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Prolonged Depletion of AH Receptor without Alteration of Receptor mRNA Levels after Treatment of Cells in Culture with 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin*

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ABSTRACT. Previous experiments have shown that the total cellular content of the AH receptor (AHR) drops rapidly after exposure of mouse hepatoma cells (Hepa-1) to the potent AHR ligand 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD); within 6 hr after treatment, less than 20% of the original cell content of AHR can be detected by radioligand binding or by immunoblotting. The goals of our current study were to determine the duration of receptor depletion following treatment with ligand and to determine if depletion is due to decreased expression of the *Ahr* gene that encodes the AHR. We found that depletion of AHR persisted for at least 72 hr after exposure to TCDD. Treatment with 3-methylcholanthrene caused a transient drop in total cell AHR, but the AHR levels returned to near pretreatment levels within 72 hr after the first exposure. TCDD treatment did not alter the levels of AHR mRNA as assessed by reverse transcription–polymerase chain reaction or slot blot assays. Thus, the decrease in AHR protein cannot be attributed to depression of transcription of the *Ahr* gene by TCDD. TCDD treatment did not alter the levels of the dimerization partner of the AHR, the AH receptor nuclear translocator protein (ARNT), or ARNT mRNA. In the presence of TCDD, both the AHR and the ARNT protein can be maintained at high levels in the nucleus if transcription is inhibited with actinomycin-D. In the absence of actinomycin-D, the AHR protein was lost rapidly, but the ARNT protein level in the cell was maintained. Together, these results suggest that the AHR protein is degraded through a selective mechanism that spares the ARNT protein and that the degradation pathway involves a protein that itself has a short half-life. BIOCHEM PHARMACOL 55;4:489–497, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. AH receptor; AH receptor nuclear translocator protein (ARNT); 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; TCDD; 3-methylcholanthrene; Hepa-1 hepatoma cells

The AHR‖ mediates toxic effects of dioxin-like halogenated aromatic hydrocarbons [1–3] and also is a transcriptional regulator of several Phase I enzymes such as CYP1A1, CYP1A2, and CYP1B1, as well as certain Phase II enzymes such as some species of UDP-glucuronosyltransferase and GST [4–7]. The AHR-signaling pathway has considerable implications for the responses of humans and other vertebrates to potentially harmful xenobiotic chemicals [4, 8].

The AHR mechanism has been explored most thoroughly in a mouse hepatoma cell system employing Hepa-1 cells and their mutants [9]. In this model system, a specific sequence of events has been established: ligands bind to the receptor in cytoplasm; the receptor dimerizes with the ARNT protein; the ligand · AHR · ARNT complex binds to specific AHREs located in the 5′-flanking region of genes whose expression is altered by AHR ligands [7].

The steps leading to DNA binding are complex and are not understood completely. Even less is known about the fate of the AHR following its nuclear binding to AHREs. We and others [10–13] previously found that the AHR population is rapidly depleted in mouse Hepa-1 cells treated with TCDD in culture and that receptor levels, as measured by radioligand binding or immunoblotting, remain depressed for up to 24 hr. The mechanism by which ligand-induced receptor depletion takes place is unknown. In the current study, we sought to determine the duration of AHR

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‖ *Abbreviations:* AHR, aromatic hydrocarbon receptor; AHRE, AH responsive element (also known as dioxin responsive element, DRE); ARNT, AH receptor nuclear translocator protein; DTT, dithiothreitol; GST, glutathione *S*-transferase; Hepa-1, Hepa-1c1c9 cells; MC, 3-methylcholanthrene; RT–PCR, reverse transcription–polymerase chain reaction; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; and TCDF, 2,3,7,8-tetrachlorodibenzofuran.

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depletion in Hepa-1 cells and to determine if the loss of receptor from TCDD-treated cells might be due to ligand-induced depression of mRNA levels encoding the receptor. We found that the content of cytosolic + nuclear AHR remains depressed for up to 72 hr after treatment with TCDD but that TCDD treatment has no effect on receptor mRNA levels.

MATERIALS AND METHODS

Chemicals

[^{3}H]TCDD (40 Ci/mmol) was purchased from Chemsyn Laboratories. TCDF was a gift from Dr. Stephen Safe (Texas A&M University). An ECL immunochemiluminescence kit, donkey anti-rabbit antibody linked to horseradish peroxidase, and [α-^{32}P]dCTP were from Amersham Canada Ltd. M-MLV reverse transcriptase, TRIZOL, and fetal bovine serum were from Gibco/BRL. RNase-inhibitor, DNase, dNTPs, and random hexamers were from Pharmacia. Ultra-Therm DNA polymerase was from Bio/Can. Primers were synthesized by ACGT. Bradford Reagent was from Bio-Rad. All other chemicals were from Sigma.

Buffers

HEGD consisted of 25 mM HEPES, 1.5 mM EDTA, 10% glycerol, and 1 mM DTT, pH 7.4. HED was HEGD without the glycerol; HE2GD was HEGD with 20% glycerol. TNT contained 10 mM Tris, 150 mM NaCl, and 0.2% Tween-20, pH 8. BLOTTO was 3% skim milk powder in TNT. The protein sample buffer was 60 mM Tris, 2% SDS, 10% glycerol, 100 mM DTT, and 0.025% bromophenol blue. The DNA sample buffer was composed of 10% glycerol, 0.25% bromophenol blue, and 0.25% xylene cyanole FF.

Cells and Treatment of Cells in Culture

Hepa-1 cells, obtained as described previously [11], were grown in α-Minimum Essential Medium (antibiotic-free) supplemented with 10% fetal bovine serum [11]. Monolayer cultures in 100-mm diameter plates (Falcon) were exposed to medium containing 2 nM TCDD or 100 nM MC for various time intervals as shown in the figure legends. The ligand concentrations were based on the relative potency of TCDD and MC for inducing CYP1A1 in these cells [14]. The same TCDD-containing or MC-containing medium remained on the plates throughout the period of exposure. Due to our goal of testing the effect of prolonged incubation with TCDD or MC (up to 72 hr), cells were initially exposed to the ligands when the cells were approximately 25–30% confluent to ensure that the cell population did not reach a density-dependent growth limitation during the exposure period. The cells were held in standard CO_2 incubators at 37° for the times indicated in the figure legends. Subcellular fractions were prepared immediately at the end of the incubation period.

Preparation of Subcellular Fractions

Cells were washed with PBS and removed from plates with 0.025% trypsin (Gibco). The cells were collected and washed three times in cold PBS. The cell pellets were resuspended in cold HED buffer, and then were incubated on ice for 15 min. A Polytron fitted with a PT7 generator (Brinkmann Instruments) was used to homogenize the cells such that 90% were ruptured but the nuclei remained intact. Cell breakage was assessed by microscopic examination. An equal volume of HE2GD buffer was added to the homogenized cells and centrifuged at 12,000 × g for 5 min. The supernatant was centrifuged further at 105,000 × g for 1 hr; the supernatant from this step constitutes the cytosolic fraction. The low-speed pellet was washed three times with HEGD + 0.1 M NaCl, resuspended in HEGD + 0.4 M NaCl, and incubated for 1 hr on ice. This suspension then was centrifuged at 105,000 × g, and the supernatant was collected; this constitutes the nuclear extract. The method of Bradford [15] was used to determine protein concentrations of both cytosolic and nuclear fractions. All cytosols and nuclear extracts were stored in liquid nitrogen until assay.

Electrophoretic Separation and Immunoblotting

Samples were prepared in protein sample-buffer at a concentration of 8 mg/mL for cytosols and 1 mg/mL for nuclear extracts. Proteins were resolved by discontinuous polyacrylamide gel electrophoresis, and then were transferred onto nitrocellulose; the efficiency of transfer was assessed by staining with Ponceau S stain. The nitrocellulose membranes were incubated overnight with BLOTTO to block nonspecific sites. Following three 10-min washes with TNT, the blots were incubated with primary antibody.

Antibodies against the AHR protein had been raised by immunizing rabbits to a synthetic peptide corresponding to a 20-amino acid sequence near the N-terminus of the receptor from C57BL/6 mice (Ah^{b-1} allele) [11]. The anti-AHR antibodies were purified before use by affinity purification using the antigenic peptide linked to ovalbumin. The specificity and sensitivity of this antibody preparation have been described previously [11].

Antibodies against the ARNT protein had been raised by immunizing rabbits with a bacterially expressed protein containing the C-terminal sequence of amino acids 399 to 777 of the human ARNT protein fused to GST. Nonspecific antibodies were removed by incubating the crude antiserum with immobilized bacterial extract expressing GST only. Specific antibodies to the ARNT fragment were further affinity-purified using immobilized GST-ARNT protein [16].

Anti-AHR antibody was incubated with membranes for 3 hr at a 1:5 dilution in BLOTTO. Anti-ARNT antibody was incubated for 1 hr at a 1:5000 dilution in BLOTTO. Following three 10-min washes with TNT, the blots were incubated with anti-rabbit secondary antibody linked to horseradish peroxidase (for AHR = 1:50,000; for ARNT = 1:20,000 dilution of secondary antibody in BLOTTO) for 1 hr. The blots were washed four times for 10 min each with TNT, and staining was visualized with an Amersham ECL immunochemiluminescent system (Amersham Canada Ltd.). Film exposure times varied from 5 min to 1 hr. Images were quantitated by densitometry of the film.

Radioligand Binding Assays on Sucrose Density Gradients

Cytosols were incubated for 1 hr on ice with 10 nM [^{3}H]TCDD in the presence or absence of a 100-fold molar excess of nonradiolabelled TCDF (to check specificity of binding). Excess ligand was removed with dextran-coated charcoal (0.1 mg DCC/mg cytosolic protein), and samples were loaded onto linear 10–30% sucrose gradients. The gradients were centrifuged, fractionated, and counted in a liquid scintillation counter [17].

RNA Preparation

Cells in 100-mm petri dishes were exposed to TCDD or MC for time intervals as indicated in the figure legends. Following incubation at 37°, the growth medium was removed, and the plates were washed two times with cold PBS. TRIZOL reagent (Gibco) (1.5 mL) was added per dish, and cells were scraped off and homogenized by repeated pipetting until the solution was no longer viscous. The TRIZOL was collected into tubes, and 0.3 mL of chloroform was added. Samples were centrifuged at 12,000 × *g* for 15 min, and then the clear supernatant was transferred to a new tube and an equal volume of isopropanol was added. Following a 10-min incubation at room temperature, the samples were centrifuged at 12,000 × *g* for 10 min and the supernatant was decanted. The resulting pellet was washed with 70% ethanol and centrifuged at 7,500 × *g* for 5 min. The ethanol was decanted and the pellets were allowed to dry for 10 min; then they were resuspended in water treated with diethylpyrocarbonate; 7.5 units of DNase I (Pharmacia) was added and the suspensions were incubated at 37° for 15 min. The reaction was stopped by incubating at 55° for 10 min. The concentration and purity of RNA were determined spectrophotometrically. An aliquot (equivalent to 1 μg) of total RNA was run on a 1% agarose gel to check RNA integrity by assessment of ribosomal RNA bands. The samples were stored at −70° until analysis.

Reverse Transcription and Analysis by PCR

Primers for PCR

AHR forward:	5′-GGTGCCTGCTGGATAATTCATCTG-3′.
AHR reverse:	5′-TCGTCCTTCTTCATCCGTCAGTG-3′.
ARNT forward:	5′-GAGGGGAACTGGCAACAC-3′.
ARNT reverse:	5′-CTCTGGGTCATCATCTGGG-3′.
β-Actin forward:	5-CTACAAGAGCTGCGTGTGG-3′.
β-Actin reverse:	5′-TAGCTCTTCTCCAGGGAGGA-3′.
CYP1A1 forward:	5′-CCCACAGCACCACAAGAGATA-3′.
CYP1A1 reverse:	5′-AAGTAGGAGGCAGGCACAATGTC-3′.

The reverse transcriptase mixture contained the following components: 1 mM of each dNTP, 20 units RNase inhibitor, 140 pmol pN6 (random hexamer), 10 μM DTT, 1 μg RNA, and 200 units of M-MLV reverse transcriptase in 1 × RT buffer (50 mM KCl, 20 mM Tris, 2.5 mM $MgCl_2$, 0.1 mg/mL BSA). The mixture was incubated at room temperature for 10 min, and then at 37° for 1 hr. Samples were stored at −70°.

The PCR mixture contained 50 pmol of each primer, 200 μmol of each dNTP, 1 μCi [α-^{32}P]dCTP, 0.5 units of Ultra Therm thermophilic DNA polymerase in manufacturer-supplied buffer (Bio/Can). Each PCR reaction corresponded to 1 μg of initial RNA that had been reverse-transcribed. The samples were first denatured at 95° for 5 min. Thermocycling conditions were: denaturing at 95° for 20 sec; annealing at gene-specific temperature for 20 sec (AHR = 53°; ARNT = 65°; β-actin = 53°; CYP1A1 = 61°) and extension at 72° for 40 sec. A final extension step at 72° for 15 min followed cycling. Cycle number was gene-specific (AHR = 30; ARNT = 30; β-actin = 25; CYP1A1 = 25). Preliminary experiments were performed to determine the optimum conditions for quantitative measurement of each specific gene product by RT–PCR. We also performed some preliminary quantitations of the PCR product by slot blot analysis using a probe directed against the same region as the primers.

Sample loading buffer was added, and the entire PCR reaction was loaded onto a 10% polyacrylamide gel. The gel was run at 200 V for 2–3 hr with cooling. The gels were dried and exposed to phosphor screens for 3 hr. Phosphor screens were then scanned on the STORM phosphoimaging system (Molecular Dynamics) and quantitated using IPLabGel Software (Signal Analytics).

All data shown in the figures are representative of at least three independent replicates of each experiment.

Throughout this report we refer to the AHR and ARNT proteins according to the subcellular fraction in which they are localized following cell homogenization and differential centrifugation as described above. Immunofluorescence experiments by Pollenz and coworkers [13, 18] indicate that the ARNT protein is localized within the nucleus of the intact cell, even in the absence of ligand.

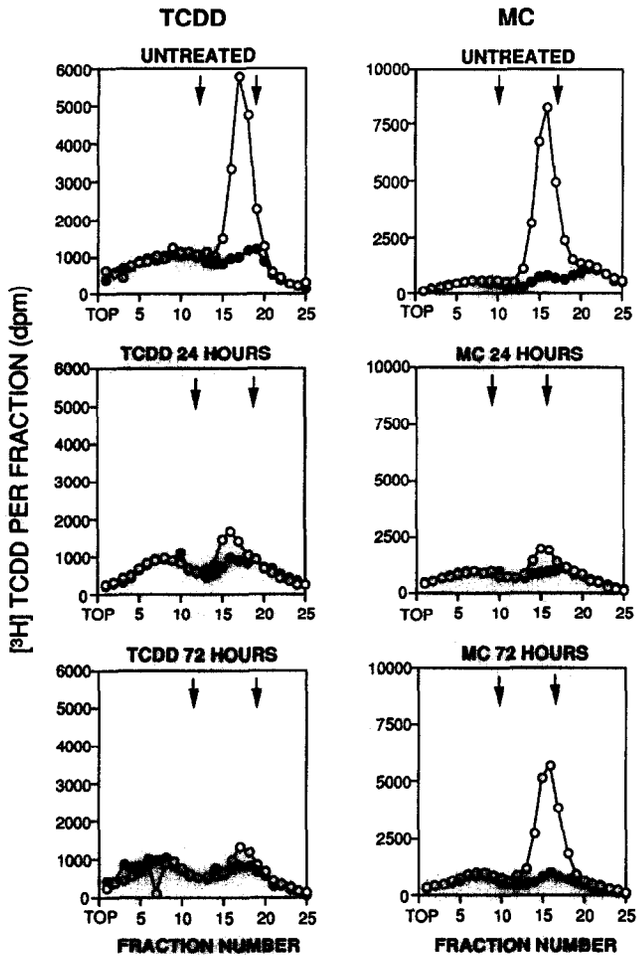

FIG. 1. Effect of TCDD or MC on cytosolic AHR levels as measured by radioligand binding. Hepa-1 cells were exposed in culture to TCDD (2 nM) or MC (100 nM) beginning at time zero. At the times indicated from zero to 72 hr after the addition of TCDD or MC, cells were harvested from plates in each treatment group. Cytosolic fractions were prepared and further incubated with 10 nM [^{3}H]TCDD (—○—) or 10 nM [^{3}H]TCDD plus 1 μM TCDF (—●—) for 1 hr on ice. These samples were charcoal-treated to remove excess ligand, and then were loaded onto sucrose gradients. ^{14}C-Labeled markers were included corresponding to BSA (4.4 S; near fraction-10) and catalase (11.3 S; near fraction-17) in the positions shown by the vertical arrows. Samples were separated by velocity sedimentation, and then were fractionated as described in Materials and Methods. The actual concentration of [^{3}H]TCDD-binding sites detected at time zero (before treatment with TCDD or MC) was 455 fmol/mg cytosolic protein in the sample illustrated, which is representative of three independent experiments with different batches of cells.

RESULTS

In earlier experiments, we found that the cytosolic content of AHR was depressed for up to 24 hr after treatment with TCDD [10, 11]. Our current experiments demonstrated that the depression of AHR in cytosol persists for at least 72 hr as reflected in the decrease in specific binding peaks for [^{3}H]TCDD on sucrose gradient separations (Fig. 1; left-hand panels). Binding peaks for [^{3}H]TCDD also were

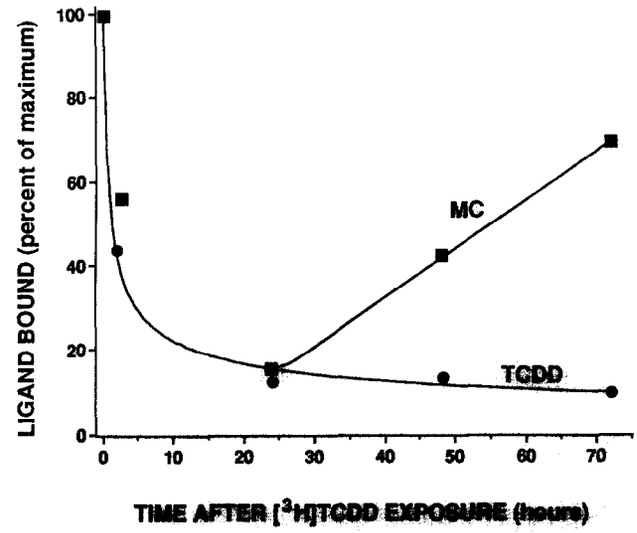

FIG. 2. Time–course of the effect of TCDD or MC on cytosolic AHR levels as measured by radioligand binding. Hepa-1 cells were treated, and cytosolic samples were harvested and analyzed as described in the legend to Fig. 1. Data are representative of at least three independent replicates of each experiment.

diminished at 24 hr in cytosols harvested from cells that had been treated with MC in culture; however, by 72 hr after the addition of MC, the binding peaks for [^{3}H]TCDD had recovered substantially (Fig. 1; right-hand panels). The full time–course for depletion of cytosolic binding sites by TCDD and MC and the recovery in MC-treated cells are illustrated in Fig. 2.

Although radioligand binding with [^{3}H]TCDD is a highly sensitive method for detecting AHR, the radioligand can only detect those sites that are not already occupied by a ligand. In cytosols from cells treated with TCDD or MC, it is possible that the ligand incubated with the cells might mask binding sites on the receptor and cause an underestimation of the receptor content. Hence, in addition to radioligand binding, we also measured the content of AHR protein in cytosols and nuclear extracts by immunoblotting with mono-specific polyclonal antibodies to the receptor. As shown in Fig. 3 (upper left panels), the immunoblots confirmed that the decrease in apparent AHR content in cytosol was not just the product of masking of receptor sites; rather there was a decrease in immunodetectable AHR protein over the same time–course where the [^{3}H]TCDD-binding assay indicated a decrease in receptor content. Within 1 hr following addition of TCDD to the cell culture, there was a decrease in the cytosolic AHR content and a concomitant increase in AHR in the nuclear extract from the same cells. By 24 hr, the cytosolic immunodetectable AHR protein was diminished dramatically, and the signal in the nuclear extract was also beginning to wane.

Treatment of cells with MC also caused an initial depletion of AHR in cytosol accompanied by the appearance of AHR in nuclear extract by 1 hr (Fig. 3; upper right panels); however, in contrast to TCDD, cytosolic AHR immunodetectable protein rebounded by 72 hr post-MC treatment.

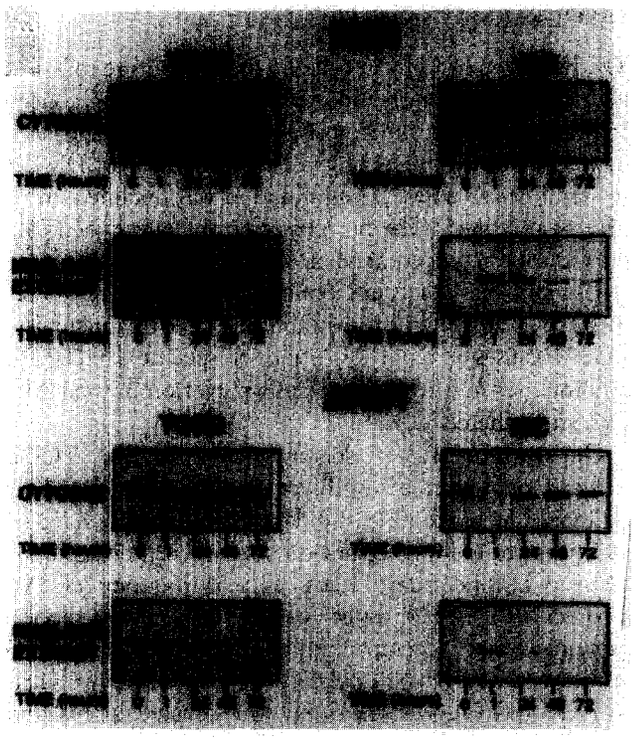

FIG. 3. Effect of TCDD or MC on cytosolic and nuclear AHR and ARNT levels as measured by immunoblotting. Hepa-1 cells were exposed to TCDD or MC as described in the legend to Fig. 1. At 0, 1, 24, 48 and 72 hr, cells were harvested from plates in each group. Cytosols and nuclear extracts were prepared and subjected to immunoblotting after SDS–PAGE as described in "Materials and Methods." Amounts loaded: cytosols, 160 μg protein/lane; nuclear extracts, 20 μg/lane. The intensities of the signals in films from each immunoblot were quantitated by densitometry. Optimum linear conditions for measurement by densitometry were determined before final quantitation of blot densities.

It also is relevant to track the fate of ARNT, the dimerization partner of the AHR, after ligand treatment of intact cells in culture since both proteins are required to form a transcriptionally active complex in the nucleus. In our experiments with conventional cellular homogenization and preparation of cell fractions by differential centrifugation, ARNT appeared totally in the cytosolic fraction of cells prior to treatment with AHR agonists. Immunofluorescent microscopic experiments by Pollenz and coworkers [13, 18] indicate that in the intact cell ARNT is localized exclusively within the nucleus. Our cell fractionation procedures revealed that ARNT was easily mobilized out of the nucleus unless the cell has been exposed to ligand. The retention of ARNT in the nucleus following treatment with TCDD or MC likely reflects the dimerization of ARNT with AHR and the strengthening of interaction of the liganded dimeric pair with DNA. In any case, both TCDD and MC promoted rapid and substantial relocation of ARNT from the cytosolic fraction into the nuclear fraction within 1 hr (Fig. 3; lower panels). Within the first hour, MC caused a greater decrease in ARNT within the

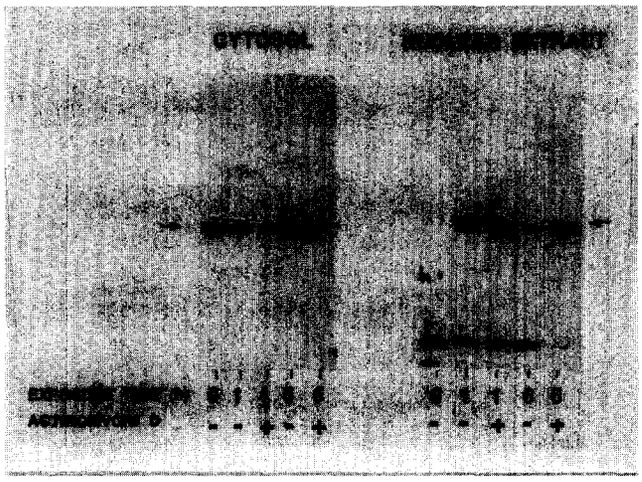

FIG. 4. Maintenance of ARNT protein in the nucleus of cells treated with TCDD and actinomycin-D. Cells were incubated in culture at 37° with 2 nM TCDD in the presence or absence of 10 μM actinomycin-D for 0, 1, or 6 hr. Cytosols and nuclear extracts were prepared and analyzed by immunoblotting as described in "Materials and Methods."

cytosolic fraction and a greater increase in ARNT in the nuclear fraction than did TCDD; this may be due to a more rapid uptake and distribution within the cells for MC than for TCDD. At later time points (48 or 72 hr), when the level of cytosolic AHR remained substantially depressed, the ARNT signal in the cytosol was near pretreatment levels.

In previous experiments [11], we found that treatment of cells with actinomycin-D, a blocker of transcription, could preserve the nuclear pool of AHR for at least up to 6 hr after TCDD treatment. Our current experiments (Fig. 4) demonstrated that actinomycin-D also caused ARNT levels in the nucleus to be much higher at 6 hr post-TCDD than in the absence of actinomycin-D. Although the nuclear level of ARNT was lower without actinomycin-D than when actinomycin-D was present, ARNT was not lost from the cell in the absence of actinomycin-D; rather, ARNT levels in the cytosol were reciprocal to those in the nuclear extract (Fig. 4), suggesting that the effect of actinomycin-D is not to prevent destruction of ARNT but simply to cause retention of ARNT within the nucleus. Since in the absence of actinomycin-D the AHR was degraded rapidly, the most likely explanation for the reappearance of the ARNT protein back in the cytosolic fraction (in the absence of actinomycin-D) is that as the AHR is degraded its dimerization partner, ARNT, is released and can no longer bind with high affinity to nuclear sites, as previously suggested by Pollenz [13].

Our next goal was to determine if the depletion of AHR following treatment with TCDD or MC might be due to inhibition of transcription of the *Ahr* gene and a drop in receptor mRNA levels. Figure 5 shows the bands obtained for RT–PCR products over the time–course from 0 to 72 hr following treatment with TCDD or MC. As expected, CYP1A1 mRNA, the prototypical gene product induced by TCDD and MC via the AHR mechanism, was elevated

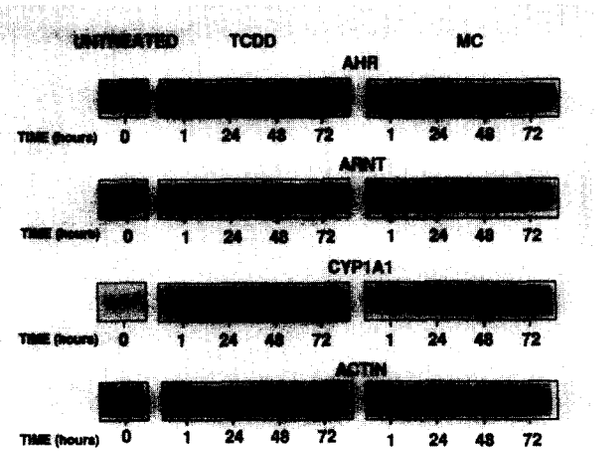

FIG. 5. mRNA levels for AHR, ARNT, β-actin, and CYP1A1 in Hepa-1 cells treated with TCDD or MC. RNA was prepared from cells treated with TCDD or MC for the indicated time intervals. One microgram of the RNA was reverse-transcribed and subjected to "hot PCR" as described in "Materials and Methods." The entire PCR reaction was loaded onto a 10% acrylamide gel and run at 200 V for 2–3 hr. The gels were dried and exposed to phosphor screens for 3 hr. The screens were scanned on the STORM phosphoimaging system to quantitate blot intensities. For quantitation of each blot, the blot intensities were expressed as a ratio to the β-actin loading control.

markedly by 1 hr after treatment with either TCDD or MC and remained highly induced for up to 72 hr. The mRNA levels for the AHR and for ARNT were unaffected by treatment of the cells with either TCDD or MC. In pilot experiments, mRNA levels for the AHR were assessed by a slot blot assay. This assay also did not reveal any decrease in AHR mRNA levels for up to 3 days after TCDD treatment of Hepa-1 cells (data not shown).

Figure 6 summarizes the changes in protein levels and mRNA levels after TCDD or MC treatment over the time–course from 0 to 72 hr. As can be seen in Fig. 6A, both the cytosolic and nuclear content of AHR protein were reduced dramatically within 24 hr after TCDD, and the levels were less than 5% of pretreatment levels for as long as 72 hr in culture. In contrast, AHR mRNA did not decrease at any time following TCDD treatment. MC (Fig. 6B) caused an initial drop in cytosolic + nuclear AHR-protein levels within the first 24 hr, but by 72 hr the cytosolic AHR level had returned to near normal; as was the case with TCDD treatment, MC did not reduce AHR mRNA levels.

ARNT protein underwent an apparent "redistribution" from the cytosolic extract before treatment, into nuclear extract within 1 hr after TCDD or MC, and then redistributing back into cytosol within 24 hr after treatment with either TCDD or MC (Fig. 6C and D). As was the case with mRNA for the AHR, the mRNA for ARNT also was not altered significantly by treatment of the cells with AHR agonists.

DISCUSSION

The rapid loss of AHR in cells exposed in culture to TCDD is in contrast to effects reported *in vivo* where the concentration of cytosolic binding sites for [^{3}H]TCDD appears to increase after chronic exposure to low levels of TCDD [19]. In the *in vivo* studies, no information was obtained on the level of receptor in the nucleus or on the effects of TCDD on the total tissue content of AHR protein.

Previous studies in our laboratory and others [10–13, 20] in Hepa-1 cells in culture established that treatment with TCDD causes a rapid depletion of specific [^{3}H]TCDD-binding sites in cytosol as well as a depletion in total AHR protein in the cells.

Our current studies demonstrated that the TCDD-induced depletion of AHR protein in Hepa-1 cells is prolonged for up to at least 3 days in culture. We have not attempted to follow the time–course of receptor depletion/recovery beyond 3 days. It also is clear from our RT–PCR analyses and slot blot assays that the depletion of receptor protein is not accompanied by a decrease in mRNA encoding the AHR. Nor did there appear to be any increase in AHR mRNA over the 3-day time–course of this study after treatment with either TCDD or MC.

Previously, Swanson and Perdew [20] reported that the half-life of the AHR in Hepa-1 cells after treatment with β-naphthoflavone or α-naphthoflavone is increased rather than decreased. The conclusion of increased half-life was based on actual turnover of the receptor by a density shift method, but in their experiments the ligands also caused a decrease in the total amount of cytosolic receptor.

It is not clear what pathways are responsible for AHR degradation following exposure of cells to TCDD. The cytosolic AHR is susceptible to proteolytic cleavage with exogenous proteases such as trypsin, chymotrypsin, or V8 protease [21–23]. With isolated cell fractions it appears that the proteolytic products are the same for transformed cytosolic receptor and for nuclear receptor, but the proteolytic products differ according to what specific chemical ligand has been used to transform the receptor [23]. These experiments with receptor outside the context of the cell show that the receptor can be cleaved proteolytically and that the sites of attack may depend to some extent upon the nature of the ligand that is bound to the receptor, but they do not illuminate pathways of degradation within the cell.

In whole cells, if protein synthesis is blocked with cycloheximide or if mRNA synthesis is blocked with actinomycin-D, the nuclear content of AHR can be preserved for at least up to 6 hr [11, 12]. These experiments indicate that the factor responsible for AHR degradation within the intact cell has, itself, a short half-life. Blocking of protein synthesis with cycloheximide also causes superinduction of CYP1A1 mRNA, an AHR-regulated gene product [24], but it is not clear if superinduction is due to increased nuclear AHR levels or to a removal of a different factor that represses CYP1A1 transcription.

The depletion of AHR by TCDD treatment is not

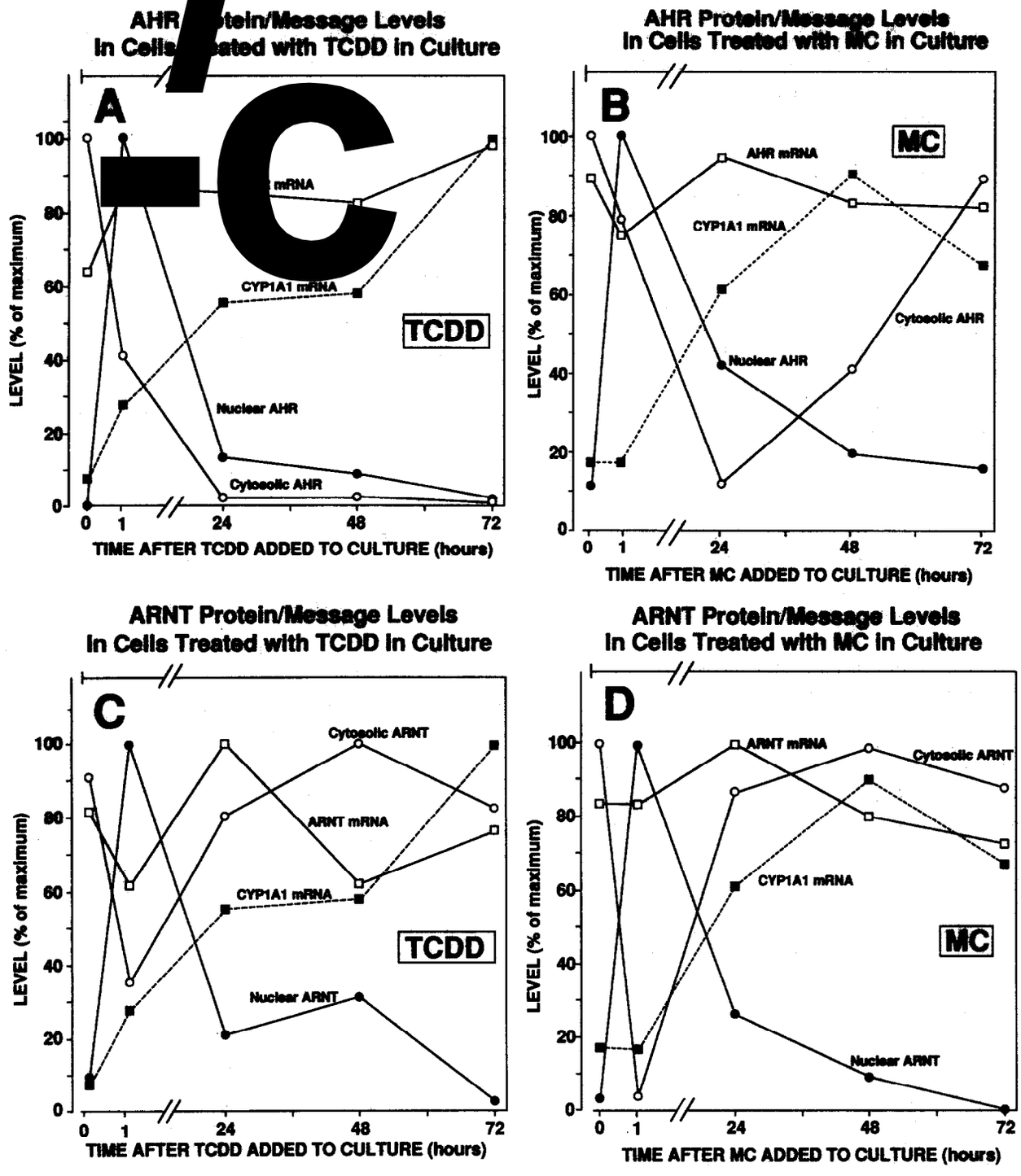

FIG. 6. Time–course summary of protein and mRNA levels in Hepa-1 cells treated with TCDD or MC. Relative levels of protein or of mRNA are expressed as a percentage of the maximum level that occurred at any time point for that product. Key: (—○—) protein level in cytosol as assayed by immunoblotting; (—●—) protein level in nuclear extracts as assayed by immunoblotting; (—□—) mRNA as measured by RT–PCR; and (---■---) mRNA of CYP1A1 as measured by RT–PCR. For quantitation of each blot, the blot intensities were expressed as a ratio to the β-actin loading control. The time–course data shown here are representative of three independent experiments with different batches of Hepa-1 cells.

peculiar to mouse Hepa-1 cells. Pollenz [13] observed a similar phenomenon in mouse embryonic fibroblasts (3T3), rat aortic smooth muscle cells (A7), and murine skeletal muscle cells (C2C12). ARNT protein levels were not affected by TCDD treatment in any of these cells or in the Hepa-1 cells in our experiments. Although AHR and ARNT form a functional partnership at the level of DNA, their fates diverge at later times following ligand treatment. The total cell content of AHR remained depleted for up to 72 hr following TCDD treatment, whereas the ARNT

signal temporarily was stronger in cytosol than in nuclear extract within a few hours but the total cellular pool of ARNT seemed to be maintained at pre-ligand levels. Actinomycin-D caused prolonged retention of both AHR and ARNT in the nuclear extract. Without actinomycin-D, the ARNT protein reappeared in the cytosolic fraction within 6 hr. With actinomycin-D, ARNT levels were maintained in the nucleus along with the AHR. This suggests that the AHR may be degraded in the nucleus and that once the receptor protein is destroyed, ARNT is released intact back into the non-DNA-bound pool.

It is not clear if TCDD and other ligands induce the factor responsible for degradation of their own receptor. The duration of depletion of AHR was much longer following exposure of cells to the persistent ligand, TCDD, than with the metabolizable ligand, MC; this could be interpreted to mean that AHR ligands induce a transiently expressed protease or other factor that disappears upon removal of the ligand. It could also mean, however, that the only vulnerable form of the receptor is receptor that has been transformed to the DNA-binding state and that the apparent induction of degradation by TCDD and other ligands is simply due to their ability to transform the receptor and remove it from protection by its chaperone protein, hsp90.

In these experiments, we also measured induction of CYP1A1 mRNA as an index to the uptake and downstream biological effectiveness of the ligands. It is interesting that CYP1A1 levels remain elevated at 72 hr post-treatment for both TCDD-treated and MC-treated cells despite the very low nuclear AHR levels that exist at that time for both ligands. This suggests that only a small fraction of the cell's total AHR pool is sufficient to drive transcription at near maximal rates.

Finally, turnover and degradation of the AHR could, itself, constitute a level of regulation in AHR signalling pathways as has been observed recently for other proteins that act as transcription factors or transcriptional enhancers [25–28].

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References

1. Okey AB, Riddick DS and Harper PA, The Ah receptor: Mediator of the toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin and related compounds. *Toxicol Lett* **70:** 1–22, 1994.
2. Pohjanvirta R and Tuomisto J, Short-term toxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in laboratory animals: Effects, mechanisms, and animal models. *Pharmacol Rev* **46:** 483–549, 1994.
3. Fernandez-Salguero PM, Hilbert DM, Rudikoff S, Ward JM and Gonzalez FJ, Aryl-hydrocarbon receptor-deficient mice are resistant to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-induced toxicity. *Toxicol Appl Pharmacol* **140:** 173–179, 1996.
4. Nebert DW, Puga A and Vasiliou V, Role of the Ah receptor and the dioxin-inducible [*Ah*] gene battery in toxicity, cancer, and signal transduction. *Ann NY Acad Sci* **685:** 624–640, 1993.
5. Bock KW, Aryl hydrocarbon or dioxin receptor: Biologic and toxic responses. *Rev Physiol Biochem Pharmacol* **125:** 1–42, 1993.
6. Schmidt JV and Bradfield CA, AH receptor signaling pathways. *Annu Rev Cell Dev Biol* **12:** 55–89, 1996.
7. Whitlock JP Jr and Denison MS, Induction of cytochrome P450 enzymes that metabolize xenobiotics. In: *Cytochrome P450: Structure, Mechanism, and Biochemistry* (Ed. Ortiz de Montellano PR), pp. 367–391. New York, Plenum Press, 1995.
8. Nebert DW, McKinnon RA and Puga A, Human drug-metabolizing enzyme polymorphisms: Effects on risk of toxicity and cancer. *DNA Cell Biol* **15:** 273–280, 1996.
9. Hankinson O, The aryl hydrocarbon receptor complex. *Annu Rev Pharmacol Toxicol* **35:** 307–340, 1995.
10. Prokipcak RD and Okey AB, Downregulation of the Ah receptor in mouse hepatoma cells treated with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Can J Physiol Pharmacol* **69:** 1204–1210, 1991.
11. Giannone JV, Okey AB and Harper PA, Characterization of polyclonal antibodies to the aromatic hydrocarbon receptor. *Can J Physiol Pharmacol* **73:** 7–17, 1995.
12. Reick M, Robertson RW, Pasco DS and Fagan JB, Down-regulation of nuclear aryl hydrocarbon receptor DNA-binding and transactivation functions: Requirement for a labile or inducible factor. *Mol Cell Biol* **14:** 5653–5660, 1994.
13. Pollenz RS, The aryl-hydrocarbon receptor, but not the aryl-hydrocarbon receptor nuclear translocator protein, is rapidly depleted in hepatic and nonhepatic culture cells exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Mol Pharmacol* **49:** 391–398, 1996.
14. Riddick DS, Huang Y, Harper PA and Okey AB, 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin versus 3-methylcholanthrene: Comparative studies of Ah receptor binding, transformation, and induction of CYP1A1. *J Biol Chem* **269:** 12118–12128, 1994.
15. Bradford M, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72:** 248–254, 1976.
16. Probst MR, Reisz-Porszasz S, Agbunag RV, Ong MS and Hankinson O, Role of the aryl hydrocarbon receptor nuclear translocator protein in aryl hydrocarbon (dioxin) receptor action. *Mol Pharmacol* **44:** 511–518, 1993.
17. Tsui HW and Okey AB, A rapid vertical tube rotor assay for binding of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin to the Ah receptor. *Can J Physiol Pharmacol* **59:** 927–931, 1981.
18. Pollenz RS, Sattler CA and Poland A, The aryl hydrocarbon receptor and aryl hydrocarbon receptor nuclear translocator protein show distinct subcellular localizations in Hepa 1c1c7 cells by immunfluorescence microscopy. *Mol Pharmacol* **45:** 428–438, 1994.
19. Sloop TC and Lucier GW, Dose-dependent elevation of Ah receptor binding by TCDD in rat liver. *Toxicol Appl Pharmacol* **88:** 329–337, 1987.
20. Swanson HI and Perdew GH, Half-life of aryl hydrocarbon receptor in Hepa 1 cells: Evidence for ligand-dependent alterations in cytosolic receptor levels. *Arch Biochem Biophys* **302:** 167–174, 1993.
21. Prokipcak RD, Denison MS and Okey AB, Nuclear Ah receptor from mouse hepatoma cells: Effect of partial proteolysis on relative molecular mass and DNA-binding properties. *Arch Biochem Biophys* **283:** 476–483, 1990.

22. Wang X, Santostefano M, Lu Y and Safe S, A comparison of the mouse versus human aryl hydrocarbon (Ah) receptor complex: Effects of proteolysis. *Chem Biol Interact* **85:** 79–93, 1992.
23. Santostefano M and Safe S, Characterization of the molecular and structural properties of the transformed and nuclear aryl hydrocarbon (Ah) receptor complexes by proteolytic digestion. *Chem Biol Interact* **100:** 221–240, 1996.
24. Lusska A, Wu L and Whitlock JP Jr, Superinduction of *CYP1A1* transcription by cycloheximide: Role of the DNA binding site for the liganded Ah receptor. *J Biol Chem* **267:** 15146–15151, 1992.
25. Boyer SN, Wazer DE and Band V, E7 protein of human papilloma virus-16 induces degradation of retinoblastoma protein through the ubiquitin-proteasome pathway. *Cancer Res* **56:** 4620–4624, 1996.
26. Kim TK and Maniatis T, Regulation of interferon-γ-activated STAT1 by the ubiquitin-proteasome pathway. *Science* **273:** 1717–1719, 1996.
27. Yoshida H, Kitamura K, Tanaka K, Omura S, Miyazaki T, Hachiya T, Ohno R and Naoe T, Accelerated degradation of PML-retinoic acid receptor α (PML-RARA) oncoprotein by all-*trans*-retinoic acid in acute promyelocytic leukemia: Possible role of the proteasome pathway. *Cancer Res* **56:** 2945–2948, 1996.
28. Musti AM, Treier M and Bohmann D, Reduced ubiquitin-dependent degradation of c-Jun after phosphorylation by MAP kinases. *Science* **275:** 400–402, 1997.