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Interaction of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) with induced adipocyte differentiation in mouse embryonic fibroblasts (MEFs) involves tyrosine kinase c-Src

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Abstract

Adipocyte differentiation of mouse embryonic fibroblasts (MEFs) derived from c-Src wild-type or c-Src-deficient (abbreviated as MEF+/+ and MEF−/− hereafter) C57BL/6 mice was induced by ascorbic acid (A) and β-glycerophosphate (G). TCDD clearly suppressed differentiation of MEF+/+, but not MEF−/−, as measured by increased accumulation of triglycerides associated with increased expression of adipocyte differentiation-specific genes such as peroxisome proliferators activated receptor (PPAR)γ, stearoyl-CoA desaturase (SCD-1). Studies on inducibility of TCDD-activated genes such as cytochrome P450 (CYP)1A1 and CYP1B1 revealed a comparable dose response in both MEF+/+ and MEF−/−. Furthermore, the binding activity of AhR complexes to xenobiotic response elements (XREs) was similar in both cell lines. We further studied the effect of TCDD on CCAAT/enhancer binding proteins (C/EBP), which are known to be important regulators of cell differentiation. TCDD induced C/EBPβ and C/EBPδ mRNA expression and DNA binding activity in a time- and dose-dependent manner in MEF+/+ but not in MEF−/−. The levels of C/EBPβ and C/EBPδ were still elevated in differentiated MEF+/+ after 10 days of treatment with TCDD. In MEF−/−, C/EBPβ and C/EBPδ are highly expressed constitutively. In contrast to MEF+/+, TCDD does not cause any significant change of these transcription factors in MEF−/−. These data indicate that suppression of differentiation by TCDD in MEF requires a functional c-Src activity and induced levels of C/EBPβ and C/EBPδ, including their maintenance at high levels by TCDD, rather than ultimate high levels of these C/EBP isoforms.

Keywords: Aryl hydrocarbon receptor (AhR); 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD); Mouse embryonic fibroblasts (MEFs); Differentiation; CCAAT/enhancer binding protein (C/EBP); c-Src

1. Introduction

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD, dioxin) is considered the most toxic member of a large family of halogenated polyaromatic hydrocarbons including polychlorinated dioxins, furans, biphenyls and naphthalenes. Exposure of animals to TCDD leads to characteristic toxicological and biochemical responses including wasting, immune suppression, reproductive and developmental alterations, endocrine disruption, and enzyme activation [1,2]. Most of the actions of TCDD are believed to be mediated by the binding of TCDD to a cytosolic protein, the aryl hydrocarbon receptor (AhR). The activated AhR subsequently mediates transcriptional activation of genes for drug metabolizing enzymes such as CYP1A1, CYP1B1, glutathione *S*-transferase Ya, and NAD(P)H: menadione oxidoreductase [3]. TCDD-dependent activation of these genes involves binding of AhR and the AhR nuclear translocator (Arnt) as a dimer 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 (Hsp90). In addition to two Hsp90s, other proteins seem to be involved in the formation of high affinity ligand binding form(s) of the AhR [4,5]. They are: the co-chaperone p23, which has been shown to play a part in the activation process of AhR to the DNA-binding complex [6], and an immunophilin-like protein, XAP-2 [7,8]. The precise role of these proteins in the activation process of AhR-regulated genes is currently not well understood.

Besides the regulation of xenobiotic metabolizing enzymes, the AhR is postulated to play an important role

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in normal cellular growth and differentiation processes even without an exogenous ligand [9]. Thus, AhR knockout mice exhibit decreased liver size as well as decreased body size over the first 4 weeks relative to their wild-type littermates [10,11]. Numerous *in vitro* studies have also shown the interaction of activated AhR with developmental and differentiation processes [12–15].

One characteristic effect of TCDD exposure is the reduction of adipose tissue mass, increase in serum triglycerides, alteration of lipid and carbohydrate metabolism, and onset of diabetic-like symptoms [16,17]. There are several lines of evidence from *in vitro* experiments suggesting the AhR as a negative regulator of adipocyte differentiation [18]. Previous reports have already shown that TCDD suppresses the differentiation of 3T3-L1 cells into adipose cells [14,19–21]. This cell line was used in our laboratory to investigate the effects of TCDD on lipid metabolism and adipocyte function (e.g. [22]). Structure activity analysis of different TCDD congeners and the use of α-napthoflavone, an AhR antagonist, revealed that suppression of 3T3-L1 differentiation by TCDD is an AhR-dependent mechanism [14]. Our findings agree with the observation reported by Shimba *et al*. [23] showing that the AhR acts as a negative regulator of 3T3-L1 adipose differentiation independent of ligand binding.

The transcriptional regulation of adipogenesis is believed to be mainly controlled by two families of transcription factors, C/EBP and PPAR [24]. The central role of C/EBP proteins was clearly shown in C/EBP knock out animals and in the 3T3-L1 model [25]. From these findings, it was originally proposed that C/EBPα acts as a key factor for adipocyte differentiation. However, C/EBPβ and C/EBPδ are the first transcription factors induced following exposure to differentiation reagents. According to Darlington *et al*. [25] C/EBPβ and C/EBPδ synergistically act as the inducer of C/EBPα at the early stage of adipocyte differentiation. Embryonic fibroblasts from mice lacking both C/EBPβ and C/EBPδ also demonstrate an inability to differentiate into adipocytes [25].

Since the tyrosine kinase c-Src has been shown to be involved in TCDD-mediated serum triglyceride accumulation [26,27], we performed parallel studies in MEFs from wild-type and c-Src-deficient mice to provide further insight into the regulatory mechanisms of TCDD-mediated inhibition of adipogenesis. A commonly used model for adipogenesis is the mouse 3T3-L1 cell line which can easily undergo differentiation from fibroblasts into adipocytes when preadipocytes are treated with insulin, isobutylmethylxanthine (IBMX), and dexamethasone (Dex) [28]. In the case of MEFs, Alexander *et al*. [18] have shown that the same differentiation inducers could also trigger adipogenesis providing MEFs as a useful vitro system.

In the current study we found that in contrast to MEF+/+, differentiation of MEF−/− is not suppressed by TCDD. In view of the striking difference in response to TCDD, we judged that it is worthwhile to investigate the underlying mechanism for this differential sensitivity in MEF+/+ and MEF−/−. Results from the present study showed that the differential suppression of adipocyte differentiation by TCDD in MEF+/+ and MEF−/− is not dependent on the difference in the AhR or a general TCDD unresponsiveness; rather, the main difference appears to be the induced expression of C/EBPβ and C/EBPδ, which are likely to be responsible for the differential sensitivities of these cells to TCDD.

2. Materials and methods

2.1. Chemicals

TCDD (>99% purity) was originally obtained from Dow Chemicals Co. DMSO was obtained from Aldrich Chemical Co. [y-^{32}P]ATP (6000 Ci/mmol) was purchased from ICN. Antibodies for C/EBP isoforms were purchased from Santa Cruz Biotechnology. Oligofectamine reagent was from Invitrogen. Troglitazone was a kind gift from Dr. J. Ohsumi of Sankyo Pharmaceutical Co. Other molecular biological reagents were purchased from Qiagen and Roche.

2.2. MEF isolation

Primary MEFs were isolated from 14-day post coitus (d.p.c.) c-Src+/+ and c-Src−/− C57BL6 mouse embryos [29]. Embryos were surgically removed, and separated from maternal tissues and the yolk sack. The bodies were minced finely and then incubated in a solution of trypsin:EDTA (0.05% trypsin; 1 mM EDTA) at 37° for 30 min. The supernatant was centrifuged for 5 min at 1000 *g*. The resulting pellet was resuspended in culture medium and cells were plated in 100 mm culture dishes.

2.3. Cell culture and differentiation

C3H10T1/2 cells were purchased from ATCC (Manassas, VA) and maintained in Basal Modified Eagle (BME, Gibco BRL) medium. Primary MEFs from both wild-type and c-Src-deficient mice were maintained in Dulbecco's Modified Eagle's Medium:F-12 nutrient mix (DMEM:F12, Gibco BRL). Cell culture medium contained 10% fetal bovine serum (FBS, Gemini) and 100 units of penicillin and 100 μg/mL streptomycin. Confluent plates of cells were induced to differentiate with BME or DMEM:F12 containing 10% FBS. For adipocyte differentiation in MEF four different protocols were tested. Protocol #1: culture medium supplemented with 0.5 mM IBMX, 0.25 μM Dex and 10 μg/mL insulin for the first 3 days [30]. Subsequently, cultured cells were maintained in standard medium supplemented with 5 μg/mL insulin only. Protocol #2: standard medium was supplemented with 125 μM indomethacine and 5 μg/mL insulin. After the first 3 days, medium was supplemented with 5 μg/mL insulin only.

Protocol #3: standard medium including 2 nM triiodothyronine (T_3) and 17 nM insulin throughout the differentiation period. Protocol #4: standard culture medium, containing 82 μg/mL ascorbic acid (A) and 10 mM β-glycerophosphate (G) throughout the differentiation period. In some cases, Protocol #4 was modified by adding the PPARγ agonist Troglitazone (TRO, 10 μM) to differentiation medium where indicated. To investigate TCDD's action on differentiation cells were pretreated with 10 nM TCDD 1 hr before adding the differentiation medium. TCDD treatment was renewed when differentiation agents were added (every 2 days). Control samples received an equal volume of the vehicle DMSO alone.

2.4. Cell transfection

Antisense and control sense oligodeoxynucleotides (ODN) with a length of 20 bases were synthesized and purified (Molecular Structure Facility). The following ODN sequence with phosphorothioate linkages throughout the entire ODN molecule was used: antisense-c-Src (AS-c-Src) 5′-TTG CTC TTG TTG CTG CCC AT-3′. C3H10T1/2 cells were transfected with c-Src sense (S-c-Src) and antisense ODN (AS-c-Src) (200 nM final concentration) in 6-well plates 24 hr prior to the addition of differentiation inducing agents or TCDD. At day of transfection, cells were about 30–50% confluent. Control cells received a sense ODN at equal concentrations. Cells were transfected with Oligofectamine reagent (Invitrogen) according to the protocol of the manufacturer. Cells were transfected with S-c-Src and AS-c-Src every second day.

2.5. Oil Red O staining

Ten days after the induction of differentiation, cells were stained with Oil Red O (ORO) according to Kasturi and Joshi [31]. Briefly, cells were washed twice with PBS and fixed with 10% formalin in PBS for 1 hr; then they were washed an additional three times with water and dried. Cells were stained with ORO [six parts of saturated ORO dye (0.6%) in isopropanol and four parts of water] for 15 min. Excess stain was removed by washing with 70% ethanol; stained cells were then washed with water. In some experiments, spectrophotometrical quantification of the stain was performed by dissolving the stained oil droplets in the cell monolayers with 4% Nonidet P-40 in isopropanol for 5 min. The absorbance was then measured at 520 nm.

2.6. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from MEFs using a high pure RNA isolation kit (Roche). RT-PCR analysis was carried out as previously described [27]. Briefly, reverse transcription was performed with 1 μg of total RNA, 1 μg of oligo (dT_{15}), 1 mM dithiothreitol, and 4 U Omniscript™ reverse transcriptase (Qiagen) in a 40 μL-reaction volume. PCR amplifications were performed in a 50-μL reaction volume containing 2.0 μL of the reverse transcriptase reaction, 10× PCR buffer, 200 μM desoxynucleoside triphosphates, 2.5 U Taq DNA polymerase (Qiagen), and 200 nM of each primer. Mouse-specific primers for detection of Ahr, Arnt, CYP1A1, CYP1B1, C/EBPα, C/EBPβ, C/EBPδ, SCD-1, PPARγ, PPARγ-1, PPARγ-2, and GAPDH were selected from published sources and cDNA sequences [32–37]. The conditions for PCR amplification were 2 min at 94° before the first cycle, 45 s at 94° for denaturation, 1 min for primer annealing, 1 min at 72° for primer extension, and 7 min at 72° after the final cycle. The following annealing temperatures and cycle numbers were used for amplification: AhR and Arnt: 60°, 28 cycles; CYP1A1: 60°, 30 cycles; CYP1B1: 60°, 26 cycles; C/EBPα: 57°, 27 cycles; C/EBPβ: 57°, 27 cycles; C/EBPδ: 60°, 28 cycles; GAPDH: 57°, 20 cycles; SCD-1: 60°, 28 cycles; PPARγ: 60°, 28 cycles; PPARγ-1: 60°; 30 cycles; PPARγ-2: 60°, 30 cycles. Linearity of amplifications was controlled by three different cycle numbers for one cDNA concentration. The PCR products were resolved by agarose gel electrophoresis and stained with ethidium bromide using standard methods. For semiquantitative analysis, respective bands were quantified using a ChemiImager™ 4400 (Alpha Innotech Corporation); the data were analyzed by using the manufacturer's software and normalized to the amount of GAPDH signals.

2.7. Electromobility shift assays (EMSA)

Nuclear extracts were isolated from primary MEF+/+ and MEF−/− according to Dennler *et al*. [38]. In brief, 5×10^6 cells were harvested in Dulbecco's PBS containing 1 mM phenylmethanesulfonyl fluoride (PMSF) and 0.05 μg/μL of aprotinin. After centrifugation, the cell pellets were gently resuspended in 1 mL of hypotonic buffer (20 mM HEPES, 20 mM NaF, 1 mM Na_3VO_4, 1 mM $Na_4P_2O_7$, 1 mM EDTA, 1 mM EGTA, 0.5 mM PMSF, 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 by 25 strokes of a Dounce-homogenizer. After centrifugation for 1 min at 16,000 *g* nuclear pellets were resuspended in 300 μL 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°. The nuclear lysates were microcentrifuged at 16,000 *g* for 20 min, aliquoted and stored at −70°. Protein concentrations were determined by the method of Bradford (Bio-Rad, Hercules, CA).

For EMSA, double-stranded oligonucleotides were used containing consensus sequences (underlined) for the C/EBP (5′-TGCAGATTGCGCAATCTGCA-3′) (Santa Cruz Biotechnology) or the XRE (3′-CCAGGCTCTTCT-CACGCAACTCCGGGGC-3′) binding sites [39]. DNA–protein binding reactions were carried out in a 20 μL total

volume containing 15 μg nuclear protein, 40,000 cpm of DNA oligonucleotide, 25 mM Tris buffer (pH 7.5), 50 mM NaCl, 1 mM $MgCl_2$, 1 mM EDTA, 0.5 mM dithiothreitol, 5% glycerol, and 1 μg poly (dI-dC). The samples were incubated at room temperature for 20 min. Supershift analyses were performed by addition of 2 μg of polyclonal C/EBPα, C/EBPβ or C/EBPδ antibodies to the reaction mixtures. Competition experiments were done in the presence of a 200-fold molar excess of unlabeled DNA fragments. Protein–DNA complexes were resolved on a 5% nondenaturating polyacrylamide gel and visualized by exposure of the dehydrated gels to X-ray films. For quantitative analysis, respective bands were analyzed by using a ChemiImager™ 4400.

2.8. Statistical analysis

All experiments were repeated a minimum of three times and results are expressed as means ± standard deviations. Statistical differences were determined by Student's *t*-test and for significance between pairs of mean values the Bonferroni post hoc test was employed.

3. Results

3.1. Inhibitory action of TCDD on differentiation of MEFs

One standard marker of adipocyte differentiation is the accumulation of triglyceride droplets in the cytosol which can be stained by ORO. To examine TCDD's action on differentiation of MEF, four different protocols were tested to induce differentiation as described in Section 2. All four treatment regimes were found to be capable of inducing differentiation in MEFs (Fig. 1A). The extent of differentiation of MEF+/+ and MEF−/−, determined by ORO staining, was comparable. However, the most significant

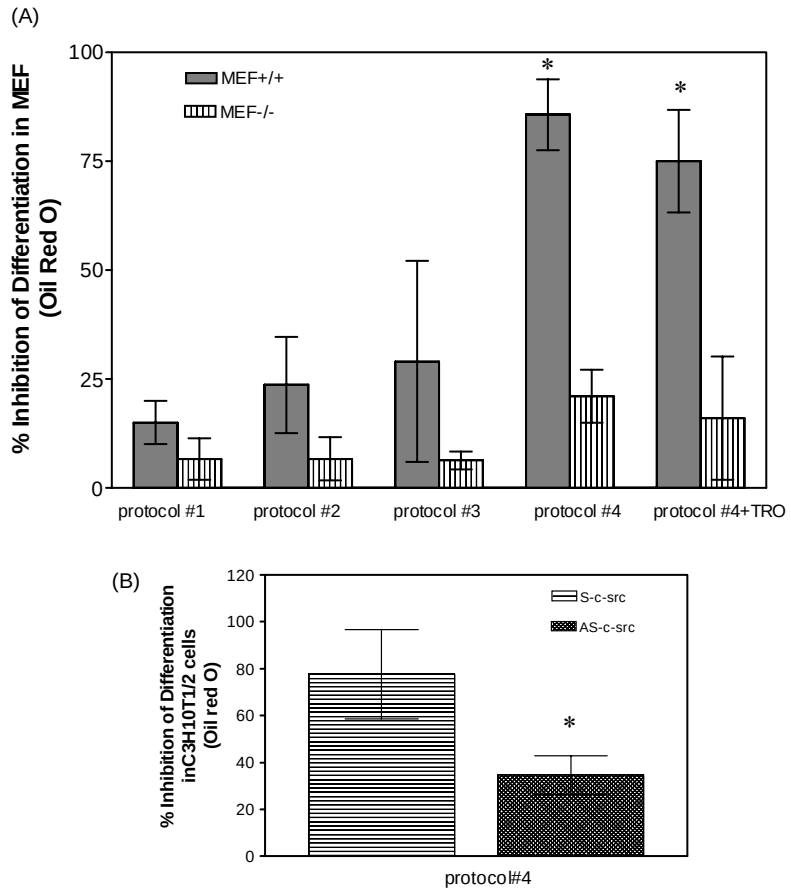

Fig. 1. Inhibition of adipocyte differentiation by TCDD. (A) To induce cell differentiation, four different treatment regimens (Protocols #1–4) were tested in MEF+/+ (shaded bars) and MEF−/− (striped bars). Cells were pretreated for 1 hr with 10 nM TCDD followed by a 10 days treatment of differentiation inducing agents. Protocol #1: insulin (10 μg/mL) + dexamethasone (Dex, 0.25 μM) + isobutylmethylxanthine (IBMX, 500 μM); Protocol #2: indomethacin (125 μM) + insulin (5 μg/mL); Protocol #3: triiodothyronine (T_3, 2 nM) + insulin (10 ng/mL); Protocol #4: ascorbic acid (A, 82 μg/mL) + β-glycerophosphate (G, 10 mM); Protocol #4 + TRO: ascorbic acid (82 μg/mL) + β-glycerophosphate (10 mM) + 10 μM troglitazone. (B) Effect of an antisense ODN against c-Src mRNA on TCDD-inhibited adipocyte differentiation in C3H10T1/2 cells. Cells were transfected with either a sense (S-c-Src) or an antisense (AS-c-Src) ODN against c-Src mRNA, and 24 hr later the cells were exposed 10 nM TCDD during 1 hr followed by treatment with A/G for 10 days. Cells were fixed and stained by ORO. Extracted ORO was quantified in a spectrophotometer at 520 nm. The percentage of inhibition of differentiation in TCDD-pretreated cells relatively to cells treated with differentiation inducing agents alone are depicted; (*) significantly different from control ($P < 0.005$).

inhibitory effect of TCDD in cell differentiation was observed in Protocol #4 (abbreviated as A/G, hereafter) as depicted in Fig. 1A. TCDD was found to inhibit approximately 80% of the differentiation of MEF+/+ cells assessed by spectrophotometric measurement of eluted ORO staining. The additional treatment with troglitazone, a known agonist for PPARγ receptor, led to an increased ORO staining of MEFs by about 30% in comparison to A/G alone. Despite the presence of troglitazone, TCDD was still capable of suppressing the differentiation by 70%. In contrast, MEF−/− differentiation was not significantly suppressed by TCDD. In some experiments on MEF+/+, TCDD was removed after 10 days of differentiation but cells were continuously treated with A/G for a further 5 days (data not shown). At that time point we noticed that the inhibitory action of TCDD became partially reversible.

To test the relevance of c-Src in the differentiation process we transfected another mouse embryonic fibroblast cell line C3H10T1/2 with an antisense-c-Src ODN (AS-c-Src). The inhibitory action of TCDD on adipocyte differentiation of C3H10T1/2 is significantly reduced by the treatment of cells with AS-c-Src by about 50% (Fig. 1B). This result confirms previous studies with AS-c-Src in 3T3-L1 cells [40]. Treatment with this AS-c-Src ODN led to a reduced protein expression by about 70% as described previously [41].

Examination by light microscopy revealed that the decrease in lipid staining in the TCDD-treated MEF+/+ cells is due to a decrease in the total number of cells that differentiate to adipocytes, rather than uniform decrease in the content of lipid per cell.

3.2. Inhibition of PPARγ and SCD-1 expression by TCDD

Differentiation to adipocytes, as a result of A/G treatment, is associated with induction of SCD-1 and PPARγ, which are well known as differentiation-specific marker genes. RT-PCR analysis of total RNA revealed that the mRNA expression of both, PPARγ and SCD-1, is upregulated in A/G-treated differentiated MEF+/+ and MEF−/− cells. However, TCDD prevents the increase of the aforementioned differentiation marker PPARγ and SCD-1 only in MEF+/+ cells by about 60 and 75%, respectively (Fig. 2), but not in MEF−/−.

3.3. TCDD responsiveness of c-Src+/+ and c-Src−/− MEFs

Since these data indicated a lower responsiveness of MEF−/− than MEF+/+ toward TCDD, we first compared the expression of AhR and Arnt in both cell lines. The results showed that AhR and Arnt were expressed at similar levels in both cell lines (Fig. 3A). Since these results alone cannot prove that the function of the AhR from these two cell lines is comparable, we investigated the functionality of the AhR by

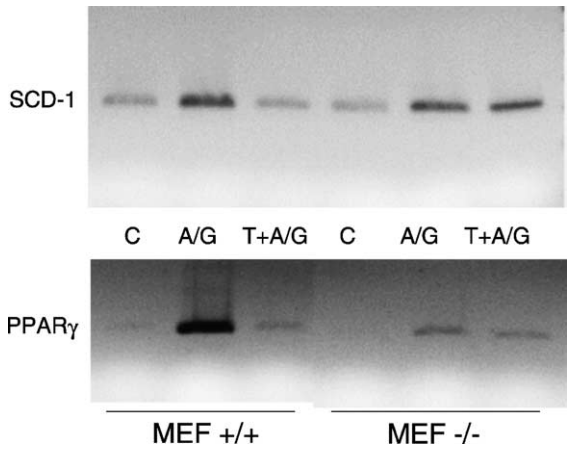

Fig. 2. Expression of SCD-1 and PPARγ mRNA in MEF+/+ and MEF−/−. Cells were treated with ascorbic acid and β-glycerophosphatate (A/G) or pretreated with 10 nM TCDD followed by A/G treatment for 10 days. SCD-1 and PPARγ mRNA expression was analyzed by RT-PCR. PCR products were separated on a 1% agarose gel and stained by ethidium bromide.

the induction of CYP1A1 and CYP1B1. It was found that the expression of CYP1A1 and CYP1B1 were significantly induced by 1 nM TCDD in both +/+ and −/− cell lines after 6 hr of treatment, reflecting a similar dose-responsiveness (Fig. 3B). We further investigated the ability of the AhR to recognize and bind to its specific DNA recognition site through EMSA, using nuclear extracts from TCDD or DMSO-treated MEF+/+ and MEF−/− cells (Fig. 4). The results clearly showed that nuclear extracts from both +/+ and −/− cells contain the activated AhR protein (lanes 2 and 5) which binds in a TCDD-specific manner to the XRE oligonucleotide.

3.4. Effect of TCDD on C/EBP mRNA expression in MEFs

In view of our failure to detect any difference in the AhR properties between these MEF strains, we next turned our attention to C/EBP proteins. Previous studies from this laboratory have shown that C/EBPα, C/EBPβ, and C/EBPδ are involved in the TCDD-mediated suppression of adipocyte differentiation [14]. Therefore, we examined the expression of C/EBP isoforms in MEFs. TCDD induces mRNA expression of C/EBPβ as well as C/EBPδ in MEF+/+ in a time- and dose-dependent manner (Figs. 5 and 6). The earliest significant TCDD-induced increase of C/EBPβ was observed at 3 hr, and this effect was sustained until 24 hr after TCDD treatment. C/EBPδ was significantly increased after 6 hr (3-fold) and remained elevated after 24 hr (Fig. 5). As a result of a dose–response study, it was found, that at a dose of 1 nM, TCDD induced C/EBPβ and C/EBPδ in MEF+/+ significantly as compared to vehicle control (Fig. 6). In contrast, TCDD did not change the expression of C/EBPβ or C/EBPδ in MEF−/−. Instead, we observed a higher constitutive expression of C/EBPβ

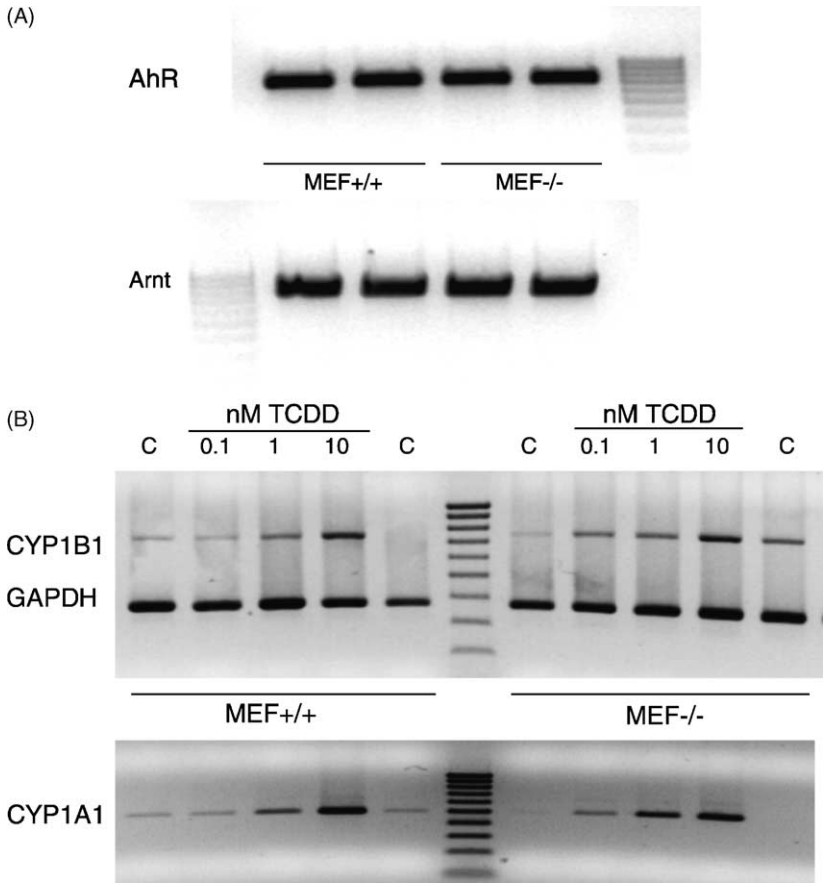

Fig. 3. Expression level of AhR and Arnt mRNA in untreated MEF+/+ and MEF−/− and dose-dependent induction of CYP1A1 and CYP1B1. (A) MEFs were grown under standard condition and mRNA expression of AhR and Arnt was analyzed as in Fig. 2. (B) MEFs were treated with 0.1, 1, and 10 nM TCDD for 6 hr. Control cells received 0.1% DMSO and mRNA was analyzed as in Fig. 2.

and C/EBPδ in MEF−/− as compared to MEF+/+. The mRNA level of C/EBPα remained unchanged by TCDD in both MEF+/+ and MEF−/− (Figs. 5 and 6).

3.5. C/EBP expression pattern in MEFs during differentiation

At the end of the differentiation period, we determined the effect of TCDD on the expression of the three C/EBP isoforms. As shown in Fig. 7 mRNA levels of C/EBPβ as well as C/EBPδ were still elevated at day 10 in differentiated MEF+/+ cells which were pretreated with TCDD compared to cells which were treated with the differentiation inducing agents alone. The expression pattern of C/EBP isoforms was unchanged in MEF−/− (Fig. 7).

3.6. Altered DNA binding activity of C/EBP after TCDD treatment

To verify the effect of TCDD on the DNA binding activity of the C/EBP proteins gel mobility shift analyses were conducted. The oligonucleotide probe used was a 20-mer containing the consensus C/EBP-binding site sequence. Nuclear extracts of MEF+/+ and MEF−/− were prepared from control and TCDD pretreated cells at 3 hr, 6 hr, 3 day, 9 day, and 12 day of the differentiation process. The effect of TCDD on DNA binding activity of C/EBP proteins after 3 and 6 hr of treatment are presented in Fig. 8. Nuclear extracts from MEF+/+ cells treated with TCDD (Fig. 8B, lanes 2 and 4) showed 2-fold higher binding activity of C/EBP proteins compared to the respective controls (Fig. 8B, lanes 1 and 3). In contrast, TCDD did not significantly affect binding activity of C/EBP proteins in MEF−/− cells (Fig. 8B, lanes 7 and 9).

Since all of C/EBP isoforms can bind to the same consensus C/EBP binding site, super-shift analyses were performed to assess the composition of DNA-bound C/EBP proteins. The effect of A/G and TCDD on binding activity of C/EBP isoforms was then investigated after 3 days of differentiation. MEF+/+ treated with TCDD (Fig. 9, lane 2) showed 2-fold higher binding activity of C/EBP proteins as compared to the control (Fig. 9, lane 1). In MEF+/+, which were co-treated with TCDD and A/G (Fig. 9, lane 4), the binding activity of C/EBP proteins was still than that observed in cells treated with A/G only (Fig. 9, lane 3).

In contrast, although an elevated C/EBP binding activity in MEF−/− was detectable by A/G treatment (Fig. 10, lane 3),

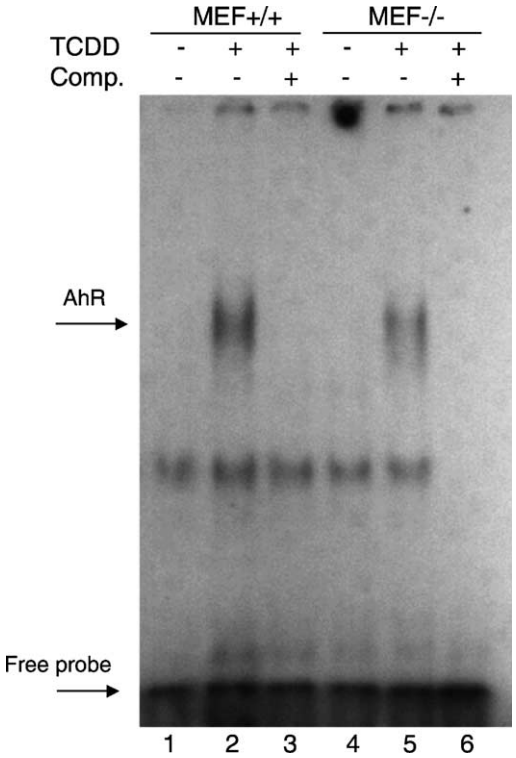

Fig. 4. Gel mobility shift assay on TCDD-inducible protein–DNA complexes formed using MEF+/+ and MEF−/−. Nuclear protein extracts were prepared from cells incubated for 1.5 hr in the absence (−) or presence (+) of 10 nM TCDD. Nuclear protein extracts from the indicated cells were incubated with [^{32}P]XRE in the absence (lanes 1, 2, 4, 5) or presence of a 200-fold molar excess of unlabeled XRE (lanes 3 and 6). The top arrow indicates the electrophoresis position of the AhR complex, and the bottom arrow shows the location of the free ^{32}P-labeled XRE oligonucleotide.

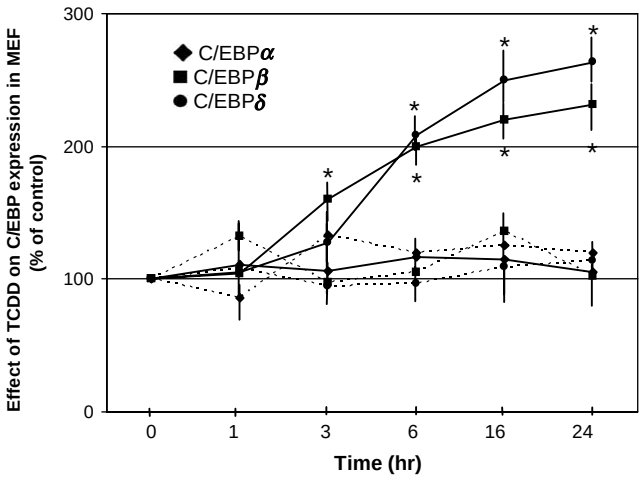

Fig. 5. Time-dependent expression of C/EBPα, C/EBPβ, and C/EBPδ. MEF+/+ (solid lines) and MEF−/− (dashed lines) were treated with 10 nM TCDD for 1, 3, 6, 16, and 24 hr. Values for C/EBP mRNA expression are normalized to the expression of GAPDH. mRNA expression is given relative to that of the respective controls; (*) significantly different from control ($P < 0.005$).

it was not further increased by TCDD (Fig. 10, lane 4). As shown in super-shift analyses, nuclear extracts from differentiating MEF+/+ cells treated for 3 days presented significantly elevated binding activity of C/EBPβ (Fig. 9B, lanes 9 and 12) in TCDD-treated cells compared to their corresponding controls (Fig. 9, lane 6). Similar to C/EBPβ, the binding capacity of C/EBPδ was also found to be about 2-fold increased by TCDD in MEF+/+ (Fig. 9B, lanes 10 and 13) but not in MEF−/− (Fig. 10, lanes 7–10). The binding activity of C/EBPα is still barely detectable at this time point in both MEF+/+ and MEF−/− (Figs. 9 and 10).

The C/EBP binding activity at day 9 of differentiation is demonstrated in Figs. 11 and 12. C/EBPβ was the most dominant isoform in both MEF+/+ (Fig. 11, lanes 2, 5, 8, and 11) and MEF−/− cells (Fig. 12, lanes 2, 5, 8, and 11) in agreement with our results from RT-PCR studies. Furthermore, it was observed that the binding capacity of C/EBPβ was elevated in nuclear extracts from TCDD-treated (Fig. 11, lane 5) or TCDD-pretreated (Fig. 11, lane 11) MEF+/+ compared to corresponding controls (Fig. 11B, lanes 2 and 8). In contrast, TCDD did not significantly change C/EBPβ binding activity in MEF−/− (Fig. 12,

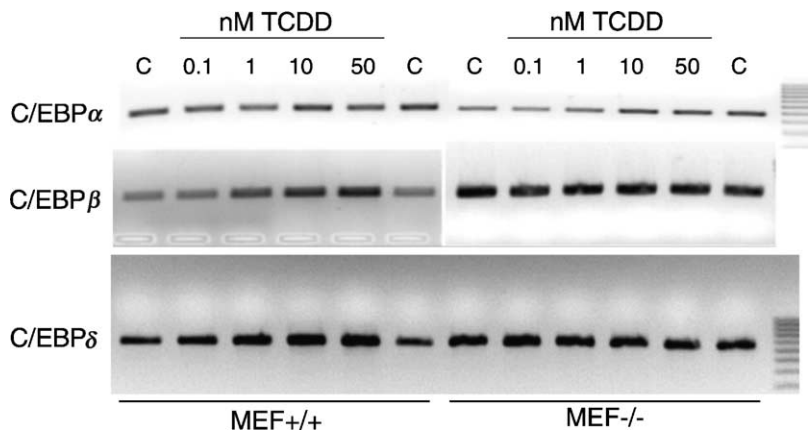

Fig. 6. Dose-dependent induction of C/EBPβ and C/EBPδ mRNA by TCDD. MEFs were treated for 24 hr with 0.1, 1, 10, and 50 nM TCDD; control cells received only vehicle solvent (C).

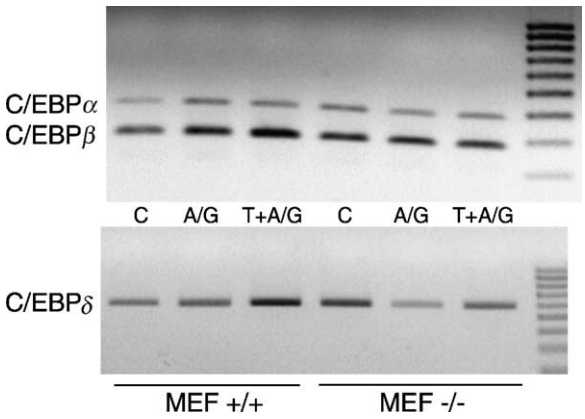

Fig. 7. Expression of C/EBPα, C/EBPβ, and C/EBPδ after differentiation of MEFs. Cells were treated for 10 days with A/G or pretreated for 1 hr with 10 nM TCDD followed by A/G (T + A/G) treatment for 10 days. TCDD treatment was renewed when differentiation agents were added. mRNA expression was analyzed as in Fig. 2.

lanes 5 and 11). The presence of C/EBPδ was barely noticeable at day 9 in MEF+/+ (Fig. 11, lanes 3, 6, 9, and 12) and MEF−/− (Fig. 12, lanes 3, 6, 9, and 12). C/EBPα binding was still weak at day 9 in MEF+/+ and MEF−/− (Figs. 11 and 12, lanes 1, 4, 7, and 10).

C/EBPα binding activity was detected at day 12 in all samples (Fig. 13A). C/EBPα was not affected by TCDD alone in MEF+/+ or MEF−/− (Fig. 13, lanes 2 and 7), despite the fact that C/EBPα was increased in differentiated cells treated with A/G (Fig. 13, lanes 3 and 8). However, the extent of reduction in the band intensity of C/EBPα was greatest in TCDD + A/G (Fig. 13, lane 4) as compared to TCDD alone (Fig. 13, lane 2) or A/G alone (Fig. 13, lane 3) in MEF+/+, but not in MEF−/− (Fig. 13A, lane 9). The analyses of supershifted band intensities corroborate this observation (Fig. 13B).

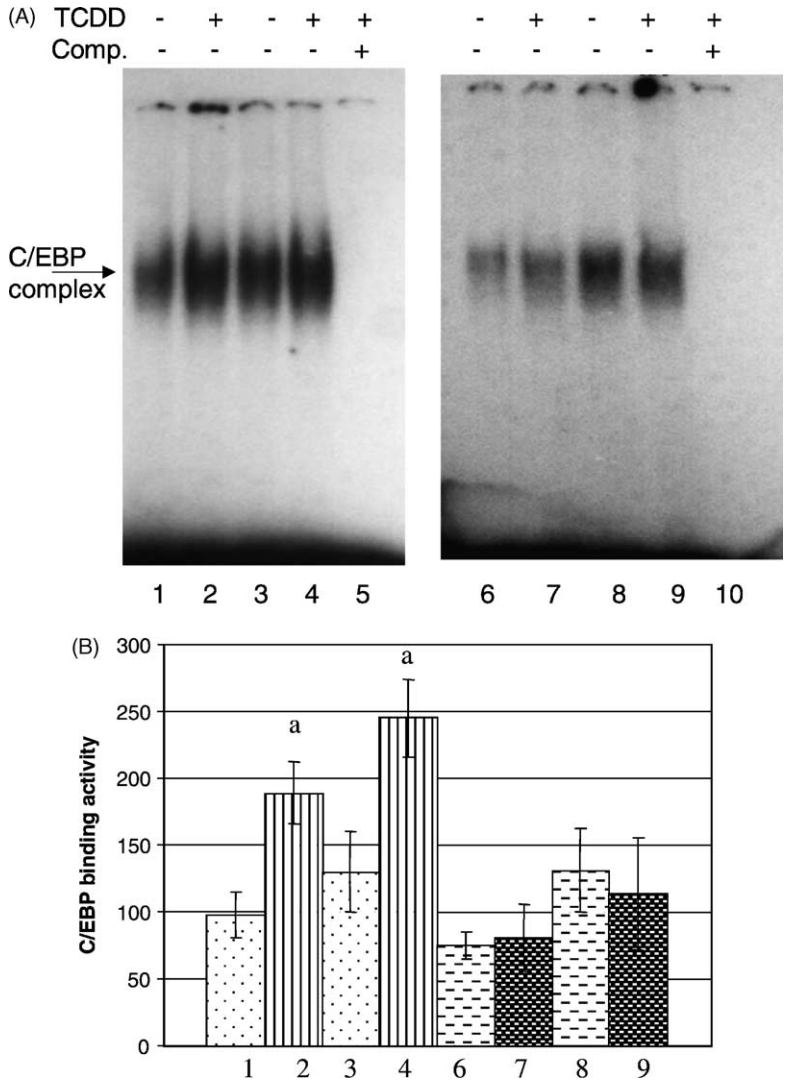

Fig. 8. DNA binding of C/EBP complexes to a ^{32}P-end-labeled oligonucleotide containing the C/EBP consensus binding site. (A) MEF+/+ (lanes 1–5) and MEF−/− (lanes 6–10) were treated with 10 nM TCDD for 3 hr (lanes 2 and 7) and for 6 hr (lanes 4 and 9) and nuclear proteins were extracted at 3 and 6 hr. Control cells received 0.1% DMSO (lanes 1, 3, 6, and 8). A 200-fold molar excess of unlabeled C/EBP was added in lanes 5 and 10. (B) Densitometric evaluation of band intensities of the total C/EBP binding activity is expressed as arbitrary units. Results of three separate experiments are presented as mean values ± SD; ([a]) significantly different from control cells ($P < 0.05$).

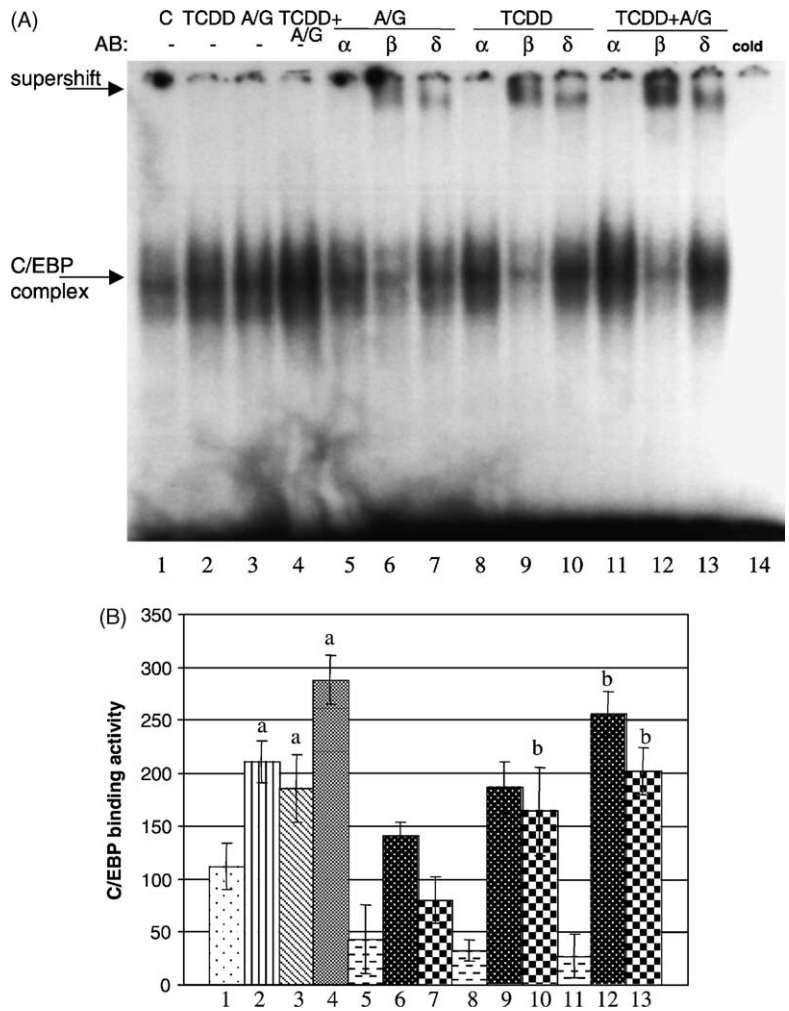

Fig. 9. DNA binding activity of C/EBP complexes in nuclear extracts from differentiating MEF+/+. (A) MEF+/+ were treated for 3 days with 10 nM TCDD (lane 2), 82 μg/mL ascorbic acid (A)/10 mM β-glycerophosphate (G) (lane 3) or pretreated for 1 hr with 10 nM TCDD followed by A/G for 3 days (TCDD + A/G) (lane 4). Controls received 0.1% DMSO (lane 1). Supershift analysis with C/EBPα- (lanes 5, 8, and 11), C/EBPβ- (lanes 6, 9, and 12), and C/EBPδ-specific antibodies (lanes 7, 10, and 13) identified isoforms of C/EBP binding complexes in A/G, TCDD and TCDD + A/G-treated cells. (B) Densitometric evaluation of band intensities of the total (lanes 1–4) or upshifted (lanes 5–13) C/EBP binding activity is presented. Results of three separate experiments are shown as mean values ± SD; ([a]) significant different from control cells ($P < 0.05$); ([b]) significant different from A/G-treated cells ($P < 0.05$).

4. Discussion

In this study we investigated the inhibitory action of TCDD on induced MEF differentiation into adipocytes. To examine the involvement of the tyrosine kinase c-Src, we performed parallel experiments with MEFs derived from embryos of wild-type and c-Src-deficient C57BL/6 mice. Our results showed that TCDD inhibits cell differentiation of MEF+/+ but not of MEF−/− cells. A comparison of the two cell lines indicates that their respective AhR and Arnt expression were similar, based upon mRNA levels. EMSA and RT-PCR analysis revealed that DRE binding and the induction of CYP1A1 or CYP1B1 occur equally in both cell lines upon TCDD treatment. Since the above data suggest that the classical AhR–DRE-mediated pathway must be functional equally in both cell lines, the differential effect of TCDD observed between MEF+/+ and MEF−/− with respect to suppression of differentiation, support our interpretation that such a differential effect depends specifically on the presence of c-Src rather than a general TCDD non-responsiveness. We are aware of the possibility that, besides c-Src, other cell type characteristics of MEF−/− may differ from those of MEF+/+, since the absence of c-Src in −/− mice would force those cells to adopt some compensatory adjustments for their maintenance of normal physiology. Nevertheless, additional evidence for a c-Src-specific effect is indicated by the observation in a standard mouse embryonic fibroblast cell line, C3H10T1/2 cells, which were transfected with a c-Src-specific antisense oligonucleotide (Fig. 1B). The inhibitory effect of TCDD on adipocyte differentiation induced by A/G was significantly reduced by 50% in C3H10T1/2 cells when c-Src antisense was present. The antagonizing action of c-Src antisense on TCDD-mediated inhibition of adipogenesis was also found in a recent study from this laboratory with 3T3-L1 cells [40].

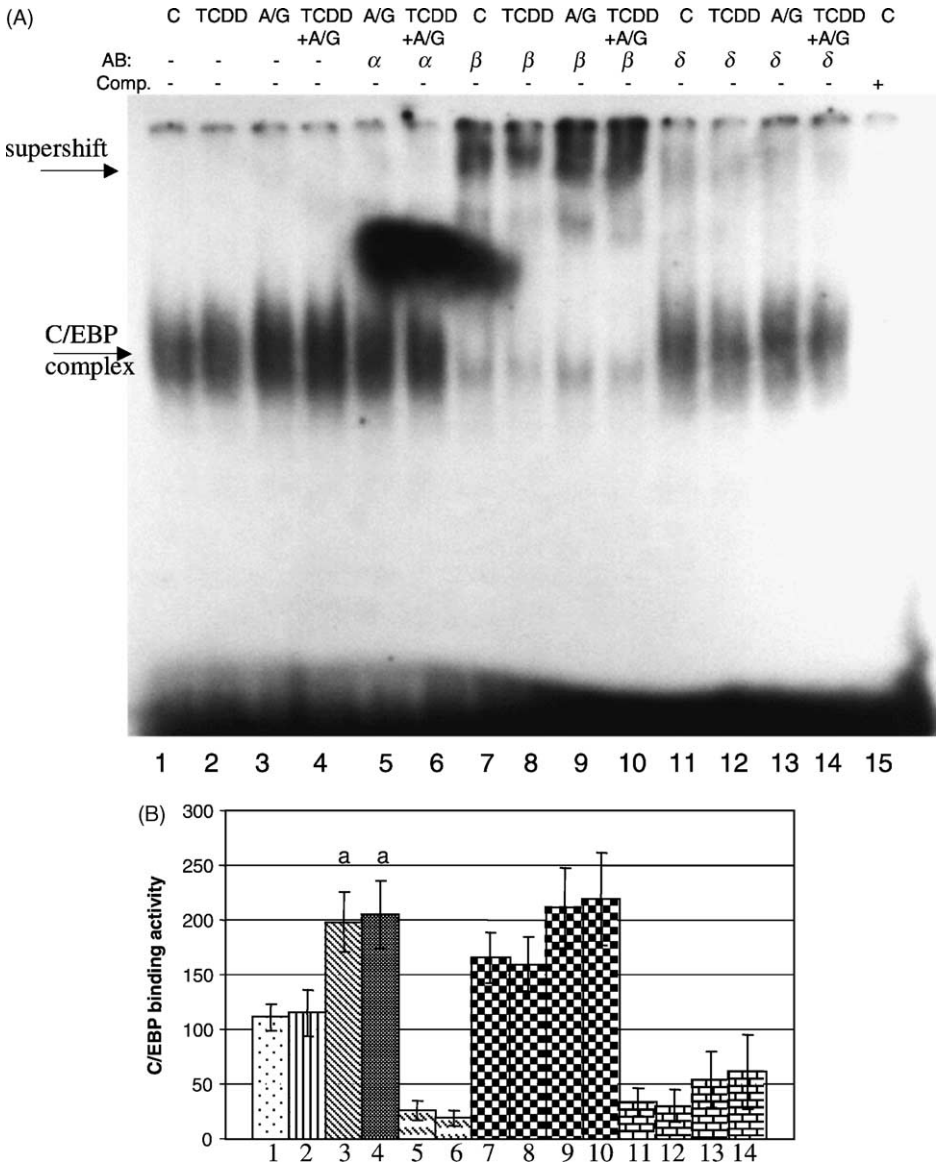

Fig. 10. DNA binding activity of C/EBP complexes in nuclear extracts from MEF−/−. (A) MEF−/− were treated for 3 days with 10 nM TCDD (lane 2), 82 μg/mL ascorbic acid (A)/10 mM β-glycerophosphate (G) (lane 3) or pretreated for 1 hr with 10 nM TCDD followed by A/G for 3 days (*TCDD + A/G*) (lane 4). Controls received 0.1% DMSO (lane 1). Supershift analysis with C/EBPα- (lanes 5 and 6), C/EBPβ- (lanes 7–10), and C/EBPδ-specific antibodies (lanes 11–14) identified isoforms of C/EBP binding complexes in A/G, TCDD and TCDD + A/G-treated cells. A 200-fold molar excess of unlabeled C/EBP was added in lane 15. (B) Densitometric evaluation of band intensities of the total (lanes 1–4) or upshifted (lanes 5–14) C/EBP binding activity is presented. Results of three separate experiments are shown as mean values ± SD; ([a]) significant different from control cells ($P < 0.05$).

C/EBP proteins are known to play a key role in determining the differentiation of cells as shown in different C/EBP knockout animals [25]. Confirming previous studies in liver and hepatoma cells [41], this study shows that TCDD induces C/EBPβ and C/EBPδ mRNA expression as well as their binding activity significantly in MEF+/+ cells. No TCDD-induced alteration of C/EBPβ and C/EBPδ was found in MEF−/−; this fact appears to be crucial in providing a basic explanation for the c-Src limited effect. It is interesting to note that this lack of action of TCDD in MEF−/− cells is accompanied with high constitutive expression levels of the two C/EBP isoforms which in turn might cause the insensitivity to the action of TCDD. Therefore, the increased level of C/EBPβ and/or C/EBPδ but not the ultimate level of these C/EBP isoforms might be critical to affect the differentiation process.

It was recently reported from this laboratory [40], that the F442A cell line, clonally derived from 3T3-L1 cells, is resistant to anti-adipogenic action of TCDD as well as exogenously added EGF, unlike the case of the original 3T3-L1 cells. It was found that 3T3-F443A cells express constitutively high levels of C/EBPβ and C/EBPδ as compared to 3T3-L1, where C/EBPβ and C/EBPδ expression is low before their induction by IBMX and dexamethasone. Such a pattern of differential responses of adipogenic cells to TCDD appears to be very similar to

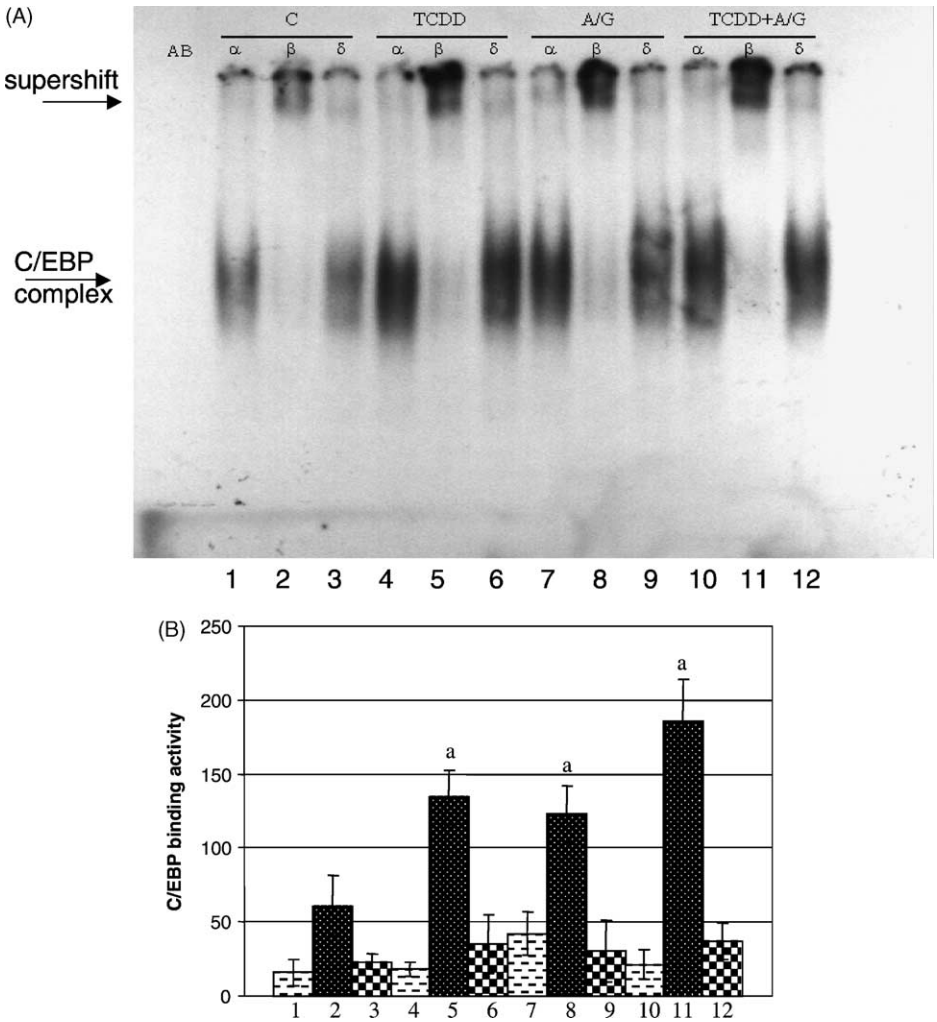

Fig. 11. DNA-bound C/EBP proteins upshifted with C/EBPα-, C/EBPβ-, and C/EBPδ-specific antibodies. (A) MEF+/+ were treated for 9 days with 10 nM TCDD (lanes 4–6), 82 μg/mL ascorbic acid (A)/10 mM β-glycerophosphate (G) (lanes 7–9) or pretreated for 1 hr with 10 nM TCDD followed by treatment with A/G for 9 days (TCDD + A/G) (lanes 10–12). Control cells received 0.1% DMSO (lanes 1–3). (B) Densitometric evaluation of band intensities of the upshifted C/EBP binding activity is presented. Results of three separate experiments are shown as mean values ± SD; ([a]) significant different from control cells ($P < 0.05$).

the case of MEF+/+ and MEF−/− cells, indicating the importance of C/EBPβ and C/EBPδ in the anti-adipogenic action of TCDD.

Besides the altered expression of C/EBPβ and C/EBPδ, we also found a suppressed C/EBPα binding activity by TCDD only in MEF+/+. In contrast to C/EBPβ and C/EBPδ which are directly increased by TCDD, C/EBPα was only affected by TCDD in differentiated cells under the treatment of A/G. Down-regulation of C/EBPα DNA binding activity was previously reported in different studies [14,19]; considering C/EBPα as a powerful promoter of adipogenesis and cellular differentiation, its down regulation seems to be an additional essential event and/or molecular endpoint for the inhibitory action on differentiation by TCDD. Several reports [30,34] have shown that the increase in the levels of C/EBPβ as well as C/EBPδ precedes the rise in the level of C/EBPα during adipogenesis. It is well known, that the levels of C/EBPβ and C/EBPδ decline as induced cells become fully differentiated and C/EBPα reaches its maximum.

If we hypothesize that the primary mechanism of action of TCDD in acting as an anti-adipogenic agent, is the untimely and inappropriate upregulation of C/EBPβ and C/EBPδ, one can explain why we found in the current study the immediate response of MEF+/+ to TCDD was upregulation of C/EBPβ and C/EBPδ (Fig. 5A). In that case, the eventual effect of TCDD to suppress C/EBPα upregulation by the adipogenesis inducers could be considered as an end-result of C/EBPβ and C/EBPδ over-expression. In support of the above hypothesis, it was previously shown by Liu *et al.* [41], that in Hepa 1C1C7 cells, which is devoid of any C/EBPα expression, TCDD still caused a clear cut increase in C/EBPβ without being coupled to C/EBPα.

Besides C/EBP proteins, the PPARγ is known to play a crucial role in the process of adipocyte differentiation; for

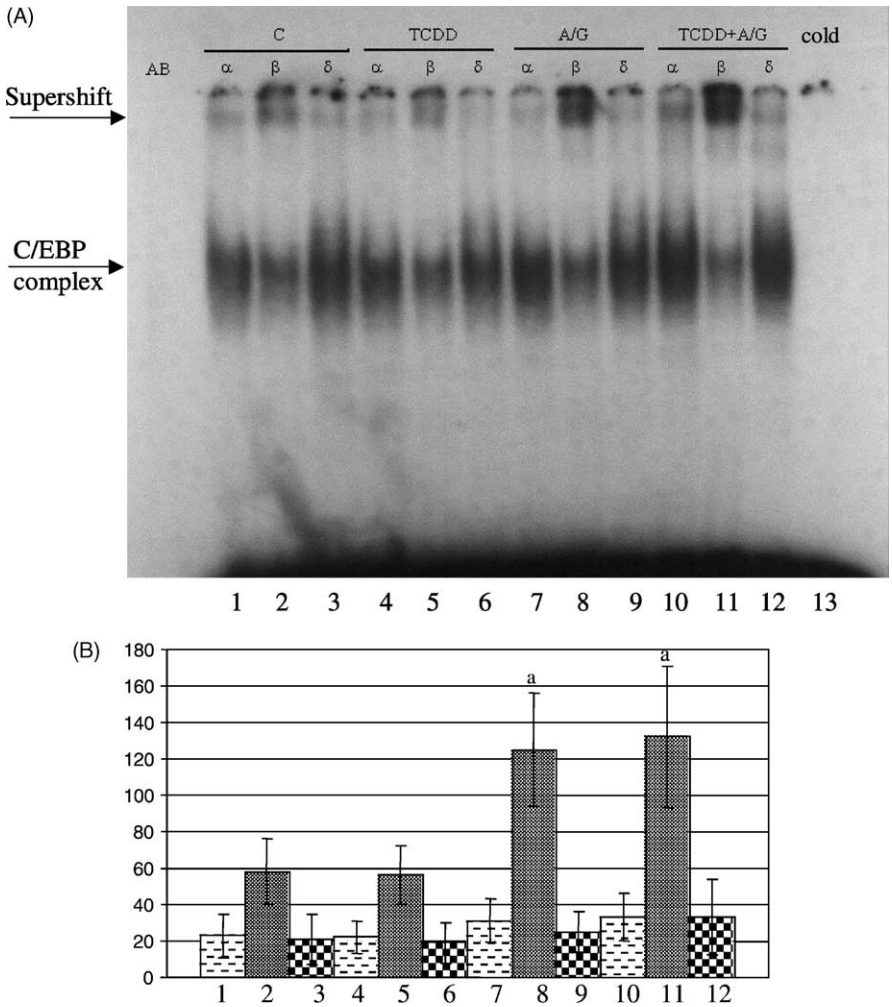

Fig. 12. DNA-bound C/EBP proteins upshifted with C/EBPα-, C/EBPβ-, and C/EBPδ-specific antibodies. (A) MEF−/− were treated for 9 days with 10 nM TCDD (lanes 4–6), 82 μg/mL ascorbic acid (A)/10 mM β-glycerophosphate (G) (lanes 7–9) or pretreated for 1 hr with 10 nM TCDD followed by treatment with A/G for 9 days (TCDD + A/G) (lanes 10–12). Control cells received 0.1% DMSO (lanes 1–3). (B) Densitometric evaluation of band intensities of the upshifted C/EBP binding activity is presented. Results of three separate experiments are shown as mean values ± SD; ([a]) significant different from control cells ($P < 0.05$).

instance, by elevating C/EBPα protein. In line with previous findings TCDD downregulates significantly the mRNA expression of PPARγ in MEF+/+ cells, which were incubated with differentiation inducing agents A/G. However, the additional treatment of MEF+/+ cells with the PPARγ agonist troglitazone could not abrogate the inhibitory action of TCDD on cell differentiation, showing that TCDD does not cause a direct antagonistic action on PPARγ ligands.

One major question remaining to be addressed is the relationship between c-Src and C/EBP (proteins). Previously it was shown by Dunlap *et al.* [26,27] that the degree of TCDD downregulation of C/EBPα protein in liver was much lower in c-Src−/− and c-Src−/+ than that of c-Src+/+ mice, indicating that these two events are tightly coupled. Another relevant observation made by Liu *et al.* [40] is that the same phenomenon of TCDD-induced C/EBPβ level and downregulation of C/EBPα can be reproduced by exposing 3T3-L1 cells to EGF instead of TCDD. Both, TCDD and EGF are capable to block hormone-induced adipogenesis. Furthermore, F442A cells, overexpressing C/EBPβ, are also resistant to EGF as well as TCDD. These pieces of evidence clearly support the interpretation that the signal, triggered by TCDD, which is mediated by c-Src and EGF receptor activation is one of the main causes of disturbance of the C/EBP protein expression. Thus, deficiency in c-Src contributes to the decreased responsiveness by removing this important mediator of toxic signaling component and by forcing the cells to readjust C/EBP protein expression.

In summary, we could clearly establish in the current study that the inhibitory action of TCDD on adipocyte differentiation process was absent in c-Src−/− MEF unlike MEF+/+ cells. Such an observation supports the view that c-Src kinase plays an important role in mediating the antagonistic action of TCDD on the adipocyte differentiation process.

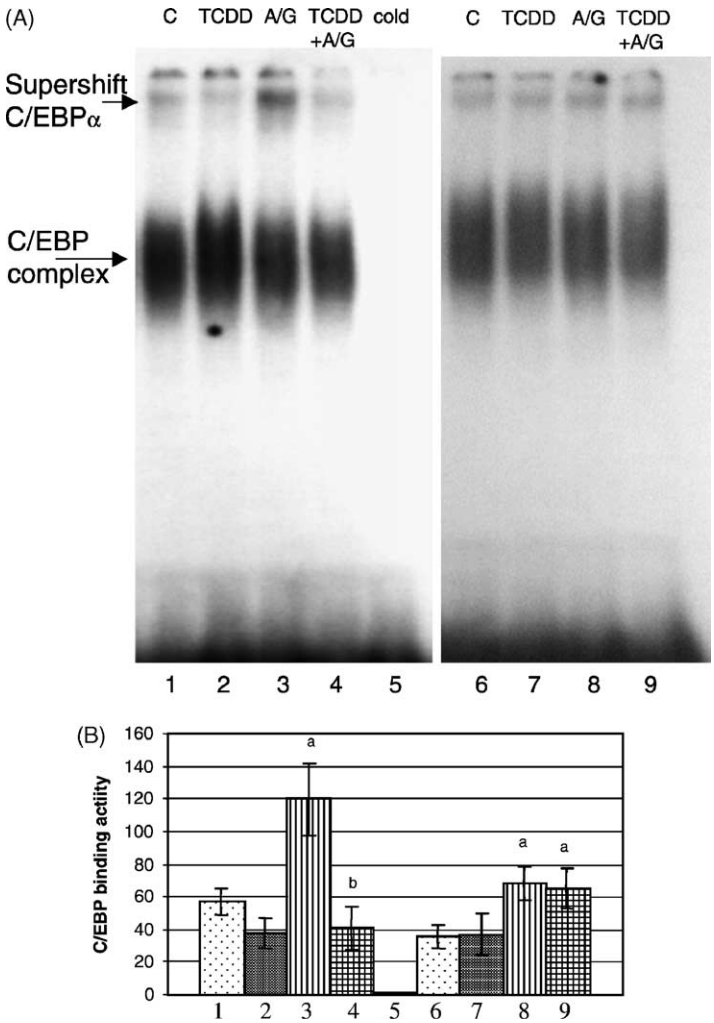

Fig. 13. C/EBPα binding activity in MEF after 12 days. MEF+/+ (lanes 1–5) and MEF−/− (lanes 6–9) were treated with TCDD (lanes 2 and 7), with A/G (lanes 3 and 8), or pretreated with 10 nM TCDD (TCDD + A/G, lanes 4 and 9). A 200-fold molar excess of unlabeled C/EBP was added in lane 5. (B) Densitometric evaluation of the upshifted band intensities is presented. Results of three separate experiments are shown as mean values ± SD; ([a]) significant different from control cells ($P < 0.05$); ([b]) significant different from A/G-treated cells ($P < 0.05$).

Acknowledgments

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