Aryl Hydrocarbon Receptor Imported into the Nucleus following Ligand Binding Is Rapidly Degraded via the Cyttoplasmic Proteasome following Nuclear Export*

Nikos A. Davarinos and Richard S. Pollenz‡
From the Department of Biochemistry and Molecular Biology, Medical University of South Carolina, Charleston, South Carolina 29403

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor that dimerizes with the AHR nuclear translocator protein to mediate gene regulation. However, the AHR protein is rapidly depleted in vitro and in vivo following exposure to ligands. The purpose of the studies in this report was to characterize the mechanism of AHR degradation and determine the consequence of blocking the degradation process. Western blot and immunological analysis of rat smooth muscle (A7), murine Hepa-1, and human HepG2 cells show that ligand-induced degradation of AHR is blocked when the proteasome is inhibited by MG-132. AHR degradation is also blocked in Hepa-1 and HepG2 cells when nuclear export is inhibited with leptomycin B. Mutation of a putative nuclear export signal present in the AHR results in the accumulation of AHR in the nucleus and reduced levels of degradation following ligand exposure. In addition, inhibition of AHR degradation results in an increase in the concentration of AHR: AHR nuclear translocator complexes associated with DNA and extends the duration that the complex resides in the nucleus. These findings show that nuclear export and degradation of the AHR protein are two additional steps in the AHR-mediated signal transduction pathway and suggest novel areas for regulatory control.

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‡ To whom correspondence should be addressed: Dept. of Biochemistry and Molecular Biology, Medical University of South Carolina, 173 Ashley Ave., Charleston, SC 29403. Tel.: 843-792-6801; Fax: 843-792-4322; E-mail: pollenz@musc.edu.

The abbreviations used are: AHR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocator; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; GAR-HRP, goat anti-rabbit horse-radish peroxidase; GAR-TR, goat anti-rabbit Texas Red; ALLM, N-acetyl-Leu-Leu-methioninal; XRE, xenobiotic response element; LMB, leptomycin B; NES, nuclear export signal; mAHR, mouse AHR.

protein in vivo and in vitro. AHR protein is reduced by 80–95% in numerous cell culture models within 4 h of TCDD treatment and does not recover to basal levels as long as ligand is present in the medium (8–10). AHR protein is also dramatically reduced in the male reproductive tissues, spleen, thymus, liver, and lung of rats given a single oral dose of TCDD (11, 12) and in male reproductive tissues of rats exposed to TCDD in utero and lactationally (13).

At the molecular level, Western blot analysis shows that the concentration of the AHR and ARNT protein detected in nuclear lysates of culture cells is highest following 1 h of TCDD exposure but then rapidly declines (3, 8). These findings are supported by gel mobility shift analysis showing that the association of the AHR:ARNT complex with the XRE is transient and becomes greatly reduced in cell culture models within 2–6 h of the initial agonist stimulation (14, 15). In addition, studies in rat LCS7 and mouse Hepa-1 cells indicate that protein association at the endogenous CYP1A1 promoter and CYP1A1 transcription are reduced in a time-dependent manner following TCDD exposure (14). However, earlier studies utilizing the Hepa-1 cell line suggest that CYP1A1 transcription and promoter occupancy by the AHR:ARNT complex are maintained following 16 h of TCDD exposure (16, 17). Therefore, a correlation between the degradation of AHR protein and control of the magnitude and duration of AHR-mediated gene regulation has yet to be formally established.

The purpose of the studies detailed in this report was to determine the mechanism of AHR protein degradation and evaluate the consequence of blocking the degradation process. The results indicate that the liganded AHR is degraded in the cytoplasm via the proteasome after being exported from the nuclear compartment. Blockage of AHR degradation results in an increase in the concentration of AHR:ARNT complexes associated with DNA and extends the duration that the complex resides in the nucleus. These findings show that the degradation of AHR protein is a critical component of the AHR-mediated signal transduction pathway and suggest novel areas that may be involved in the control of AHR signaling.

EXPERIMENTAL PROCEDURES

Chemicals—TCDD (98% stated chemical purity) was obtained from the Radian Corp. (Austin, TX) and was solubilized in dimethyl sulfoxide (Me2SO). MG-132 (purity, >90% by high pressure liquid chromatography) and ALLM were purchased from Calbiochem. Leptomycin B (LMB) was a generous gift from Dr. M. Yoshida (Osaka University Medical School, Osaka, Japan).

Buffers—Phosphate-buffered saline is 0.8% NaCl, 0.02% KCl, 0.14% Na2HPO4·0.02% KH2PO4, pH 7.4. 2× gel sample buffer is 125 mM Tris, pH 6.8, 4% SDS, 25% glycerol, 4 mM EDTA, 20 mM dithiothreitol, 0.005% bromphenol blue. Tris-buffered saline is 50 mM Tris, 150 mM NaCl, pH 7.5. Tris-buffered saline with Tween 20 is 50 mM Tris, 0.2% Tween 20, 150 mM NaCl, pH 7.5. Tris-buffered saline with Tween 20 is 50 mM Tris, 0.5% Tween 20, 300 mM NaCl, pH 7.5. BLOTTO is 5% dry

The aryl hydrocarbon receptor (AHR)1 is a ligand-activated transcription factor that belongs to the growing family of basic helix-loop-helix/PER-ARNT-SIM proteins (1, 2). The AHR is ubiquitously expressed and is usually localized in the cytoplasm of cells in an inactive multiprotein complex that contains hsp90 (3–5). Upon ligand binding, the AHR complex translocates to the nucleus where the AHR can dimerize with the ARNT protein to mediate gene regulation through direct binding to xenobiotic response element (XRE) enhancer sequences (reviewed in Refs. 6 and 7). In addition, recent studies also show that ligand binding results in rapid depletion of the AHR
milk in Tris-buffered saline with Tween 20. 2× lysis buffer is 50 mM Hapes, pH 7.4, 40 mM sodium molybdate, 10 mM EGTA, 6 mM MgCl2, 20% glycerol. 5× gel shift buffer is 50 mM Hapes, pH 7.5, 15 mM MgCl2, 50% glycerol. 0.5× Tris borate-EDTA is 45 mM Tris borate, 1 mM EDTA. 

Antibodies—Specific antibodies against either the AHR (A-1, A-1A) or ARNT (R-1) are identical to those described previously (3, 8). All antibodies are affinity-purified IgG fractions. For Western blot analysis, goat anti-rabbit IgG conjugated to horseradish peroxidase (GAR-HRP) were utilized. For immunohistochemical studies, goat anti-rabbit IgG conjugated to Texas Red (GAR-TR) was used. Both of these reagents were purchased from Jackson ImmunoResearch (West Grove, PA). Polyclonal rabbit β-actin antibodies were purchased from Sigma.

Cell Culture Lines and Growth Conditions—Wild type Hepa-1c1c7 (Hepa-1) and type II Hepa-1 variants were a generous gift from Dr. James Whitlock, Jr. (Department of Pharmacology, Stanford University). These cells were propagated in Dulbecco’s minimum essential medium supplemented with 5% fetal bovine serum. E36 cells were a generous gift from Dr. Alan Schwartz (St. Louis University). Cells were propagated at 30°C in α-MEM supplemented with 10% fetal bovine serum and 4.5 g/liter glucose. All other cells were obtained from American Type Culture Collection (ATCC, Rockville, MD). HepG2 cells were propagated in Dulbecco’s minimum essential medium supplemented with 10% fetal bovine serum. A7 cells were propagated in MEM supplemented with 10% fetal bovine serum. All cells were passaged 1-week intervals and used in experiments during a 2-month period. For treatment regimens, TCDD, MG-132, and leptomycin B were administered directly into growth medium for the indicated incubation times. The vehicle used for TCDD and MG-132 was Me2SO, and the final concentration of Me2SO ranged from 0.2 to 1.0%.

Preparation of Cell Lysates, Cytosol, and Nuclear Lysates—Following treatment, cell monolayers were washed twice with phosphate-buffered saline and detached from plates by trypsinization (0.05% trypsin, 0.5 mM EDTA). Cell pellets were then washed with phosphate-buffered saline and suspended in ice-cold 1× lysis buffer supplemented with Nonidet P-40 (0.5%), leupeptin (10 μg/ml), and aprotinin (20 μg/ml). Cell suspensions were immediately sonicated for 10 s, supplemented with phenylmethylsulfonyl fluoride (100 μM, final concentration), and sonicated an additional 10 s. A small portion of the lysate was then removed for protein determination, and the remainder was combined with an equal volume of 2× gel sample buffer, vortexed, and immediately heated for 5 min at 100°C. Cytosol and total nuclear lysates were prepared essentially as detailed previously (3, 8). Protein concentrations were determined by the Coomassie Blue Plus assay kit (Pierce) with bovine serum albumin as the standard.

Quantitative Western Blot Analysis of AHR and ARNT—The linear range of the AHR, ARNT, and β-actin antibodies for detection of target proteins and the quantitative Western blotting procedure has been described previously (4, 8, 11, 18). Briefly, ECL exposures were scanned into a Power Macintosh computer utilizing an HP ScanJet IIcx/T with Adobe Photoshop 4.0 software. Images were quantified utilizing NIH Image 1.55 software. The raw level of AHR protein was then divided by the level of β-actin protein to generate normalized values for the concentration of the AHR in each sample. In all studies, the trend of the data was never affected by the normalization procedure.

Immunofluorescence Staining and Microscopy—All immunocytochemical procedures (cell plating, fixation, staining, and photography) were carried out as described previously (3, 4, 8, 18). Cells were observed on a Zeiss Axiophot microscope using the 568-nm filter. On average, 15–20 fields (5–20 cells each) were evaluated on each coverslip, and 3–4 fields were photographed to generate the raw data. Experiments were repeated at least two times.

Electrophoretic Mobility Shift Assay—A double-stranded XRE fragment corresponding to the consensus XRE-1 of the CYPIA1 promoter (19) was labeled with [32P]dCTP by Klenow fill in (20). 20–100 μg of nuclear extract were then incubated at 22°C for 15 min in 1× gel shift buffer supplemented with KCl (80 mM) and poly(dI-dC) (0.1 mg/ml). Approximately 4 ng of [32P]-labeled XRE were then added to each sample, and the incubation was continued for an additional 15 min at 22°C. The samples were resolved on 5% acrylamide, 0.5% Tris borate-EDTA gels, dried, and exposed to film.

In Vitro Mutagenesis and Eukaryotic Transfections—Leucine 70 and leucine 72 of the mAHR were changed to alanine residues by the Quick Change® in vitro mutagenesis kit as detailed by the manufacturer (Stratagene, Palo Alto, CA). Selected clones were then sequenced to confirm the presence of the mutation and the expression plasmid termed pSportM’AHRA5ES. For transfection, approximately 5 × 10⁶ E36 cells were plated into 60-mm culture dishes and incubated at 30°C for 16–24 h. 1.25 μg of pSportM’AHRA5ES or pSportM’AHRA were then transfected into cells with LipofectAMINE™ reagent as detailed by the manufacturer (Life Technologies, Inc.). Following a 24–36-h recovery period, cells were incubated in the presence of 2 nM TCDD or Me2SO for 6 or 16 h. Cells were harvested from plates by trypsinization, and total cell lysates were prepared as detailed above. For immunological studies of transfected cells, 5 × 10⁶ E36 cells were plated into 60-mm culture dishes containing poly-l-lysine-coated glass coverslips, and then they were transfected, treated, and fixed as detailed above.

Luciferase Reporter Gene Assay—Hepa-1 cells stably integrated with an XRE-luciferase reporter (Promega, Madison, WI), and luciferase activity was quantified as detailed by the manufacturer (Promega). Basal levels of luciferase activity associated with MG-132 were subtracted from the MG-132 + TCDD values prior to plotting the data. Protein concentration was determined by the Coomassie Blue Plus assay kit (Pierce). The raw luciferase activity was normalized to the level of cellular protein and plotted.

RESULTS AND DISCUSSION

AHR Is Rapidly Degraded by the Proteasome following Exposure of Culture Cells to TCDD—Previous studies have demonstrated that the AHR accumulates in the nucleus of Hepa-1 cells within 60–90 min of TCDD exposure, whereas depletion of the AHR lags behind the translocation event (3, 8). Because redistribution of the AHR to the cytoplasm has never been observed, these findings resulted in the hypothesis that AHR was degraded in the nuclear compartment (8, 10). To gain insight into the pathway responsible for reducing AHR protein levels, AHR degradation was evaluated in several cell culture lines incubated with MG-132 or calpain inhibitor II (ALLM). MG-132 has been extensively utilized to implicate the multiple peptidase activities of the proteasome in protein degradation (22–27).

The effect of TCDD and MG-132 on AHR protein levels was analyzed by Western blot analysis of total cell lysates. A representative experiment with the A7 cell line is shown in Fig. 1. Treatment of A7 cells with TCDD resulted in nearly complete degradation of the AHR by 6 h, whereas treatment of cells with 1.0, 5.0, or 7.5 μM MG-132 alone did not change the concentration of AHR protein (Fig. 1A). In contrast, cells treated with TCDD in the presence of increasing concentrations of MG-132 showed a dose-dependent inhibition of AHR degradation (Fig. 1B). A final concentration of 7.5 μM, MG-132 completely blocked the TCDD-induced degradation of AHR. Treatment of Hepa-1 or HepG2 cells with 5–10 μM MG-132 also blocked the TCDD-induced degradation of AHR protein by approximately 90%, and in all cell types there was no effect on ARNT protein concentration. Because MG-132 has been reported to inhibit the activity of lysosomal and Ca2+-activated proteases (calpains) to a limited degree, studies were repeated with a calpain inhibitor II (ALLM) that has less inhibitory activity of the proteasome (reviewed in Refs. 24 and 28). Treatment of A7 cells with ALLM did not block the TCDD-induced degradation of the AHR (Fig. 1C). Thus, the ability of MG-132 but not ALLM to block degradation of the AHR implicates the proteasome in the degradation of ligand-bound AHR.

To confirm the Western blotting results, the subcellular distribution of the AHR was evaluated in the presence of MG-132, TCDD, or Me2SO. A7 cells treated with Me2SO exhibited diffuse cytoplasmic and nuclear staining for AHR that was not significantly changed following treatment with MG-132 for 8 h (Fig. 2, A–C). However, AHR staining became predominantly nuclear following 1 h of TCDD exposure (Fig. 2E) but was dramatically reduced following 6 h of TCDD exposure (Fig. 2F).
In contrast, A7 cells incubated with MG-132 for 2 h and then exposed to TCDD for an additional 6 h exhibited intense fluorescence within the nuclear compartment (compare Fig. 2, G and H with F). These results show directly that MG-132 inhibits the degradation of the AHR following ligand binding. Interestingly, the AHR did not appear to redistribute to the cytoplasmic compartment in the presence of MG-132 and TCDD but remained predominantly nuclear. This pattern of localization was not specific to the A7 cells, as treatment of wild type and HepG2 cells with MG-132 and TCDD also resulted in persistent AHR staining within the nuclear compartment.

Degradation of the AHR Requires a Nuclear Export Signal (NES) and Redistribution from the Nucleus to the Cytoplasm—

The proteasome is a large multiprotein complex that has been implicated in the degradation of proteins within the cytoplasm, endoplasmic reticulum, and nucleus (reviewed in Refs. 24, 28, and 29). However, there is limited information concerning the subcellular distribution of the proteasome and the activity of the complex within the nucleus. Thus, it was pertinent to investigate whether the redistribution of liganded AHR from the nucleus to the cytoplasm was a requirement for degradation. A comparison of the amino acid sequences of mammalian AHR revealed that a putative NES (30, 31) was present in the helix 2 domain that was 100% conserved in all AHR sequences evaluated. The leucine-rich sequence spanned amino acids 63–73 and is shown in Fig. 3. This sequence has recently been shown to function in nuclear export when fused to green fluorescent protein and injected into bovine kidney cells (31); however, the function of the NES in the native AHR protein is uncharacterized. Thus, it was pertinent to evaluate the function of nuclear export in the context of AHR degradation. To evaluate this process, the nuclear export pathway was blocked...
Fig. 3. Nuclear export sequence of AHR. Amino acid sequence of putative NES and of alanine substitutions present in mAHRNES. Numbers represent the amino acid number of mAHR. Bold amino acids and arrows show leucine to alanine changes.

by exposure of cells to the fungal antibiotic, LMB. LMB has been shown to specifically inhibit nuclear export of proteins by direct interaction with the nuclear export receptor, CRM1 (32, 33).

The effect of TCDD and LMB on AHR protein levels was analyzed by Western blot analysis of total cell lysates. Fig. 4A shows representative results for the HepG2 cell line. As demonstrated in numerous rodent cell lines (8–10), treatment of HepG2 cells with TCDD for 4 h resulted in >80% reduction in AHR protein. However, the TCDD-induced degradation of the AHR was completely inhibited in cells pretreated with 5 and 25 nM LMB. In Hepa-1 cells, AHR degradation was also blocked by treatment with LMB (Fig. 4B). However, the mouse Hepa-1 cells required a concentration of 50 nM LMB to significantly inhibit AHR degradation. The reduced sensitivity may be related to the specificity of the LMB for human and murine CRM1 as higher concentrations of LMB were also needed to inhibit AHR degradation. The reduced sensitivity may be related to the specificity of the LMB for human and murine CRM1 as higher concentrations of LMB were also needed to inhibit AHR degradation. In addition, nuclear export is required for AHR degradation and that the degradation process occurs within the cytosolic compartment. To further validate this idea, studies next focused on the subcellular location of AHR in cells treated with LMB.

To confirm the Western blotting results, the subcellular distribution of the AHR was evaluated in the presence of LMB, TCDD, or Me2SO. Consistent with previous results (3, 8), cells treated with Me2SO showed predominant cytoplasmic AHR staining that became nuclear after 1 h of TCDD exposure (Fig. 5, A and C) but was dramatically reduced following 4 h of TCDD exposure (Fig. 5D). Cells treated with LMB for 9 h showed a similar pattern of staining to Me2SO-treated cells with predominant cytoplasmic staining and no evidence that significant levels of AHR accumulated within the nucleus (compare Fig. 5B with A). However, Hepa-1 cells incubated with LMB for 5 h and then exposed to TCDD for 4 h exhibited intense staining within the nuclear compartment that was in direct contrast to the reduced staining observed with TCDD alone (Fig. 5, E and F). These results confirm the Western blot data (Fig. 4B) and provide direct support to the hypothesis that LMB blocks nuclear export of the liganded AHR and that nuclear export is required for AHR degradation. In addition, the cytoplasmic distribution of AHR following treatment of LMB for 9 h, indicates that the AHR-hsp90 complex does not shuttle between the nucleus and cytoplasm in Hepa-1 cells. Identical results were observed when AHR localization was evaluated in HepG2 cells treated with LMB.2

To extend these studies further, the putative NES of the mAHR was mutated, and the degradation and subcellular localization of the expressed mAHRNES protein was determined in transfected cells. The mutation of the NES changed both leucine 70 and leucine 72 of the NES to alanine residues and the expression vector was termed pSportMAHRNES (Fig. 3). These exact mutations have been shown to block the ability of the NES to direct the cytoplasmic localization of nuclear green fluorescent protein (31). pSportMAHR or pSportMAHRNES was transfected into E36 cells, allowed to recover for 24–36 h, and treated with TCDD (2 nM) for 4 or 6 h. Total cell lysates were then evaluated for AHR and β-actin levels by quantitative Western blotting. Fig. 6 shows a representative experiment. E36 cells did not contain high basal levels of AHR, whereas cells transfected with pSportMAHR or pSportMAHRNES expressed a protein that migrated at approximately 95 kDa and was of similar molecular mass to the AHR expressed in Hepa-1 cells. When the transfected cells were treated with TCDD, mAHR was reduced by 39 and 53% compared with Me2SO-treated cells after 6 and 16 h of treatment, respectively. In contrast, mAHRNES protein was de-
Fig. 5. Subcellular localization of AHR in Hepa-1 cells exposed to LMB and TCDD. Hepa-1 cells were grown on glass coverslips, exposed to the compounds detailed below, and then fixed as detailed previously (3, 4, 8). Coverslips were then incubated with A-1 IgG (1.0 μg/ml) and visualized with GAR-TR IgG (1:750). A, cells exposed to Me2SO (0.1%) for 9 h and stained for AHR. B, cells exposed to LMB (50 nm) for 9 h and stained for AHR. C, cells exposed to TCDD (2 nm) for 1 h and stained for AHR. D, cells exposed to TCDD (2 nm) for 4 h and stained for AHR. E and F, cells exposed to LMB (50 nm) for 5 h followed by TCDD (2 nm) for an additional 4 h and stained for AHR. All panels were exposed and printed for identical times.

Fig. 6. Western blot analysis of recombinant AHR protein expression in E36 cells exposed to TCDD. E36 cells were transfected with either pSportmAHR or pSportmAHRΔNES. Duplicate plates were exposed to Me2SO (DMSO) (0.1%) or TCDD (2 nm) for 6 or 16 h, and 18 μg of total cell lysate were resolved by SDS-polyacrylamide gel electrophoresis. Blots were stained with A-1A IgG (1.0 μg/ml) and β-actin IgG (1:1000) and visualized by ECL with GAR-HRP IgG (1:10,000). Bands were quantified and normalized as detailed (4, 8, 11, 18). Data are expressed as the percentage of AHR protein compared with Me2SO-treated controls. A sample of Hepa-1 total lysate is included as a control. Bars represent the average ± S.E. of two independent samples.
fraction of cells treated with MG-132 and TCDD suggest that a high fraction of the AHR protein pool remains tightly associated with nuclear structures when proteolysis is inhibited. These results are consistent with the immunostaining data showing that the AHR does not redistribute to the cytoplasm in the presence of MG-132 and TCDD (Fig. 2, G and H).

To extend these results, HepG2 cells were treated with MeSO or TCDD in the presence or absence of MG-132, and nuclear extracts were evaluated for AHR-ARNT complexes by electrophoretic mobility shift assays. Consistent with previous studies (14, 15), a specifically shifted AHR-ARNT-XRE band was maximally detected after 1 h of TCDD exposure but was reduced to near basal levels after a 5-h exposure (Fig. 8B). In contrast, nuclear extracts prepared from cells treated with MG-132 and TCDD appeared to accumulate AHR-ARNT complexes over the time course of the experiment and showed the highest level of AHR-ARNT-XRE complex at the 5-h time point (Fig. 8B). Similar results were observed when identical experiments were carried out in the Hepa-1 cell line. These results are consistent with the detection of high levels of nuclear AHR in the nucleus by Western blotting (Fig. 8A) and immunofluorescence (Figs. 2, 5, and 7) and suggest that AHR-ARNT complexes will continue to associate with XRE sequences as long as the concentration of nuclear AHR is maintained at a high level.

Whereas the previous studies showed that high levels of AHR-ARNT were present in the nucleus of Hepa-1 and HepG2 cells treated with MG-132, electrophoretic mobility shift assay and immunofluorescence staining provide no information about the functionality of the AHR-ARNT complex. Therefore, studies were carried out to determine whether MG-132 treatment would enhance the gene induction by TCDD. Following TCDD exposure, luciferase activity peaked at 5 h and then slowly decreased. The maximal level of TCDD-induced luciferase activity was 113-fold higher than untreated cells at the 5-h time point. In contrast, cells treated with MG-132 and then exposed to TCDD induced luciferase activity more rapidly and to a much greater magnitude than cells treated with TCDD alone. Luciferase activity was induced over 1300-fold after 8 h of TCDD exposure in the presence of MG-132. This represents a 24-fold increase above cells treated with TCDD alone. In addition, cells treated with TCDD and MG-132 did not show the characteristic plateau of luciferase activity (21) but continued to rise throughout the time course. These results are...
consistent with the presence of increased levels of AHR-ARNT in the nucleus (Fig. 9) and suggest that TCDD-mediated gene regulation can be dramatically affected by inhibiting the degradation of the AHR. It is important to note, however, that the proteolytic mechanism responsible for the degradation of luciferase protein has not been determined, although the protein has a half-life of approximately 3 h (34, 35). Therefore, it is possible that some of the luciferase activity associated with MG-132 and TCDD may be related to reduced turnover of the luciferase enzyme.

Conclusion and Implications—AHR-mediated signaling has been extensively investigated in numerous model systems in an attempt to define the components of the pathway, understand protein and DNA interactions, and define specific changes in gene expression (reviewed in Refs. 6 and 7). There has been considerably less emphasis placed on the fate of the AHR and ARNT proteins following ligand exposure and the mechanism involved in turning the signaling pathway off. The results presented in this report provide strong evidence for at least two additional events in the AHR signal transduction pathway: nuclear export of liganded AHR and rapid proteolysis by the cytoplasmic proteasome complex. These findings result in a model of AHR-mediated signaling that is shown in Fig. 10.

In this model, the AHR exists as an AHR-hsp90 complex in which putative nuclear localization signals and NES are masked by the presence of hsp90, other proteins, or protein conformation. Thus, nuclear localization signals and NES would not be used to shuttle the inactive AHR complex between the nucleus and cytoplasm. Following ligand binding, the nuclear localization signal present in the N-terminal region of the AHR (31) would be exposed, and the entire AHR-hsp90 complex would then translocate to the nucleus prior to the dissociation of hsp90. This mechanism takes into account the observation that nuclear translocation precedes AHR degradation (3, 8), the isoulation of AHR-hsp90 complexes from the nucleus (36, 37), the finding that AHR translocates to the nucleus in cells that lack ARNT but is isolated in the 9S conformation (3, 38, 39), and the observation that dissociation of the AHR from hsp90 by treatment of cells with geldanamycin results in rapid proteolysis of the AHR (40). Once in the nucleus, the AHR would dissociate from hsp90 through unknown mechanisms (phosphorylation state?) that expose the NES sequence present in helix 2 (AHRNES). A role for ARNT in this process is unlikely as AHR is rapidly imported, exported, and degraded in Hepa-1 cells that lack high levels of ARNT protein (3, 8). Ligand-bound nuclear AHRNES would then have the potential to associate with ARNT and bind DNA or become a substrate for nuclear export receptors. However, it is likely that AHRNES dimerization with ARNT would block the NES because helix 2 has been shown to function in dimerization (41). The equilibrium of AHRNES interactions would be influenced by the level of AHRNES affinity for ARNT compared with export receptors, and possibly post-translational modifications. For example, changes in the phosphorylation state of AHR and ARNT have been shown to affect AHR-mediated signaling (42–44), whereas the phosphorylation state is known to be critical in the nuclear-cytoplasmic shuttling of the PHO4 and NF-AT4 transcription factors (45, 46). Thus, the model presented above suggests several novel areas for regulatory control including AHR-hsp90 dissociation, AHR-ARNT dimerization, and nuclear export of AHR.

The implications of AHR degradation on cell function are wide ranging but can be considered in the context of basal cellular metabolism or with respect to unprogrammed changes in AHR protein concentration following exposure to exogenous xenobiotics. In the first instance, the degradation of AHR may play two important roles. First, the process may be involved in controlling the magnitude and duration of transcriptional induction or repression by the AHR-ARNT complex. Indeed, degradation of proteins involved in signal transduction pathways is an established mechanism of regulation (reviewed in Ref. 47). Proteolysis has been shown to be involved in such divergent signaling systems as NF-κB (48), glucocorticoid-mediated signaling (49), and the basic helix-loop-helix/PER-ARNT-single

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minded protein hypoxia-inducible factor-1α (27, 51, 52). For the AHR, proteolysis may be critical in helping turn off or reduce the response to ligands that are not readily metabolized by the cell. Second, because ARNT is involved in hypoxia signaling (53, 54), single minded signaling (55, 56), and can also function as an ARNT-ARNT homodimer (57–60), degradation of AHR may play a role in modulating the amount of activated AHR so that appropriate concentrations of ARNT are maintained for ARNT-dependent signaling pathways. Studies have shown that AHR protein concentration is generally in excess of ARNT in numerous cell culture models and various tissues (4, 11), thus, the potential exists for the liganded AHR to sequester a fraction of the ARNT pool. However, only about 15% of the ARNT pool is actually utilized during AHR-mediated signaling in cell culture lines in part because 85–95% of the AHR protein is degraded (3, 8). The concentration of hypoxia-inducible factor-1α is also modulated by proteolysis under normoxic conditions with no affect on ARNT protein levels (27, 51). The importance of maintaining ARNT protein levels is underscored by the finding that ARNT –/+ mouse models die by GD-10 (61, 62).

What might be the effect of unprogrammed changes in AHR protein concentration following exposure to xenobiotics such as TCDD? Previous studies have shown that reductions in AHR protein appear to affect subsequent stimulation of AHR-mediated signaling (11) and that reductions of AHR protein also appear to affect growth in cell culture models (63). In addition, AHR knockout mice exhibit a variety of growth defects including immune system impairment (64), reduced mammary gland development (65), lower incidence of large interfrontal bones (66), liver fibrosis (64), impaired reproductive outcome, and fetal viability (50, 64). These findings suggest that the AHR is involved in important aspects of growth and development and make it possible that unprogrammed reductions in AHR protein may disrupt endogenous signaling pathways that influence transcriptional events involved in growth and differentiation. Studies are in progress to evaluate the importance of AHR protein levels and the degradation pathway in AHR-mediated signaling cascades.

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