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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

RICHARD S. POLLENZ

Department of Biochemistry and Molecular Biology, Medical University of South Carolina, Charleston, South Carolina 29425

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SUMMARY

Western blot analysis and indirect immunofluorescence microscopy were used to evaluate the fate of the aryl-hydrocarbon receptor (AhR) and aryl-hydrocarbon receptor nuclear translocator (Arnt) protein in culture cell models exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). In wild-type (WT) murine Hepa-1c1c7 cells, AhR protein was depleted by 85% after 4 hr of TCDD treatment as measured in total cell lysates. In contrast, the concentration of Arnt protein was unaffected by TCDD treatment in WT cells. Analysis of the AhR with immunofluorescence microscopy revealed that nuclear translocation of the liganded AhR preceded its depletion from cells. AhR protein was depleted from Hepa-1 type I variants (that contain a concentration of AhR that is 10% of WT) with a similar time course and to the same maximal level observed in WT cells (85%). The EC_{50} for AhR depletion in Hepa-1 cells was 39 pM TCDD and corresponded to the EC_{50} for induction of P4501A1 protein. Murine embryonic fibroblasts (NIH-3T3), rat aortic smooth muscle cells (A7), and murine skeletal muscle cells (C2C12) all exhibited >90% depletion of the AhR after 2–4 hr of TCDD treatment. Arnt concentration was not affected by TCDD in these cell lines. These results indicate that the liganded AhR is rapidly depleted within the nuclear compartment of hepatic and nonhepatic cells in a manner independent of the Arnt protein.

The AhR and Arnt protein are basic-helix-loop-helix transcription factors that mediate biological response to HAHs typified by TCDD (1–3). These proteins have been identified in numerous cell culture lines and animal tissues and appear to be ubiquitous (3–5). The model of AhR-mediated signal transduction proposes that the unliganded AhR resides in the cytoplasm as a complex containing hsp90 and other unidentified proteins (6–8). After ligand binding, the AhR dissociates from hsp90 and translocates to the nucleus where it associates with the nuclear Arnt protein through the helix-loop-helix domain (8–12). The AhR/Arnt complex then binds specific DNA sequences termed XREs to regulate gene expression through powerful transactivation domains (12–17). Tissue-specific changes in gene expression are hypothesized to be the mechanism by that HAHs ultimately mediate a biological response (1–3, 18).

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An important area of research with regard to the AhR-mediated signal transduction pathway concerns the fate of the AhR and Arnt protein subsequent to ligand binding. For ligand-activated transcription factors such as steroid hormone receptors, the level of receptor is often depleted after ligand binding as a means of controlling cellular response (19). Unlike labile biological ligands, TCDD and its related congeners have the potential to activate the AhR to a form that can bind Arnt and regulate transcription for sustained periods because they are biologically stable and are not readily metabolized or easily cleared from body (1–3). Therefore, changes in the level of AhR or Arnt protein could affect the ability of HAHs to induce or sustain a response. Indeed, a reduction in the concentration of the AhR in certain Hepa-1 cell variants results in reduced levels of P4501A1 expression after ligand exposure. The reduced response allows the cells to grow in high concentrations of benzo[*a*]pyrene that would normally be lethal (20–22).

Down-regulation in the AhR-mediated signal transduction

ABBREVIATIONS: P450 or CYP, cytochrome P450; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; XRE, xenobiotic-responsive element; AhR, aryl-hydrocarbon receptor; Arnt, aryl-hydrocarbon receptor nuclear translocator; GAR-TR, goat anti-rabbit IgG conjugated to Texas Red; GAR-HRP, goat anti-rabbit IgG conjugated to horseradish peroxidase; ECL, enhanced chemiluminescence; HAH, halogenated aromatic hydrocarbon; WT, wild-type.

pathway has been studied after exposure of hepatoma cell models to [^{3}H]TCDD (23–26). In these studies, specific [^{3}H]TCDD binding was reduced by 90% in high-speed cytosolic fractions of Hepa-1 cells after 4–8 hr of exposure. These results suggested that AhR was down-regulated, but because ligand binding was used to measure the concentration of the AhR, it was not possible to determine whether the decreased binding represented actual protein loss, decreased binding capacity, or movement to cellular compartments that were not assayed. A recent study has provided insight into these questions by using Western blot analysis to evaluate the fate of the AhR in TCDD-treated Hepa-1 cells (26). The results show that AhR is depleted from both high-speed cytosol and nuclear extracts after 6 hr of TCDD treatment. These results provide the first direct evidence that the AhR may be depleted from cells after exposure to saturating levels of TCDD.

The experiments in this report were designed to further investigate the fate of the liganded AhR. The results show that the AhR but not Arnt protein is rapidly depleted from both hepatic and nonhepatic culture cells after ligand binding. These results indicate that the loss of AhR staining represents an actual reduction in the number of AhRs within the cell because Western blot analysis of total cell lysates was used to evaluate the entire pool of AhR and Arnt protein. Importantly, the basal concentration of AhR or Arnt protein did not affect the rate or degree of its depletion. In addition, loss of AhR protein was strictly dependent of the concentration of TCDD and could be observed at concentrations in the parts per trillion range. These results imply that the stability of the liganded AhR may be the limiting factor in formation of AhR/Arnt complexes and cellular response to TCDD and its related congeners.

Materials and Methods

Buffers. PBS contained 0.8% NaCl, 0.02% KCl, 0.14% Na_2HPO_4, and 0.02% KH_2PO_4, pH 7.4. The 2× gel sample buffer contained 125 mM Tris, pH 6.8, 4% SDS, 25% glycerol, 4 mM EDTA, 20 mM dithiothreitol, and 0.005% Bromphenol blue. TBS contained 50 mM Tris and 150 mM NaCl, pH 7.5. TTBS contained 50 mM Tris, 0.2% Tween-20, and 150 mM NaCl, pH 7.5. TTBS+ contained 50 mM Tris, 0.5% Tween-20, and 300 mM NaCl, pH 7.5. BLOTTO is 5% dry milk in TTBS. The 2× lysis buffer contained 50 mM HEPES, pH 7.4, 40 mM sodium molybdate, 10 mM EGTA, 6 mM $MgCl_2$, and 20% glycerol.

Cells and growth conditions. WT Hepa 1c1c7 cells, types I and II variants, were a generous gift from Dr. Jim Whitlock, Jr. (Department of Pharmacology, Stanford University, Stanford, CA). The cells were propagated in DMEM containing 5% fetal bovine serum. NIH-3T3, A7, and C2C12 cells were purchased from American Type Culture Collection. C2C12 and A7 cells were propagated in DMEM containing 10% fetal bovine serum. NIH-3T3 cells were propagated in DMEM containing 10% calf serum. All cells were passaged at 1-week intervals and used in experiments during a 2-month period.

Antibody preparation and nomenclature. Specific antibodies against either the AhR (A-1) or Arnt protein (R-1) are identical to those described previously (8). All antibodies are affinity-purified IgG fractions. For Western blot analysis, GAR-HP were used. For immunohistochemical studies, GAR-TR were used. Both of these reagents were purchased from Jackson Immunoresearch (West Grove, PA). Polyclonal rabbit β-actin antibodies were purchased from Sigma Chemical Co. (St. Louis, MO). Polyclonal rabbit antibodies against mammalian P4501A1 were a generous gift from Dr. Colin Jeffcoat (University of Wisconsin).

Experimental design and administration of TCDD. Stock flasks of culture cells were harvested as detailed above, counted, and aliquoted into the appropriate number of 100-mm culture plates in 7 ml of media. The cells were generally seeded at high density so that they were 80–90% confluent after 2 days of growth at 37°. An 8× stock TCDD was prepared in fresh media, and 1 ml was applied directly to the culture dish with gently swirling to mix. For long term incubations (16–24 hr), cells were treated before shorter time points so that all treatments were terminated at the same time. Each experiment was repeated at least three times. TCDD was a gift of Dow Chemical Co. and was solubilized in dimethylsulfoxide.

Preparation of cell lysates. After treatment, cell monolayers were washed twice with PBS and detached from plates by trypsinization (0.05% trypsin/0.5 mM EDTA). Cell pellets were then washed with ice-cold PBS and then suspended in ice-cold 1× lysis buffer supplemented with NP-40 (5%), leupeptin (10 μg/ml), and aprotinin (20 μg/ml). Suspensions were then sonicated for 10 sec and supplemented with phenylmethylsulfonyl fluoride (100 μM final concentration) and 200 units of DNase (Promega, Madison, WI). The lysate was incubated on ice for 4 min and then sonicated for an additional 10 sec. At this time, 10 μl of sample was removed for protein determination, and the remainder of the sample was combined with an equal volume of 2× gel sample buffer. Samples were heated at 100° for 5 min and stored at -20°. Cytosol and total nuclear extracts were prepared essentially as described (8), except for the following modifications. Nuclei were suspended in one half the volume of cytosol and were sonicated two times for 10 sec each. In addition, at the time of preparation, all samples were combined with one volume 2× gel sample buffer and heated at 100° for 5 min. Protein concentrations were determined by the Coomassie Blue Plus assay (Pierce, Rockford, IL) with bovine serum albumin as the standard.

Gel electrophoresis and Western blotting. Protein samples were resolved by denaturing electrophoresis on discontinuous polyacrylamide slab gels (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) and were electrophoretically transferred to nitrocellulose as described (8). Immunochemical staining was carried out with varying concentrations of primary antibody (see text and Fig. legends) in BLOTTO buffer for 1–2 hr at 22°. Blots were washed with three changes of TTBS+ for a total of 45 min. The blot was then incubated in BLOTTO buffer containing a 1:10,000 dilution of GAR-HRP for 1 hr at 22° and washed in three changes of TTBS+ as above. Before detection, the blots were washed in TBS for 5 min. Bands were visualized with the ECL kit as specified by the manufacturer (Amersham, Arlington Heights, IL). Multiple exposures of each set of sample were produced.

Quantification of protein expression. ECL exposures were scanned into a Power Macintosh computer with an HP Scanjet II cx/T and Adobe 2.51 software. Images were then quantified with the use of National Institutes of Health Image 1.55 software. Quantification of protein bands was performed as follows. A rectangular tool was produced that surrounded the largest band of interest. The size of this tool was held constant for all measurements within a specific set of samples (i.e., all AhR bands in a seven-point dose-response experiment). The mean value of the intensity within the tool was then determined for (i) the band of interest, (ii) the area directly above the band, and (iii) the area directly below the band. The intensities of the area above and below the band of interest were then averaged and subtracted from the band of interest. The resulting value represented the raw level of AhR, Arnt, P4501A1, or β-actin expression. Because the relative concentration of AhR and Arnt protein in each cell line was known, the amount of sample and concentration of antibodies were controlled so that exposure was within the linear range determined for that protein (see below). To control for the amount of protein loaded on each gel, blots were also probed with antibodies against β-actin (Sigma). The raw level of target protein expression was divided by the level of β-actin expression to generate normalized values for the concentration of each

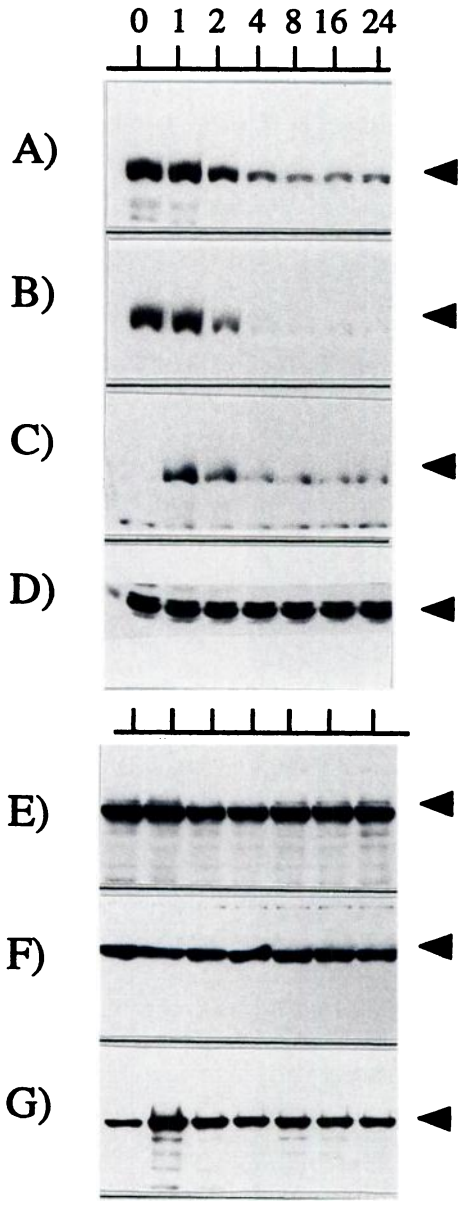

Fig. 1. Representative Western blots of TCDD-treated WT Hepa-1 cells stained for AhR and Arnt. WT Hepa-1 cells were treated with TCDD (1 nM) for 0, 1, 2, 4, 8, 16, and 24 hr at 37°. Cells were harvested, and total cell lysate, cytosol, and total nuclear lysate were prepared as described in Experimental Procedures. Fractions were blotted and stained with 0.75 μg/ml A-1 (A–C), 0.75 μg/ml R-1 (E–G), or a 1:500 dilution of anti-β-actin (D). The secondary antibody was GAR-HRP (1:10,000). A, 12 μg of total cell lysate. *Arrow*, 95-kDa AhR. B, 8 μg cytosol. *Arrow*, 95-kDa AhR. C, 16 μg total nuclear lysate. *Arrow*, 95-kDa AhR. D, 12 μg total cell lysate. *Arrow*, 45-kDa actin. E, 12 μg of total cell lysate. *Arrow*, 89-kDa Arnt protein. F, 8 μg cytosol. *Arrow*, 89-kDa Arnt protein. G, 16 μg total nuclear lysate. *Arrow*, 89-kDa Arnt protein.

protein. In nearly all experiments, the amount of protein loaded in all wells was within 10%, and thus the trend of the data was not affected by the normalization procedure (see the consistency of actin staining in Fig. 1D). The linearity of the ECL technique was determined for each antibody by staining graded dilutions of cytosol or purified bacterial-expressed proteins.[1] In all cases, the curves were linear through zero ($r^2 > 0.9$) over ~1 order of magnitude. Before gels were stained with primary antibodies, blots were stained with Ponceau S to evaluate the efficiency of transfer and the gel loading. If areas of the gel did not transfer or if large differences in gel loading were apparent, the blot was not used.

Immunofluorescence staining and microscopy. All immunocytochemical procedures (cell plating, fixation, staining, and photography) were carried out as previously described (8). Cells were stained with 1 μg/ml A-1 or R-1 antibodies and a 1:750 dilution of GAR-TR. The cells were observed with a Zeiss Axiophot microscope with the use of the 568-nm filter. On average, 15–20 fields (5–20 cells each) were evaluated on each slip, and 3 were photographed to generate the raw data. Experiments were repeated at least three times.

Results and Discussion

AhR protein but not Arnt is rapidly depleted in Hepa-1 cells exposed to TCDD. WT Hepa-1 cells were treated with 1 nM TCDD for 0–24 hr. Cells were harvested, and total cell lysate, cytosol, and total nuclear lysate were prepared. These lysates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, blotted, and then stained with the A-1 antibody. Fig. 1 shows representative blots from each lysate. In total cell lysates, the AhR concentration was decreased by 85% after exposure to TCDD for 4 hr and remained reduced for the duration of exposure (Fig. 1A). In cytoplasmic fractions (Fig. 1B), AhR concentration was essentially undetectable after 4 hr of TCDD exposure (1–4% of AhR present in untreated cells). In contrast, the AhR concentration in nuclear lysates (Fig. 1C) was maximal after 1 hr of TCDD treatment and then declined to ~5% of the total AhR pool between 4–24 hr. To control for differences in sample loading and potential proteolysis occurring during sample preparation, blots were also probed with antibodies against β-actin. Fig. 1D shows the β-actin staining of the identical total cell lysate fractions represented in Fig. 1A. The concentration of actin did not vary by >10% between the samples and showed no indication of degradation. In contrast to the results observed with the AhR, TCDD exposure did not appear to affect the concentration of Arnt in Hepa-1 cells. When evaluated in the identical cell lysates shown in Fig. 1A, Arnt concentration remained essentially constant (Fig. 1E). In cytoplasmic fractions, Arnt showed a 5–10% decrease after 1 hr of TCDD treatment, but by 2 hr, levels were essentially equal to those observed in untreated cells (Fig. 1F). In nuclear fractions, Arnt could be detected in untreated cells as previously reported (8) but showed a 2–3-fold increase after 1-hr TCDD exposure that then declined to near-untreated levels by 2–4 hr. The increase in nuclear Arnt at 1 hr corresponded to the increase observed with the AhR (Fig. 1C). Nuclear extracts prepared from cells treated with TCDD for 2, 4, and 8 hr specifically shifted XRE oligonucleotides in gel mobility shift experiments, confirming that the small fraction of AhR and Arnt tightly associated with nuclear structures represented AhR/Arnt complexes.[2]

Because the AhR was quantified in total cell lysates with specific polyclonal antibodies, the loss of AhR staining represents an actual reduction in the number of AhRs within the cell. The decrease in AhR protein could be the result of

[1] R. S. Pollenz, unpublished observations.

[2] R. S. Pollenz, unpublished observations.

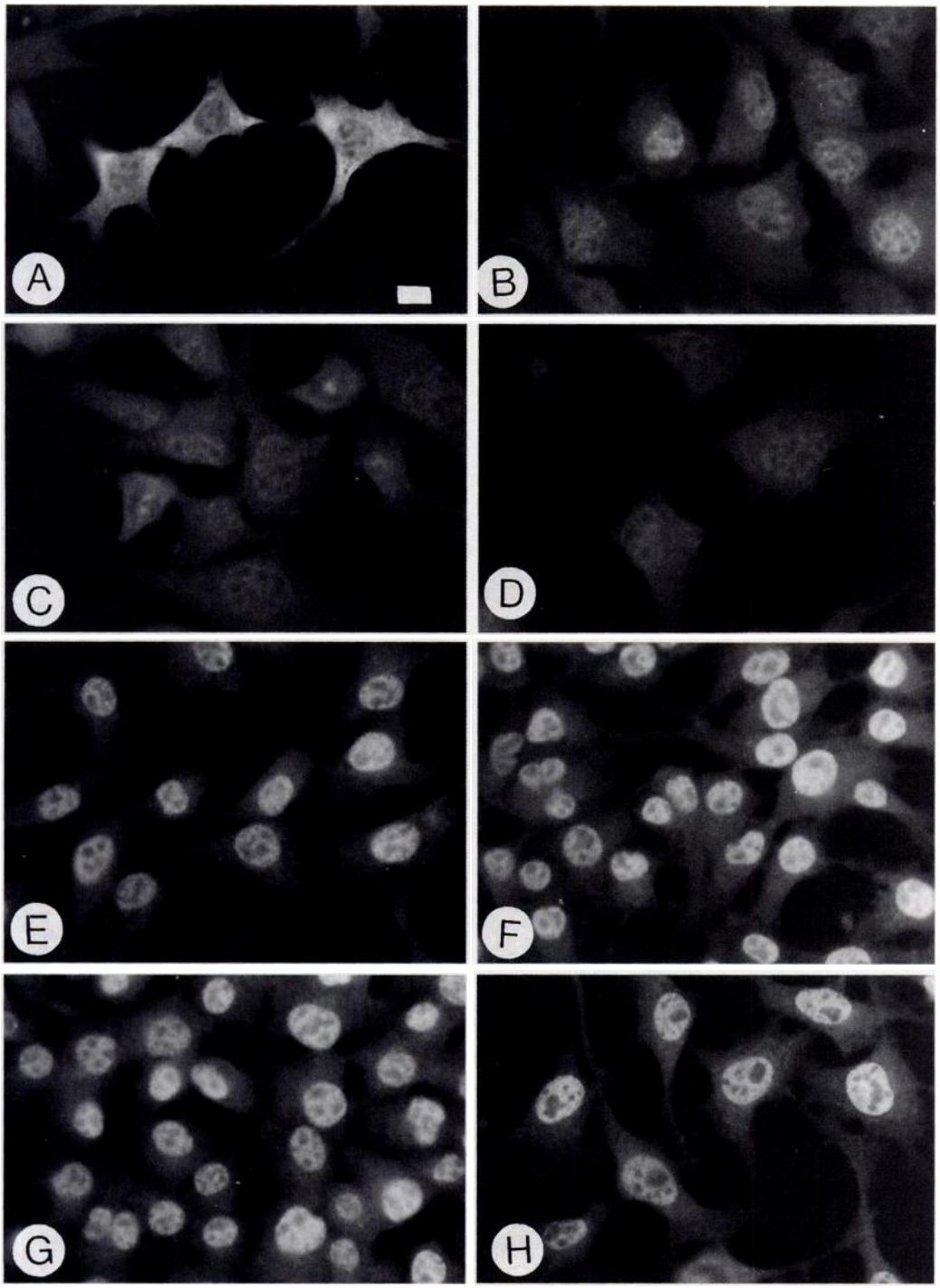

Fig. 2. Immunofluorescence microscopy of WT Hepa-1 cells after treatment with TCDD. WT Hepa-1 cells were incubated with TCDD (1 nM) for the indicated times, fixed, and stained with 1 μg/ml A-1 (A–D) or R-1 (E–H) and GAR-TR (1:750). A, untreated; B, 2 hr; C, 8 hr; D, 24 hr; E, untreated; F, 2 hr; G, 8 hr; and H, 24 hr. *Bar*, 10 μm.

reduced biosynthesis or increased degradation. However, decreased biosynthesis alone cannot account for the 85% reduction in AhR within 4 hr of exposure because the half-life of the AhR in Hepa-1 cells is reported to be 8 hr (24). Therefore, it is likely that the rapid depletion of the AhR from Hepa-1 cells reflects proteolytic digestion of the protein as observed with ligand-bound glucocorticoid receptors (19). Down-regulation of AhR transcription may also play as role in AhR depletion (27), but decreased message levels of a protein with a half-life in excess of 8 hr would likely function to sustain a lower level of protein once it was depleted. The lack of coordinate Arnt depletion was unexpected because it has been proposed that the two proteins form stable heterodimers once the AhR is dissociated from hsp90 and translocated to the nuclear compartment (3, 9, 10, 12). The dramatic change in AhR but not Arnt concentration suggests that AhR/Arnt dimerization is a dynamic process *in vivo* or that the AhR can be preferentially dissociated from an AhR/Arnt complex and degraded independently.[3]

[3] Preliminary results indicate that the half-life of the Arnt protein is longer than that observed for the AhR. Therefore, it is unlikely that Arnt is rapidly turned over with the liganded AhR and kept at constant level by increased biosynthesis.

A reduction in AhR but not Arnt can be observed in TCDD treated Hepa-1 cells with immunofluorescence microscopy. The AhR can be detected with immunofluorescence microscopy in the nucleus of Hepa-1 cells within 15 min of TCDD treatment and becomes exclusively localized to the nuclear compartment after 1–2-hr exposure (8). Experiments were carried out to determine whether reductions in the AhR protein could be observed by indirect immunofluorescence microscopy of WT Hepa-1 cells treated with 1 nM TCDD for 0–24 hr. The AhR was cytoplasmic in untreated cells and became predominately nuclear within 2 hr of TCDD treatment (Fig. 2, A and B). Importantly, when cells were treated with TCDD for 8 or 24 hr, the intensity of the AhR signal in the nucleus was markedly diminished (Fig. 3, C and D). No concomitant increase in cytoplasmic staining was observed at these times. In contrast, Arnt staining was strongly nuclear at all time points and did not exhibit any changes in intensity or distribution in the presence of TCDD (Fig. 2, E–H).

These results are consistent with the hypothesis that AhR is rapidly depleted from Hepa-1 cells after ligand exposure. The immunofluorescence results also suggest that depletion of the AhR occurs in the nuclear compartment because the majority of AhR becomes nuclear before its loss from the cell (see Fig. 1A). The mechanism by which proteins are depleted within the nuclear compartment is not well understood, but a number of recent reports have identified proteases in the nuclear compartment by biochemical and immunological methods (28, 29). Whether the levels of liganded AhR are regulated by proteases is not clear. However, due to the rapid loss of AhR observed in Hepa-1 cells, modulation of protease activity could have profound effects on the concentration of liganded AhR capable of interacting with Arnt and then binding to DNA.

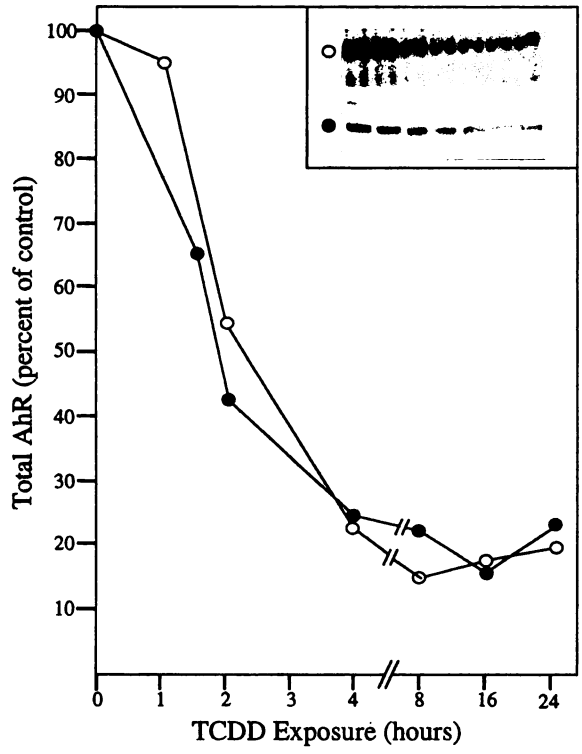

Fig. 3. Time course of AhR depletion in total cell lysates of TCDD-treated WT and type I Hepa-1 cells. Cells were incubated with TCDD (1 nM) for 0, 1, 2, 4, 8, 16, and 24 hr at 37°. Total cell lysates were prepared, blotted, and stained with A-1 (0.75 μg/ml) and GAR-HRP (1:10,000). Blots were then reprobed with β-actin antibodies and GAR-HRP (1:10,000). AhR and β-actin bands were quantified as detailed in Experimental Procedures. All normalized AhR values were divided by the time 0 sample (100% AhR) to generate percentage of total AhR. Each data point represents a single normalized sample. ○, WT Hepa-1. ●, type I Hepa-1. *Inset*, Western blots from WT Hepa-1 cells and type I Hepa-1 cells. *Lanes* correspond to 0-, 1-, 2-, 4-, 8-, 16-, and 24-hr TCDD exposure (*left to right*).

Depletion of AhR is independent of initial AhR concentrations or stoichiometry to Arnt. WT cells express concentrations of AhR that are ~10-fold higher than those observed in the liver of the C57 mouse stain from that the cells were derived.[4] Therefore, it is unclear whether the AhR concentration in these cells is physiological and represents a specific subset of hepatocytes or is the result of cell culture conditions. Experiments were carried out to evaluate the possibility that the reduction of the AhR was related to its concentration. Total cell lysates were prepared from WT Hepa-1 cells and the Hepa-1 type I variants that had been treated with 1 nM TCDD for 0–24 hr. The concentration of AhR in type 1 cells is ~10-fold lower than that found in WT Hepa-1 cells (20–22). The AhR and β-actin concentrations were quantified and normalized from Western blots as described above. The results are expressed as the percentage of total AhR measured in untreated cells. Fig. 3 shows that the pool of AhR in both the WT and type I cells is depleted by ~80–85% after 4 hr of TCDD treatment. Between 4–24 hr, the AhR level remained reduced by 80% in both cell types as observed in Fig. 1. Representative blots are given to highlight the difference in AhR concentration in these cells.

These results show that (i) depletion of the AhR is independent of its initial concentration, (ii) the ratio of AhR to Arnt does not influence the maximal level of depletion, and (iii) the availability of nuclear binding sites does not influence AhR loss (the total pool of AhR expressed in type I cells approximates the amount of AhR bound to nuclear structures in TCDD-treated WT Hepa-1 cells). The implication of these results is that the total number of AhRs in type 1 cells are reduced from ~1800 to 360 within 4 hr of TCDD treatment.[5] It is well established that type 1 cells do not sustain high levels of *CYP1A1* expression (and P4501A1 activity) in the presence of saturating doses of TCDD (20–22). The molecular mechanism behind these findings has not been detailed. The rapid loss of liganded AhR could account for the inability of type I cells to maintain a transcriptional response to AhR agonists. In addition, a reduction in AhR levels could also explain the loss of both AhR-mediated DNA binding activity and *CYP1A1* transcription observed in TCDD-treated rat LCS7 cells (30). In these studies, nuclear extracts of LCS7 did not bind to XRE sequences *in vitro*, and protein binding to XRE sequences in the *CYP1A1* promoter could not be detected

[4] The concentration of AhR in mouse liver cytosol is reported to be ~100–150 fmol/mg (33). WT Hepa-1 cells contain ~1000 fmol AhR/mg cytosol as determined by saturation binding studies with 2-[^{125}I]iodo-7–8-dibromodibenzo-*p*-dioxin (E. Glover, personal communication). The difference in AhR concentration between Hepa-1 cells and liver can also be observed by Western blot analysis with the A-1 antibody (R. S. Pollenz, unpublished observations).

[5] The number of AhRs per cell is based on *in vivo* saturation binding studies with [^{3}H]TCDD (R. S. Pollenz, unpublished observations). These numbers can also be interpolated from previous reports (20).

in vivo after 60 min of TCDD treatment. Collectively, these results make it likely that the stability of the liganded AhR may be the limiting factor in formation of stable AhR/Arnt complexes and the responses they mediate.

Depletion of AhR is dose dependent and shows an inverse relationship to induction of P4501A1 protein. The apparent K_D for TCDD binding to the mouse AhR is 1–10 pM (31). Previous studies of AhR down-regulation have used saturating concentrations of TCDD of ≥1 nM (23–26). Studies were performed to determine the effective concentration of TCDD required for a 50% reduction in AhR protein levels (EC_{50}). Eight graded doses of TCDD were applied to culture dishes of WT cells for 16 hr. Total cell lysate was evaluated for AhR, P4501A1, and β-actin expression by Western blots and the levels of each protein were quantified and normalized as described above. For the AhR, results are expressed as the percentage of AhR measured in untreated cells. For P4501A1, data are plotted as the percentage of maximal P4501A1 expression. Fig. 4 shows that the depletion of the AhR is strictly dependent on the concentration of TCDD. The EC_{50} value determined from three independent experiments was 39 ± 11.2 pM . This value is similar to published values for the induction of aryl-hydrocarbon hydroxylase activity by

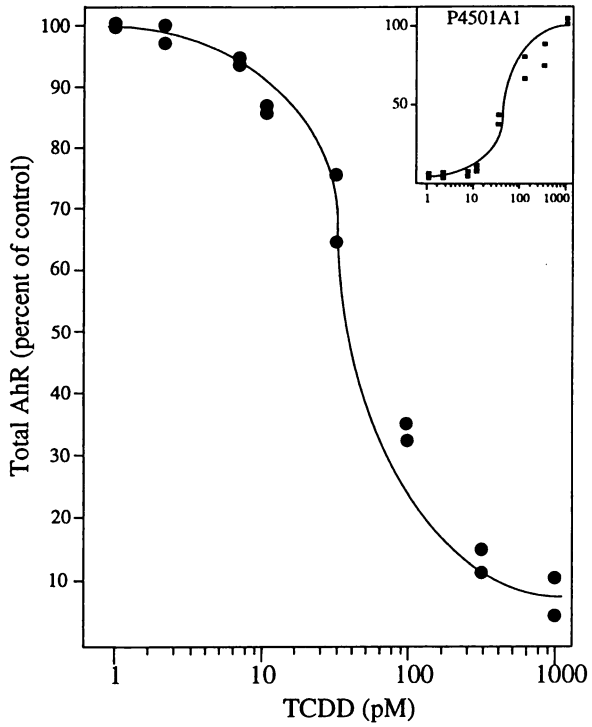

Fig. 4. Depletion of the AhR in WT Hepa-1 cells treated with graded concentrations of TCDD. Duplicate plates of WT Hepa-1 cells were incubated at the following concentrations of TCDD for 16 hr at 37° (all values are molar concentration): 10^{-9}, 4×10^{-10}, 1×10^{-10}, 4×10^{-11}, 1×10^{-11}, 7.5×10^{-12}, 2.5×10^{-12}, and 1×10^{-12}. Total cell lysates were prepared, blotted, and stained with both A-1 (0.75 μg/ml) and β-actin (1:750) followed by GAR-HRP (1:10,000). Blots were then reprobed with P4501A1 antibodies (1:500) and GAR-HRP (1:10,000). AhR and actin bands were quantified as detailed in Experimental Procedures. All normalized AhR values were divided by the average of the duplicate untreated samples (100% AhR) to generate percentage of total AhR. All P450 values were divided by the average of the two 1 nM samples. Each data point represents a single normalized sample. *Inset*, induction of P4501A1 protein. Note that both sets of data result in an EC_{50} of 39 pM.

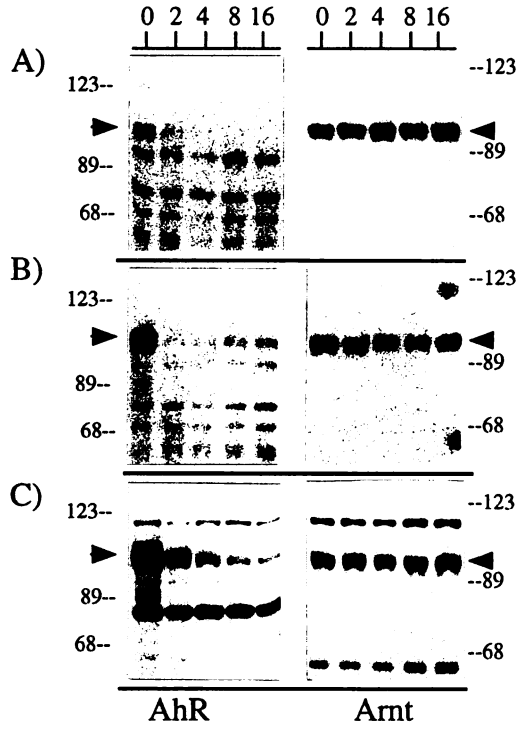

Fig. 5. Representative Western blots of TCDD-treated NIH 3T3, A7, and C2C12 cells stained for AhR and Arnt. Cells were treated with TCDD (1 nM) for 0, 2, 4, 8, and 16 hr. Total cells lysates were prepared, blotted, and stained with 0.75 μg/ml A-1 or R-1 followed by GAR-HRP (1:10,000). *Left*, AhR blots; *right*, Arnt blots. A, 12 μg of NIH 3T3 total cell lysate. B, 12 μg of C2C12 total cell lysate. C, 12 μg of A7 total cell lysate. *Arrows*, 105-kDa AhR or 89-kDa Arnt band. Molecular weight markers were β-galactosidase (123 kDa), fructose-6-phosphate kinase (89 kDa), and pyruvate kinase (67 kDa).

TCDD in Hepa-1 cells (5). Indeed, the loss of AhR was reciprocal to the induction of P4501A1 in the cells (see Fig. 4, *inset*). These results indicate that depletion of AhR will occur at concentrations of TCDD in the parts per trillion range and that the level of AhR protein may be a novel biomarker of TCDD exposure.

AhR but not Arnt is depleted in culture cells derived from nonhepatic tissues. It was pertinent to investigate whether cells derived from nonhepatic sources showed the same patterns of AhR depletion as observed in Hepa-1 cells. Three cell lines were chosen: mouse embryonic fibroblasts (NIH-3T3), rat smooth muscle cells (A7), and murine skeletal muscle myoblasts (C2C12). The choice of these cells was based on (i) their nonhepatic origin, (ii) the fact that they do not express the same allele of AhR found in Hepa-1 cells (4, 32), and (iii) that each cell has a different basal concentration of AhR. Each cell line was treated with 1 nM TCDD for 0–16 hr. Total cell lysates were prepared, blotted, and stained with the A-1 or R-1 antibody (Fig. 5). NIH 3T3 cells express levels of AhR that are similar to Hepa-1 type 1 cells (~15% of WT Hepa-1).[6] In these cells, the AhR band was rapidly depleted within 2

[6] In all nonhepatic cell lines, lower molecular weight bands that reacted with the A-1 antibody were consistently detected in whole-cell lysates and nuclear fractions but not cytosol. The bands did not exhibit changes related to the duration of TCDD incubation and, thus, likely represent nonspecific binding to a nuclear component and not AhR degradation. It would be expected that degraded fragments of the AhR would be detected in the cytosol as observed for a 70-kDa proteolytic fragment observed by Poland *et al.* (33).

hr of TCDD treatment and was undetectable at 4, 8, or 16 hr (Fig. 5A). The Arnt concentration in these cells was essentially unchanged after TCDD treatment (Fig. 5A). C2C12 cells showed rapid loss of nearly the entire AhR pool by 2 hr of TCDD treatment (Fig. 5B). There appeared to be a very small recovery of AhR protein at 8 and 16 hr; however, AhR remained reduced by >90% at these times. The expression of Arnt was not significantly affected by TCDD exposure (Fig. 5B). A-7 smooth muscle cells express levels of AhR comparable to those found in Hepa-1 cells. A7 cells exhibited a rapid loss of AhR that continued to decrease through 16 hr of TCDD exposure (Fig. 5C). The maximal amount of AhR depleted in these cells was >95% at 16 hr. As observed in all other cell lines evaluated in the study, Arnt concentration was essentially unaffected by TCDD in the A7 cells (Fig. 5C).

These results show that the reduction in AhR protein is not exclusive for cells of hepatic origin and may be a universal mechanism for controlling the level of activated AhR. As observed in the WT Hepa-1 and type 1 cells, the initial concentration of AhR did not influence the degree of maximal reduction. The finding that the AhR was depleted more rapidly (within 2 hr) and more extensively (>95% loss) in these nonhepatic cells may reflect the fact that all three cell types express an AhR protein that is distinct from the AhR expressed in Hepa-1 cells (32, 34). The AhR expressed in NIH 3T3 and C2C12 cells is the Ah^{b-2} allele that appears to be more labile *in vitro* than the Ah^{b-1} allele expressed in Hepa-1 cells and is more representative of the AhR expressed in human cells (32, 34, 35). This property could affect the function of these AhRs *in vivo* and make them more susceptible to proteolysis.

Conclusions. The rapid change in AhR but not Arnt concentration is similar to results reported for the basic-helix-loop-helix zip proteins myc and max (36, 37). For these proteins, max is constitutively expressed in the nuclear compartment of most cells. Its has a half-life of >18 hr and is maintained at a constant level. In contrast, myc is transcriptionally regulated by numerous signals. Once induced, the majority of myc protein becomes associated with max, and this heterodimer can then bind specific core DNA sequences to mediate gene expression. However, myc has a half-life of only 20–30 min, and once its biosynthesis is turned off it is rapidly depleted from the cell in a manner independent of max. These results indicate that the highly regulated myc biosynthesis is the limiting factor in formation of myc/max complexes and cellular response. It is intriguing to speculate that a similar theme may be important in AhR-mediated signal transduction and that tissue-specific regulation of liganded AhR levels may be a mechanism by that cells control responsiveness to exogenous and endogenous ligands.

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Send reprint requests to: Dr. Richard S. Pollenz, Department of Biochemistry and Molecular Biology, Medical University of South Carolina, 171 Ashley Avenue, Charleston, SC 29425. E-mail: pollenzr@musc.edu