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Suppression of C/EBPα and Induction of C/EBPβ by 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin in Mouse Adipose Tissue and Liver

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ABSTRACT. We examined the effect of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on two transcription factors, CAAT/enhancer binding protein-alpha (C/EBPα) and beta (C/EBPβ), involved in the coordination of gene expression in adipose and liver. A single dose of TCDD (100 μg/kg) to male C57BL mice resulted in a time- and dose-dependent decrease in the level of C/EBPα mRNA in adipose tissue and liver, and a reciprocal increase in C/EBPβ mRNA. Gel shift analysis using hepatic nuclear extracts from control and TCDD-treated mice and an oligonucleotide containing a C/EBP recognition element revealed a time-dependent change in DNA–protein complexes formed. Bands corresponding to C/EBPα, as determined by supershift analysis, diminished in TCDD-treated animals over a 7-day time period, whereas two new bands corresponding to C/EBPβ, not present in control extracts, were increased significantly in treated samples. TCDD induced C/EBPβ mRNA in wild-type mouse hepatoma cells, but not in aryl hydrocarbon receptor (AhR) nuclear translocator-deficient hepatoma cells. Induction in wild-type hepatoma cells was antagonized effectively by a molar excess of α-naphthoflavone. These results showed that TCDD caused rapid, reciprocal changes in C/EBPα and C/EBPβ mRNAs and DNA binding in the adipose and liver of male C57BL mice and induced C/EBPβ in hepatoma cells in an AhR-dependent manner. C/EBPs play vital roles in the coordination of energy homeostasis, and their alteration by TCDD may provide insight into the mechanism by which TCDD perturbs energy storage and utilization *in vivo*. BIOCHEM PHARMACOL **55**;10:1647–1655, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. TCDD; Ah receptor; DNA-binding proteins; C/EBPα; C/EBPβ

TCDD‡ and related halogenated aromatic hydrocarbons comprise a class of pollutants that are found ubiquitously in the environment. In animal studies, TCDD has been shown to elicit diverse toxicological and biochemical responses including immune suppression, reproductive and developmental toxicity, carcinogenesis, endocrine modulation, and enzyme induction (reviewed in [1]). One phenomenon that is characteristic of acute TCDD exposure is the perturbation of energy storage and utilization (reviewed in [1, 2]). Both the liver and adipose tissue, which are the primary sites of carbohydrate and lipid metabolism, respectively, show impairment of synthesis and storage of metabolic reserves following exposure to TCDD. In adipose tissue, glucose transport [3, 4], lipoprotein lipase activity [5], and fatty acid synthesis [6] are inhibited. Moreover, the levels of mRNA for several adipose-specific genes including the insulin-responsive glucose transporter (GLUT4), lipoprotein lipase, and FABP (or aP2) are selectively reduced compared with those of pair-fed control animals [4]. These changes indicate a coordinated shift from a lipogenic to lipolytic or antilipogenic mode.

Regulation of energy metabolism in adipose tissue and liver requires the coordinated expression of genes encoding lipogenic and gluconeogenic enzymes in response to hormonal stimuli. The molecular mechanisms by which these complex events occur have begun to be elucidated only recently. One putative regulator of lipogenesis and gluconeogenesis is the CAAT/enhancer binding protein (C/EBPα), a transcription factor containing a basic leucine zipper motif [7]. A close relationship between C/EBPα and energy homeostasis has been postulated, based on several lines of biochemical and genetic evidence [8]. C/EBPα has been shown to *trans*-activate several genes, including stearoyl-CoA desaturase 1, GLUT4, FABP, and acetyl CoA carboxylase, involved in the uptake and synthesis of metabolic fuels [9–11]. Expression of C/EBPα mRNA is highest in adipose, liver, and intestine, tissues involved in metabolic fuel uptake, synthesis, and storage, and is increased dramatically just prior to birth [12]. Finally, analysis of mice that had the *c/epbα* gene deleted has provided compelling

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‡ *Abbreviations:* TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; C/EBP, CAAT/enhancer binding protein; GLUT, facilitative glucose transporter; FABP, fatty acid binding protein; AhR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; LPS, lipopolysaccharide; and TNF, tumor necrosis factor.

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evidence for a link between C/EBPα and energy metabolism. The *c/ebp*α knockout mice did not accumulate either lipid or glycogen and suffered from severe metabolic disorders that led to death by hypoglycemia [13].

C/EBPα is one member of a family of related transcription factors. Other members include C/EBPβ [14] (also called NF-IL6 [15], AGP/EBP [16], LAP [17], and IL-6DBP [18]), and CRP2 [19]; C/EBPδ [14]; and C/EBPϵ [20] (gadd153, CHOP-10). These proteins can form homo- and heterodimers resulting in a large number of complexes that recognize and bind to the same nucleotide sequences to modulate gene transcription. While it is now believed that C/EBPα plays a key role in energy metabolism [8, 21], specific roles for other C/EBP members are not as clearly defined. It has been proposed that C/EBPβ plays an important role in the transcription of liver-specific genes and in mediating the coordinated acute phase response to inflammatory agents, including LPS, TNFα, and interleukins. In response to these inflammatory agents, levels of C/EBPβ and C/EBPδ mRNA and protein are induced dramatically, while the level of C/EBPα is reduced [22, 23].

In view of the coordinated inhibition of lipid and carbohydrate synthesis by TCDD and some similarities between acute symptoms of TCDD toxicity and acute response to inflammatory agents, we postulated that some of the effects of TCDD could be mediated by an alteration in the activities of C/EBP proteins, particularly in tissues engaged in energy homeostasis. We previously observed that TCDD suppresses the levels and activity of C/EBPα while stabilizing C/EBPβ in 3T3-L1 cells during adipocyte differentiation, and that this suppression of C/EBPα directly correlates with inhibition of triglyceride accumulation and fat cell differentiation [24]. In the present report, we demonstrated that exposure of male C57BL mice to TCDD resulted in a rapid decrease in C/EBPα mRNA levels in adipose tissue and liver and in reduced DNA binding by C/EBPα. Additionally, we observed that C/EBPβ is induced by TCDD in these same tissues and mouse hepatoma cells, and its induction is dependent upon the presence of functional AhR.

MATERIALS AND METHODS

Animal Care

Male C57BL/6N mice, age 6–8 weeks upon delivery, were purchased from Simonsen Laboratories and housed in pairs in a climate-controlled facility with a 12-hr photo period and allowed free access to standard rodent chow and water. Animals were acclimated for at least 1 week prior to use. TCDD (a gift from the Dow Chemical Co., >99.9% purity) was administered by a single i.p. injection in acetone:corn oil (1:9), whereas control mice received an equal volume (7.5 mL/kg) of vehicle alone. Animals were killed by cervical dislocation, and the epididymal adipose tissue and liver were dissected and weighed. A 100- to 200-mg section from the largest lobe of the liver was removed for RNA isolation; the remainder of the liver was cut into small pieces in ice-cold PBS. Tissues were snap-frozen in liquid nitrogen and stored at −80° until used.

Cell Culture

The mouse hepatoma cell lines Hepa 1 (wild type) and Hepa c4 (ARNT-deficient mutant) were gifts from Dr. O. Hankinson (University of California, Los Angeles). They were grown in α-Modified Eagle's Medium containing 10% fetal bovine serum and supplemented with penicillin, streptomycin, and fungizone and cultured at 37° under an atmosphere of 5% CO_2. Treatments were made by diluting TCDD or DMSO vehicle alone (control) into the medium [0.1% (v/v) final solvent concentration].

RNA Isolation and Northern Blot Analysis

RNA isolation and northern blotting were performed as described previously [24]. Band intensities were quantified with an Ambis radioscanning instrument. Full-length cDNA clones for C/EBPα, -β, and -δ were gifts from Dr. Stephen McKnight (Tularik); a cDNA clone for Cyp1A1 [25] was obtained from the American Type Culture Collection; an oligonucleotide probe for the 18S rRNA [23] was synthesized at the Protein Structure Laboratory (University of California, Davis).

Preparation and Immunoblot Analysis of Nuclear Protein Extracts

Nuclei were isolated from tissues by ultracentrifugation according to the method of Hattori and colleagues [26], and extracts were prepared as described previously [24]. Protein concentrations were measured according to the method of Bradford [27], using bovine serum albumin standards. Immunoblotting of samples was performed as previously described [24], using a polyclonal anti-C/EBPβ antibody from Santa Cruz Biotechnology.

Electrophoretic Mobility Shift Assay

Mobility shift assays were performed as described previously [24] with double-stranded oligonucleotide probes containing sequences for the C/EBP binding site (see Fig. 3A for sequences) from the C/EBPα [28], GLUT4 [10], or FABP [9] promoter. Supershift assays were performed by the addition of polyclonal anti-C/EBPα and/or anti-C/EBPβ antibodies after incubation of protein samples with the labeled DNA probes. Protein:DNA complexes were resolved on a 4% nondenaturing polyacrylamide gel. For some experiments, a 3–5% acrylamide gradient gel was used and run for 3.5 hr at 9 V/cm to achieve maximum resolution between multiple complexes. After being dried, the gel was exposed to x-ray film in the presence of an intensifying screen at −80°.

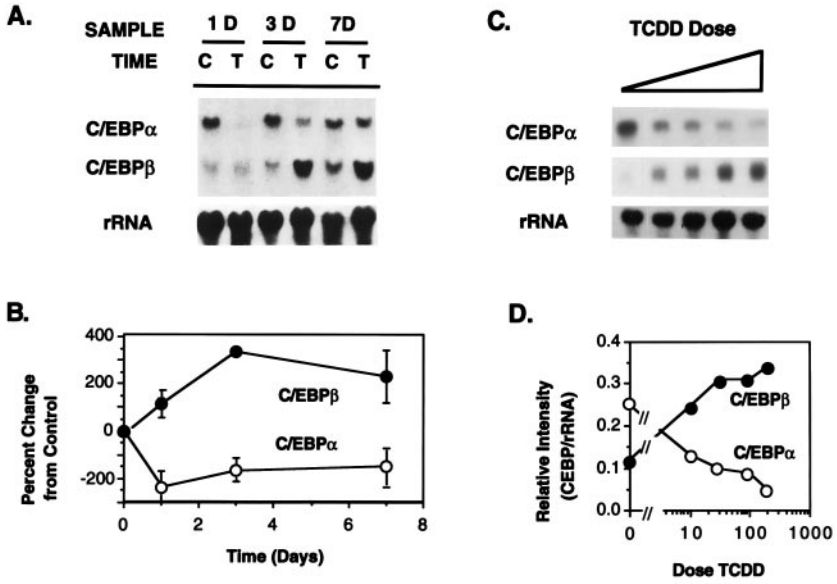

FIG. 1. Effect of TCDD on C/EBPα and C/EBPβ mRNA in adipose tissue. Animals were dosed with a single injection of TCDD or acetone vehicle alone. Total RNA was isolated, electrophoresed, and subjected to Northern blot analysis as described in Materials and Methods. (A) Time course of TCDD (100 μg/kg) effect. Hybridization to the 18S ribosomal band (rRNA) was used for normalization of loading. A representative blot of three individual experiments is shown. (B) Quantification of the blots by densitometry. Values for C/EBP intensity are divided by the corresponding rRNA value and expressed as a percent of the control group for each time point. Each bar represents the average ± SD of three animals. (C) Effect of TCDD dose on levels of C/EBP mRNAs. From left, the doses were 0 (corn oil control), 10, 30, 100, and 300 μg/kg. Animals were treated for 3 days. (D) Densitometric analysis of dose–response blots.

RESULTS

Suppression of C/EBPα and Induction of C/EBPβ mRNAs by TCDD In Vivo

We examined the steady-state levels of C/EBPα and C/EBPβ mRNA by Northern blotting analysis in the adipose and liver of C57BL mice after a single dose (100 μg/kg) of TCDD or vehicle alone. Within 24 hr of TCDD treatment, C/EBPα mRNA was decreased in adipose tissue, and levels remained lower than in control animals through day 7 (Fig. 1, A and B). The effect of TCDD on the levels of C/EBPα mRNA was dose-dependent (Fig. 1C) with an approximate ED_{50} of 30 μg/kg (Fig. 1D). In contrast, TCDD treatment resulted in a 2- to 3-fold induction of C/EBPβ mRNA over the same period, and induction was observed at all doses tested. Neither C/EBPα nor C/EBPβ was affected at 6 hr (data not shown).

A similar pattern of effect on C/EBPα and C/EBPβ mRNAs by TCDD treatment was observed in liver samples isolated from the same animals (Fig. 2, A and B). Steady-state levels of C/EBPα mRNA were decreased by TCDD, although there was some variability in the level of hepatic C/EBPα even among control animals. A larger induction of C/EBPβ mRNA (up to eight-fold higher than control) was observed in the liver than in adipose tissue and occurred within 6 hr. The time course of C/EBPβ stimulation was similar to the induction of Cyp1A1. Changes in both C/EBPα and C/EBPβ mRNAs were dose dependent (Fig. 2, C and D).

Reciprocal Changes in Protein:DNA Binding by C/EBPα and β

We next sought to determine whether TCDD treatment altered functional DNA-binding activity of C/EBPs by gel

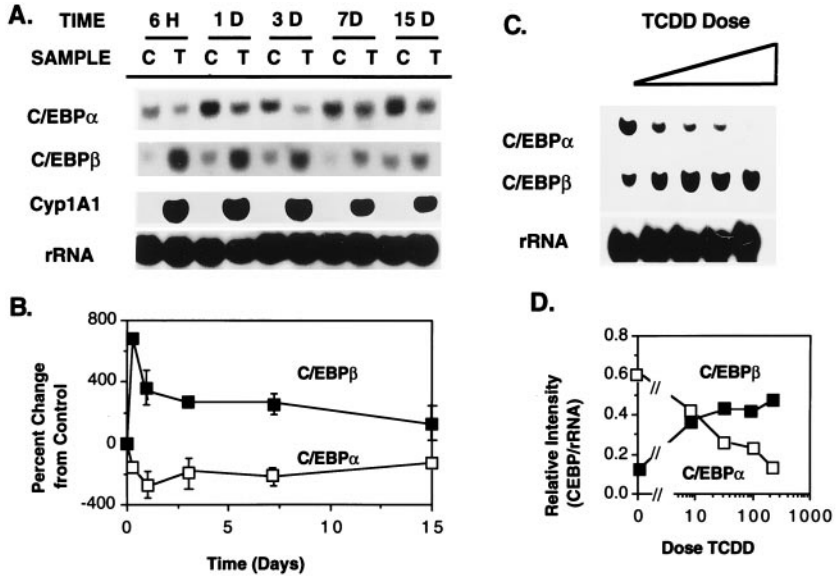

FIG. 2. Effect of TCDD on C/EBPα and C/EBPβ mRNA in liver. For experimental details, see the legend to Fig. 1. (A) Time course of the effect of TCDD (100 μg/kg). (B) Densitometric analysis of hybridization normalized to rRNA values. Each bar represents the average ± SD of three animals. (C) Effect of TCDD dose on expression of C/EBP mRNAs. From left, the doses were 0 (corn oil control), 10, 30, 100, and 300 μg/kg. (D) Densitometric analysis of the dose–response blot.

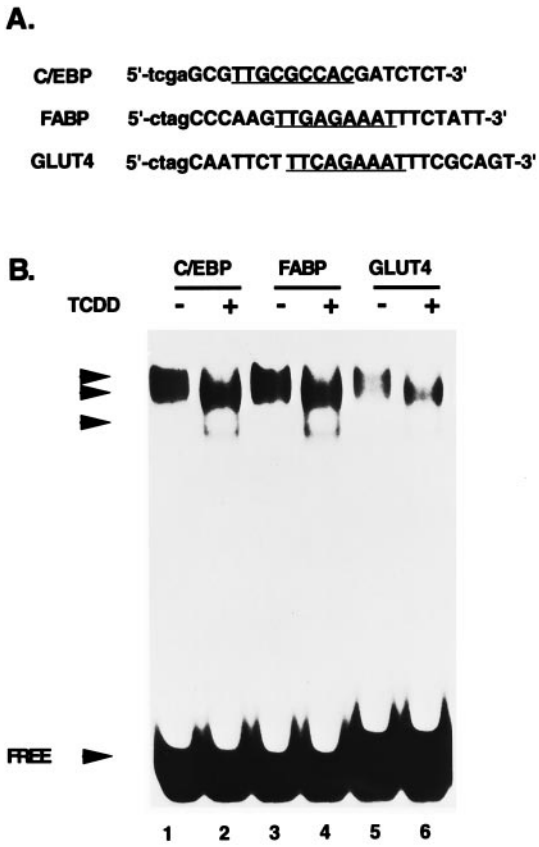

FIG. 3. Detection of C/EBP protein–DNA complexes by mobility shift assay. (A) Synthetic oligonucleotides used in the current study. The underlined bases correspond to the putative C/EBP binding sites contained within the 5′-regulatory sequences (capital letters) from the FABP [9], GLUT4 [10], and C/EBPα [28] genes. (B) Comparison of C/EBP binding to three oligonucleotides shown in part A. EMSA analysis was performed using hepatic nuclear extracts isolated 3 days after dosing the animals with 100 μg/kg of TCDD (lanes 2, 4, and 6) or acetone vehicle alone (lanes 1, 3, and 5) and ^{32}P-labeled FABP (lanes 1 and 2), C/EBP (lanes 3 and 4) or GLUT4 (lanes 5 and 6) oligonucleotides. The three upper arrowheads indicate specific C/EBP-containing complexes, while the lower arrowhead denotes the position of the free probe.

shift analysis. Because C/EBP recognizes and binds to divergent DNA sequences, we compared protein–DNA binding using three bona fide recognition sequences (Fig. 3A) from the promoters of *glut*4 [10], *fabp* [9], and *c/ebp*α [28]. The patterns of DNA–protein complexes formed using extracts from control livers were similar for all three oligonucleotide probes (Fig. 3B, lanes 1, 3, and 5). In addition, EMSA analysis revealed that nuclear extracts from TCDD-treated mouse livers resulted in the appearance of more rapidly migrating complexes (Fig. 3B, lanes 2, 4, and 6) that were not present in control samples. For further experiments, we chose the oligonucleotide from the C/EBPα promoter. Additionally we optimized electrophoresis conditions, as detailed in Materials and Methods, to achieve better resolution between the multiple retarded complexes.

To determine the temporal pattern of effect, we isolated nuclear extracts from liver samples of TCDD or vehicle-treated mice over a 7-day time course and conducted gel shift analysis (Fig. 4A). These experiments revealed a time-dependent shift in the pattern of complex formation. Specifically, extracts from all of the control animals (lanes 1, 3, and 5) showed a consistent pattern of two retarded bands (A and A′). In TCDD-treated animals, two additional bands (B and B′) appeared as early as day 1 and became increasingly intense over time. In particular, band B′, which was not visible in any of the control samples, was increased significantly after 7 days of treatment. The uppermost band (A) also appeared to decrease in TCDD-treated samples, whereas band A′ remained relatively constant over time in both control and treated extracts. A 100-fold excess of unlabeled C/EBP oligonucleotide abolished all of the bands, whereas a 100-fold molar excess of a nonspecific oligonucleotide had no effect on binding of any of the complexes, indicating that these bands represented specific binding to the oligonucleotide.

To identify the specific protein components of each retarded band, we incubated the protein:DNA mixture with antiserum against C/EBPα and/or C/EBPβ. The results of these supershift assays (Fig. 4B) showed that in control extracts both bands (A and A′) were quantitatively shifted by antiserum against C/EBPα, but not affected by antiserum against C/EBPβ, indicating that these complexes were composed of C/EBPα. Supershift analysis of TCDD-treated extracts, which contained bands A′, B, and B′, revealed that band A′ was shifted by C/EBPα antiserum, as in control extracts. In contrast, the two lower bands (B and B′) were mostly shifted by antiserum against C/EBPβ. A complete supershift of these bands occurred only in the presence of antibodies against both C/EBPα and C/EBPβ, suggesting that some heteromeric complexes may have co-migrated with the C/EBPβ homodimers. Thus, TCDD treatment resulted in the complete disappearance of a C/EBPα-containing complex (band A) and the appearance of two new complexes containing C/EBPβ (bands B and B′), consistent with a shift in the pool of DNA-binding competent forms of C/EBP proteins in the liver.

To determine if increased levels of C/EBPβ:DNA complexes in TCDD-treated liver samples were due to increased amounts of C/EBPβ protein, we performed immunoblotting with nuclear protein extracts. The blot shown in Fig. 4C indicates that only a single C/EBPβ species corresponding to a 37-kDa protein was detected with the antibody. Surprisingly, the steady-state level in the TCDD-treated sample was lower after 1 day of treatment, but increased after 7 days. Thus, increased levels of DNA-binding competent forms of C/EBPβ did not correlate with higher levels of the p37 form of C/EBPβ at early times after TCDD treatment.

Comparison of the Effects of TCDD on C/EBPα and C/EBPβ to Acute Phase Mediators

The effects of TCDD on C/EBPα and C/EBPβ mRNA levels and DNA binding activity were similar to the

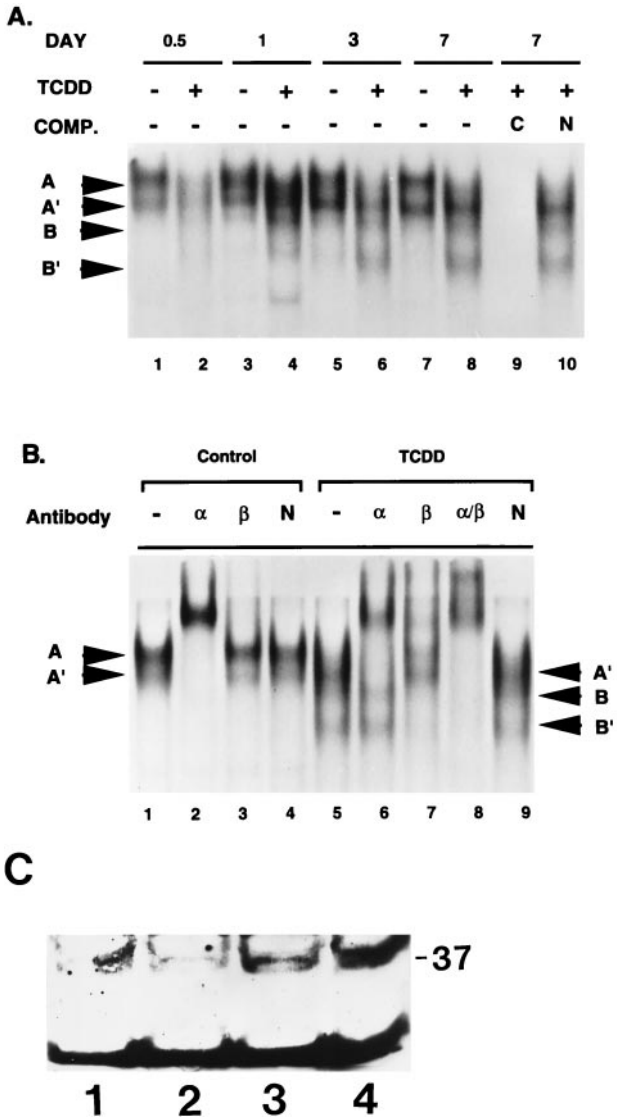

FIG. 4. TCDD-inducible changes in C/EBP binding in nuclear extracts. (A) Time course of effect. Nuclear extracts were prepared as described in Materials and Methods from the livers of vehicle-treated control or TCDD (100 μg/kg)-treated mice on the indicated days. Mobility shift assay was performed using a ^{32}P-labeled oligonucleotide from the C/EBPα promoter (see Fig. 3A) and 8 μg of protein. Electrophoresis through a 2–4% gradient nondenaturing acrylamide gel and autoradiography was performed as described. Lanes 9 and 10 show the effect of adding a 100-fold molar excess of either unlabeled competitor oligonucleotide (C) or a nonspecific oligonucleotide (N), respectively. These results are representative of two independent experiments. (B) Identification of retarded bands by supershift analysis. After incubation of nuclear extracts with ^{32}P-labeled probes, antibodies (1 μL) specific to C/EBPα, C/EBPβ, both antibodies (α, β, or 2 μL of nonimmune rabbit serum (N) containing the same amount of protein (1 μg) were added to the mixture and incubated for an additional 20 min. Electrophoresis was conducted as described in part A. Assignments of specific complexes based on this experiment are indicated on the side. (C) Immunoblot for steady-state levels of 37 kDa C/EBPβ protein in nuclear extracts from control and TCDD-treated animals. (Lane 1) control day 1, (lane 2) TCDD-treated day 1, (lane 3) control day 10, and (lane 4) TCDD-treated day 10. Densitometric readings were 102.2 ± 1.1, 48.2 ± 2.1, 174.4 ± 4.9, and 316 ± 9.8 (all in arbitrary units).

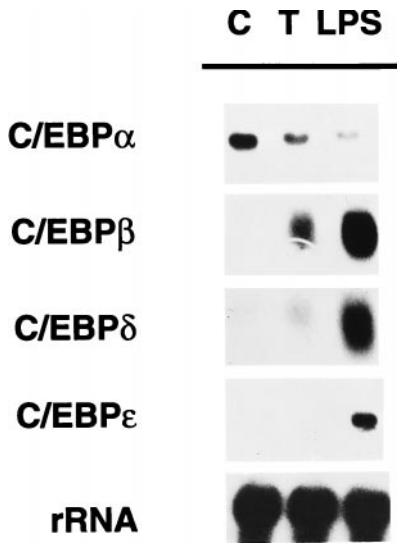

FIG. 5. Comparison of TCDD- and LPS-induced changes on C/EBP mRNAs. Northern blot analysis of RNA isolated from the liver of mice treated with corn oil:acetone vehicle, 100 μg/kg of TCDD, or 100 μg of bacterial LPS. Total RNA was isolated after 6 hr of treatment and analyzed by Northern blotting as described in Materials and Methods.

reciprocal regulation of these factors by inflammatory cytokines during the acute phase response [22]. Some of the acute toxicity of TCDD has been attributed to activation of inflammatory mediators; therefore, we compared the effects of TCDD to bacterial LPS on the induction of hepatic C/EBP-related genes: C/EBPα, C/EBPβ, C/EBPδ, and C/EBPε (Fig. 5). In agreement with previous findings [22, 23], we found that LPS suppressed C/EBPα and induced a large stimulation of C/EBPβ, C/EBPδ, and C/EBPε. While TCDD affected the steady-state levels of C/EBPα and C/EBPβ mRNA by 6 hr, it had no detectable effect on either C/EBPδ or C/EBPε at this time point (Fig. 5) or later times (data not shown).

Requirement of Functional AhR for Induction of C/EBPβ

To determine if the induction of C/EBPβ mRNA was mediated by the AhR, we compared the effect of TCDD in two mouse hepatoma cell lines [Hepa 1 (wild type) and ARNT-deficient hepatoma cells (c4 mutant)], which have been shown previously to be defective in specific TCDD-inducible DNA binding and gene activation by the AhR [2]. Induction of C/EBPβ in the wild-type cell line was concentration dependent (Fig. 6A) with an estimated EC_{50} of 0.1 nM (Fig. 6B). Induction occurred within 1 hr of treatment and reached a maximum induction by 6 hr (Fig. 6C). We detected C/EBPβ mRNA in both control and TCDD-treated ARNT-deficient cells; however, we did not observe TCDD-inducible expression of C/EBPβ mRNA in the mutant cell line (Fig. 6D, lanes 1 and 2). Induction in wild-type cells could be antagonized by pretreating them with 1 μM of α-naphthoflavone 1 hr prior to TCDD

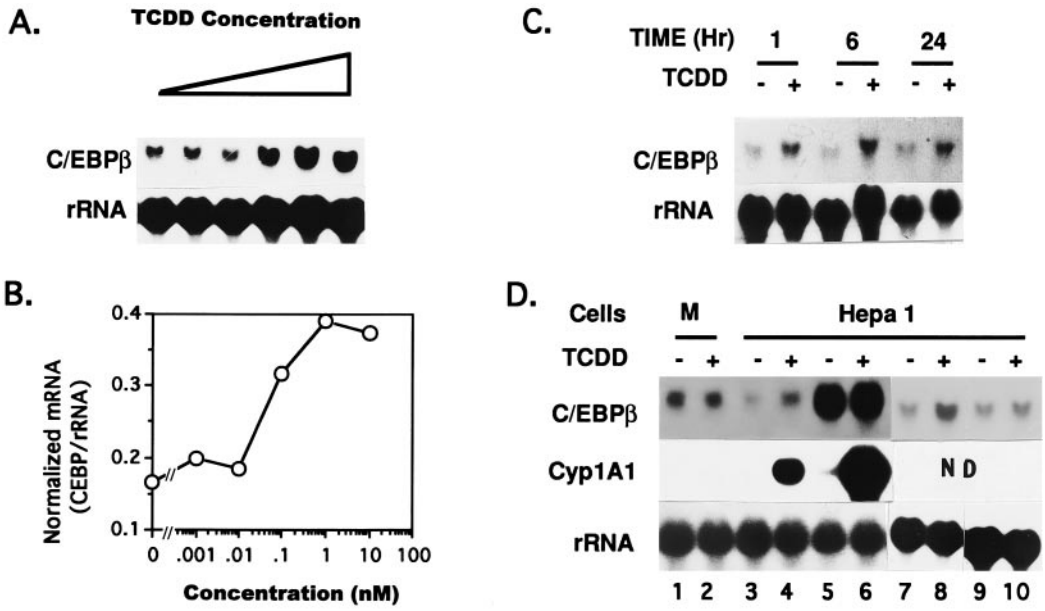

FIG. 6. Induction of C/EBPβ by TCDD in mouse hepatoma cells. (A) Effect of TCDD concentration on the induction of C/EBPβ in Hepa 1 (wild type) cells. Cells were treated with TCDD [from left the concentrations are 0 (DMSO control), 0.001, 0.01, 0.1, 1, and 10 nM of TCDD], and total RNA was isolated after 16 hr and subjected to Northern blot analysis. (B) Concentration–response curve. Amount of C/EBP probe hybridized to the membrane was quantitated by densitometry and normalized to the amount of rRNA hybridized to the same blot. Values are representative of two experiments. (C) Time course of induction by TCDD. Cells were treated as in part A, and their RNA was isolated at the indicated time points. A representative blot of two independent experiments is shown. The induction of C/EBPβ after normalization to rRNA was 180, 350, and 230% of control for 1, 6, and 24 hr., respectively. (D) AhR dependence of the induction of C/EBPβ. ARNT-defective mutant (M) (lanes 1 and 2) or wild-type hepatoma (Hepa 1) cells (lanes 3 through 10) were treated for 16 hr with or without TCDD (10 nM). Lanes 3 and 4 represent Hepa 1 cells treated with solvent only or TCDD, respectively. In lanes 5 and 6, cycloheximide was added 1 hr prior to TCDD, and the cells were harvested after 3 hr of total incubation. In lanes 7–10, cells were exposed for 6 hr to DMSO (lanes 7 and 9) or 10 nM of TCDD (lanes 8 and 10) and in the absence (lanes 7 and 8) or presence (lanes 9 and 10) of 1 μM of α-naphthoflavone. After hybridization to C/EBPβ, the blot were stripped and rehybridized with a cDNA probe for mouse cytochrome P4501A1 (Cyp1A1). ND = not determined.

addition (Fig. 6D, lanes 9 and 10). These data indicate that induction of C/EBPβ required the presence of functional AhR complexes. To test whether this effect was a direct effect that did not require protein synthesis, we pretreated Hepa 1 cells with cycloheximide prior to the addition of TCDD; however, in both control and TCDD-treated samples, a superinduction of C/EBPβ mRNA was observed (Fig. 6D, lanes 5 and 6). We were unable to ascertain the AhR dependence of the suppression of C/EBPα by TCDD using this system because C/EBPα mRNA was not detected in either cell line (data not shown), in agreement with previous findings that its expression is limited to well-differentiated hepatocytes [29].

DISCUSSION

The role of C/EBPα in the regulation of genes involved in nutritional homeostasis has been documented using both biochemical and genetic analysis [8, 21]. Recent analyses of two independently derived *c/ebp*α-knockout mouse strains revealed significant impairments in the ability to synthesize and store metabolic fuels [13, 30]. Specifically, these mice showed abnormally small depots of adipose tissue, suppressed fatty acid synthesis and glycogen storage, and delayed expression of genes involved in gluconeogenesis. Intriguingly, many of the symptoms exhibited by *c/ebp*α-deficient mice are similar to the symptoms observed in TCDD-treated animals. Expression of several adipogenic genes are selectively decreased in the adipose tissue of TCDD-treated mice [4]. Moreover, there was a gradual depletion of fat stores with a decreased ability to synthesize fatty acid [6]. In the liver, not only was fat synthesis decreased by TCDD, but gluconeogenesis was significantly lower than in control animals [27, 31]. It is an attractive hypothesis that a decrease in C/EBPα could mediate some of the metabolic alterations in these two tissues. In the current study, we report a rapid decrease in the level of C/EBPα and a reciprocal increase in C/EBPβ in the adipose and liver of mice exposed to TCDD.

The current results demonstrated a rapid change in levels of C/EBP mRNA in response to TCDD treatment *in vivo*. Induction of C/EBPβ mRNA is dependent upon functional AhR complexes, as determined by the ability to block the induction with α-napthoflavone in Hepa 1 cells and by a lack of induction in ARNT-deficient Hepa cells. The absence of response of C/EBPβ mRNA to TCDD in ARNT-deficient cells, however, could mean either that the process of AhR-ARNT dimerization and probably a complete gene transactivation function of the AhR is necessary to express this effect of TCDD, or that these ARNT-

deficient cells have some functional alteration from normal cells that prevents them from responding to TCDD. We have observed previously that two similar preadipocyte cell lines, 3T3-L1 and 3T3-F442A from mouse fibroblast cells, responded to TCDD in a very different manner despite their similar response capabilities in terms of CYP1A1 induction: i.e. in the former cell line, C/EBP mRNA and the entire process of adipogenesis were not responsive to TCDD, but those of the latter were quite responsive to the same treatment of TCDD (Liu PCC, Phillips MA, and Matsumura F, unpublished data). Thus, it appears to be prudent to withhold judgement until we accumulate more data on the characteristics of the ARNT-deficient Hepa 1 cells. Surprisingly, the increased levels of mRNA in liver samples from treated animals did not correlate with increased levels of the p37 kDa form of the protein on day 1, but correlated well at a later time point. Whether smaller translational products from the C/EBP mRNA, such as p21 (LIP), were produced and not detected by the specific antibody used in these experiments is not known.

C/EBP proteins share a common leucine zipper domain, which facilitates the formation of homo- and heterodimeric DNA-binding complexes [14–19]. Using gel shift analysis, we found a time-dependent transition in the pattern of C/EBP complexes bound to an oligonucleotide containing a native C/EBP recognition element. In particular, TCDD treatment resulted in the disappearance of the uppermost band (Fig. 4, band A) corresponding to C/EBPα homodimers. A concomitant appearance of two new bands (bands B and B′), which corresponded to C/EBPβ homodimers and C/EBPα/β heterodimers, was observed in the TCDD-treated samples. Thus, the consequence of TCDD treatment was an overall shift in the distribution of DNA-binding competent forms of C/EBP from only C/EBPα in control animals to a significant increase in C/EBPβ-containing complexes in treated animals. Overall, the levels of C/EBPβ protein did not increase substantially in spite of increased DNA binding by C/EBPβ. One possibility is that post-translational modifications to existing C/EBPβ proteins resulted in activation of DNA binding. Phosphorylation by cAMP-dependent protein kinase or protein kinase C has been shown to activate the p37 form of C/EBPβ [32, 33]. Since C/EBPα and C/EBPβ differ in their putative transactivation domains, it is likely that these complexes mediate different biological responses. Thus, these experiments reveal two potential levels of regulation of C/EBPβ by TCDD. First, TCDD induces the mRNA of C/EBPβ in an AhR-dependent manner. Second, increased DNA-binding competent forms of C/EBPβ accumulate in TCDD-treated animal livers as a result of post-translational modification that is independent of new protein synthesis.

The functional significance of decreased C/EBPα and increased C/EBPβ in response to TCDD remains to be determined; however, this pattern of change is similar to the known response of these genes to physical tissue injury, such as partial hepatectomy [34], and exposure to mediators of injury, such as inflammatory cytokines [22]. In both cases, induction of C/EBPβ is coupled to decreased expression of C/EBPα. Analysis of C/EBP recognition sites in the gene promoters of several acute phase proteins such as α_1-acid glycoprotein revealed that LPS-induced inflammation correlates to a replacement of C/EBPα by C/EBPβ [35]. Differential control of gene expression by C/EBPs could not be fully accounted for by simple replacement of one C/EBP type for another but was also influenced by interactions with proximal *cis*-acting elements and their corresponding DNA binding proteins. A shift in the C/EBPα to C/EBPβ ratio may also indicate an overall change in cellular state. Expression of C/EBPα is generally limited to well-differentiated cells [12, 36]. Reduced levels of C/EBPα relative to C/EBPβ is associated with dedifferentiation of adipocytes by TNFα [37] and dedifferentiation of primary hepatocytes in culture under conditions favoring cell proliferation [29]. These data suggest that the ratio of C/EBPα to C/EBPβ may be one important factor in regulating cellular programs of proliferation and differentiation, but specific responses in gene expression may also depend on promoter-specific sequences and interactions between C/EBP proteins and other transcription factors.

As there are similarities between acute TCDD effects and exposure to TNFα, one possible mechanism for the alterations in C/EBPs by TCDD is that they occur subsequent to TNFα activation. TNFα hypersensitivity has been observed in mice dosed with TCDD and then challenged with LPS [38] or sheep red blood cells [39]. In one study, cotreatment of mice with anti-TNFα antibodies or dexamethasone protected them from the acute effects of TCDD [40]. These data indicate that inflammatory cytokines may modulate some symptoms of TCDD's acute toxicity and hyperinflammation, although a direct activation of cytokines by TCDD has not been reported [39]. Our data, on the other hand, showed that LPS and TCDD had different effects on the expression of C/EBPs (Fig. 5). Specifically, C/EBPδ, which showed the strongest response to TNFα in mouse liver [22], was only minimally induced by TCDD *in vivo* and in Hepa 1 cells (Fig. 5 and data not shown). Alternatively, TCDD may alter C/EBPβ activity through its effects on signal transduction pathways involved in the endogenous regulation of this factor. A likely candidate is cyclic AMP (cAMP), which has been shown to induce the expression of C/EBPβ in adipocytes [14, 41]. In view of our previous report that TCDD could rapidly enhance cAMP-dependent protein phosphorylation in adipose tissue *in vivo* [42], we propose that TCDD may indirectly increase expression of C/EBPβ by altering cAMP levels, at least in adipocytes. Moreover, this model would be consistent with activation of C/EBPβ DNA binding by phosphorylation via cAMP-dependent kinases. Increased levels of C/EBPβ could contribute to TCDD-induced hypersensitivity to antigens because C/EBPβ itself has been shown to regulate the expression of several cytokines [43, 44].

The alterations by TCDD of C/EBPα and C/EBPβ may provide a molecular basis for understanding some of the effects of TCDD on intermediary metabolism. We propose

that rapid changes in C/EBPα and C/EBPβ after exposure to TCDD may be an important trigger for the coordinated shift in metabolic homeostasis and an early indicator of changes in overall cellular programs in these tissues. We are working to clarify the mechanism by which TCDD regulates these transcription factors. Understanding the regulation and interactions of such nuclear factors by TCDD will provide insight into the ability of these chemicals to cause complex changes in cellular programs of growth and maintenance.

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