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# Analysis of Aryl Hydrocarbon Receptor-Mediated Signaling during Physiological Hypoxia Reveals Lack of Competition for the Aryl Hydrocarbon Nuclear Translocator Transcription Factor

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## ABSTRACT

The aryl hydrocarbon nuclear translocator (ARNT) protein functions as a transcription factor after dimerization with other basic helix-loop-helix proteins. Thus, dimerization of ARNT within one pathway may limit the availability of this protein to others. To investigate this issue, aryl hydrocarbon receptor (AHR)-mediated signaling was investigated in mouse (Hepa-1), rat (H4IIE), and human (HepG2) hepatoma cell lines undergoing physiologically induced hypoxia ($<$1% $O_2$). Basal levels of ARNT protein were not affected by hypoxia in any cell line, and ARNT remained exclusively nuclear. Furthermore, quantitative Western blotting revealed that the concentration of ARNT sequestered during hypoxia represented a small fraction of the total ARNT protein pool (12 and 15% in Hepa-1 and H4 cells, respectively). When the AHR-mediated signaling pathway was activated during hypoxia by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, the induction of P4501A1 protein was reduced by 55% without changes in the level of mRNA in Hepa-1 cells, whereas the levels of induction of both P4501A1 protein and *CYP1A1* mRNA were reduced by $>$80% in the H4 cell line. Importantly, gel mobility shift analysis and Western blotting showed that the same level of AHR/ARNT complexes could be detected in cells treated with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin during hypoxia and normoxia. These data suggest that the effects of hypoxia on AHR-mediated gene regulation occur distal to the formation of AHR/ARNT complexes and imply that functional interference between hypoxia and AHR-mediated signaling does not occur through competition for ARNT protein.

The aryl hydrocarbon nuclear translocator [ARNT/hypoxia factor-1$\beta$ (HIF-1$\beta$)] protein is a member of the basic helix-loop-helix/PER-ARNT-SIM (bHLH/PAS) family of transcription factors (Huang et al., 1993; Whitlock, 1993; reviewed in Hankinson, 1995). ARNT protein is constitutively expressed in all cell culture models evaluated to date and is localized exclusively within the nucleus of these cells (Pollenz et al., 1994; Holmes and Pollenz, 1997). The ARNT protein does not appear to bind a ligand or require activation but functions as a heterodimeric binding partner for other bHLH/PAS proteins to mediate several signal transduction pathways. ARNT forms a heterodimer with the aryl hydrocarbon receptor (AHR), and the AHR/ARNT complex associates with the xenobiotic-responsive elements (XREs) to mediate many of the biological effects of halogenated aromatic hydrocarbons (Whitlock, 1993; Hankinson, 1995). In addition, ARNT forms a heterodimer with HIF-1$\alpha$ to regulate hypoxia-inducible genes such as vascular endothelial growth factor, erythropoietin, and numerous glycolytic enzymes and transporters (reviewed in Bunn and Poyton, 1996; Semenza, 1998). ARNT also appears to regulate genes through the CACGTG E-box element (Antonsson et al., 1995; Sogawa et al., 1995; Swanson et al., 1996) and can interact with mouse SIM (Ema et al., 1996; Moffett et al., 1997). Thus, ARNT appears to be a protein critical to the function of at least three distinct signaling pathways that are activated by different stimuli. Indeed, the expression of ARNT appears to be essential for normal development in the mouse because a genetic knockout of the ARNT gene results in the death of animals by gestation day 10 (Kozak et al., 1997; Maltepe et al., 1997).

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**ABBREVIATIONS:** ARNT, aryl hydrocarbon nuclear translocator; AHR, aryl hydrocarbon receptor; bHLH, basic helix-loop-helix; HIF-1$\alpha$, hypoxia-inducible factor-1$\alpha$; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; PER, periodicity; SIM, single minded; PAS, PER-ARNT-SIM; GAR-HRP; goat anti-rabbit horseradish peroxidase; ECL, enhanced chemiluminescence; DFO, desferrioxamine; TTBS, Tris-buffered saline with Tween 20; CYP or P450, cytochrome P-450; PAGE, polyacrylamide gel electrophoresis; EMSA, electrophoretic mobility shift assay; XRE, xenobiotic-responsive element.

Therefore, it is of interest to determine 1) how ARNT-dependent pathways function when more than one is activated, 2) whether one pathway is dominant over the others, and 3) whether some of the biological affects observed after exposure to compounds such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) are related to recruitment of ARNT to the AHR-mediated signaling pathway and away from others.

To begin to address this question, it is critical to know the concentration and subcellular location of ARNT in cells so the amount used by each signaling pathway can be calculated. Recent studies from this laboratory have used quantitative Western blotting procedures to show that the concentration of ARNT in nine different cell culture lines averages ~156 fmol/mg cell lysate (~21,000 ARNTs/cell; Holmes and Pollenz, 1997). In total tissue lysates derived from the spleen, liver, and thymus of female Sprague-Dawley rats, ARNT levels were ~50 fmol/mg total tissue lysate (Pollenz et al., 1998). Thus, the ARNT protein represents a modest 0.001 to 0.002% of total cellular protein and appears to be susceptible to sequestration if a binding partner was expressed at sufficiently high levels or if the level of ARNT was reduced during signaling. Indeed, the concentration of AHR protein in the same cell lines used to evaluate ARNT averaged >700 fmol/mg cell lysate (Holmes and Pollenz, 1997). These findings established the hypothesis that saturating levels of ligand might activate the entire pool of AHR resulting in binding to the entire pool of ARNT and inhibition of other ARNT-dependent signaling pathways. However, such a scenario does not appear likely because the vast majority of liganded AHR is rapidly degraded both in vitro and in vivo without affecting the level of ARNT protein (Giannone et al., 1995; Pollenz, 1996; Pollenz et al., 1998; Roman et al., 1998). Thus, although the AHR is 4- to 10-fold higher than ARNT in vivo and in vitro, only ~15% of the ARNT pool is used when the AHR-signaling pathway is saturated. Similar studies with HIF-1$\alpha$ have not been performed; thus, a current area of research in numerous laboratories concerns the interplay of AHR and hypoxia signaling pathways through ARNT.

In the HepG2 human hepatoma cell line, results indicate that during hypoxic signaling stimulated by $CoCl_2$, the expression of TCDD-inducible reporter genes and the induction of the endogenous cytochrome P4501A1 (*CYP1A1*) gene were reduced by ~50% (Gradin et al., 1996). It was also noted that there was a functional reduction in the level of AHR/ARNT heterodimer as detected by gel shift analysis and that HIF-1$\alpha$ appeared to have a greater affinity for ARNT than the AHR. Similar results were observed in the Hep3B cell line where treatment with $CoCl_2$ reduced agonist-induced activity of XRE-driven reporter genes and endogenous *CYP1A1* by ~45% (Chan et al., 1999). In contrast, experiments in the mouse Hepa-1c1c7 cell line showed that AHR-mediated *CYP1A1* expression and DNA binding of AHR/ARNT heterodimers were only minimally reduced under hypoxia (Gassmann et al., 1997). Taken together, these three reports suggest that hypoxic signaling may partially affect gene regulation through AHR-mediated signaling but do not provide direct insight into the mechanism of the response. Specifically, it is unclear 1) whether similar results are observed under physiological hypoxia (<1% $O_2$), 2) whether the ratio of AHR to ARNT protein is important in observing functional interference between the two pathways, 3) whether the timing of the hypoxic signal during activation of the AHR is critical to the biological outcome, 4) whether the hypoxic state of the cell contributes to reduced levels of gene induction, and 5), most important, whether ARNT is the limiting factor in the reduced response to AHR agonists.

To answer some of these questions, studies were initiated to specifically evaluate the AHR-mediated signaling pathway under various hypoxic stimuli in cell lines that express defined concentrations of AHR and ARNT protein. AHR-mediated signaling was evaluated by measuring the induction of the endogenous *CYP1A1* mRNA and P4501A1 protein, the presence of AHR/ARNT heterodimer, and the cellular concentration of AHR and ARNT protein, with a focus on whether ARNT was a limiting factor in AHR signaling during hypoxia. The results indicate that reductions in AHR-mediated gene regulation under hypoxia are not due to functional interference of hypoxia and AHR-mediated signaling through competition for the ARNT protein.

## Materials and Methods

**Chemicals.** TCDD (98% stated chemical purity) was obtained from Radian Corp. (Austin, TX). Desferrioxamine (DFO) were purchased from Sigma Chemical Co. (St. Louis, MO). All other chemicals used in this study were of the highest grade available from commercial sources.

**Antibodies.** Specific antibodies against the mouse ARNT (R-1) and AHR (A-1A) have been described previously (Holmes and Pollenz, 1997; Pollenz et al., 1994; Pollenz et al., 1998). All antibodies are affinity-purified IgG fractions. Antibodies specific to $\beta$-actin were purchased from Sigma Chemical Co. Anti-HIF-1$\alpha$ antibodies were generously provide by Dr. C. Bradfield (University of Wisconsin) and Dr. L. Poellinger (Karolinska Institute). Antibodies specific for rat P4501A1 were purchased from Zenotech (St. Louis, MO). Goat anti-rabbit IgG conjugated to horseradish peroxidase (GAR-HRP) or Texas Red was purchased from Jackson ImmunoResearch (West Grove, PA).

**Buffers.** PBS consists of 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 consisted of 125 mM Tris, pH 6.8, 4% SDS, 25% glycerol, 4 mM EDTA, 20 mM dithiothreitol, and 0.005% bromphenol blue. TBS consists of 50 mM Tris and 150 mM NaCl, pH 7.5. TTBS consists of 50 mM Tris, 0.2% Tween 20, and 150 mM NaCl, pH 7.5. TTBS+ consists of 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 consisted of 50 mM HEPES, pH 7.4, 40 mM sodium molybdate, 10 mM EGTA, 6 mM $MgCl_2$, and 20% glycerol.

**Cell Culture Lines and Growth Conditions.** Wild-type Hepa-1c1c7 cells (Hepa-1) were a generous gift from Dr. James Whitlock Jr. (Department of Pharmacology, Stanford University). These cells were propagated in Dulbecco's modified Eagle's medium supplemented with 5% FBS. All other cells were obtained from American Type Culture Collection (Rockville, MD). H4IIE cells were propagated in Dulbecco's modified Eagle's medium supplemented with 10% calf serum and 5% FBS. All experiments on each cell line were completed within a 2-month period.

**Generation of Hypoxic Conditions.** Hypoxia was generated by incubating cells in a hypoxia chamber (Billups Rothberg, San Diego, CA) perfused with a gas mixture of 1% $O_2$/5% $CO_2$/94% $N_2$ for 30 min at 3 liters/min. The chamber was then sealed and placed in a $CO_2$ incubator at 37°C for the indicated times. Hypoxia was monitored with a gas meter and was <5% $O_2$ within 4 min and <2% $O_2$ within 6 min of start of the gas flow. When cells were dosed with TCDD after hypoxia, the chamber was opened, TCDD was applied to the cultures, and the chamber was sealed and perfused with 1% $O_2$ as described earlier and returned to the 37°C incubator. In these in-

stances, cells were dosed with TCDD and returned to the chamber within 1 min.

**Preparation of Total Cell and Nuclear Lysates.** Total cell lysates for Western blot analysis were prepared by sonicating cell pellets in 1× lysis buffer supplemented with Nonidet P-40 (0.5%) essentially as detailed previously (Pollenz et al., 1994; Pollenz, 1996; Holmes and Pollenz, 1997). Total nuclear lysates were prepared by vortexing cells in 1× lysis buffer supplemented with Nonidet P-40 (1%). The solution was then centrifuged for 2 min at 700*g* to pellet nuclei. The supernatant (cytosol) was removed and supplemented with an equal volume of 2× gel sample buffer, and the nuclei were washed with 500 $\mu$l of 1× lysis buffer. Nuclei were pelleted through centrifugation for 2 min at 700*g* and then sonicated in the presence of 1× lysis buffer supplemented with Nonidet P-40 (1%). Nuclear lysates were then combined with an equal volume of 2× gel sample buffer and heated at 95°C for 5 min. Protein concentrations of all samples were determined with the Coomassie Plus Protein assay (Pierce, Rockford, IL). All samples were stored at −20°C.

**Gel Electrophoresis and Western Blotting.** Total cell or nuclear lysates were resolved by denaturing electrophoresis on discontinuous polyacrylamide slab gels [SDS-polyacrylamide gel electrophoresis (PAGE)] and were electrophoretically transferred to nitrocellulose. Immunochemical staining was carried out with varying concentrations of primary antibody (see text and figure legends) in BLOTTO buffer supplemented with DL-histidine (20 mM) for 1 to 2 h at 22°C. 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 h at 22°C and washed in three changes of TTBS+ as earlier. Before detection, the blots were washed in TBS for 5 min. Bands were visualized with the enhanced chemiluminescence (ECL) kit as specified by the manufacturer (ECL Kit; Amersham, Arlington Heights, IL). Multiple exposures (autoradiographs) of each blot were produced to ensure linearity.

**Quantification of AHR, ARNT, and P4501A1.** The linearity of the R-1, A-1A, P4501A1, and $\beta$-actin antibodies for detection of target proteins has been described in detail previously (Pollenz, 1996; Holmes and Pollenz, 1997; Pollenz et al., 1998; Roman et al., 1998). To calculate the actual concentration of AHR or ARNT in a given sample, known amounts of protein (i.e., nuclear lysate, total cell lysate, and so on) were resolved by SDS-PAGE with known concentrations of Hepa-1 cell lysates that contain 2200 fmol of AHR/mg total lysate and 231 fmol of ARNT/mg total cell lysate. Western blots were developed by the ECL technique as described by the manufacturer (Amersham), and multiple exposures of autoradiographs were evaluated by regression analysis as previously detailed (Pollenz, 1996; Holmes and Pollenz, 1997; Pollenz et al., 1998; Roman et al., 1998). Because it has previously been determined that the total amount of protein in the nuclear lysate fraction represents 25% of the total cellular protein pool (Pollenz et al., 1994), it is possible to determine the actual amount of target protein retained within each cellular fraction.

**Electrophoretic Mobility Shift Assay (EMSA).** Oligonucleotides XRE-1 (5′-CGGCTCGGAGTTGCGTGAGAAGAG) and XRE-2 (5′-CGGCTCTTCTCACGCAACTCCGAG) were annealed and labeled with $^{32}$P-dCTP by Klenow fill-in (Sambrook et al., 1989). The double-stranded fragment corresponds to the consensus XRE-1 of the *CYP1A1* promoter as previously described (Shen and Whitlock, 1992). From 5 to 15 $\mu$g of nuclear extract was incubated at 22°C for 15 min in 1× gel shift buffer supplemented with 80 mM KCl and 0.1 mg/ml poly(dI/dC). In some instances, 0.5 to 1.0 $\mu$g of affinity-purified IgG was included in the sample. Approximately 4 ng of $^{32}$P-labeled XRE was 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/boric acid/EDTA gels, dried, and exposed to film.

**RNA Isolation and Northern Blot Analysis.** Total RNA was isolated from cells with the RNAeasy kit essentially as detailed by the manufacturer (Qiagen, Studio City, CA). RNA was resolved on formaldehyde gels and transferred to nitrocellulose via vacuum blotting. Blots were probed with $^{32}$P-labeled cDNA fragments, washed, and exposed to film as described previously (Sambrook et al., 1989).

**Cell Growth Assay.** Stock cells were harvested, and $10^5$ cells were then plated onto 12 dishes of 60 mm and allowed to attach for 12 h. After this time period, cells were harvested from four plates, counted, and lysed with 1 M NaOH to assess the total cellular protein. Four plates were then placed under hypoxia (1% $O_2$) or normoxia for 19 to 24 h. After the incubation, cells were counted and lysed as above. Cell growth rates (doubling time) was then determined by as Doubling time (h) = 0.693/[ln cell count at harvest − ln cell count at time 0/total growth time (h).

## Results and Discussion

**Hypoxia Stimulation Does Not Affect Location or Concentration of ARNT Protein in Hepa-1 Cells.** Studies in vitro have demonstrated that HIF-1$\alpha$ protein appears to have a higher affinity for ARNT than the AHR (Gradin et al., 1996); thus, it has been hypothesized that there may be competition for the ARNT protein when hypoxia and AHR-mediated signaling pathways are simultaneously stimulated (Gradin et al., 1996; Chan et al., 1999). However, the validity of this hypothesis requires an understanding of 1) how hypoxia affects the basal level of ARNT protein in cells, 2) the importance of timing in the stimulation of both pathways, and 3) calculation of the actual amount of ARNT protein used by both signaling pathways. To answer these questions, it was first necessary to establish that the level of ARNT present in nuclear lysates was an accurate measure of hypoxic signaling and correlated to the presence of HIF-1 $\alpha$.

Hepa-1 cells were incubated under hypoxia (1% $O_2$) for 8 h or incubated under hypoxia for 8 h and then returned to normoxia for an additional 30 min. Cells held under normoxic conditions served as controls. The levels of ARNT and HIF-1$\alpha$ protein present in nuclear lysates and cytosol were then determined by Western blotting. A representative experiment is shown in Fig. 1. The blots show that HIF-1$\alpha$ protein is predominately present in the nuclear lysate fraction of hypoxic cells and is not detected at high levels in the cytosolic fraction (Fig. 1A). The ARNT protein is also detected in the nuclear fraction of hypoxic cells but is detected in the cytosolic fraction as well (Fig. 1B). Importantly, the level of both ARNT and HIF-1$\alpha$ protein detected in the nuclear lysate fraction is markedly decreased as hypoxic cells are returned to normoxia. However, although HIF-1$\alpha$ is no longer detected in any fraction, ARNT protein is present in the cytosol at the same level as in normoxic controls (Fig. 1B). It is important to note that previous immunological studies have demonstrated that ARNT is localized exclusively within the nucleus of Hepa-1 cells and that only ARNT tightly associated with nuclear structures (such as AHR/ARNT complexes associated with XREs) is retained in the nucleus after lysis (Pollenz et al., 1994; Pollenz, 1996; Holmes and Pollenz, 1997). Indeed, immunological staining of fixed cells shows that the subcellular location of ARNT is exclusively nuclear before, during, and after hypoxic treatment (Fig. 1C). Thus, these results are consistent with previous findings suggesting that the basal level of ARNT protein is unaffected by hypoxia (Pugh et al., 1997; Salceda and Carol, 1997; Huang et al., 1998). To confirm this hypothesis, total cell lysates were prepared from Hepa-1 cells that were incubated under hypoxia (1% $O_2$) for

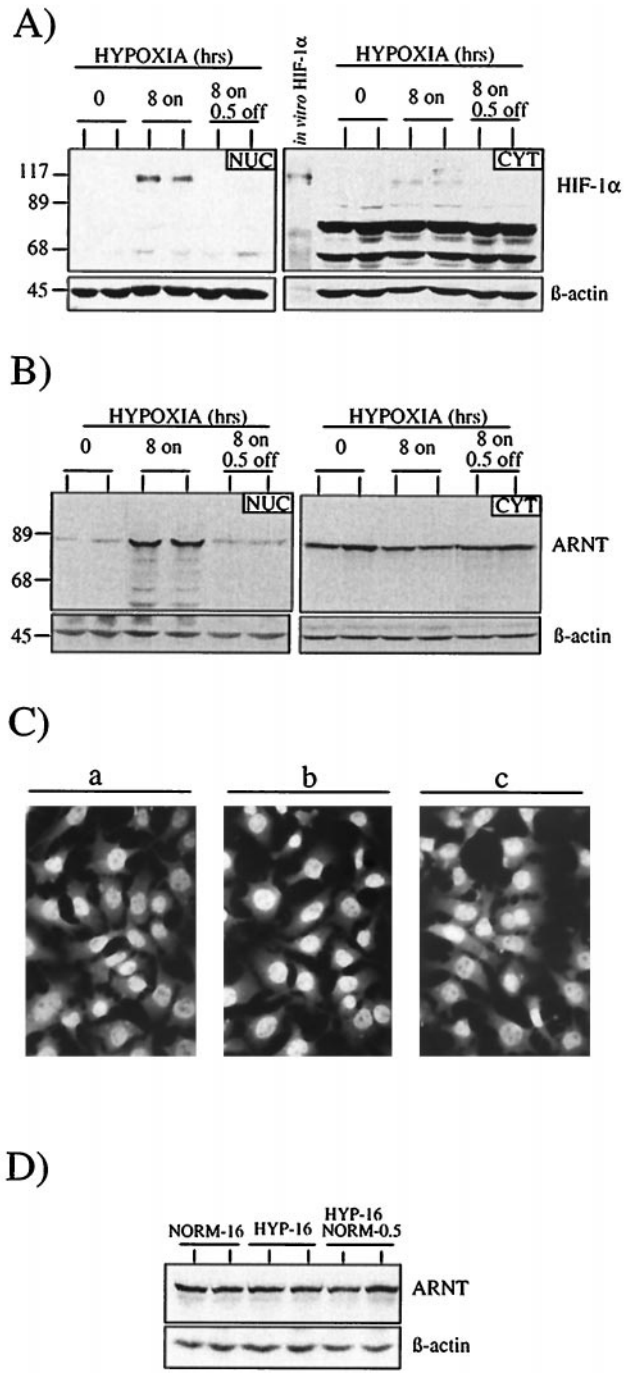


**Fig. 1.** Analysis of HIF-1$\alpha$ and ARNT protein in Hepa-1 cells under hypoxia. Either 12 $\mu$g of Hepa-1 cytosol or 23 $\mu$g of nuclear lysate sample was resolved by SDS-PAGE and blotted. Identical blots were stained with either 1:1000 dilution of anti-mouse HIF-1$\alpha$ IgG (A) or 1 $\mu$g/ml R-1 IgG (B), followed by GAR-HRP (1:10,000). Anti-$\beta$-actin antibodies were also included in the staining of each blot and bands visualized by ECL. 0, normoxic controls. 8 on, Hepa-1 cells incubated under hypoxia (1% $O_2$) for 8 h. 8 on-0.5 off, cells incubated under hypoxia for 8 h and then returned to normoxia for an additional 30 min. The molecular mass of standard proteins is indicated on the left (in kDa). HIF-1$\alpha$ protein produced in vitro was loaded as a molecular mass and staining control. Each lane represented an individual plate of cells. C, Hepa-1 cells were incubated under normoxia (a) or hypoxia (b) for 16 h. Cells were also incubated under hypoxia for 16 h and returned to normoxia for 30 min (c). Cells were fixed, stained with 1 $\mu$g/ml R-1 IgG followed by goat anti-rabbit IgG conjugated to Texas Red (1:750), and visualized by fluorescence microscopy. D, total cell lysates (15 $\mu$g) derived from Hepa-1 cells were resolved by SDS-PAGE, blotted, and stained with 1 $\mu$g/ml R-1 followed by GAR-HRP (1:10,000).

16 h or incubated under hypoxia for 16 h and returned to normoxia for an additional 30 min. Equal amounts of protein were evaluated for ARNT and $\beta$-actin by Western blotting. Figure 1D shows that the level of ARNT protein remains constant during and after hypoxic stimulation.

Several findings are important here. First, the data show that hypoxia does not affect the basal concentration of ARNT in Hepa-1 cells. Second, the level of ARNT detected in the nuclear lysate fraction is consistent with the presence of HIF-1$\alpha$ and an active hypoxic signaling pathway (the hypoxia-responsive genes aldolase and glucose transporter 1 were also induced 4- to 6-fold in hypoxia-stimulated Hepa-1 cells). Third, the subcellular location of ARNT is unaffected by hypoxia, and therefore interaction with HIF-1$\alpha$ must occur in this compartment. Last, the rapid loss of HIF-1$\alpha$, but not ARNT, from the nuclear lysate fraction under normoxia indicates that HIF-1$\alpha$ and ARNT protein levels are regulated distinctly in the Hepa-1 cell line. Thus, ARNT present in the nuclear lysate fraction likely represents ARNT/HIF-1$\alpha$ complexes, whereas cytosolic ARNT represents that fraction of the ARNT protein pool that has redistributed from the nucleus and is an approximation of ARNT protein pool that may be available to other dimerization partners (if the entire pool is accessible). The rapid loss of HIF-1$\alpha$ after normoxia is consistent with the hypothesis that HIF-1$\alpha$ protein concentration is controlled primarily at the level of protein stability and is rapidly degraded under normoxic conditions (Pugh et al., 1997; Salceda and Carol, 1997; Huang et al., 1998). Interestingly, these findings parallel the interaction of the AHR and ARNT during AHR-mediated signaling, where AHR is rapidly degraded after ligand binding without changes in concentration or subcellular localization of ARNT (Pollenz, 1996; Pollenz et al., 1998; Roman et al., 1998).

**Small Fraction of ARNT Protein Pool Is Used during Hypoxic Signaling in Hepa-1 and H4IIE Cells.** Having established that ARNT detected in nuclear lysate fractions of hypoxic cells correlated to the presence of HIF-1$\alpha$, studies were next focused on the level of the ARNT pool used during physiologically induced hypoxia and the time course of the response. For these studies, mouse Hepa-1 and rat H4IIE (H4) cells were used. The choice of the H4 cell line was based on a number of criteria. First, the concentration of AHR in H4 cells (70 fmol of AHR/mg total cell lysate; Holmes and Pollenz, 1997) is closer to the concentration of AHR in rat liver (236 fmol of AHR/mg liver lysate; Pollenz et al., 1998) than the Hepa-1 cell (2300 fmol of AHR/mg cell lysate; Holmes and Pollenz, 1997) and may represent a more physiologically relevant model system for analysis of AHR-mediated signaling. Second, the molecular mass and biochemical properties of the AHR expressed in H4 cells are more consistent with other mammalian AHRs than the AHR expressed in Hepa-1 cells (Poland and Glover, 1990; Dolwick et al., 1993). Finally, the H4 cell represents a hepatic model in which the AHR/ARNT ratio is $<1.0$ as opposed to 10 in the Hepa-1 cell line (Holmes and Pollenz, 1997). Thus, it becomes possible to evaluate the importance of AHR and ARNT protein concentration on AHR-mediated signaling during hypoxic stimulation.

NORM-16, cells incubated under nor- moxia for 16 h. HYP-16, Hepa-1 cells incubated under hypoxia (1% $O_2$) for 16 h. HYP-16 NORM-0.5, cells incubated under hypoxia for 16 h and then returned to normoxia for an additional 30 min.

Hepa-1 or H4 cells were placed under hypoxia (1% $O_2$) for 0 to 16 h, and nuclear lysates were evaluated for ARNT and β-actin by Western blotting. Figure 2 shows a representative blot from each cell line. Hepa-1 cells incubated in 1% $O_2$ show significant levels of ARNT protein in nuclear lysates within 2 h of treatment that are maintained at a similar level for the duration of the hypoxic stimulation (Fig. 2A). In the H4 cell line, significant levels of ARNT are also detected within 2 h of hypoxia and are maintained for the duration of the stimulation (Fig. 2B). In addition, as observed for the Hepa-1 cell line (Fig. 1B), the level of ARNT protein in nuclear lysates is rapidly lost from the fraction when cells are returned to normoxia. Thus, it appears that these cells sequester a fraction of the ARNT protein pool during hypoxia and maintain the level used as long as hypoxia is maintained. Similar results were obtained when mouse 10T1/2 fibroblasts and human HepG2 hepatoma cells were evaluated in identical experiments (R. S. Pollenz, unpublished results).

It was next of interest to calculate the actual amount of the ARNT protein pool sequestered during hypoxic signaling in Hepa-1 and H4 cells. Nuclear lysates were prepared from hypoxia-treated cells and resolved along with a standard curve of Hepa-1 or H4 total cell lysate. The concentration of ARNT was then determined by quantitative Western blotting and computer densitometry as described previously (Pollenz, 1996; Holmes and Pollenz, 1997; Roman et al., 1998). Previous studies have established that a Hepa-1 cell contains ~33,000 molecules of ARNT, whereas H4 cells contain ~19,000 molecules (Pollenz, 1996; Holmes and Pollenz, 1997). It has also been established that the amount of protein present in the nuclear lysate fraction of these cells represents ~25% of total cellular protein (Pollenz et al., 1994; Pollenz, 1996). [Nuclear lysates, cytosol, and total cell lysates were produced from a defined number of cells. Protein assays revealed that the percentage of cellular protein contained within the nuclear lysate fraction was ~25% of the total and that the remaining protein could be recovered in the supernatant (cytosol) fraction (Pollenz et al., 1994).] Therefore, it is possible to determine the concentration of ARNT protein used during hypoxia by comparing the amount of ARNT in a known concentration of nuclear lysate with that of the total cell. The results indicate that the amount of ARNT sequestered in Hepa-1 cells after the exposure to physiological hypoxia represents 12 ± 5% of the total ARNT protein pool (~4000 molecules). For H4 cells, the data show that the amount of ARNT sequestered after hypoxia exposure represents 15 ± 5% of the total ARNT pool (2800 ARNT molecules). Thus, the results of these experiments indicate that the concentration of ARNT tightly associated with nuclear structures (i.e., associated with HIF-1α or other isoforms) under physiological hypoxia is a relatively small fraction of the total ARNT pool (as shown in Fig. 1B). However, a caveat to these results is the fact that it is not known whether the entire pool of ARNT is "accessible" to the various dimerization partners. The calculation that ~15% of the ARNT pool is sequestered by the AHR or HIF-1α may in fact be higher if the entire pool is not available for dimerization.

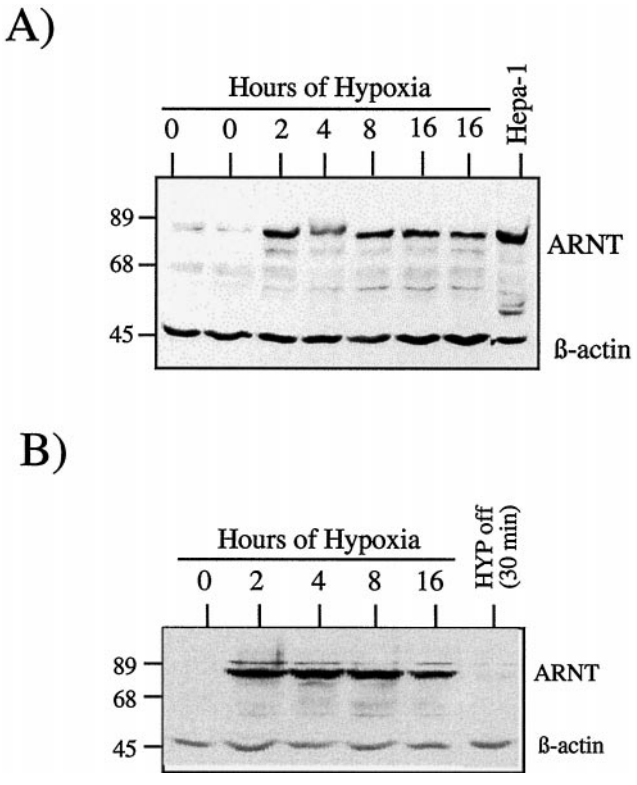


**Fig. 2.** Western blot analysis of ARNT accumulation of in nuclear lysates of hypoxic cells. Hepa-1 and H4 cells were exposed to hypoxia (1% $O_2$) for 0 to 16 h. H4 cells were also exposed to hypoxia for 16 h and then returned to normoxia for 30 min [HYP off (30 min)]. Nuclear lysates were prepared, 18 μg was resolved by SDS-PAGE, and gels were blotted. Blots were stained with 1 μg/ml R-1 IgG and anti-β-actin IgG (1:1000), followed by GAR-HRP (1:10,000), and visualized by ECL. A, results for Hepa-1 cells. Hepa-1 lane is 18 μg of total cell lysate. B, results for H4 cells. The molecular mass of standard proteins is indicated on the left (in kDa). Each lane represents an individual plate of cells. The reduced level of ARNT in nuclear lysates of control treated cells indicates that the detection of ARNT in these samples is directly related to the experimental stimulus and not the result of contamination of unlysed cells.

**Hypoxia Stimulation of Hepa-1 Cells Results in Reduced Levels of TCDD-Induced P4501A1 Protein but Not *CYP1A1* mRNA.** Because hypoxia-mediated signaling appeared to use modest levels of ARNT protein pool, the next set of studies focused on whether hypoxia affected AHR-mediated signaling through ARNT. Hepa-1 cells were preincubated under physiological hypoxia (1% $O_2$) for either 2 or 16 h and then exposed to a single dose of TCDD (1 nM) for either 6 or 16 h while still under the hypoxic stimulation. Total cell lysates were prepared and evaluated for P4501A1 and β-actin protein by Western blotting. Representative blots are shown in Fig. 3. The results show that P4501A1 levels are reduced by 55% when cells are preincubated under 1% $O_2$ for 16 h (Fig. 3A). However, TCDD-inducible P4501A1 protein is not reduced when TCDD treatment occurs immediately after return of the cells to normoxia. Similar results were observed when the hypoxic preincubation was reduced to 2 h. Thus, hypoxia resulted in reduced levels of TCDD-induced P4501A1 protein, and the effect correlated temporally to the recruitment of ARNT by HIF-1α (Fig. 2).

The expression of P4501A1 protein requires induction of *CYP1A1* mRNA through direct binding of an AHR/ARNT heterodimer to the *CYP1A1* promoter (Whitlock, 1993; Hankinson, 1995). Therefore, studies were carried out to evaluate whether hypoxia affected the TCDD-mediated induction of *CYP1A1* mRNA. Hepa-1 cells were incubated in an atmosphere of 1% $O_2$ for 16 h and then treated with TCDD for 6 h while still under hypoxia. In addition, plates of cells were removed from hypoxia after 16 h and treated with

TCDD for 6 h under normoxia. Total RNA was prepared, and the expression of *CYP1A1* and β-actin was determined by Northern blotting. The results show that the induction of *CYP1A1* is identical whether cells are treated with TCDD under hypoxic or normoxic conditions (Fig. 3B). Similar results were observed when the hypoxic preincubation was reduced to 2 h (R. S. Pollenz, unpublished results). These results are in sharp contrast to those observed for induction of P4501A1 protein (Fig. 3A). It appears that the reduced level of TCDD-inducible P4501A1 protein in hypoxia-treated cells does not correlate to the level of *CYP1A1* mRNA and the recruitment of ARNT by HIF-1α. Reductions in P4501A1 appear to be distal to the induction of *CYP1A1*.

**Hypoxia Stimulation Does Not Affect Formation of AHR/ARNT Heterodimers in Hepa-1 Cells.** The finding that TCDD-inducible P4501A1 protein, but not *CYP1A1* mRNA, was reduced by hypoxia in Hepa-1 cells suggested that the formation of the AHR/ARNT heterodimer and binding at the *CYP1A1* promoter were occurring normally in cells

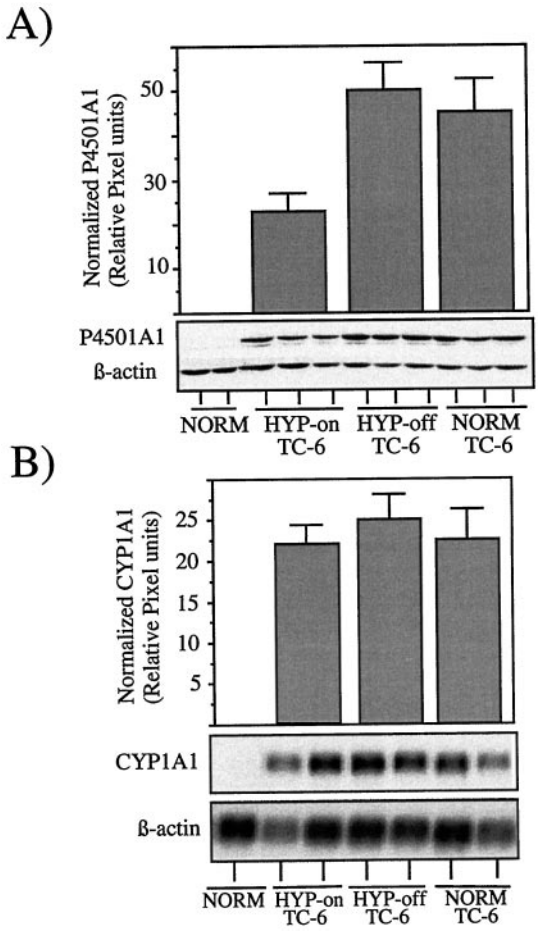


**Fig. 3.** Analysis of *CYP1A1* expression in TCDD-treated Hepa-1 cells under hypoxic stimulation. A, NORM, Hepa-1 cells exposed to normoxia for 22 h. HYP-on TC-6, cells exposed to hypoxia (1% $O_2$) for 16 h and then exposed to TCDD (1 nM) for an addition 6 h while still under hypoxia. HYP-off TC-6, cells exposed to hypoxia (1% $O_2$) for 16 h and then exposed to TCDD (1 nM) for an addition 6 h under normoxia. NORM TC-6, cells exposed to TCDD (1 nM) for 6 h. After each incubation, 15 μg of total cell lysate was resolved by SDS-PAGE and blotted. Blots were stained with anti-P4501A1 IgG (1:1000) and anti-β-actin IgG (1:1200) followed by GAR-HRP (1:10,000). Bands were analyzed by computer densitometry, and P4501A1 was normalized to the level of β-actin. Bar graph represents mean ± S.D. of the three samples shown in the Western blot. B, NORM, Hepa-1 cells exposed to normoxia for 22 h. HYP-on TC-6, cells exposed to hypoxia (1% $O_2$) for 16 h and then exposed to TCDD (1 nM) for an addition 6 h while still under hypoxia. HYP-off TC-6, cells exposed to hypoxia (1% $O_2$) for 16 h and then exposed to TCDD (1 nM) for an addition 6 h under normoxia. NORM TC-6, cells exposed to TCDD (1 nM) for 6 h. after each incubation, total RNA was extracted from cells and evaluated by Northern blotting as described in *Materials and Methods*. Bands were analyzed by computer densitometry, and *CYP1A1* was normalized to the level of β-actin. Bar graph represents average ± S.D. of the three samples shown in the Western blot.

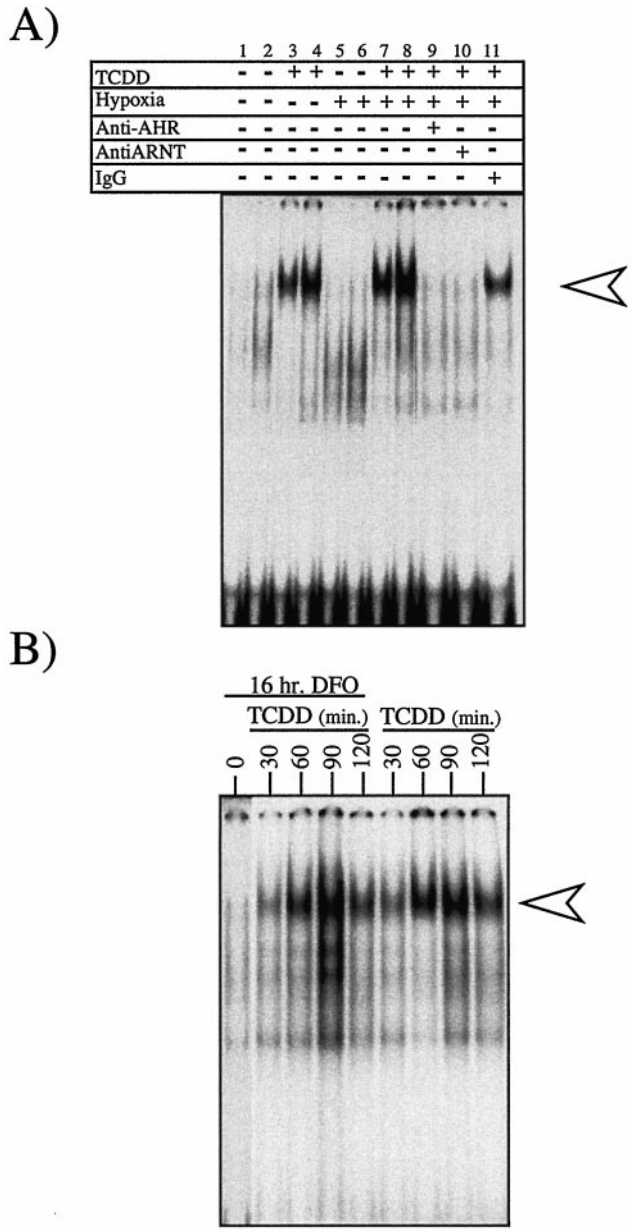


**Fig. 4.** EMSA of AHR/ARNT complexes. A, Hepa-1 cells were incubated under normoxia or hypoxia (1% $O_2$) for 16 h. After this time period, cells were treated with TCDD (1 nM) for 1 h under hypoxic or normoxic conditions. At the end of each treatment, cells were harvested, and nuclear extracts were prepared. EMSA was then performed in the absence or presence of specific antibodies as described in *Materials and Methods*. Then, 5 μg of nuclear extract was used in lanes 1, 3, 5, 7, 9, 10, and 11, whereas 10 μg of nuclear extract was used in lanes 2, 4, 6, and 8. B, Hepa-1 cells were incubated in the presence or absence of DFO (100 μM) for 16 h and then treated with TCDD (1 nM). Cells were harvested 30, 60, 90, and 120 min later, and nuclear extracts were prepared. EMSA was then performed with 10 μg of nuclear extract. The open arrow indicates the specific shift representing AHR/ARNT complexes.

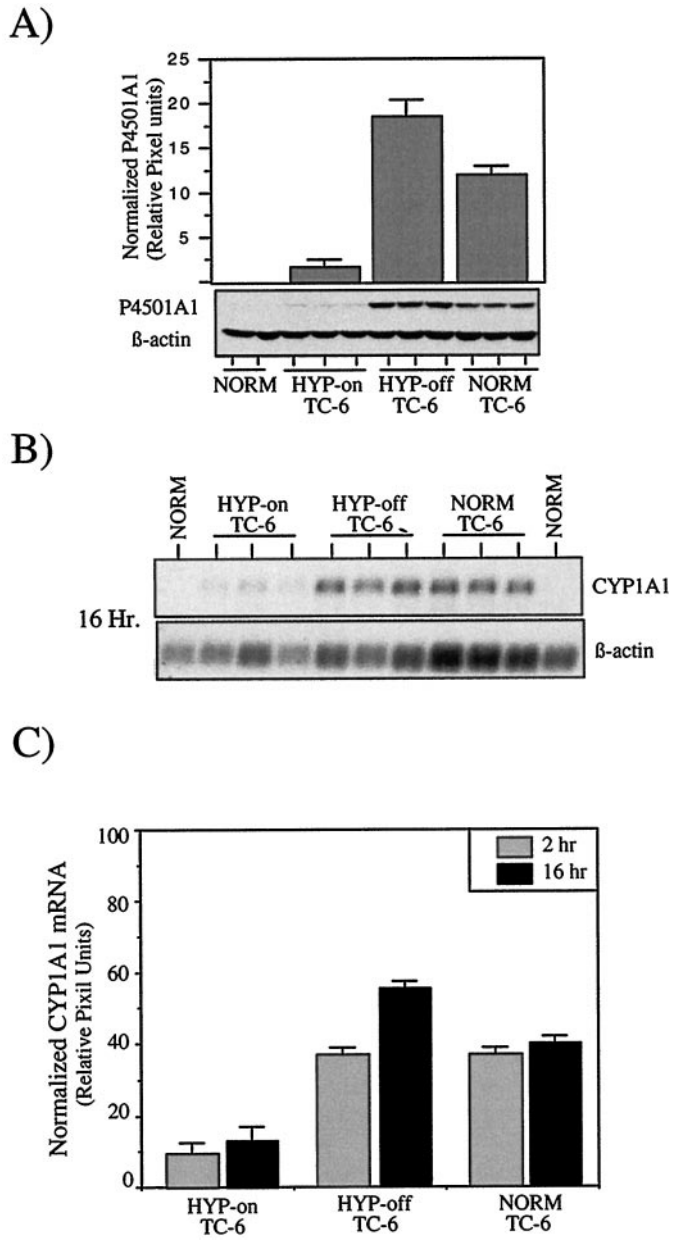


**Fig. 5.** Analysis of *CYP1A1* expression in TCDD-treated H4 cells under hypoxic stimulation. A, NORM, H4 cells exposed to normoxia for 22 h. HYP-on TC-6, cells exposed to hypoxia (1% $O_2$) for 16 h and then exposed to TCDD (1 nM) for an addition 6 h while still under hypoxia. HYP-off TC-6, cells exposed to hypoxia (1% $O_2$) for 16 h and then exposed to TCDD (1 nM) for an addition 6 h under normoxia. NORM TC-6, cells exposed to TCDD (1 nM) for 6 h. After each incubation, 15 $\mu$g of total cell lysate was resolved by SDS-PAGE and blotted. Blots were stained with anti-P4501A1 IgG (1:1000) and anti-$\beta$-actin IgG (1:1200) followed by GAR-HRP (1:10,000). Bands were analyzed by computer densitometry and P4501A1 normalized to the level of $\beta$-actin. Bar graph represents mean ± S.D. of three independent experiments ($n$ = 12 independent samples), and data are expressed as the percent of maximal P4501A1 protein. B, NORM, H4 cells exposed to normoxia. HYP-on TC-6, cells exposed to hypoxia (1% $O_2$) for 16 h and then exposed to TCDD (1 nM) for an additional 6 h while still under hypoxia. HYP-off TC-6, cells exposed to hypoxia for 16 h then exposed to TCDD (1 nM) for an additional 6 h under normoxia. NORM TC-6, cells exposed to TCDD (1 nM) for 6 h. After each incubation, total RNA was extracted from cells and evaluated for *CYP1A1* and $\beta$-actin expression by Northern blotting as described in *Materials and Methods*. Each lane represents an independent plate of cells. C, Northern blots were quantified by computer densitometry, and the level of *CYP1A1* was normalized to the level of $\beta$-actin. The data shown in B are represented by the filled column, whereas data from cells preincubated under hypoxia for only 2 h are represented by the shaded columns. Each bar represents mean ± S.D. of the three independent samples.

undergoing hypoxic signaling. To confirm this hypothesis, Hepa-1 cells were incubated under normoxia or hypoxia, and nuclear extracts were evaluated by EMSAs. Figure 4A shows a representative experiment. A specific shift of similar intensity is observed in all samples treated with TCDD regardless of whether the nuclear extracts were isolated from cells exposed to hypoxia (Fig. 4A, compare lane 3 with lane 7 and lane 4 with lane 8). Quantification of the shifted bands showed them to be within 10% of each other when normalized to the amount of nuclear extract used. To confirm the presence of the AHR and ARNT in the shifted bands, IgG specific for AHR or ARNT was included in the reaction before the addition of XRE. The specifically shifted band is lost with the addition of specific antibodies but not with preimmune rabbit IgG (Fig. 4A, lanes 9–11). In addition, Western blot analysis of the identical nuclear lysate fractions used for the EMSA showed the presence of similar levels of AHR protein in all samples treated with TCDD (R. S. Pollenz, unpublished results).

To dose the hypoxic cells with TCDD, the hypoxia chamber is opened, the cells are dosed, and the chamber is sealed and then re-equilibrated with 1% $O_2$. Due to the rapid turnover of HIF-1$\alpha$ protein (Pugh et al., 1997; Salceda and Carol, 1997; Huang et al., 1998), it was pertinent to confirm that the technical aspects of the experiment did not influence the EMSA results. Hepa-1 cells were treated with DFO to induce hypoxia-mediated signaling. DFO has been used in numerous laboratories as a chemical method of inducing hypoxia (reviewed in Bunn and Poyton, 1996; Semenza, 1998), and studies in Hepa-1 and H4 cells have confirmed that treatment with DFO results in recruitment of ARNT to nuclear lysate fractions and the induction of hypoxia-responsive genes (R. S. Pollenz, unpublished results). Hepa-1 cells were treated with 100 $\mu$M DFO for 16 h and then treated with TCDD (1 nM) for 0, 30, 45, 90, and 120 min. The results of a representative EMSA are shown in Fig. 4B. A specific shift of similar intensity is observed at each time point along the same time course. In addition, DFO alone does not produce a gel shift. The ability to observe specific shifts in this study supports the use of physiological hypoxia as a stimulus (Fig. 4A) and indicates that the technical aspects of the use of physiological hypoxia do not influence the results. In summary, the results of these experiments are consistent with previous studies in the Hepa-1 cell line (Gassmann et al., 1997) and support the hypothesis that the formation of AHR/ARNT complexes capable of binding DNA is not affected by conditions in which up to 15% of the ARNT protein pool has been recruited to the hypoxia signaling pathway.

**Hypoxia Stimulation of H4 Cells Results in Reduced Levels of TCDD-Induced P4501A1 Protein and *CYP1A1* mRNA.** It was next pertinent to determine whether hypoxia affected AHR-mediated signaling in H4 cells because these cells express 30-fold lower levels of AHR than Hepa-1 cells. H4 cells were incubated under hypoxia (1% $O_2$) and exposed to TCDD, and total cell lysates were evaluated for P4501A1 protein by Western blotting. The results show that TCDD-inducible P4501A1 protein is reduced by >80% when H4 cells are preincubated under hypoxia for 16 h (Fig. 5A). However,

TCDD-inducible P4501A1 protein was not reduced if TCDD exposure was initiated immediately on return of the cells to normoxia. In addition, as observed in the Hepa-1 cells, the results did not change when the hypoxic stimulation was reduced to 2 or 6 h before the addition of TCDD (R. S. Pollenz, unpublished results).

Studies were next focused on whether hypoxia affected the TCDD-mediated induction of *CYP1A1* mRNA. H4 cells were incubated in an atmosphere of 1% $O_2$ for 2 or 16 h and treated with TCDD (1 nM) for 6 h while still under hypoxia. In addition, plates of cells were removed from hypoxia after 2 or 16 h and treated with TCDD for 6 h under normoxia. Total RNA was then prepared, and the expression of *CYP1A1* and $\beta$-actin was determined by Northern blotting. In sharp contrast to the results observed in the Hepa-1 cell line, TCDD-inducible *CYP1A1* mRNA was reduced by ~75% whether cells were incubated under hypoxia for 2 or 16 h before TCDD treatment (Fig. 5, B and C). However, TCDD-inducible *CYP1A1* was not reduced if TCDD treatment occurred immediately after return of the cells to normoxia (Fig. 5, B and C). Thus, reductions in P4501A1 protein are supported by reduced levels of *CYP1A1* and suggest that hypoxia is affecting function at the *CYP1A1* promoter region in the H4 cell line.

**Hypoxic Stimulation of H4 Cells Results in Reduced Levels of AHR Protein, but Formation of AHR/ARNT Heterodimers Is Not Affected.** Because the TCDD-mediated induction of *CYP1A1* mRNA was dramatically reduced during hypoxia in H4 cells, studies were initiated to determine whether formation of the AHR/ARNT heterodimer was also affected. To begin the analysis of this question, it was first of interest to evaluate whether hypoxia affected the endogenous level of AHR or ARNT protein in the H4 cells because this could account for reductions in response to TCDD. Hepa-1 cells were incubated under normoxia, incubated under hypoxia (1% $O_2$) for 16 h, or incubated under hypoxia for 16 h and then returned to normoxia for an additional 30 min. Total cell lysates were then evaluated for ARNT, AHR, or $\beta$-actin. The results show that hypoxia does not significantly change the level of ARNT protein but results in a 2-fold reduction in the level of AHR protein (Fig. 6A). This is the first evidence that hypoxia may affect the basal level of a transcription factor and is consistent with the response of cells to TCDD under hypoxia (Fig. 5).

To further evaluate these findings, H4 cells were exposed to DFO (100 $\mu$M) for 16 h to induce hypoxia as described for Hepa-1 cells. Cells were then treated with TCDD (1 nM) for 0, 60, 80, and 120 min. At the end of each treatment, cells were harvested, nuclear extracts were prepared, and the samples were evaluated for AHR and $\beta$-actin protein by Western blotting. Figure 6C shows a representative blot. Importantly, AHR protein is detected in all samples treated with TCDD regardless of whether they were exposed to DFO. Because AHR protein is not detected in cells incubated with DFO alone, the presence of AHR protein cannot be due to the presence of the chemical or to contamination of the nuclear extract with nonlysed cells. The reduced level of AHR observed in samples treated with TCDD for 60 min may represent a delay in the timing of the AHR-signaling cascade; however, identical levels of AHR are observed in cells treated with DFO or not treated with DFO after 80 and 120 min. The presence or ARNT in each sample was confirmed by Western blotting, and both ARNT and AHR were detected in the same fraction (elution at ~200 kDa), when evaluated by gel filtration chromatography (R. S. Pollenz, unpublished results). Collectively, this set of experiments support the hypothesis that AHR/ARNT complexes capable of interacting with DNA are present in H4 cells during hypoxic signaling with DFO. Similar findings were observed when hypoxia was used as the stimulus (R. S. Pollenz, unpublished results). It is important to note, however, that given the reduction observed in TCDD-inducible *CYP1A1* and P4501A1, the functionality and timing of the formation of the AHR/ARNT heterodimer may be dramatically affected by the hypoxic stress to the cell. Indeed, analysis of H4 cells after 24 h of hypoxia revealed that the doubling time of the culture increased from 11.9 $\pm$ 2 to 28.4 $\pm$ 5 h, whereas the concentration of total cellular protein in each cell was unchanged. Thus, the H4 cell line appears particularly sensitive to hypoxia, and this condition results in greatly reduced transcription and translation of *CYP1A1* after exposure to TCDD.

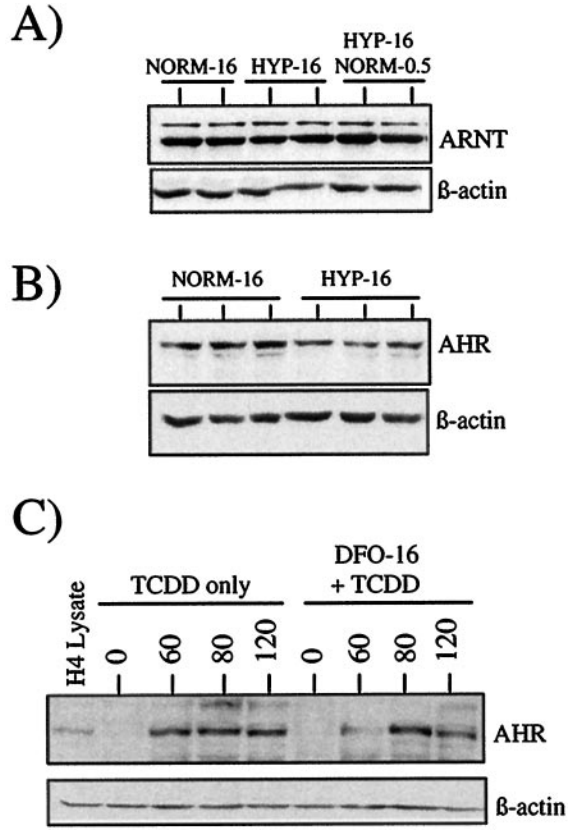


**Fig. 6.** Western blot analysis of AHR and ARNT protein levels in H4 cells exposed to hypoxia. A, H4 cells were exposed to the indicated conditions, and total cell lysates were evaluated for ARNT by Western blotting. Blots were stained with R-1 IgG (1 $\mu$g/ml) and anti-$\beta$-actin IgG (1:1200) followed by GAR-HRP (1:10,000). NORM-16, H4 cells exposed to normoxia for 16 h. HYP-16, cells exposed to hypoxia (1% $O_2$) for 16 h. HYP-16 NORM-0.5, cells exposed to hypoxia (1% $O_2$) for 16 h and then exposed to normoxia for 30 min. Each lane represents an independent plate of cells. B, H4 cells were exposed to normoxia (NORM-16) or hypoxia (HYP-16) for 16 h, and total cell lysates were evaluated for AHR by Western blotting. Blots were stained with A-1A IgG (1 $\mu$g/ml) and anti-$\beta$-actin IgG (1:1200) followed by GAR-HRP (1:10,000). Each lane represents an independent plate of cells. C, H4 cells were incubated in the presence or absence of DFO (100 $\mu$M) for 16 h and then treated with TCDD (1 nM) for the indicated times. Nuclear extracts were prepared, and 18 $\mu$g was resolved by SDS-PAGE, blotted, and stained with A-1A IgG (1 $\mu$g/ml) and anti-$\beta$-actin IgG (1:1200) followed by GAR-HRP (1:10,000). Then, 15 $\mu$g of H4 total cell lysate was loaded as a control. Note that the level of $\beta$-actin is consistent in all samples and that AHR is detected only in samples derived from TCDD-treated cells.

**Hypoxia Does Not Affect Formation of AHR/ARNT Complexes in Human Cell Lines.** Although the Hepa-1 and H4 cell line has been used extensively for analysis of AHR-mediated signaling, other studies on the interplay of AHR and hypoxia signaling have used human cell lines (Gradin et al., 1996; Chan et al., 1999). Therefore, studies were performed to assess the formation of AHR/ARNT complexes in two human cell lines under physiological hypoxia. HepG2 hepatoma and MCF-7 breast cancer cells were exposed to hypoxia (1% $O_2$) or normoxia and treated with TCDD, and nuclear extracts were evaluated by EMSA. The results of representative experiments are shown in Fig. 7. For the HepG2 cell line, a specific shift is observed in all samples treated with TCDD regardless of whether the nuclear extracts were isolated from cells exposed to hypoxia (Fig. 7A, lanes 2 and 4). In three independent experiments, the intensity of the specific shifts was 80 ± 12 relative densitometry units for TCDD alone compared with 85 ± 15 relative densitometry units for TCDD exposed under hypoxic conditions. To confirm the presence of the AHR and ARNT in the shifted bands, IgG specific for AHR or ARNT was included in the reaction before the addition of XRE. The major shifted band was lost with the addition of specific antibodies but not with preimmune rabbit IgG (Fig. 7A, lanes 5–7). These results are identical to those presented for Hepa-1 cells (Fig. 4A). For the MCF-7 cell line, specifically shifted bands were also observed in all samples from TCDD-treated cells (Fig. 7B). The relative intensity of the bands in hypoxia-treated cells (52 ± 10 relative densitometry units) were slightly reduced compared with TCDD-treated controls (65 ± 15 relative densitometry units) in two separate experiments, but the values were not statistically different. For both cell lines, the presence of AHR in nuclear extracts was confirmed by Western blotting and showed no significant changes whether isolated from cells exposed to hypoxia or normoxia (R. S. Pollenz, unpublished results). These results are consistent with the findings in Hepa-1 and H4 cells and support the hypothesis that the formation of AHR/ARNT complexes is not affected by the use of ARNT during hypoxia-mediated signaling.

**Conclusions and Implications.** The ARNT protein serves as a dimerization partner for AHR, HIF-1$\alpha$, HIF-2$\alpha$, mouse SIM, and other proteins in the bHLH/PAS family (Ema et al., 1996; Swanson et al., 1996; Moffett et al., 1997; Probst et al., 1997; Pugh et al., 1997; Semenza, 1998). The importance of ARNT-dependent signaling is highlighted by several gene targeting studies. *ARNT*$^{-/-}$ and *HIF*$^{-/-}$ mice exhibit an embryonic lethal phenotype most probably caused by the lack of vascularization mediated through HIF-1$\alpha$/ARNT complexes (Kozak et al., 1997; Maltepe et al., 1997; Ryan et al., 1998). Gene targeting knock-outs of the AHR on the other hand result in viable animals that exhibit significant defects in immune system development, liver fibrosis (Fernandez-Salguero et al., 1995), and mammary gland development (Hushka et al., 1998). In addition, reduced expression of AHR in cell culture models also suggests important endogenous functions for the AHR signaling pathway in cell growth (Ma and Whitlock, 1996). Thus, an important issue with regard to bHLH/PAS protein signaling is whether recruitment of ARNT to one pathway limits the availability of this protein for other bHLH/PAS factors. Analysis of this issue has the potential to provide insight into the interaction of signaling pathways and the mechanism of toxicity for aryl hydrocarbons.

Previous studies suggest that chemically induced hypoxia can affect agonist induced regulation of reporter genes in human cell lines (Gradin et al., 1996; Chan et al., 1999). Thus, it has been hypothesized that these findings indicate that a limiting cellular factor is shared by these pathways, with the ARNT protein as the most likely candidate (Chan et al., 1999). The data presented in this report strongly suggest that functional interference between hypoxia and AHR-mediated signaling does not occur through competition for ARNT protein. This hypothesis is supported by several experiments. First, hypoxia does not affect the distribution or

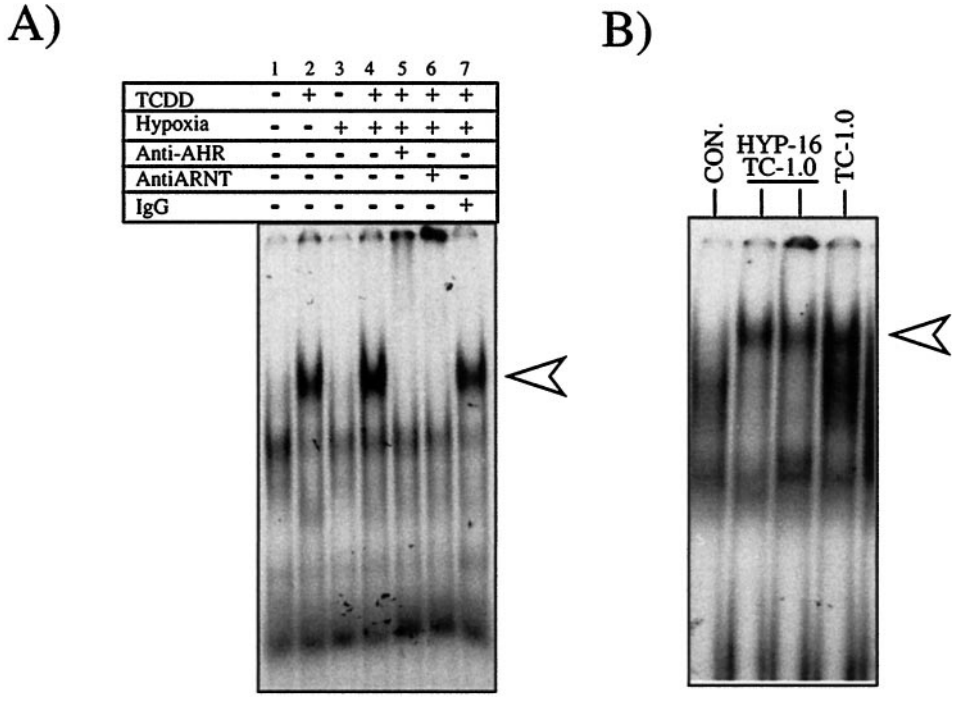


**Fig. 7.** EMSA of AHR/ARNT complexes. A, Hepa-G2 cells were incubated under normoxia or hypoxia (1% $O_2$) for 16 h. After this time period, cells were treated with TCDD (1 nM) for 1 h under hypoxic or normoxic conditions. At the end of each treatment, cells were harvested, and nuclear extracts were prepared. EMSA was then performed in the absence or presence of specific antibodies as described in *Materials and Methods*. B, EMSA of MCF-7 cells. CON, nuclear extracts from normoxic cells. HYP-16-TC-1.0, nuclear extracts from cells incubated under hypoxia (1% $O_2$) for 16 h and exposed to TCDD (1 nM) for 1 h. TC-1.0, nuclear extracts from cells exposed to TCDD (1 nM) for 1 h. Arrowheads indicate the specific shift representing AHR/ARNT complexes.

concentration of ARNT protein present in either rat or mouse hepatoma cells, and the ARNT protein that is sequestered is returned to the ARNT pool once the hypoxic signal is removed. Second, the formation of AHR/ARNT complexes should be the most sensitive endpoint if functional interference is occurring through the use of ARNT (especially because the liganded AHR becomes limiting due to rapid proteolysis). Physiological hypoxia does not affect the formation of AHR/ARNT complexes capable of binding DNA in four distinct cell lines from three different species even though each cell line expresses different levels of AHR and ARNT protein. Finally, quantitative Western blotting shows that the amount of ARNT protein actually sequestered during physiological hypoxia represents only 12 to 15% of the ARNT pool in Hepa-1 and H4 cells, and thus the majority of the pool appears to be available to other protein factors. It is important to note that even if this percentage is higher due to the possibility that the entire ARNT pool is not available for dimerization, the ability to detect AHR/ARNT complexes during hypoxia indicates that AHR and HIF-1$\alpha$ are not competing for the ARNT protein during simultaneous signaling and that ARNT is clearly not the limiting factor in the observed reductions in AHR-mediated signaling. How, then, does hypoxia stimulation result in reductions in TCDD-mediated induction of *CYP1A1* protein and mRNA in certain cells?

A possible answer to this question is that the formation of AHR/ARNT complexes and binding to DNA do not guarantee that AHR-mediated signaling will proceed through gene regulation. As elegantly shown in the steroid hormone receptor signaling cascades, coactivators may be required to obtain maximal transcriptional response (Horwitz et al., 1996), and such a factor may be limiting within the context of AHR or hypoxia-mediated signaling. Alternatively, because hypoxia is a stress response and the AHR/ARNT complexes appear to have formed normally, the reductions in transcription and translation of the *CYP1A1* gene may be related to metabolic state of the cell and a shift toward maintenance of regulation of essential genes. Recent studies support this view by clearly demonstrating that hypoxia can induce apoptosis and affect cellular growth (Bunn and Poyton, 1996; Carmeliet et al., 1998). Indeed, the H4 cell line exhibited dramatic effects of hypoxia on *CYP1A1* induction, and these results appear to correlate with greatly reduced growth rates of this cell during hypoxic stress. In contrast, Hepa-1 cells did not exhibit sensitivity to hypoxia either in cellular growth or the magnitude of changes in TCDD-mediated signaling.

In summary, the results presented in this report are consistent with previous studies that have shown hypoxia-induced reductions in AHR-mediated gene regulation (Gradin et al., 1996; Chan et al., 1999) but support a model in which ARNT is not the limiting factor. It appears that the competition for ARNT is minimized in part because the AHR and HIF-1$\alpha$ proteins capable of binding ARNT are kept in check by proteolysis. In the case of AHR, ligand binding results in rapid degradation of the majority of the protein pool (>85%), creating a situation in which the AHR actually becomes the limiting dimerization partner for ARNT in most cell lines (Pollenz, 1996; Pollenz et al., 1998; Roman et al., 1998). For HIF-1$\alpha$, the protein is rapidly turned over during normoxia (Pugh et al., 1997; Salceda and Carol, 1997; Huang et al., 1998) and appears to be the limiting binding partner for ARNT during the physiological hypoxia stimulation based on the levels of ARNT actually used during signaling (<15% of the ARNT pool). Once normoxia is reestablished, HIF-1$\alpha$ is rapidly degraded, allowing the release of ARNT back to the pool (Fig. 1). A proteolytic mechanism for the regulation of transcription factors appears to be a developing biological theme and is important in the function of p53 (Ciechanover et al., 1994), c-JUN (Trier et al., 1994), nuclear factor-$\kappa$B (Palombella et al., 1994; Traencker et al., 1994), and glucocorticoid receptors (Hoeck et al., 1989). The next challenge is to determine whether biological conditions exist (i.e., developmental stages, expression of additional ARNT partners) to perturb this model, which appears to allow multiple bHLH/PAS signaling pathways to operate simultaneously.

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**References**

Antonsson C, Arulampalam V, Whitelaw MA, Petersson S and Poellinger L (1995) Constitutive function of the basic-helix-loop-helix/PAS factor ARNT: Regulation of target promoters via the E-box motif. *J Biol Chem* **270:**13968–13972.

Bunn HF and Poyton RO (1996) Oxygen sensing and molecular adaptation to hypoxia. *Physiol Rev* **76:**839–885.

Carmeliet P, Dor Y, Herbert J-M, Fukumura D, Brusselmans K, Dewerchin, Neeman M, Bono F, Abromovitch R, Maxwell P, Koch CJ, Ratcliffe P, Moons L, Jain RK, Collen D and Keshet E (1998) Role of HIF-1alpha in hypoxia-mediated apoptosis, cell proliferation and tumour angiogenesis. *Nature (Lond)* **394:**485–490.

Chan WK, Yao G, Gu Y-Z and Bradfield CA (1999) Cross-talk between the aryl hydrocarbon receptor and hypoxia inducible signaling pathways. *J Biol Chem* **274:**12115–12123.

Ciechanover A, Shkedy D, Oren M and Bercovich B (1994) Degradation of the tumor suppressor protein p53 by the ubiquitin-mediated proteolytic system requires a novel species of ubiquitin-carrier protein, E2. *J Biol Chem* **269:**9582–9589.

Dolwick KM, Schmidt JV, Carver LA, Swanson HI and Bradfield CA (1993) Cloning and expression of a human Ah receptor cDNA. *Mol Pharmacol* **44:**911–917.

Ema M, Morita M, Ikawa S, Tanaka M, Matsuda Y, Gotoh O, Saijoh Y, Fujii H, Hamada H, Kikuchi Y and Fuji-Kuriyama Y (1996) Two new members of the murine Sim gene family are transcriptional repressors and show different expression patterns during mouse embryogenesis. *Mol Cell Biol* **16:**5865–5875.

Fernandez-Salguero P, Pineau T, Hilbert DM, McPhail T, Lee SS, Kimura S, Nebert DW, Rudikoff S, Ward JM and Gonzalez FJ (1995) Immune system impairment and hepatic fibrosis in mice lacking the dioxin binding Ah receptor. *Science (Wash DC)* **268:**722–726.

Gassmann M, Kvietikova I, Rolfs A and Wenger RH (1997) Oxygen and dioxin-regulated gene expression in mouse hepatoma cells. *Kidney Int* **51:**567–574.

Giannone JV, Okey AB and Harper PA (1995) Characterization of polyclonal antibodies to the aromatic hydrocarbon receptor. *Can J Physiol Pharmacol* **73:**7–17.

Gradin K, McGuire J, Wenger RH, Kvietikova I, Whitelaw ML, Toftgard R, Tora L, Gassmann M and Poellinger L (1996) Functional interference between hypoxia and dioxin signal transduction pathways: Competition for recruitment of the Arnt transcription factor. *Mol Cell Biol* **16:**5221–5321.

Hankinson O (1995) The Aryl hydrocarbon receptor complex. *Annu Rev Pharmacol Toxicol* **35:**307–340.

Hoeck W, Rusconi S and Groner B (1989) Down regulation and phosphorylation of glucocorticoid receptors in cultured cell. *J Biol Chem* **264:**14396–14402.

Holmes JL and Pollenz RS (1997) Determination of aryl hydrocarbon receptor nuclear translocator protein concentration and subcellular localization in hepatic and nonhepatic cell culture lines: Development of quantitative Western blotting protocols for calculation of aryl hydrocarbon receptor and aryl hydrocarbon receptor nuclear translocator protein in total cell lysates. *Mol Pharmacol* **52:**202–211.

Horwitz KB, Jackson TA, Bain D, Richer JK, Takimoto GS and Tung L (1996) Nuclear receptor coactivators and corepressors. *Mol Endocrinol* **10:**1167–1177.

Huang LE, Gu J, Schau M and Bunn HF (1998) Regulation of hypoxia inducible factor 1$\alpha$ is mediated by an oxygen-dependent domain via the ubiquitin-proteasome pathway. *Proc Natl Acad Sci USA* **95:**7987–7992.

Huang ZJ, Edery I and Robash M (1993) PAS is a dimerization domain common to *Drosophila* period and several transcription factors. *Nature (Lond)* **364:**259–262.

Hushka LJ, Williams JS and Greenlee WF (1998) Characterization of 2,3,7,8-tetrachlorodibenzofuran-dependent suppression and AH receptor pathway gene expression in the developing mouse mammary gland. *Toxicol Applied Pharmacol* **152:**200–210.

Kozak KR, Abbott B and Hankinson O (1997) ARNT-deficient mice and placental differentiation. *Dev Biol* **191:**297–305.

Ma Q and Whitlock JP (1996) The AHR modulates the Hepa-1c1c7 cell cycle and differentiated state independently of dioxin. *Mol Cell Biol* **16:**2144–2150.
Maltepe E, Schmidt JV, Baunoch D, Bradfield CA and Simon MC (1997) Abnormal angiogenesis and response to glucose and oxygen deprivation in mice lacking the protein ARNT. *Nature (Lond)* **386:**403–406.
Moffett P, Reece M and Pelletier J (1997) The murine Sim-2 gene product inhibits transcription by active repression and functional interference. *Mol Cell Biol* **17:** 4933–4947.
Palombella VJ, Rando OJ, Goldberg AL and Maniatis T (1994) The ubiquitin-proteasome pathway is required for processing the NF-κB1 precursor protein and the activation of NF-κB. *Cell* **78:**773–785.
Poland AP and Glover E (1990) Characterization of the strain distribution pattern of the Ah receptor specifies by the $Ah^d$ and $Ah^{b-3}$ alleles. *Mol Pharmacol* **38:**306–312.
Pollenz RS (1996) 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.
Pollenz RS, Santostefano MJ, Klett E, Richardson VM, Necela B and Birnbaum LS (1998) Female Sprague Dawley rats exposed to a single oral dose of 2,3,7,8-tetrachlorodibenzo-p-dioxin exhibit sustained depletion of aryl hydrocarbon receptor protein in liver, spleen, thymus and lung. *Toxicol Sci* **42:**117–128.
Pollenz RS, Sattler CA and Poland A (1994) The aryl hydrocarbon receptor and aryl hydrocarbon receptor nuclear translocator protein show distinct subcellular localization in Hepa 1c1c7 cell by immunofluorescence microscopy. *Mol Pharmacol* **45:**428–438.
Probst MR, Fan CM, Tessier-Lavigne M and Hankinson O (1997) Two murine homologs of the *Drosophila* single-minded protein that interact with the mouse aryl hydrocarbon receptor nuclear translocator protein. *J Biol Chem* **272:**4451–4457.
Pugh CW, O'Rourke JF, Nagao M, Gleadle JM and Ratcliffe PJ (1997) Activation of hypoxia inducible factor 1: Definition of regulatory domains within the alpha subunit. *J Biol Chem* **272:**11205–11214.
Roman BL, Pollenz RS and Peterson RE (1998) Responsiveness of the adult male rat reproductive tract to 2,3,7,8-tetrachlorodibenzo-p-dioxin exposure: Ah receptor and ARNT expression, CYP1A1 induction, and Ah receptor downregulation. *Toxicol Appl Pharmacol* **150:**228–239.
Ryan HE, Lo J and Johnson RS (1998) HIF-1α is required for solid tumor formation and embryonic vascularization. *EMBO J* **1:**7:3005–3015.
Salceda S and Caro J (1997) Hypoxia-inducible factor 1α protein is rapidly degraded via the ubiquitin-proteasome system under normoxic conditions. *J Biol Chem* **272:**22642–22650.
Sambrook J, Fritsch EF and Maniatis T (1989) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Semenza GL (1998) Hypoxia-inducible factor 1: Master regulator of $O_2$ homeostasis. *J Lab Clin Med* **131:**207–214.
Shen ES and Whitlock JP (1992) Protein-DNA interactions at the dioxin responsive enhancer. *J Biol Chem* **267:**6815–6819.
Sogawa K, Nakano R, Kobayashi A, Kikuchi Y, Ohe N, Matsushita N and Fuji-Kuriyama Y (1995) Possible function of Ah receptor nuclear translocator (ARNT) homodimer in transcriptional regulation. *Proc Natl Acad Sci USA* **92:**1936–1940.
Swanson HI, Chan WK and Bradfield CA (1996) DNA binding specificities and pairing rules of the Ah receptor, ARNT, and SIM proteins. *J Biol Chem* **270:**26292–26302.
Traencker EM-B, Wilk S and Baeuerle PA (1994) A proteasome inhibitor prevents activation of NF-kappa B and stabilizes a newly phosphorylated form of I kappa B-alpha that is still bound to NF-kappa B. *EMBO J* **13:**5433–5441.
Trier M, Staszewski LM and Bohmann D (1994) Ubiquitin-dependent c-Jun degradation in vivo is mediated by the gamma domain. *Cell* **76:**787–798.
Whitlock JP (1993) Mechanistic aspects of dioxin action. *Chem Res Toxicol* **6:**754–763.

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