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Depletion of Arylhydrocarbon Receptor during Adipose Differentiation in 3T3-L1 Cells

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Arylhydrocarbon receptor (AhR) is the receptor for 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and related compounds. Although a physiological ligand for AhR has yet to be identified, several lines of evidence suggest that AhR may play an important role not only in the regulation of xenobiotic metabolism but also in the maintenance of homeostatic functions. When TCDD is administrated in vivo, this compound is primarily deposited in adipose tissue. Therefore, it is critical to know the states of AhR in adipose cells for assessing the expression of toxicities of TCDD and related compounds in vivo. In this report, we examined the levels of AhR protein and its associated protein (Arnt) during the adipose differentiation in 3T3-L1 cells. The level of AhR protein was found to decrease with ongoing adipose differentiation in 3T3-L1 cells. The binding activity to the xenobiotic response element and the cellular response to TCDD were also lowered as a result of adipose differentiation. These results indicate that the depletion of AhR is a novel event associated with adipose differentiation in 3T3-L1 cells and that the magnitude of the depletion of AhR is sufficient for 3T3-L1 cells to lose the functional response to xenobiotics. We also found a population of 3T3-L1 cells which have an adipose differentiation capability in the presence of high doses of TCDD. These cells lack nuclear AhR but not cytoplasmic AhR, suggesting a possible negative role of liganded nuclear AhR in adipose differentiation. The level of the Arnt protein also decreased as a result of the differentiation. However, the pattern of the depletion of the Arnt protein was distinct from that of the AhR protein. The data presented in this study will provide opportunities to carry out studies to better understand the roles of AhR in adipose cells which are the primary targets of TCDD.

Arylhydrocarbon receptor (AhR) is the receptor for 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and related compounds. The AhR contains basic helix-loop-helix structure (bHLH)/PAS domain and function as transcription factor (1, 2). Unliganded AhR exists in the cytoplasm in a form of a complex with 90-kDa heat shock protein (HSP90) and FKBP-like protein (3-6). Upon binding to the ligand, AhR dissociates from the complex and translocates to the nucleus followed by the formation of a heterodimer with the AhR nuclear translocator (Arnt) (7). This heterodimer binds to specific enhancer sequence (termed xenobiotic response element; XRE) within the promoter region of various genes (8-10). Therefore, most, if not all, of the toxic effects of TCDD and its related compounds are due to the modulation of gene expressions.

Although a physiological ligand for AhR has yet to be identified, several reports have shown the constitutive activation of the AhR in the absence of exogenous ligand (11-13). The precise physiological roles of AhR are unknown. Studies using AhR-knock out mice showed that AhR is involved in the normal development of the liver (14-16). AhR-defective mouse hepatoma cells exhibit a different morphology, a decreased albumin synthesis and a prolonged doubling time compared with wild-type cells. Interestingly, introduction of AhR cDNA into the AhR-defective cells alters these characteristics such that the cells resemble wild-type cells, suggesting a physiological function of AhR in mice livers (17). In cell differentiation, the level of AhR is elevated during HaCat-keratinocyte differentiation (18) and monocytic differentiation of HL 60 cells (19). Collectively, the AhR may play important roles not only in the regulation of xenobiotic metabolism but also in the maintenance of homeostatic functions.

TCDD, a highly toxic compound that has recently attracted much attention as an environmental contaminant, elicits a variety of toxic responses. These include chloracne, tumor promotion, thymic involution, hydronephrosis, cleft palate and wasting syndrome (20-23). Because of its high lipophilicity, TCDD is primarily deposited into adipose tissue in vivo. In adipose tissue, the inhibition of glucose transport (24, 25), lipoprotein

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lipase activity (26) and fatty acid synthesis (27) have been reported as a result of the presence of TCDD. This compound inhibits the expression of adipose differentiation-specific transcription factors such as C/EBP α and PPAR γ2 in 3T3-L1 cells (28). Furthermore, TCDD increases the binding of COUP-TF, a negative regulator of adipose differentiation, to a PPAR/RXR binding sequence (29). These inhibitory effects of TCDD in adipose differentiation may be the result of interaction with AhR. However, there is little information concerning the states of AhR during adipose differentiation.

In this report, we examined the level of AhR during the adipose differentiation in 3T3-L1 cells and observed a reduction in AhR during differentiation followed by a reduction in DNA binding activity in vitro.

MATERIALS AND METHODS

Cell culture. 3T3-L1 cells, obtained from Human Science Research Resources Bank (Osaka), were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum (CS). For induction of adipose differentiation, cells were plated at a density of 1 x 10^4 cells/35 mm dish and passaged just before confluence. The cells were then fed with a differentiation medium (mixture of DMEM and Ham's F12 (3:1) containing 10% CS, 1.6 μM insulin, 0.0005% transferrin, 180 μM adenine, 20 pM triiodothyronine, 0.25 μM dexamethasone (DEX) and 500 μM isobutylxanthine (IBMX)). After 3 days, the cells were re-fed with fresh differentiation medium without DEX and IBMX and maintained for following days. Hep G2 cells, obtained from the Cancer Cell Repository, Tohoku University, were propagated in DMEM supplemented with 10% fetal bovine serum.

Cell fractionation. The cells grown in 35 mm dishes were rinsed with ice-cold phosphate buffered saline (PBS). The rinsed cells were scraped off the dish, placed in a microcentrifuge tube, and centrifuged at 5000 x g for 1 min. The resulting pellets were lysed in 400 μl of buffer A (10 mM Hepes-KOH (pH 7.8), 10 mM KCl, 0.1 mM EDTA, 1 mM DTT and 0.1% Nonidet P-40), vortexed and centrifuged at 5000 x g for 1 min. The supernatants (cytosolic fraction) were frozen after the concentrations of KCl and glycerol were brought to 100 mM and 10%, respectively. The resulting pellets were resuspended in 100 μl of the buffer B (50 mM Hepes-KOH (pH 7.8), 420 mM KCl, 0.1 mM EDTA, 5 mM $MgCl_2$, 1 mM DTT, 0.5 mM PMSF, 0.0002% leupeptin, and 20% glycerol) and were rocked at 4°C for 60 min. The suspensions were centrifuged and the resulting supernatants (nuclear fraction) were then frozen until further analysis. To prepare the whole cell extract, the cells washed with PBS were suspended in the buffer B, vortexed, and centrifuged for 10 min at 10000 xg. The resulting supernatants were subjected to the western blotting analysis. Protein concentration of the extracts was determined according to the method of Bradford, using bovine serum albumin as standard (31).

Western blotting. Protein samples were denatured by heating to 90°C in SDS-reducing buffer and were resolved by electrophoresis on 10% SDS-polyacrylamide gel. After transfer to a nitrocellulose membrane, the filters were probed with antibodies against AhR or Arnt (Affinity Bioregents, Inc.). Color visualization was performed using the secondary antibodies conjugated with alkaline phosphatase and nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate substrate solution (Promega). Protein expression was quantitated with the use of National Institutes of Health Image 1.61 software as described previously (32).

Electrophoretic mobility shift assays (EMSA). XRE binding reactions were performed at a volume of 30 μl containing 25 mM Hepes-NaOH (pH 7.9), 150 mM NaCl, 1.5 mM EDTA, 1 μg of poly(dI-dC), 10% glycerol, and 5 μg of the nuclear extract protein. The mixture was incubated for 30 min at room temperature prior to the addition of double stranded oligonucleotides containing the XRE sequence (33). The incubation was continued for a further 30 min after the addition of the probe. The mixtures were loaded onto 4.5 % nondenaturing polyacrylamide gel in 0.5 x TBE buffer and electrophoresed.

Oil red O staining. To judge the states of adipose differentiation by visual inspection, cultures were fixed with 10% formalin in PBS for 2 h, rinsed 3 times with distilled water and then air dried. The fixed cells were stained with 0.5% Oil red O solution for 1 h. After staining, the cultures were rinsed with 70% ethanol several times.

Glycerophosphate dehydrogenase (GPDH) activity. Cells grown in 35 mm culture dishes were rinsed twice with ice cold PBS, scraped into 0.2 ml of the extraction buffer (25 mM Tris-HCl (pH 7.5), 1 mM EDTA), and homogenized with a Teflon/ pestle drill apparatus. The homogenate was centrifuged for 10 min at 4°C. The supernatant obtained was subjected to an assay for GPDH activity. The activity was assayed by monitoring the decrease in absorbance at 340 nm of NADH in the presence of dihydroxyacetone phosphate (30).

Immunofluorescence staining. Cells were grown on glass slides coated with collagen, fixed with 2% paraformaldehyde in PBS, and permeabilized in methanol at -30°C. Cells were then stained with anti-AhR antibodies (Affinity Bioregents, Inc.) and the secondary antibodies labeled with FITC. Experiments were performed at least three times, and , on average, 20 fields were evaluated on each slip.

RESULTS

The Level of AhR Decreased in Adipose 3T3-L1 Cells

Preadipose 3T3-L1 cells were differentiated to adipocytes by the addition of DEX and IBMX. The adipocytes were confirmed by staining with Oil red O and measurement of GPDH activity following 168 h induction (data not shown). The expression of the AhR protein in preadipose and adipose cells was analyzed by western blotting using specific antibodies against AhR. As shown in Fig. 1, the density of the 95 kDa protein in the extract of the differentiated cells was one-tenth of that in the preadipose cells. To exclude the possibility that the depletion of AhR is due to the direct effects of DEX and IBMX, Hep G2 cells were treated with these inducers. The level of AhR in Hep G2 cells was unchanged by treatment with the adipose inducers (Fig. 1).

AhR and Arnt Are Depleted during Differentiation

We examined the kinetics of AhR and Arnt expression following induction of differentiation. Immunoblot analysis showed that the level of AhR began to decrease after 24 h of the induction and was markedly diminished in the differentiated cells (168 h) (Fig. 2A). We also determined the GPDH activity, a marker enzyme in adipose differentiation, to evaluate the differentiation states of the cells. The induction of the GPDH activity showed an inverse relationship to the depletion of AhR (Fig. 2B). By comparison, the Arnt protein was hardly detectable with the system used after 168 h of induction as in the case of AhR. However, the expres-

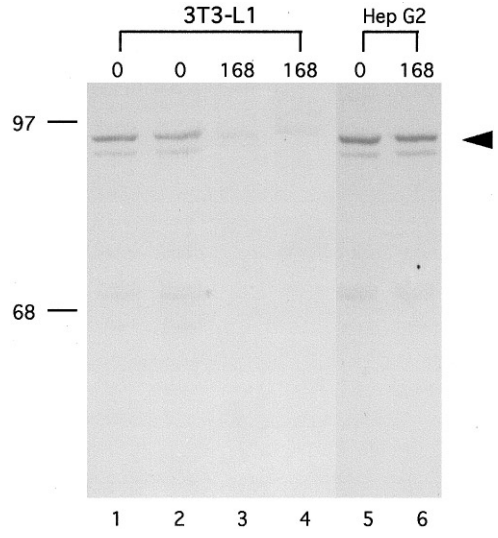

FIG. 1. Depletion of the AhR protein in differentiated 3T3-L1 cells. The cells were cultured in the differentiation medium for 168 h. Western blot analysis of AhR was performed on the whole cell extracts (10 μg) that were resolved by electrophoresis on a 10% SDS/polyacrylamide gel. Lanes 1 and 3 were from a different experiment from lanes 2 and 4.

sion pattern of the Arnt protein was distinct from that of AhR, i.e., Arnt protein was induced and was maximal by 48 h after the addition of DEX and IBMX. Interestingly, Arnt protein rapidly disappeared in the subsequent differentiation process (Fig. 2A).

Loss of XRE Binding Activity during Differentiation

In a further set of experiments, the changes in XRE binding activity during adipose differentiation were examined. Nuclear extracts prepared from the cells at various stages of differentiation were tested for XRE binding activity by EMSA. As shown in Fig. 3A, XRE binding activity began to decrease following treatment with the inducers for 48 h, and the activity in the adipose cells (168 h treatment) was one-tenth of that in the preadipose cells. The binding activity was observed without an exogenous ligand and was substantially unchanged by the treatment of cells with 100 nM TCDD or the addition of 100 nM TCDD to the reaction mixture (data not shown). The specificity of binding was confirmed by the competition with excess amounts of nonlabeled wild type XRE oligonucleotides or of mutant XRE oligonucleotides (Fig. 3B). The band identified was supershifted in the presence of the anti-AhR antibody (Fig. 3B).

TCDD Is Ineffective to the Cells in the Late Stages of Differentiation

We examined whether the differentiation-associated changes in AhR level were of sufficient magnitude to affect the inhibitory effect of TCDD on adipose differentiation in 3T3-L1 cells. TCDD was added to the cultures at various time intervals following the addition of DEX and IBMX. After induction for 168 h, the extent of the inhibitory effect of TCDD on adipose differentiation was estimated by the staining of the lipid droplets with Oil red O and by measuring the GPDH activity. The cells exposed to TCDD within the first 24 h of induction failed to differentiate to adipose cells as judged by the adipose staining (Fig. 4A) and the enzyme activity of the GPDH (Fig. 4B). In contrast, later addition (>48 h) of TCDD was ineffective, and the cells were differentiated as well as control cells (Fig. 4). The GPDH activity appeared higher than expected based on the staining of the droplets with Oil red O. One possible reason is that TCDD may inhibit the differentiation process between the induction of GPDH and the accumulation of lipids. Alternatively, TCDD may induce the GPDH independence of differentiation.

TCDD-Resistant Cells Lack Nuclear AhR

During this study, we accidentally found a population of 3T3-L1 cells that are resistant to TCDD. The cells grow normally and are able to differentiate to adipose cells (data not shown). However, the cells differentiated even in the presence of 100 nM TCDD (Fig. 5A).

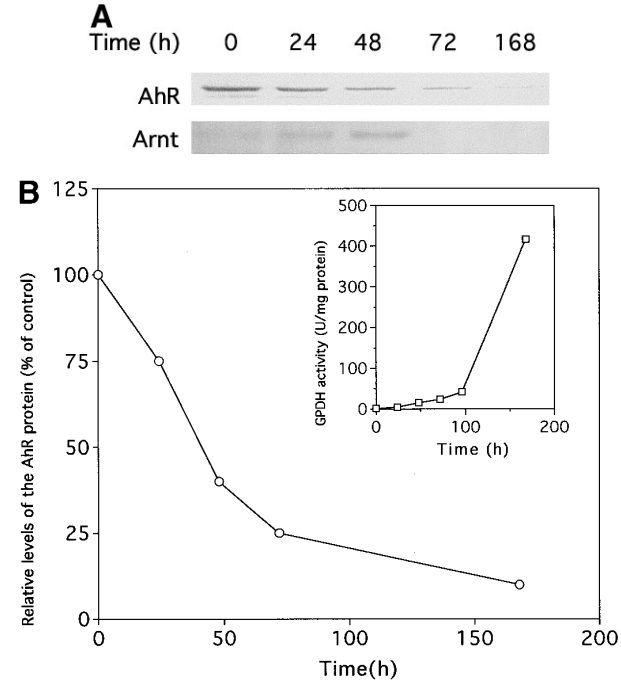

FIG. 2. Kinetics of depletion of the AhR protein and Arnt protein. (A) Western blots of AhR and Arnt in the whole cell extracts prepared at various stages of differentiation. (B) The magnitude of AhR was assessed by densitometry. The relative intensity for the 0 time treatment group was arbitrarily assigned a value of 100% to which to all other groups are compared. Results are representative of four separate experiments. *Insert,* induction of GPDH activity during differentiation.

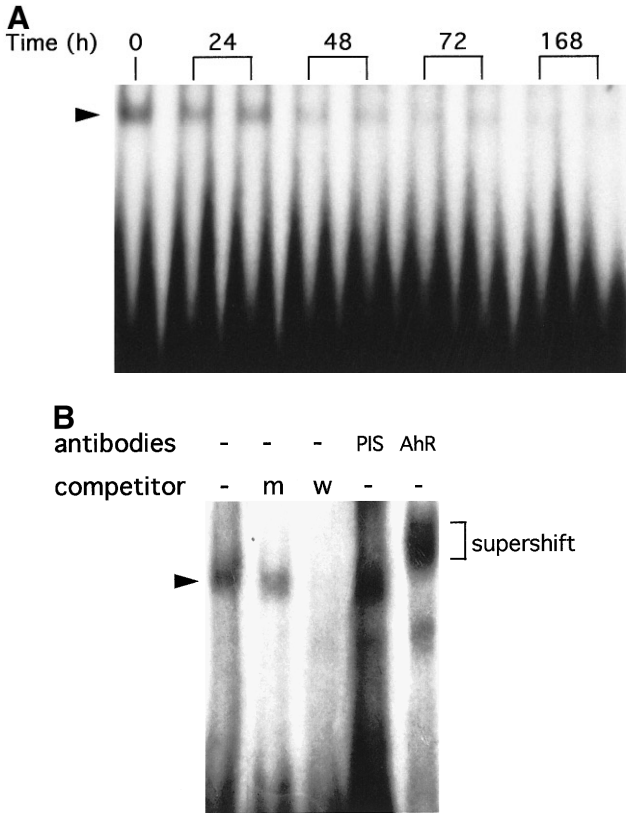

FIG. 3. Kinetics of DNA binding activity during adipose differentiation. (A) EMSA binding reactions were performed by incubating the nuclear extracts with ^{32}P-labeled double-stranded oligonucleotides specifying the XRE. The arrow indicates the position of the specific retarded band. The experiment was repeated three times with similar results. (B) Unlabeled competitor oligonucleotides (m; mutated XRE, w; wild type XRE) were present in 100-fold molar excess relative to the XRE probe. AhR antibodies or preimmune serum (PIS) were added to the performed DNA:protein complexes for 15 min prior to electrophoresis. The arrow indicates the position of the AhR complex.

To characterize these cells, we examined their expression of the AhR protein by western blotting (Fig. 5B) and immunofluorescence staining (Fig. 5C). AhR in normal cells is distributed evenly between the cytoplasm and nucleus, making the distinction between cytoplasmic and nuclear compartments difficult in cells (Fig. 5C). This staining pattern is similar to those in the recent reports (11, 12). As with resistant cells, AhR was only detectable in the cytoplasmic fraction but not in the nuclear fraction (Fig. 5B). Immunocytochemical study showed that AhR in the resistant cells is mostly localized in the cytoplasm (Fig. 5B). XRE binding activity was not found in the resistant cells even after the treatment with 100 nM TCDD, whereas the control cells have constitutive activity (Fig. 5D).

DISCUSSION

When TCDD and related compounds are administrated in vivo, they are primarily deposited in adipose tissue (34). Subsequently, most, if not all, of these compounds interact with its specific receptor, AhR. Therefore, it is critical to know the state of AhR in adipose cells for assessing the expression of toxicities of TCDD and the related compounds in vivo. We have shown here that AhR was found to decrease with ongoing adipose differentiation in 3T3-L1 cells. The depletion of AhR was accompanied by a loss of binding activity to the XRE sequence and of the functional responses of cells to TCDD, i.e., the addition of TCDD to the cells in the early stage of differentiation inhibited subsequent differentiation but the cells in the late stage of differen-

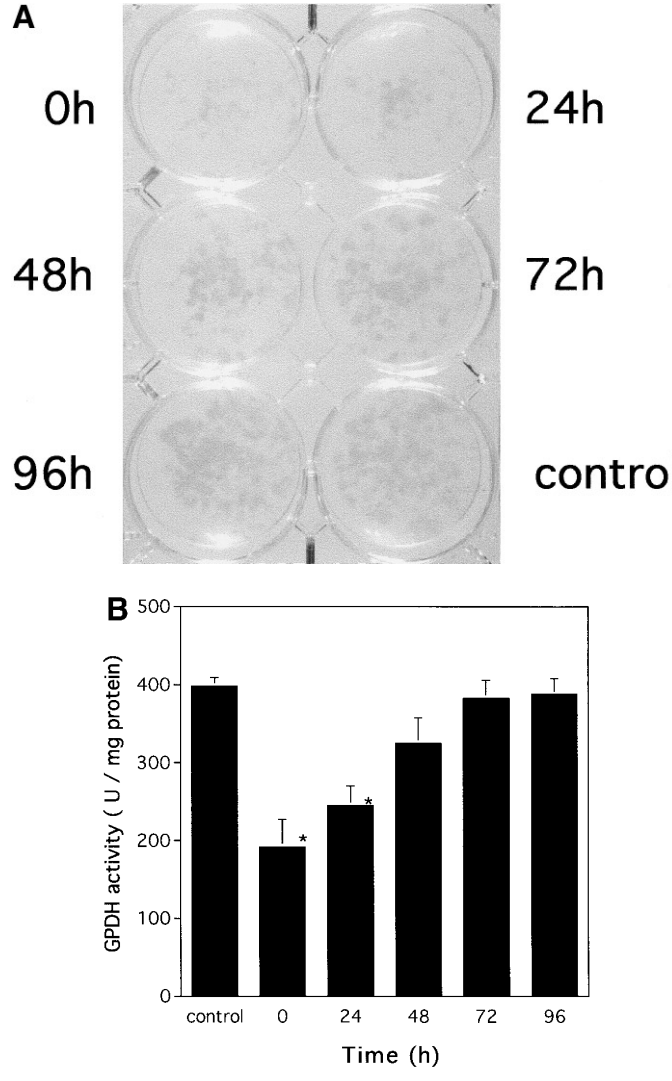

FIG. 4. Effect of time of TCDD treatment on adipocytes. Cells were treated with 1 nM TCDD at the time of DEX and IBMX addition (0 h) or later as indicated. TCDD was dissolved in dimethyl sulfoxide (DMSO), and control cells were given the same amounts (2 μl) of DMSO. Staining of adipocytes with Oil red O (A) and assays of the GPDH activity (B) were performed following 168 h induction of differentiation. The results are the means of three separate experiments. Asterisks indicate significant differences ($p < 0.01$) from the value of the control.

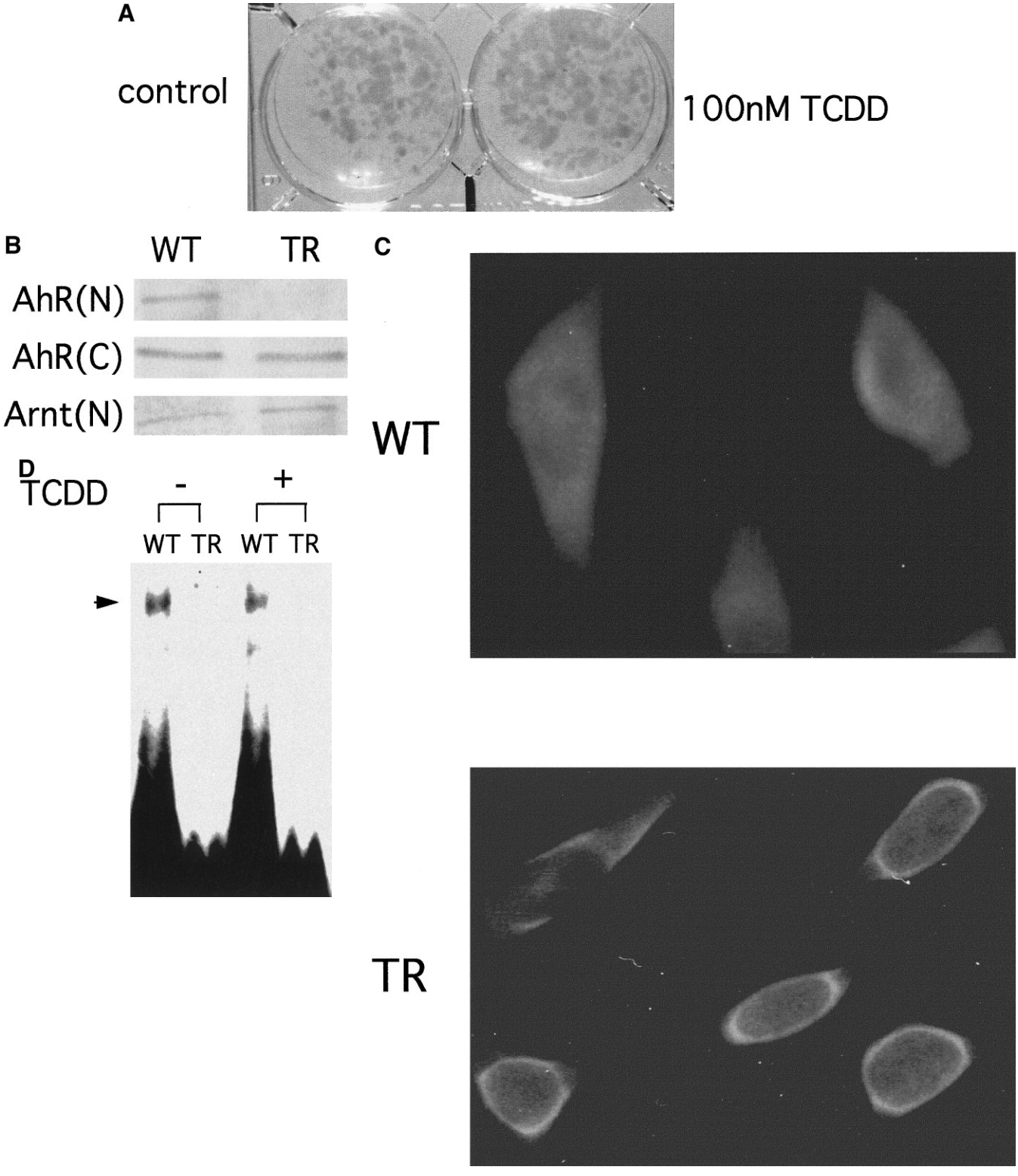

FIG. 5. Characteristics of TCDD-resistant clone of 3T3-L1 cells. (A) The cells were treated with either 100 nM TCDD or DMSO (control cells) during differentiation (168 h). The cultures were fixed and stained with Oil red O. (B) Representative western blot of TCDD-resistant 3T3-L1 cells stained for AhR and Arnt. WT; normal 3T3-L1 cells, TR; TCDD-resistant 3T3-L1 cells, N; nucleus, C; cytoplasm. (C) Immunofluorescence microscopy of 3T3-L1 cells stained for AhR. WT; normal 3T3-L1 cells, TR; TCDD-resistant 3T3-L1 cells. (D) Nuclear extracts from cells cultured for 24 h with DMSO or 100 nM TCDD were analyzed by EMSA. WT; normal 3T3-L1 cells, TR; TCDD-resistant 3T3-L1 cells.

tiation were tolerant to TCDD. The level of AhR in Hep G2 cells was unchanged by treatment with IBMX and DEX. Collectively, these results indicate that the depletion of the AhR protein is dependent on the state of the adipose differentiation process of the cells but not on the direct effect of IBMX and DEX. The means by which AhR is depleted during differentiation is unknown. Sato et al. reported that retinoid and vitamin D inhibit adipose differentiation in 3T3-L1 cells and their receptors are depleted during differentiation (35, 36). These results suggest that these receptors and AhR may show inhibitory effects on adipose differentiation by similar mechanisms. However, the molecular mechanisms remain to be elucidated.

Several lines of evidences suggest that AhR play important roles not only in the regulation of the xenobiotic metabolism but also in the maintenance of homeostatic functions. AhR is postulated to be involved in normal liver development based on studies using AhR-defective mice (14-16). Stable transfection of AhR cDNA into receptorless hepatoma cells has shown that AhR play important roles in the control of the cell cycle progression and that no exogenous ligands are required for the function (17). Other members of the bHLH/PAS gene family such as SIM are believed to participate in early embryonic development (37). The treatment of culturing embryos with AhR antisense oligonucleotides resulted in a significantly lower incidence of both blastocyst formation and mean embryo cell number (38). The AhR protein is increased during differentiation toward keratinocytes and monocytes (18, 19). Therefore, it is likely that AhR play roles in some aspects of development and differentiation. The physiological role, if any, of AhR for adipose differentiation in 3T3-L1 cells is unknown. The cells exposed to 1 nM TCDD failed to differentiate, but the cells lacking nuclear AhR were differentiated even in the presence of 100 nM TCDD (Fig. 5), suggesting that liganded nuclear AhR affect the machinery of adipose differentiation. Liu et al. reported that the induction of C/EBP α and PPAR γ2, which are adipose differentiation-associated transcription factors, is blocked in the presence of TCDD (28). The DNA binding of COUP-TF, a negative regulator of adipose differentiation, is increased by the TCDD treatment of 3T3-L1 cells (29). Interestingly, fatty metamorphosis was observed in AhR-deficient mice liver (15). Consequently, one possible role of AhR in 3T3-L1 cells could be participation in the negative regulation of adipose differentiation. However, the precise role of AhR in adipose differentiation must be further investigated.

The expression of Arnt was detectable in the undifferentiated cells but not in the differentiated cells as in the case of AhR (Fig. 2). However, the processes of the depletion of these two proteins during differentiation were distinct from each other (Fig. 2). While AhR was decreased with ongoing differentiation, the level of Arnt was increased and the expression was maximal by 48 h of induction. Recent reports have described that mice embryos lacking Arnt are not viable (39). Arnt can bind to the E-box motif as a homodimer (33) and can form a heterodimer with other bHLH/PAS proteins, SIM and HIF-1α (40-45). Interestingly, HIF-1α is induced at early stage of differentiation in 3T3-L1 cells (46). These results, together with our observations, suggest that Arnt may play other physiological roles in addition to the co-work with the AhR during adipose differentiation in 3T3-L1 cells.

In the course of this study, we found a population of 3T3-L1 cells that are resistant to TCDD. The cells grow normally (data not shown) and are able to differentiate to adipose cells even in the presence of 100 nM TCDD (Fig. 5A). Interestingly, AhR in this cells is not detectable in nucleus as judged by western blotting (Fig. 5B). Indirect immunostaining of the cells showed that AhR is mostly localized in perinuclear portion, whereas AhR in normal cells is distributed evenly between the cytoplasm and nucleus (Fig. 5C). Treatment of resistant cells with 100 nM TCDD failed to induce XRE binding activity (Fig. 5D). These results suggest that translocation of AhR may be affected in resistant cells.

In conclusion, this study is the first to report that AhR and Arnt are decreased as a result of adipose differentiation. The data presented in this study will provide opportunities to carry out studies to better understand the roles of AhR in adipose cells which are the primary targets of TCDD.

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