reprobed for two markers of adipocyte differentiation, LPL and FABP, and PPARγ. The blot shown for PPARγ did not contain RNA samples from day 12. Hybridization to the 18*S* ribosomal band (*rRNA*) was used to normalize loading variations between samples. Each experiment was repeated three independent times with similar results. *C*, DMSO control; *T*, 5 nM TCDD.

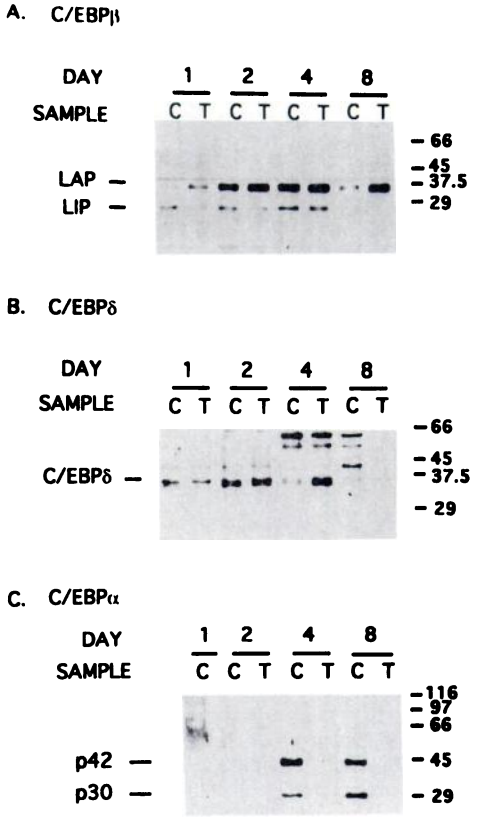

Fig. 4. Levels of C/EBP proteins in 3T3-L1 cells during the course of differentiation. Nuclear extracts were prepared on the days shown from 3T3-L1 cells treated with 5 nM TCDD or DMSO alone at the time of differentiation. The extracts (20 μg/lane) were subjected to SDS-polyacrylamide gel electrophoresis and analyzed through Western blotting for C/EBP proteins according to the procedure in Materials and Methods. Blots were incubated in diluted antiserum against C/EBPβ (A), C/EBPδ (B), or C/EBPα (C), followed by incubation with a horseradish peroxidase-linked goat anti-rabbit secondary antibody. Antibody-protein conjugates were detected with enhanced chemiluminescence reagents and visualized on X-ray film. Blots are representative of an experiment that was repeated two (C/EBPβ and C/EBPδ) or of four (C/EBPα) independent times. Higher molecular mass bands in B are nonspecific and were not observed in all experiments.

tional products of C/EBPα, p30 and p42 (40, 41), were suppressed by TCDD. Thus, TCDD treatment seemed to prolong the presence of C/EBPβ and C/EBPδ proteins while blocking the induction of C/EBPα.

The inhibition of C/EBPα was dose-dependent, with an ED_{50} of ~0.5 nM (Fig. 5A). Also, the level of C/EBPα suppression by TCDD was directly correlated with cellular accumulation of triglyceride as assessed with oil red O staining (Fig. 5B). Therefore, cells that failed to express C/EBPα protein also failed to differentiate terminally as assessed through fat accumulation and expression of genes associated with the mature adipocyte phenotype.

Effects of TCDD on the binding patterns of C/EBP proteins to DNA. Electrophoretic mobility shift assays were conducted to determine whether the differential effect of TCDD on C/EBP protein levels would be reflected in the binding of these proteins to their cognate DNA recognition sequences. The functionally active C/EBP binding site from the C/EBPα promoter was used as the oligonucleotide probe (37). In the first experiment, we isolated nuclear extracts from a time course of cells treated with TCDD (5 nM) or DMSO (Fig. 6A). The gel shift banding patterns revealed little difference between the samples on day 2. A small difference is observable in band A on day 4, and by day 8, notable differences are observed in the banding patterns. Specifically, the intensity of band A is much greater in the control (*lane 6*) than in the treated sample (*lane 7*), whereas band C shows the opposite tendency: it is higher in the treated than in the control sample. Band B did not seem to be affected by TCDD treatment. Similar results were obtained when the C/EBP binding site from FABP (16) promoter was used as the probe (data not shown).

As all three forms of C/EBP can bind to the same DNA recognition site, it was necessary to conduct supershift assays to identify the components of each shifted complex. Incubation of nuclear extracts with an antibody specific for C/EBPα (Fig. 6B, *lanes 2 and 7*) completely shifted band A, verifying that the control cells had a much greater titer of the C/EBPα isoform than the TCDD-treated cells. As a result of supershifting this predominant C/EBPα band, a fourth cryptic band was revealed (Fig. 6B, *lane 2, band A'*). This band seemed to be eliminated only when antibodies against both C/EBPα and C/EBPβ were added to the extracts (Fig. 6B, *lane 4*). Band B was supershifted by an antiserum against C/EBPβ (Fig. 6B, *lane 3*). The same patterns of supershifting were observed in the treated extracts (Fig. 6B, *lanes 6–9*) as in the control extracts. Complex C, which was prevalent in the treated extract (Fig. 6B, *lane 6*) but only weakly visible in the control (Fig. 6B, *lane 1*), seemed to be a second C/EBPβ band as it, too, was supershifted by the C/EBPβ antibody. C/EBPδ antibody did not shift any complexes present in these

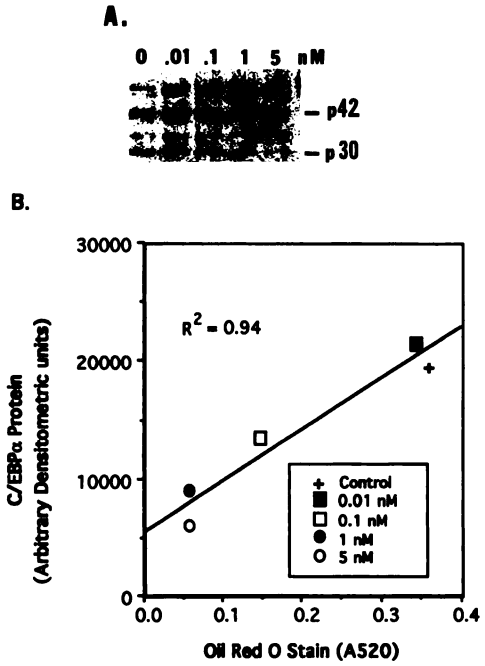

Fig. 5. Dose response for the effect of TCDD on levels of C/EBPα protein. A, 3T3-L1 cells were treated with the doses shown or DMSO alone (control) concurrently with the inducers of differentiation and harvested on day 8. Western blotting of nuclear extracts was performed as described in the legend to Fig. 4 with a polyclonal antiserum against C/EBPα. Bands were quantified with the use of an AMBIS radioanalytic imaging instrument. Higher molecular mass bands are nonspecific and were not observed in all experiments (compare with Fig. 4C). B, Correlation between levels of C/EBPα protein and oil red O staining for triglyceride. Plates of 3T3-L1 cells that were exposed to the doses of TCDD shown in A during differentiation were fixed on day 8 with formalin, dried, and stained with oil red O. Quantification of stain was performed spectrophotometrically as described in Materials and Methods.

extracts (Fig. 6B, *lanes 10–12*). Nonimmune rabbit serum had no effect on the binding of C/EBP proteins to the oligonucleotide (Fig. 6B, *lane 5*), whereas a 100-fold excess of unlabeled oligonucleotide completely eliminated the bands (Fig. 6B, *lane 14*). These data confirmed that the primary effect of TCDD on C/EBP during the differentiation of 3T3-L1 cells was a suppression of C/EBPα, but increased C/EBPβ was also evident.

Discussion

TCDD suppresses differentiation of 3T3-L1 preadipocytes in a potent and specific manner. We demonstrated that TCDD affected a very narrow window of time during the differentiation process. Previous studies have shown that activation of cellular proliferation signaling or overexpression of mitogenic factors, such as c-*myc* (42), was antagonistic to preadipocyte differentiation. TCDD, however, neither altered the response of several key transcription factor mRNAs to mitogens in the differentiation medium nor affected clonal expansion of these cells. Thus, TCDD did not seem to interfere with the earliest known events in the differentiation program.

Alternatively, TCDD could have interfered with the action of the inducers of differentiation: DEX and IBMX. TCDD has been shown to decrease glucocorticoid binding to its receptors in the liver (43). However, we previously found that TCDD treatment of differentiating 3T3-L1 cells did not alter either the amount of DEX required to induce differentiation or the *trans*-activation of a reporter gene under the control of glucocorticoid enhancer elements (30). Our observation that TCDD failed to inhibit the induction of C/EBPδ mRNA by DEX further indicated that the glucocorticoid signaling pathway was intact, at least during the first 2 days of exposure to TCDD and DEX. Similarly, the induction of C/EBPβ mRNA by IBMX was not altered by TCDD treatment (Fig. 3). Taken together, our data indicate that TCDD does not merely antagonize the actions of these inducing agents.

One intriguing observation is that the levels of protein for C/EBPδ and C/EBPβ remained elevated in TCDD-treated cells, whereas their levels were abrogated in control cells by day 4 and 8, respectively. The functional significance of the prolonged presence of C/EBPβ and C/EBPδ to the differentiation program is not yet clear. Based on the mobility shift assay data (Fig. 6), the C/EBPβ proteins in extracts from TCDD-treated cells were capable of recognizing and binding to an oligonucleotide containing the C/EBP recognition sequence. Whether this binding would result in transcriptional activation or repression in the context of a gene remains to be determined. Yeh *et al.* (26) recently reported that overexpression of LAP in 3T3-L1 preadipocytes was sufficient to induce C/EBPα expression and terminal differentiation, even in the absence of exogenous stimulants. In our experiments, TCDD-treated preadipocytes showed high levels of LAP but failed to accumulate C/EBPα or convert into mature fat cells. Consequently, we infer that the arrest of adipocyte differentiation by TCDD in the 3T3-L1 cells is downstream of the initial activation of C/EBPδ and C/EBPβ.

Our data lend further support to the hypothesis that C/EBPα is obligatory for adipocyte differentiation (20, 44) because cells that showed suppressed C/EBPα mRNA and protein also failed to accumulate lipid (Fig. 5B). Furthermore, the suppression of C/EBPα by TCDD results in a substantial decrease in the expression of *FABP* and *GLUT4*, two genes that are putatively *trans*-activated by C/EBPα in 3T3-L1 cells (16, 17). TCDD treatment also inhibited the accumulation of PPARγ during adipocyte differentiation. PPARγ, like C/EBPα, has been postulated to be a key regulator of the adipocyte differentiation program (27, 29). Although overexpression of PPARγ alone was sufficient to stimulate adipogenesis in NIH 3T3 fibroblasts, coexpression of PPARγ and C/EBPα induced much greater conversion of these cells into adipocytes, indicating a cooperative effect between the two factors (29). Recently, it was reported that ectopic expression of C/EBPβ in pluripotent NIH 3T3 fibroblasts in the presence of differentiation medium activated expression of PPARγ, but not C/EBPα, and stimulated adipogenesis in 20–30% of the cells (45). These data support a model in which activation of PPARγ and C/EBPα are independent events; therefore, TCDD may affect several distinct steps in the differentiation process.

We previously reported that the effect of TCDD on 3T3-L1 differentiation required the participation of the AhR (30) as we were able to antagonize the effect of TCDD through pretreatment of the cells with a 1000-fold molar excess of the AhR antagonist α-naphthoflavone (45). Further studies will be needed to determine whether the suppression of C/EBPα and PPARγ involves a direct interaction of the liganded AhR with *cis*-acting elements in the respective gene promoters or

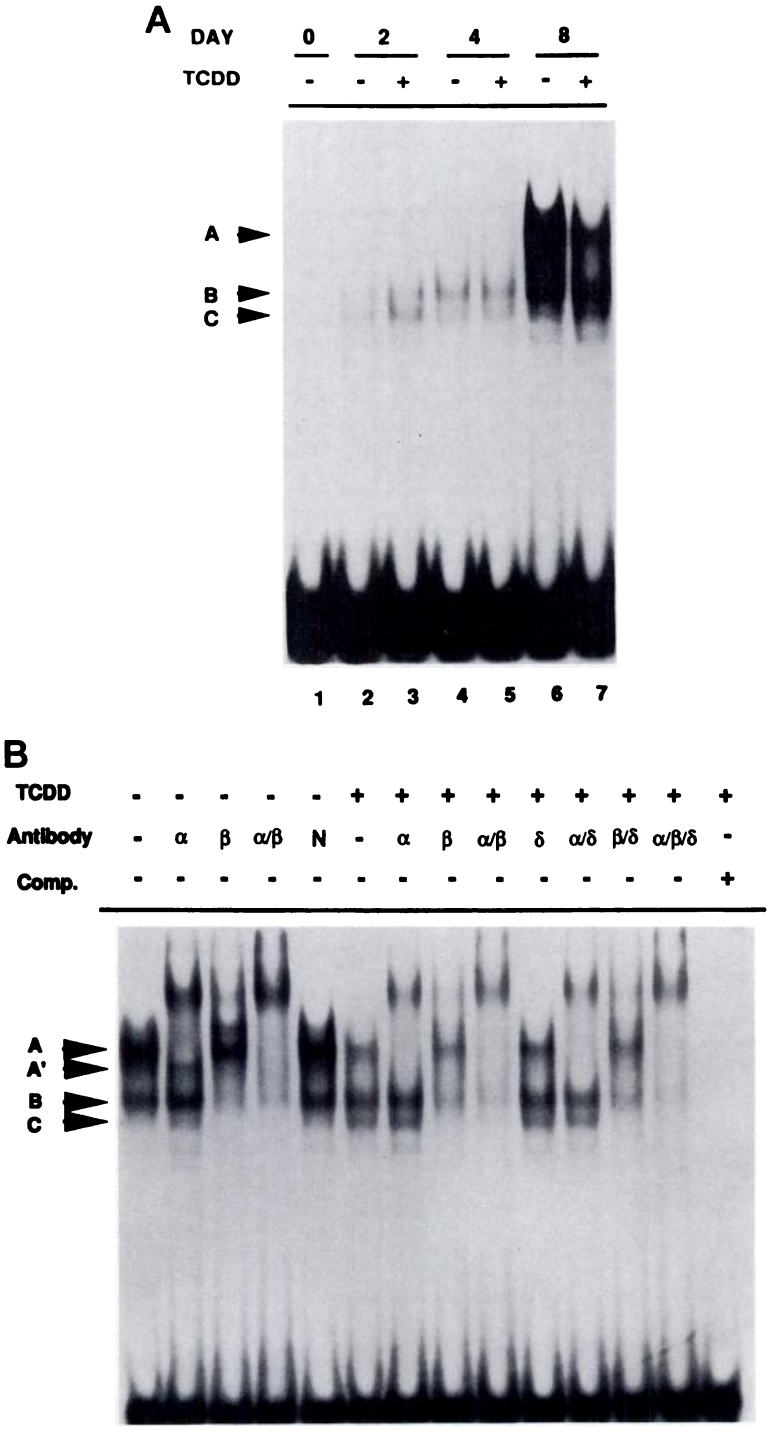

Fig. 6. DNA binding activity of C/EBP proteins in nuclear extracts from differentiating 3T3-L1 cells. Nuclear extracts were isolated from 3T3-L1 cells induced to differentiate in the presence or absence of TCDD (5 nM) and subjected to electrophoretic mobility shift analysis as described in Materials and Methods. A, Time course of effect of TCDD. Arrows, three distinct protein/DNA complexes. B, Supershift analysis of protein/DNA complexes in nuclear extracts of 3T3-L1 cells isolated 8 days after induction of differentiation. Antibodies specific for each C/EBP protein (α, β, or δ) or an equal amount of nonimmune rabbit serum (*N*) were used to shift each complex. A 100-fold excess of unlabeled oligonucleotide (*Comp.*) was used to compete away specific C/EBP/DNA complexes (*lane 14*).

involves an indirect mechanism modulated by another factor or factors. Potential mediators could include autocrine factors, such as transforming growth factor-β or tumor necrosis factor-α, which have been shown to inhibit adipocyte differentiation (46, 47).

C/EBPα, in addition to its role in stimulating adipocyte differentiation, has been postulated to be a central regulator of energy metabolism based on molecular and genetic evidence (31). A recent study of C/EBPα-knockout mice showed severely impaired energy metabolism and an inability to accumulate fat in adipose tissue, which lends strong support to this hypothesis (49). Consequently, our finding that TCDD specifically reduced expression and DNA binding of C/EBPα may have significant implications for the *in vivo* effect of TCDD on energy storage and metabolism. We previously showed that C57BL mice treated with 116 μg/kg TCDD showed a rapid decline (between 1 and 3 days) in the levels of mRNA for a number of adipose-specific genes (10). Intensity of C/EBPα mRNA was also decreased ~300% in the adipose tissues of the same animals by 24 hr.[1] More significantly, levels of C/EBPα and other mRNAs characteristic of mature

[1] P. C. C. Liu, unpublished observations.

adipocytes, such as *FABP*, *GLUT4*, and *LPL*, continued to be suppressed, even after 7 days of exposure to TCDD (10).[2]

Although we have addressed only the impact of TCDD on C/EBP in adipose tissue, this family of transcription factors is also abundant in other tissues, including the liver, where it has been shown to play a role in the transcription of several hepatic genes (50). It is plausible that impaired liver function in TCDD-treated animals could be mediated, at least in part, through altered C/EBP expression similar to that observed in adipocytes.

In conclusion, we established that TCDD differentially alters the levels and activity of three C/EBP isoforms involved in the differentiation program of 3T3-L1 preadipocytes. Significantly, one of the main targets of the action of TCDD in the 3T3-L1 model and in adipose tissue is C/EBPα, which has been demonstrated to be a key regulator of fat metabolism *in vivo* and *in vitro*. Thus, this model system allowed us to gain insights into the mechanism by which TCDD can disturb normal cellular programs of adipose development and function. By studying the regulation of the C/EBPα and C/EBPβ by TCDD, we hope to understand the processes by which TCDD and related chemicals may cause severe imbalances in lipid metabolism.

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