Dioxin Increases C/EBPβ Transcription by Activating cAMP/Protein Kinase A* 

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The environmental pollutant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD = dioxin) has been shown to increase the expression of C/EBPβ. The modulated expression of C/EBPβ has been suggested to be associated with toxic responses of TCDD such as wasting syndrome, diabetes, and inhibition of adipocyte differentiation. This study focused on the regulatory mechanism of TCDD-mediated transcriptional activation of C/EBPβ. Elevated C/EBPβ mRNA and protein levels in mouse embryonic fibroblasts (C3H10T1/2) and in mouse hepatoma cells (Hepa1c1c7) were correlated with increased binding affinity of the C/EBPβ protein. Transfection studies with different deletion constructs of the CCAAT/enhancer-binding protein promoter indicated that a small region located 60–120 bp upstream of the start site of transcription is required for activation of the C/EBPβ gene by TCDD in both cell lines tested. Further analysis by mutation constructs of the C/EBPβ promoter demonstrated that activation of the C/EBPβ promoter is mediated through incomplete cAMP-response element-binding protein (CREB) sites located close to the TATA box of the C/EBPβ gene. The protein kinase A (PKA) inhibitor H89 completely blocks the TCDD-dependent effect on C/EBPβ promoter activity, indicating that TCDD activates CREB binding via a cAMP/PKA pathway, which is supported by the increased cAMP level and PKA activity observed after TCDD treatment. Gel shift analyses demonstrated that CREB itself binds to the putative CREB motif that mediates the TCDD-dependent effect on C/EBPβ gene transcription. Cotransfection experiments with CREB and PKA expression plasmids further supported our conclusions that the TCDD-dependent effect on C/EBPβ transcription is mediated via PKA-dependent CREB activation.

C/EBPβ is a member of the C/EBP family containing a highly conserved, basic leucine zipper domain responsible for dimerization and DNA binding (1). At least six members of the C/EBP family (C/EBPα, C/EBPβ, C/EBPγ, C/EBPδ, C/EBPε, and C/EBPζ) have been characterized (2). Besides its importance in mediating inflammatory signals through the interleukin 6 pathway, C/EBPβ is known to play a crucial role in numerous cellular processes, such as liver growth and the differentiation of adipocyte and hematopoietic cells (3). These processes can be modulated by post-transcriptional and transcriptional regulation of the C/EBPβ gene during physiological and pathophysiological conditions through hormones, cytokines, nutrients, and toxic chemicals (4). There is also evidence that C/EBPβ is responsible for mediating cellular responses induced by environmental stressors like ozone (5), hypoxia (6, 7), H2O2, and asbestos (8).

Induction of C/EBPβ in mouse adipose tissue and liver after exposure to the environmental pollutant TCDD was described earlier by Liu et al. (9). However, the molecular mechanism by which TCDD induces C/EBPβ expression remained unclear. In contrast, the mechanisms of transcriptional activation of genes for drug-metabolizing enzymes like those encoding CYP1A1, CYP1B1, glutathione S-transferase Ya, and NAD(P)H:menadione oxidoreductase by TCDD are well documented (10). TCDD-dependent activation of these genes involves binding of a heterodimer consisting of the aromatic hydrocarbon receptor (AhR) and the nuclear translocator (Arnt) protein to xenobiotic-responsive elements (XREs) located in the 5‘-flanking regions of the respective genes. In the absence of an activating ligand, the AhR resides in the cytosol as a complex with heat-shock protein 90. In addition to heat-shock protein 90, other proteins seem to be involved in the formation of cytosolic high affinity ligand-binding form(s) of the AhR (11, 12). The co-chaperone p23 plays a part in the activation process of AhR to the DNA-binding complex (13) and to an immunophilin-like protein, ARA-9/XAP-2/AIP (11, 12, 14, 15).

Promoters of the human, mouse, rat, chicken, and Xenopus laevis C/EBPβ genes have already been characterized (16–20). A complete XRE-binding sequence is located at position –68 to –64, but so far it is unclear whether recruitment of the AhR/Arnt dimer to the XRE-binding site located in the C/EBPβ promoter is necessary for transcriptional activation of C/EBPβ by TCDD. Two potential binding sites for CREB near the TATA box have been already identified as the important elements regulating the expression of C/EBPβ (19). The CREB-like sequences play a pivotal role for interleukin-6-mediated induction of C/EBPβ transcription during the acute phase response in

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EMSA, electrophoretic mobility shift assay; H89, N-(2-[(p-bromocinnamyl)amino]-ethyl)-5-isouquinolinesulfonamide-2HCl, wt, wild type; mut, mutant; LAP, liver-enriched transcriptional activator protein.
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A sustained elevation of C/EBPβ level was also found in adipocytes after treatment with TCDD, which consequently leads to an inhibitory effect on adipocyte differentiation (21, 22). Recently we reported that TCDD-induced transcription of the cyclooxygenase-2 gene is mainly mediated through increased binding of C/EBPβ on the cyclooxygenase-2 promoter (22). It has been shown to play an important role in gene activation-mediated toxic responses caused by TCDD, the investigation on the molecular mechanism underlying the TCDD-mediated activation of C/EBPβ has been undertaken.

By using deletion and mutation constructs of the C/EBPβ promoter, our present analysis revealed that TCDD induces C/EBPβ gene transcription via protein kinase A through a C/EBP motif located in its promoter in close proximity to the TATA box.

EXPERIMENTAL PROCEDURES

Chemicals—TCDD (>99% purity) was originally obtained from Dow Chemicals Co. (Midland, MI). Dimethyl sulfoxide (MeSO) was obtained from Aldrich. [γ-32P]ATP (6000 Ci/mmol) was purchased from ICN (Costa Mesa, CA). Antibodies for C/EBP isoforms were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide-2HCl (H89) was purchased from Calbiochem. Other molecular biological reagents were purchased from Qiagen (Valencia, CA) and Roche Applied Science.

Cell Culture, Transfection Experiments, and Luciferase Assays—C3H10T1/2 cells were purchased from ATCC (Manassas, VA) and maintained in basal modified Eagle’s medium. The mouse hepatoma cell line Hepa1c1c7 was a gift from Dr. O. Hankinson (University of California, Los Angeles) and maintained in a minimum essential medium (Invitrogen). Both cell culture media contained 10% fetal bovine serum (Gemini, Woodland, CA) and 100 units of penicillin and 100 μg/ml streptomycin. For transfection transfection experiments C3H10T1/2 or Hepa1c1c7 cells were plated in 6-well culture plates (5 × 104/well) and medium was added before transfection complexes were applied drop by drop to the cells. Cells were transiently transfected for 16 h by using 10 μl per well Effectene (Qiagen, Valencia, CA) with 0.5 μg per well of respective luciferase reporter constructs of the LAP/C/EBPβ promoter according to the manufacturer’s instructions. To control the transfection efficiency, cells were cotransfected with 0.1 μg per well β-galactosidase reporter construct. After transfection, cells were incubated for 24 h with 10 nM TCDD, 10 μM forskolin (FSK), or 0.1% MeSO (control). Cells were washed twice with PBS and lysed with 300 μl of passive lysis buffer. Luciferase activities were measured with the Luciferase Reporter Assay System (Promega, Madison, WI) using a luminometer (Berthold Lumat LB 9501/16, Pittsburgh, PA). Relative light units were normalized to β-galactosidase activity and to protein concentration, using Bradford dye assay (Bio-Rad). Experiments were repeated three times. Three wells of cells were analyzed per experiment.

The LAP/C/EBPβ deletion and mutation luciferase reporter constructs containing the 5′-upstream regulatory sequence of the C/EBPβ promoter and the PAK( wt) and PAK( mut) expression vectors were used as described earlier (19, 24). The CREB expression plasmid and the dominant negative CREB-A vector were kindly provided by Dr. M. Montminy (Salk Institute, La Jolla, CA) and Dr. C. Vinson (National Institutes of Health, Bethesda, MD). Quantitative Real Time Reverse Transcriptase-PCR—Total RNA was isolated from C3H10T1/2 and Hepa1c1c7 cells using a high pure RNA isolation kit (Roche Applied Science), and cDNA synthesis was carried out as described previously (23). Quantitative detection of glyceraldehyde-3-phosphate dehydrogenase (GapDH) and C/EBPβ was performed using a LightCycler Instrument (Roche Diagnostics) using the LightCycler analysis software according to the manufacturer’s instructions. Calculation of cAMP concentrations (pmol/mg protein) is based on a standard curve for each experiment. PKA activity was determined in cell lysates using a PKA assay kit (Upstate Biotechnology Inc., Lake Placid, NY). Briefly, C3H10T1/2 cells in 10-cm culture dishes were washed once with ice-cold PBS and scraped into 0.5 ml of 50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 0.1% Triton X-100. Samples were passed through a 21-gauge needle, and insoluble material was removed by centrifugation at 800 × g for 2 min. The supernatant was assayed in the presence of 50 μM of [γ-32P]ATP using synthetic PKA C substrates. Total PKA activity was measured by the addition of 2 μM cAMP; basal activity, a measure of active PKA at the time of harvest, was measured in the absence of exogenous cAMP. Unincorporated 32P was removed by spotting samples on phosphocellulose paper and washing three times with dilute H3PO4 (0.75%) and one acetone wash. The amount of 32P was quantified by scintillation counting. Nonspecific activity, determined in the presence of PKA inhibitor peptide, was subtracted.

To determine whether effects on cAMP level and PKA activity are coupled to pertussis toxin-sensitive G proteins, cells were pretreated with 100 ng/ml PTX (Calbiochem) for 16 h. To examine AhR-dependent effects, cells were treated with 100 ng/ml PTX (Sigma) and 10 nM TCDD. 7-Ketocholesterol has been identified as an endogenous modulator that inhibits transactivation by the AhR through competitive binding against xenobiotic ligands (25).

Antibodies and Western Blotting—Polyclonal rabbit antisera against C/EBPβ (C-19) and a horseradish peroxidase-conjugated secondary antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Nuclear extracts were separated on a 10% SDS-polyacrylamide gel and blotted onto a polyvinylidene difluoride membrane (Immunoblot, Bio-Rad). The antigen-antibody complexes were visualized using the chemiluminescence substrate SuperSignal®, West Pico (Pierce), as recommended by the manufacturer. For quantitative analysis of specific bands, chemiluminescent bands were quantified using a ChemiImager™4400 (Alpha Innotech Corp., San Leandro, CA).

Gel Mobility Shift Assays (EMSAs)—Nuclear extracts were isolated from murine C3H10T1/2 and Hepa1c1c7 cells according to Dennler et al. (26). In brief, 5 × 106 cells were treated with 10 nM TCDD for 1–3 h and harvested in Dulbecco’s PBS containing 1 mM phenylmethylsulfonyl fluoride and 0.05 μg/ml aprotinin. After centrifugation the cell pellets were gently resuspended in 1 ml of hypotonic buffer (20 mM HEPES, 20 mM NaF, 1 mM Na2VO4, 1 mM Na3VO4, 1 mM EDTA, 1 mM dithiothreitol, pH 7.9, and 1 μg/ml each leupeptin, aprotinin, and pepstatin). The cells were allowed to swell on ice for 15 min and then homogenized with 6 strokes of a Dounce homogenizer. After centrifugation at 16,000 × g, nuclear pellets were resuspended in 300 μl of ice-cold high salt buffer (hypotonic buffer with 420 mM NaCl and 20% glycerol). The samples were passed through a 21-gauge needle and stirred for 30 min at 4 °C. The nuclear lysates were microcentrifuged at 16,000 × g for 20 min, aliquoted, and stored at −70 °C. Protein concentrations were determined by the method of Bradford.

For EMSA double-stranded oligonucleotides were used containing the consensus sequence (underlined) for the C/EBP (5′-TGCGAGATTGCACATCGAATTCGAGTGCAGGGAAG-3′) (Santa Cruz Biotechnology, Santa Cruz, CA) or the incomplete CREB (5′-GGCGGCCGCGCAATTCGACCCGGCGCCGA-3′)-binding sites located between nucleotides −123 to −99 of the LAP/C/EBPβ promoter. DNA-protein binding reactions were performed in a total volume of 20 μl containing 15 μg of nuclear protein, 40,000 cpm of a double-stranded oligonucleotide, 25 mM Tris buffer, pH 7.5, 50 mM NaCl, 1 mM MgCl2, 1 mM EDTA, 0.5 mM dithiothreitol, 5% glycerol, and 1 μg of poly(dI-dC). The samples were incubated at room temperature for 20
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RESULTS

Induction of C/EBPβ by TCDD in C3H10T½ and Hepa1c1c7 Cells—Time response studies were conducted in C3H10T½ and Hepa1c1c7 cells. The earliest time point of a significant induction of C/EBPβ mRNA was observed after 1 h of treatment with 10 nM TCDD in C3H10T½ cells as shown by real time reverse transcriptase-PCR (shown as squares in Fig. 1). In Hepa1c1c7 cells (circles in Fig. 1) TCDD caused a significant induction of C/EBPβ after 2 h. In both cell lines C/EBPβ mRNA was maximally (2.5–3.5-fold) induced after 6 h. The mRNA level of C/EBPβ was still elevated at 24 h (Fig. 1) in both cell lines tested. All values were compared with control cells that received 0.1% Me2SO. To verify the effect of Me2SO, we compared the vehicle controls with medium controls (untreated cells). No significant effect of 0.1% Me2SO was observed in any of the cell lines tested. All values were compared with control cells as shown by supershift analyses. The levels of C/EBPβ mRNA were treated for 0.5, 1, 2, 4, 6, 12, and 24 h with 10 nM TCDD; control cells received only the vehicle solvent (0.1% Me2SO). Quantitative detection of C/EBPβ mRNA was performed using real time reverse transcriptase-PCR. Values for C/EBPβ mRNA expression are normalized to the expression of GAPDH. The induced mRNA expression is given relative to the values of Me2SO-treated vehicle controls at each time point. *, significantly different from control (p < 0.005).

TCDD Increases Protein Level and DNA Binding Activity of C/EBPβ—To test whether elevated mRNA levels correspond with an increased accumulation of the C/EBPβ protein in the nucleus, Western blot analyses were performed. In nuclear extracts of C3H10T½ cells, we detected strong staining of a 38-kDa band (Fig. 2, upper arrow), which is characteristic of the full-length isoform of C/EBPβ (27). In Hepa1c1c7 cells one additional band with a lower molecular weight (Fig. 2, lower arrow) was detected, which is probably related to the 35-kDa liver-enriched transcriptional activator protein (LAP) isoform of the C/EBPβ gene (27). TCDD treatment resulted in a 3-fold increase of the 38-kDa band at 6 h as well as 16 h in C3H10T½ cells. In Hepa1c1c7 cells band intensities for both C/EBPβ isoforms were 3-fold increased at 6 h and 2-fold increased at 16 h after TCDD treatment (Fig. 2).

In order to determine whether the elevated protein level leads to an enhanced DNA binding activity of C/EBPβ, EMSAs were performed. We found an increased binding activity in nuclear extracts of C3H10T½ (Fig. 3) as well as Hepa1c1c7 cells (Fig. 4) after TCDD exposure. After treatment for 6 and 16 h with 10 nM TCDD, the binding activity of C/EBP was significant induction of C/EBPβ were treated for 0.5, 1, 2, 4, 6, 12, and 24 h with 10 nM TCDD; control cells received only the vehicle solvent (0.1% Me2SO). Quantitative detection of C/EBPβ mRNA was performed using real time reverse transcriptase-PCR. Values for C/EBPβ mRNA expression are normalized to the expression of GAPDH. The induced mRNA expression is given relative to the values of Me2SO-treated vehicle controls at each time point. *, significantly different from control (p < 0.005).
2.0-fold elevated in C3H10T1/2 (Fig. 3B, lanes 2 and 4). The supershift analyses using an anti-C/EBPβ antibody showed that binding activity of the C/EBPβ isoform is about 2.0-fold enhanced in C3H10T1/2 cells treated for 6 or 16 h (Fig. 3B, lanes 6 and 8). Similar results are obtained from EMSA with nuclear extracts from Hepa1c1c7 cells (Fig. 4). C/EBPβ-specific complex formation in Hepa1c1c7 cells increased 2-fold in cells treated with 10 nM TCDD for 6 or 16 h (Fig. 4B, lanes 2 and 4). C/EBPβ-specific complex formation was confirmed by supershift experiments using a C/EBPβ-specific antibody (Fig. 4A, lanes 5–8).

Effect of TCDD on C/EBPβ Promoter Deletion Constructs—To identify potential DNA motifs possibly responsible for TCDD-mediated C/EBPβ activation, transfection studies with different C/EBPβ promoter deletion constructs were performed in C3H10T1/2 and Hepa1c1c7 cells, respectively. The whole 5’-flanking region starting 16 bp downstream from the start site of transcription and compromising −1.4 kb was linked to a luciferase reporter gene. Increasing 5’-terminal deletions were introduced in this promoter fragment as depicted in Fig. 5A. The nomenclature of the deletion constructs (LAPPRO 1, 2, 7, 8, and 9 for 16 h, cells were treated with 10 nM TCDD (shaded bars) or 0.1% Me2SO (open bars) for 24 h. Relative luciferase activity units are given as mean values of triplicates as a result of three independent experiments. * significantly different from control cells (p < 0.05).
TCDD-dependent activation of the mutations in the CREB1 or CREB2 site significantly abolished the H11002 core sequence of the CREB1 or CREB2 site at position 511 by using two reporter constructs containing a mutation in the CREB1 or CREB2 site. This transfection experiment indicated that the binding elements located between nucleotides −121 and −71 in the C/EBPβ promoter mediate the TCDD-dependent effect on gene transcription. Additionally, our results suggest that the putative XRE-binding site located at nucleotide −68 to −64 of the 5′-upstream regulatory sequence (Fig. 6A, underlined) seems not to be involved in the TCDD-dependent effect on C/EBPβ gene transcription.

TCDD Mediates C/EBPβ Activation via a CREB-binding Motif—Our previous study identified two CREB-binding sites essential for controlling the transcriptional activity of C/EBPβ (24). The two CREB-binding sites are located at positions −111 to −107 (CREB1, Fig. 6B, boldface letters) and position −65 to −61 (CREB2, Fig. 6B, boldface letters) in the C/EBPβ promoter. Thus, these sequences of the promoter region were investigated by using two reporter constructs containing a mutation in the core sequence of the CREB1 or CREB2 site at position −109 to −107 (LAPPRO 8 M1) or position −65 to −61 (LAPPRO 8 M2) as illustrated in Fig. 6B.

Transfection studies in C3H10T½ cells revealed that both mutations in the CREB1 or CREB2 site significantly abolished TCDD-dependent activation of the C/EBPβ promoter, suggesting an important role for both CREB sites in the TCDD-induced activation of C/EBPβ (Fig. 6C). Additionally, each mutation of the CREB1 or CREB2 site caused a significant reduction in basal promoter activity.

Protein Kinase A Mediates the TCDD-dependent Increase in C/EBPβ Promoter Activity via CREB—To investigate the role of CREB in mediating TCDD-dependent C/EBPβ transcription, we performed cotransfection studies in C3H10T½ cells with increasing concentrations (100–400 ng/ml) of a CREB expression vector with the reporter construct LAPPRO 8. Cotransfection experiments with the CREB expression vector result in a dose-dependent increase (3–5-fold) of the TCDD-mediated promoter activity (Fig. 7). Transfection studies with a dominant negative CREB expression plasmid (A-CREB) that prevents DNA binding of wild-type CREB significantly blocked the TCDD- and FSK-mediated activation of LAPPRO 8 by more than 50% (Fig. 8), supporting the relevance of CREB in mediating the transcriptional activation of the C/EBPβ gene by TCDD.

Next, transfection studies in the presence of the PKA inhibitor H89 were performed, because phosphorylation of CREB by PKA is important to activate transcription via CRE. The TCDD- and FSK-mediated induction of the luciferase reporter activity was completely abolished by the presence of 0.5 μM H89 (Fig. 8). Furthermore, C3H10T½ cells were cotransfected with a PKA wild-type (wt) or PKA mutant (mut) expression vector together with the C/EBPβ reporter construct LAPPRO 8 to get additional evidence for the role of PKA in mediating the induction of C/EBPβ. As shown in Fig. 9, cotransfection with the PKA wt expression vector increased the TCDD- and FSK-mediated promoter activity by about 150%, whereas the cotransfection with the PKA mutant vector significantly reduced the TCDD-mediated activation of LAPPRO 8 (Fig. 9). These results are clearly indicating the importance of CREB activation via PKA.

TCDD Increases the Level of cAMP and Protein Kinase A Activity—Because the results of the transfection studies were indicating the involvement of PKA in the induction of C/EBPβ, we measured the concentration of cAMP and the activity of PKA. As shown in Table I, the level of cAMP in C3H10T½ cells was about 2-fold increased to 2.7 pmol of cAMP per mg of protein after treatment with 10 nM TCDD for 30 min compared with vehicle (0.1% Me2SO)-treated control cells; similar results were obtained after 60 min of TCDD treatment (Table I). Preincubation of cells with PTX for 16 h
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![Figure 7](image)

**Fig. 7. Cotransfection with CREB expression plasmid.** C3H10T½ cells were transiently transfected with wild-type LAPPRO 8 and cotransfected with increasing amounts (100–400 ng/ml) of a CREB expression plasmid. Cells were cotransfected for 16 h then treated with 10 nM TCDD (shaded bars) or 0.1% Me₂SO (open bars). Relative luciferase activity units are given as mean values of triplicates as a result of three independent experiments. *, significantly different from control cells (p < 0.05); **, significantly higher than TCDD-treated LAPPRO 8 transfected cells (p < 0.05). ****, significantly higher than PCDD-treated cells cotransfected with LAPPRO 8 and 100 ng/ml CREB (p < 0.05).

![Figure 8](image)

**Fig. 8. Transient transfection of LAPPRO 8 in presence of the PKA inhibitor H89 or a dominant negative CREB expression plasmid A-CREB.** C3H10T½ cells were transiently transfected with wild-type LAPPRO 8 and treated with 10 nM TCDD (shaded bars) or 10 μM forskolin (FSK, striped bars) with or without pretreatment for 15 min with 0.5 μM H89. Control cells received 0.1% Me₂SO (open bars). Cotransfection of C3H10T½ cells was performed with LAPPRO 8 and the dominant negative CREB expression vector A-CREB. Relative luciferase activity units are given as mean values of triplicates as a result of three independent experiments. *, significantly different from control cells (p < 0.05); **, significantly lower than only TCDD- or FSK-treated cells (p < 0.05).

The activity of PKA was measured after addition of 10 nM TCDD or 10 μM FSK for 60 and 150 min in C3H10T½ cells. The basal PKA activity was about 4- and 7-fold increased after 60 min of treatment with TCDD or FSK, respectively (Table II). After 150 min, the basal PKA activity was diminished in all groups; however, treatment of TCDD or FSK still increased PKA activity by 5- or 7-fold, respectively, compared with vehicle (0.1% Me₂SO) controls. Total PKA activity was also increased by TCDD or FSK treatment for 60 min by about 1.5- and 1.8-fold, respectively. After 150 min of TCDD or FSK

![Figure 9](image)

**Fig. 9. Cotransfection with PKA wild-type and PKA mutant expression plasmids.** C3H10T½ cells were transiently transfected with wild-type LAPPRO 8 and cotransfected with 200 ng/ml of a PKA wild-type (wt) expression plasmid or 200 ng/ml of a PKA mutant (mut) expression plasmid. Cells were cotransfected for 16 h and then treated with 10 nM TCDD (shaded bars) or 10 μM forskolin (FSK, open bars right). Control cells received 0.1% Me₂SO (open bars left). Relative luciferase activity units are given as mean values of triplicates as a result of three independent experiments. *, significantly different from control cells (p < 0.05); **, significantly higher than only LAPPRO 8 transfected cells treated with TCDD or FSK (p < 0.05).

### Table I

| Treatment | cAMP level (pmol/mg protein) |
|-----------|-----------------------------|
| 30 min    |                             |
| Control   | 1.2 ± 0.3                   |
| TCDD      | 2.7 ± 0.5*                  |
| PTX + TCDD| 0.8 ± 0.2                   |
| 7-KC + TCDD| 1.9 ± 0.3                  |
| FSK       | 12.2 ± 2.6*                 |
| 60 min    |                             |
| Control   | 1.4 ± 0.2                   |
| TCDD      | 2.6 ± 0.4*                  |
| PTX + TCDD| 0.5 ± 0.1                   |
| 7-KC + TCDD| 1.6 ± 0.5                 |
| FSK       | 11.2 ± 3.1*                 |

*Values are the mean ± S.D. of triplicate samples and are significantly different from control (p < 0.005).

### Table II

| Treatment | PKA activity (pmol/min/mg protein) |
|-----------|-----------------------------------|
| 60 min    |                                   |
| Control   | 164 ± 12                          |
| TCDD      | 380 ± 45a                         |
| PTX + TCDD| 150 ± 20                          |
| 7-KC + TCDD| 225 ± 35                      |
| FSK       | 950 ± 160a                        |
| 150 min   |                                   |
| Control   | 2884 ± 110                        |
| TCDD      | 3105 ± 230a                       |
| PTX + TCDD| 2705 ± 85                        |
| 7-KC + TCDD| 2830 ± 130                      |
| FSK       | 3042 ± 420a                       |

*Values are the mean ± S.D. of triplicate samples and are significantly different from control (p < 0.005).
treatment, total PKA activity was increased by about 1.5-fold. If cells were pretreated with 100 ng/ml PTX for 16 h, the stimulatory effect of TCDD on basal or total PKA activity was totally suppressed (Table II), demonstrating that the increased PKA activity is a G protein-sensitive effect. The PKA activity in cells simultaneously treated with 10 μM 7-ketocholesterol and 10 nM TCDD was no more significantly increased at 60 or 150 min after treatment, indicating that TCDD increases PKA activity via an AhR-dependent pathway.

**C/EBPβ Induction by TCDD**

TCDD is mediated by C/EBP—Different members of the basic leucine zipper family of transcription factors are known to bind on CRE. The sequence analysis revealed that the CRE-binding site at position −111 to −107 is an incomplete CRE DNA-binding motif containing only five of the eight conserved nucleotides in the CREB consensus sequence. To characterize the DNA binding affinity of the putative CREB site, gel retardation assays were performed with an oligonucleotide spanning from −123 to −95 of the C/EBPβ promoter region. Gel retardation assays show that nuclear extracts from C3H10T½ and Hepa1c1c7 cells form a CRE-specific complex (Fig. 10). The DNA binding activity to CREB was about 2-fold stronger in nuclear extracts from Hepa1c1c7 cells (Fig. 10, lanes 1 and 2), compared with nuclear proteins extracted from C3H10T½ cells (Fig. 10, lanes 3 and 4). An anti-CREB1 specific antibody completely supershifted the upper band indicating that this complex contains CREB1 (Fig. 10, lanes 5 and 6). Because Fos and Jun are known to form leucine zipper dimers with ATF and CREB proteins, we also tested anti-c-Jun and anti-Fos antibodies by supershift analyses. Neither anti-Fos (lanes 7 and 8) nor anti-c-Jun (lanes 9 and 10) were capable to supershift proteins from the CREB complexes formed with nuclear proteins derived from C3H10T½ or Hepa1c1c7 cells indicating that CREB1 itself is the main factor that binds to the CREB motif located at position −111 to −107. A 200-fold excess of cold oligonucleotide (−123 to −95) (Fig. 10, wt, lanes 11 and 12) completely abolished specific complex formation, whereas a 200-fold excess of the mutant CREB oligonucleotide did not affect DNA binding (Fig. 10, MUT1, lanes 13 and 14).

**DISCUSSION**

The role of C/EBPβ in differentiation and regulation of gene transcription has been well documented (28, 29). Several studies have shown that TCDD interferes with the process of adipocyte cell differentiation, which requires the precise timing of the up- and down-regulation of C/EBPβ expression. Indeed, TCDD inhibits the process of hormone-stimulated adipocyte differentiation of 3T3-L1 cells by causing sustained up-regulation of C/EBPβ (30). In the case of mouse embryonic fibroblast cell lines including CH310T½, we have shown that their adipocyte differentiation is also inhibited by TCDD, which causes significant up-regulation of C/EBPβ (22). The induction of C/EBPβ by TCDD has also been reported in adipose tissue and liver in vivo and in Hepa1c1c7 cells in vitro (9). In the latter study, TCDD was found to cause concentration-dependent induction of C/EBPβ at the identical threshold concentration as CYP1A1 induction, indicating the sensitivity of C/EBPβ response to TCDD. The reason why we have chosen both C3H10T½ and Hepa1c1c7 cells for the current study is therefore to relate the molecular events of transcriptional activation of the C/EBPβ gene to toxic end points already found in the same cells to ascertain its toxicological significance.

In the present study we found that TCDD induces C/EBPβ in a time-dependent manner in both C3H10T½ and Hepa1c1c7 cells. Increased mRNA expression was associated with an elevated level and binding activity of nuclear C/EBPβ protein to its corresponding consensus C/EBP-responsive element, which confirms our previous findings in primary rat hepatocytes. The increased binding affinity of C/EBPβ in rat hepatocytes was found to be required for transcriptional activation of the cylooxygenase-2 gene by TCDD (23).

Another gene that can be affected by TCDD exposure is the phosphoenolpyruvate carboxykinase gene (31, 32), which has also been shown to be regulated by C/EBPβ (33, 34); thus, it seems likely that down-regulation of phosphoenolpyruvate carboxykinase in liver tissue of TCDD-treated mouse is mediated by C/EBPβ.

Earlier studies identified two incomplete CREB-binding sites in the C/EBPβ promoter region that contribute to the basal and PKA-mediated transcriptional control of the gene (24). Using a sequence homology program we identified three xenobiotic-responsive element (XRE, GCGTG) located at position −68 to −64 of the 5′-regulatory region of the C/EBPβ gene (Fig. 6). Transcriptional activation of several genes (e.g., CYP1A1, CYP1B1, and GSTya) by TCDD is controlled via binding of AhR to XRE sequences in their promoter region (10). Data from a previous study indicated that the induction of C/EBPβ by TCDD requires functional AhR (9). Therefore, we were interested to study the relevance of the CREB- and XRE-binding sites in the C/EBPβ promoter. The transfection experiments using the deletion constructs revealed that only the region located between position −121 and −71 is essential for mediating the TCDD-dependent effect on transcription of the C/EBPβ gene. Because the XRE sequence is located further downstream at position −68 to −64, the induction of C/EBPβ is unlikely to be mediated via the classical AhR/XRE pathway.

Our further experiments using reporter constructs with mutations in the CREB-binding sites demonstrated that the two CREB-binding sites mediate TCDD-dependent activation of the C/EBPβ promoter. In further experiments we were interested in identifying the proteins that bind to the corresponding CREB sites of the C/EBPβ promoter. In addition to CREB, other basic leucine zipper proteins including AP-1 (Jun/Fos) and ATF members can control gene expression via CREs (35).
A recent report (36) showed that both ATF2 and c-Jun can activate CREBβ gene expression through two different binding motifs of the CREBβ promoter using nuclear extracts from lipopolysaccharide-stimulated mouse liver. From previous studies it is well known that TCDD can stimulate c-Fos and c-Jun transcription and enhance AP-1 DNA binding (37). Although c-Fos and c-Jun are known as transcription factors that are capable of binding to CRE-binding sites, we could not detect any binding activity of either c-Fos or c-Jun on the investigated CREB site of the CREBβ gene (Fig. 10). Through supershift analysis we identified CREB-1 as the major protein binding to the CREB site of CREBβ promoter.

The finding that CREB is the major factor leading to activation of the CREBβ gene is supported by cotransfection studies with a CREB expression vector and a dominant negative CREB (Δ-CREB) plasmid. CREB expression results in enhanced activation of the CREBβ reporter construct in a dose-dependent manner. Additionally, cotransfection of the dominant negative CREB expression plasmid Δ-CREB suppresses activation significantly by about 50%, which confirms that TCDD induces CREBβ via CREB. Among other signals including stress factors (38), CREB is activated by phosphorylation in response to cAMP, mediating most cellular responses via the PKA pathway (39, 40). Data from the present study show that TCDD treatment leads to slight but significant elevated levels of cAMP and increased PKA activity in an AhR-dependent and G protein-sensitive manner. Furthermore, the induction of CREBβ by TCDD or FSK was completely abolished in the presence of the PKA inhibitor H89, supporting that CREB is activated via a TCDD or FSK was completely abolished in the presence of the PKA inhibitor H89, supporting that CREB is activated via a PKA-dependent pathway leading to enhanced transcription of CREBβ. This result is confirmed by cotransfection studies with a PKA expression vector and PKA mutant vector demonstrating the PKA-dependent effect of TCDD- and FSK-mediated induction of CREBβ.

Despite the fact that TCDD has been known to cause a significant rise in PKA activities in vitro (41) as well as in vitro (42, 43), PKA itself has not been considered to be an important factor in the overall action mechanism of TCDD. The reason may be that in some tissues the TCDD-induced rise in PKA activity either occurs only after elevation of cAMP level as found in TCDD-treated rats (32) or initial activation of tyrosine kinases and PKC as in the case of thymus (42), or does not take place at all as in the case of human luteinizing granulosa cells in culture (44). The important point to consider is that the action of TCDD is often very tissue- and cell-specific (45). Therefore, the logical conclusion based on the current study results is that in these cells, where transcriptional activation of the CREBβ gene has been clearly demonstrated, activation of the cAMP/PKA pathway plays a pivotal role in mediating its action. Additionally, recent studies (46, 47) have shown that AhR can interact with the p65 (RelA) subunit of NF-κB, which may explain some of the toxic responses after TCDD exposure. From their results the authors suggested that the AhR and NFκB may interact through a PKA-dependent pathway (48). Novel findings are also showing that the XAP2, which forms a complex with AhR in the cytosol, can functionally interact with the cyclic AMP-specific phosphodiesterase (PDE4A5) (49). Binding of XAP2 to PDE4A5 reduced enzymatic activity of PDE4A5 by about 60%, which could lead to higher cAMP levels because PDE4A5 has the ability to hydrolyze cAMP and thereby modulates transcriptional signaling. Results of the current study show that the elevation of cAMP and PKA activity is an AhR-dependent process suggesting that PDE4A5 could be affected by AhR activation and mediates the increase of cAMP and PKA.

In summary, our present analysis demonstrates that induction of CREB gene transcription by TCDD is associated with elevated levels of cAMP and is mediated via a PKA-dependent pathway. The findings clearly show the important role of the CREB- and CRE-binding site in stimulating the transcriptional activity of the CREBβ gene, whereas the XRE-binding site is not functional during this process. Gene transcription of phosphoenolpyruvate carboxykinase and cyclooxygenase-2 is controlled by cAMP, PKA, and CREBβ (33, 50), and both genes can be modulated by TCDD (23, 52). Because both enzymes could play a key role in promoting toxic responses triggered by TCDD including wasting syndrome, diabetes, and carcinogenicity, stimulation of CREBβ might be a critical factor to these pathophysiological mechanisms.

REFERENCES
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43. Phillips, M., Enan, E., Liu, P. C., and Matsumura, F. (1995) J. Cell Sci. 108, 395–402
44. Enan, E., Lasley, B., Stewart, D., Overstreet, J., and Vandevoort, C. A. (1996) Reprod. Toxicol. 10, 191–198
45. Matsumura, F. (2003) Biochem. Pharmacol. 66, 527–540
46. Tian, Y., Ke, S., Denison, M. S., Rabson, A. B., and Gallo, M. A. (1999) J. Biol. Chem. 274, 510–515
47. Kim, D. W., Gazourian, L., Quadri, S. A., Romieu-Mourez, R., Sherr, D. H., and Sonenshein, G. E. (2000) Oncogene 19, 5498–5506
48. Tian, Y., Rabson, A. B., and Gallo, M. A. (2002) Chem. Biol. Interact. 141, 97–115
49. Bolger, G. B., Peden, A. H., Steele, M. R., MacKenzie, C., McEwan, D. G., Wallace, D. A., Huston, E., Baillie, G. S., and Houslay, M. D. (2003) J. Biol. Chem. 278, 33351–33363
50. Reddy, S. T., Wadleigh, D. J., and Herschman, H. R. (2000) J. Biol. Chem. 275, 3107–3113