

Cross-talk between the Aryl Hydrocarbon Receptor and Hypoxia Inducible Factor Signaling Pathways

DEMONSTRATION OF COMPETITION AND COMPENSATION*

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The aryl hydrocarbon receptor (AHR) and the α -class hypoxia inducible factors (HIF1 α , HIF2 α , and HIF3 α) are basic helix-loop-helix PAS (bHLH-PAS) proteins that heterodimerize with ARNT. In response to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin, the AHR-ARNT complex binds to “dioxin responsive enhancers” (DREs) and activates genes involved in the metabolism of xenobiotics, e.g. cytochrome P4501A1 (*Cyp1a1*). The HIF1 α -ARNT complex binds to “hypoxia responsive enhancers” and activates the transcription of genes that regulate adaptation to low oxygen, e.g. erythropoietin (*Epo*). We postulated that activation of one pathway would inhibit the other due to competition for ARNT or other limiting cellular factors. Using pathway specific reporters in transient transfection assays, we observed that DRE driven transcription was markedly inhibited by hypoxia and that hypoxia responsive enhancer driven transcription was inhibited by AHR agonists. When we attempted to support this cross-talk model using endogenous loci, we observed that activation of the hypoxia pathway inhibited *Cyp1a1* up-regulation, but that activation of the AHR actually enhanced the induction of *Epo* by hypoxia. To explain this unexpected additivity, we examined the *Epo* gene and found that its promoter harbors DREs immediately upstream of its transcriptional start site. These experiments outline conditions where inhibitory and additive cross-talk occur between the hypoxia and dioxin signal transduction pathways and identify *Epo* as an AHR-regulated gene.

The AHR¹ regulates a variety of biological responses to environmentally ubiquitous polycyclic aromatic hydrocarbons and dioxins (1, 2). In what can be defined as an adaptive pathway, the AHR up-regulates a battery of XMEs that often metabolize many of these agonists to more soluble and excret-

able products. A classic example of this pathway is observed upon exposure to benzo[*a*]pyrene. This chemical binds to the AHR leading to the up-regulation of a battery of genes including *Cyp1a1*, *Cyp1a2*, and *Cyp1b1* (3). The enzymes encoded by these loci have metabolic activity toward benzo[*a*]pyrene and thus play an important role in its elimination (4). At present, we understand many of the molecular events in what appears to be an adaptive response to polycyclic aromatic hydrocarbon exposure. In brief, the up-regulation of genes like *Cyp1a1* are regulated by an agonist-induced heterodimerization between two bHLH-PAS proteins, the AHR and ARNT (5, 6). This heterodimeric pair interacts with DREs upstream of the regulated promoters leading to an increase in their transcription rate and a resultant increase in XME activity (7).

Although we have developed models to describe how the AHR regulates the expression of XMEs, we still have very little knowledge about how this protein mediates the toxicity of potent agonists like dioxin. The molecular mechanisms of dioxin-induced effects like lymphoid involution, epithelial hyperplasia, tumor promotion, teratogenesis, or even death remain unclear. Moreover, although genetic studies indicate the involvement of the AHR in these toxic end points, we have essentially no information that can allow us to conclude that the AHR-mediated mechanisms underlying these effects are similar to the regulation of XMEs. In fact, pharmacological evidence suggests that the mechanisms may be unique (1, 8). Taken in sum, these observations have led us to postulate the existence of a toxic response pathway that may be mechanistically distinct from the adaptive one.

In an effort to provide evidence for the existence of alternative methods of dioxin signaling, we have explored the possibility that activation of the AHR may inhibit homologous pathways by sequestering limiting cellular factors (2). This idea has its roots in the observation that most bHLH proteins function in highly complex signaling networks that involve multiple combinations of bHLH partners, with each pair having a unique effect on gene expression and the cellular environment (9–11). In its simplest form, this model predicts that the activation of the AHR may sequester ARNT, preventing this protein from fully participating in other ARNT-dependent pathways. The recent discovery that HIF1 α -ARNT complexes bind to HREs and activate the transcription of a battery of hypoxia responsive genes provided a system to test the model of dioxin toxicity described above (12). Therefore, we examined the cross-talk between the dioxin and hypoxia signal transduction pathways in both an *in vitro* and cell culture model system. Our experiments outline conditions where reciprocal inhibitory cross-talk between the hypoxia and dioxin signal transduction pathways occurs and describe a compensatory mechanism that

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¹ The abbreviations used are: AHR, Ah receptor; dioxin, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; β NF, β -naphthoflavone; bHLH, basic helix-loop-helix; PAS, Per, ARNT, AHR, SIM homology domain; ARNT, Ah-receptor nuclear translocator; HIF1 α , hypoxia inducible factor 1 α ; XME, xenobiotic metabolizing enzyme, RPA, RNase protection assay; EPO, erythropoietin; CYP1A1, the 1A1 isoform of cytochrome P450; bp, base pair; DRE, dioxin responsive enhancer; HRE, hypoxia responsive enhancer.

explains the unexpected effects of dioxin on Epo expression in cell culture.

EXPERIMENTAL PROCEDURES

Oligonucleotides—The underlined nucleotides define the core sequences of the DRE or HRE: OL73: 5'-TCGAGTAGATCAGCAATGGGCCAGC; OL74: 5'-TCGAGCTGGGCCATTGCGTGAATCTAC; OL116: 5'-GGCCACATCCGGGACATCACAGA; OL117: 5'-TGGGGATGTTGAAGGGGACGAA; OL135: 5'-GAAGATCTTCCAGTGGTCCCA-GCCTACACC; OL200: 5'-GAAGATCTTCTTAGTGATGGTGATGGTG-ATGGAAGTCTAGTTTGTGTTGGTTC; OL497: 5'-CGCCACCACCGCTCAT; OL498: 5'-AGCCCCATCCTGTCTTCAT; OL523: 5'-GCCCTACGTCGTCTCA; OL524: 5'-TGAGACAGCACGTAGGGG; OL1204: 5'-GGAGATCTGGTACCGGTGGCCAGGGACTCTGCG; OL1205: 5'-GGAGATCTGATGCCCCCAGGGGAGGTG.

Materials—The *Cyp1a1* enhancer-luciferase reporter plasmid, PL1A1N, and the HepG2-101L cells that stably express this reporter plasmid were a gift from Dr. Robert Tukey (13). The Hep3B cells were obtained from American Type Culture Collection (Manassas, VA). The plasmid pbEP-luciferase, containing the HRE and promoter from the *Epo* gene driving luciferase expression, was a gift of Jaime Caro (14). The plasmid PL412, containing the HRE from the *Epo* gene fused to the SV40 promoter driving luciferase expression, was described previously (15). The plasmid PL1018, containing the *Epo* promoter driving luciferase expression was constructed by amplifying the 327-bp *Epo* promoter from the pbEP-luciferase plasmid using OL1204 and 1205 as primers. The amplification product was cloned into the *Bgl*II site of the promoterless luciferase reporter plasmid pGL2-Basic (Promega, Madison, WI). The plasmid pCH110 containing the β -galactosidase gene, driven by the SV40 early promoter was obtained from Pharmacia Biotech Inc. (Piscataway, NJ). The plasmid pGemEpo2 harbors a 998-bp fragment of the human EPO cDNA. This cDNA was amplified using OL497 and OL498 as primers. The template was a reverse transcription reaction generated from CoCl_2 induced mRNA from Hep3B cells. The fragment of the human EPO cDNA was cloned into the pGEM-T vector in the SP6 orientation (Promega, Madison, WI). The plasmid PL449 harbors a 340-bp fragment of the human CYP1A1 cDNA. This cDNA was amplified from a HepG2 cDNA library using OL116 and OL117 as primers. The cDNA fragment was cloned into the PGEM-T vector in the SP6 orientation. The plasmid phuAHR1267 harbors the first 1267 nucleotides of the human AHR. This cDNA was amplified from phuAHR using OL135 and OL200 as primers (16). The cDNA fragment was cloned into PGEM-T in the SP6 orientation.

DNA Gel Shift Assay—Oligonucleotides containing the DRE (OL73/74) and the HRE (OL523/524) were synthesized by Life Technologies, Inc. (Grand Island, NY) (17). The double-stranded ^{32}P -labeled oligonucleotide probes were generated by kinasing a single-stranded oligonucleotide with [γ - ^{32}P]ATP (10 Ci/mmol, NEN Life Science Products Inc., Boston, MA) and then allowing it to anneal with a 10-fold excess of its complementary strand. The human HIF-1 α (PL611) and AHR (phuAHR) proteins were expressed in reticulocyte lysate as described previously (15, 18). The human ARNT protein was generated using a baculovirus expression system (19). Based upon our previous estimates, approximately 1–2 fmol of each protein was used in a gel shift assay. The AHR/ARNT and HIF-1 α /ARNT samples were “activated” by incubation at 30 °C for 10 and 30 min, respectively, followed by the addition of 200 ng of poly(dI-dC) and 500,000 cpm of the ^{32}P -labeled oligo. In experiments where ligand activation of the AHR was required, the agonist β NF was added at the beginning of the activation step. Following addition of oligo, samples were incubated for 10 min at room temperature prior to loading. Samples were loaded onto a 4% nondenaturing polyacrylamide gel buffered with 0.5 \times TBE and subjected to electrophoresis at constant voltage of 135 volts for approximately 3 h at 4 °C. Migration of DNA bound complexes were analyzed by autoradiography of the dried gels.

Nuclear Extract and Microsome Preparation—Nuclear extracts and microsomes were prepared from Hep3B cells as follows. Cells were harvested, washed with phosphate-buffered saline, and subjected to centrifugation at 100 \times g for 5 min at 4 °C. The washed cells were resuspended in “lysis buffer” (20 mM HEPES-KOH, pH 7.8, containing 1.5 mM MgCl_2 , 10 mM KCl, 0.5 mM phenylmethylsulfonyl fluoride, and 2 mg/ml leupeptin) and subjected to three cycles of freeze-thaw, followed by centrifugation at 1,000 \times g for 10 min at 4 °C. The supernatant was subjected to centrifugation at 100,000 \times g for 1 h. The microsomal pellet was resuspended in 15 mM, Tris-HCl, pH 8, containing 250 mM sucrose. Nuclear extracts were prepared by resuspending the nuclei-enriched fraction (1,000 \times g supernatant from above) in 50 μ l of

“nuclear extract buffer” (20 mM HEPES-KOH, pH 7.8, containing 0.42 M KCl, 1.5 mM MgCl_2 , 20% glycerol, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 2 mg/ml leupeptin). After incubation on ice for 30 min, the sample was subjected to centrifugation at 16,000 \times g for 30 min at 4 °C. The supernatant was used directly and defined as nuclear extract.

Western Blot Analysis—Anti-AHR monoclonal antibody (VG9) was raised in mice against the C-terminal half of the human AHR (20). Anti-ARNT polyclonal antibodies were a gift from Dr. Alan Poland (21). Anti-HIF-1 α polyclonal antibodies, R35732B, were raised in rabbits against a protein fragment corresponding to amino acids 328–527 of the human HIF-1 α (15). Rabbit anti-CYP1A1 polyclonal antibodies were purchased from Human Biologics, Inc. (Phoenix, AZ). Poly(dI-dC) was purchased from Pharmacia Biotech Inc. Western blot analyses of ARNT, AHR, and CYP1A1 were performed using alkaline phosphatase-coupled secondary antibodies and developed by a colorimetric assay, as described previously (19). The HIF-1 α Western blot was performed using horseradish peroxidase-coupled secondary antibodies and developed with the chemiluminescent “SuperSignal” substrate (Pierce, Rockford, IL).

Ribonuclease Protection Assays—The effects of 75 μM CoCl_2 and/or 10 nM dioxin on the mRNA levels from the *Epo* and *Cyp1a1* genes were monitored by an RPA assay using Hep3B cell lysates as the target (Direct Protect Lysate RPA kit, Ambion, Austin, TX) (22). A 350-nucleotide EPO riboprobe was generated with T7 RNA polymerase using the template pGemEpo2 that had been linearized with *Bgl*II. A 390-nucleotide CYP1A1 riboprobe was generated with T7 polymerase using PL449 that had been linearized with *Sal*I. As a loading control in EPO measurements, a 265-nucleotide AHR riboprobe was generated with T7 polymerase using phuAHR1267 as template that had been linearized with *Nde*I. Similarly, as a loading control in CYP1A1 measurements, a 580-nucleotide AHR riboprobe was generated with T7 polymerase using phuAHR1267 that had been linearized with *Eco*R1. Analysis was performed on lysates from cells grown in 75-cm² flasks at 37 °C at 5% CO_2 . Upon harvest, cells were washed once with 5 ml of cold phosphate-buffered saline and scraped into 5 ml of cold phosphate-buffered saline, followed by centrifugation at 100 \times g for 5 min at 4 °C. The pellet was resuspended into 1 ml of “Direct Protect Lysis Solution” and stored at –20 °C until use. The CYP1A1 and EPO riboprobes generated protected fragments of 340 and 310 bp, respectively. The 265-nucleotide AHR riboprobe generated a 225-bp protected fragment. The 580-nucleotide AHR riboprobe generated a 539-bp protected fragment. Riboprobes were synthesized using Maxiscript *in vitro* translation kit (Ambion), with a specific activity of at least 1 \times 10⁸ cpm/mg RNA and a 60–90% incorporation. The riboprobes (1 \times 10⁵ cpm each) were added to the lysates and incubated at 37 °C overnight. Following this incubation, 0.5 ml of an RNase A/T1 mixture (Ambion’s Direct Protect Digestion Buffer) was added and incubated at 37 °C for 40 min. Sodium Sarcosyl (10%, 20 μ l) and proteinase K (20 $\mu\text{g}/\text{ml}$, 10 μ l) were then added, followed by another 37 °C incubation for 30 min. Samples were precipitated by the addition of 0.5 ml of isopropyl alcohol, stored at –20 °C for 30 min, and subjected to centrifugation at 16,000 \times g for 15 min at 4 °C. The pellets were dried and resuspended into 10 μ l of buffer, before being loaded onto a 4% acrylamide gel containing 8 M urea. After electrophoresis (50 constant watts for 90 min), the gel was dried and the protected fragments were quantitated using a Fuji BAS2000 PhosphorImager.

Choice of Model Cell Lines and Pharmacology—To induce AHR activation, we chose dioxin and/or β NF. Dioxin was chosen for most *in vivo* experiments because it is one of the most potent AHR agonists ever described and it is not known to activate other cellular signal transduction pathways. The agonist β NF was used in some experiments because this molecule is known to have an affinity for the AHR that is approximately 3 orders of magnitude lower than dioxin (23). Thus, its relative potency can be used as pharmacological proof that the AHR and DREs are involved in the observed phenomenon. To up-regulate HIF1 α *in vivo*, we chose to expose cells to 75–100 μM CoCl_2 (12). Although we have reproduced most of our observations using hypoxic conditions (*i.e.* 1% O_2 , data not shown), we have found the addition of the CoCl_2 yields identical results, is less labor intensive, and allows more experimental flexibility. Our choice of Hep3B cells was based upon the observation that this hepatoma cell line displayed both the AHR and HIF1 α signal transduction pathways. The use of the HepG2 derived CYP1A1 reporter line, 101L cells, was a matter of convenience and was used sparingly, primarily as a confirmation of our data generated in Hep3B cells (13).

Transient Transfection Experiments—Hep3B cells were transiently transfected using a LipofectAMINE reagent (Life Technologies, Inc.). Cells were grown to 50% confluence in 6-well plates before transfection.

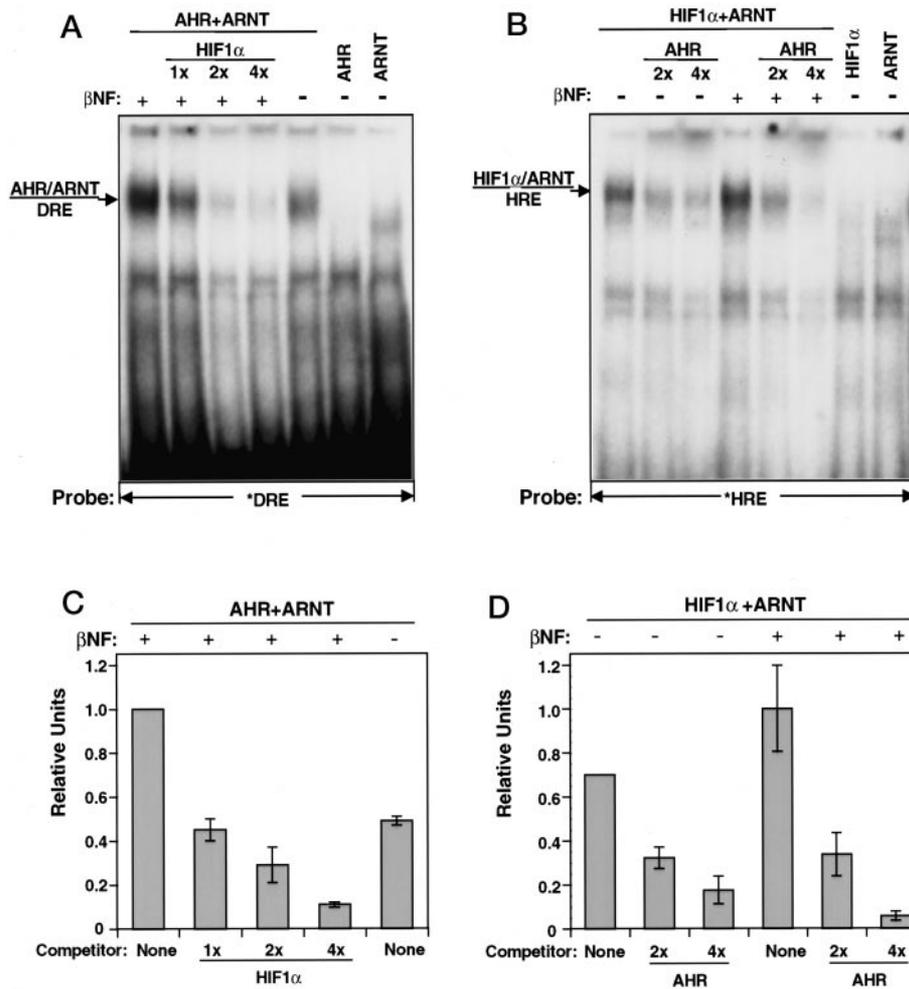


FIG. 1. Gel shift assays to examine *in vitro* competition between HIF-1 α and AHR. *A*, inhibition of the AHR-ARNT-DRE complex by the addition of HIF1 α . The arrow indicates the AHR-ARNT-DRE complex formed in the presence or absence of 10 μ M β NF. Increasing amounts of HIF-1 α (1, 2, and 4 times in excess relative to the amount of AHR) were added as competitor to the incubation mixture as illustrated. AHR or ARNT alone was included as negative controls. *B*, inhibition of the HIF1 α -ARNT-HRE complex by the addition of AHR. The arrow indicates the HIF1 α -ARNT-HRE complex. Increasing amounts of AHR (2 and 4 times in excess relative to the amount of HIF1 α) were added as competitor to the incubation mixture in the presence or absence of 10 μ M β NF as illustrated. HIF1 α or ARNT alone was included as negative controls. *C*, histogram of the relative radioactive counts of 32 P-labeled AHR-ARNT-DRE complexes from *A*. The gels as illustrated in *A* were put on a PhosphorImager overnight and the radioactive counts from the bands containing the AHR-ARNT-DRE complexes were measured. All the counts are normalized with the control (AHR + ARNT in the presence of β NF). The data shows the mean from three separate experiments and the error bars show the standard error of the mean. *D*, histogram of relative radioactive counts from the 32 P-labeled HIF1 α -ARNT-HRE complex. The gels as illustrated in *B* were put on a PhosphorImager overnight and the radioactive counts from the bands containing the HIF1 α -ARNT-HRE complexes were measured. All the counts are normalized with the control (HIF1 α + ARNT in the absence of β NF). The data shows the mean from three separate experiments and the error bars show the standard error of the mean.

A transfection solution of 1.0 ml of serum-free minimal essential medium containing plasmid (0.5 μ g of PL1A1N, 1.0 μ g of PL412, 1.0 μ g of PL1018 or 1.0 μ g of pbEP-luciferase) and 5 μ l of LipofectAMINE reagent was added to each well. Each plasmid transfection was accompanied by 0.2 μ g of the LacZ reporter pCH110, which acted as an efficiency control. Transfection was allowed to occur by incubation at 37 $^{\circ}$ C for 5 h. After this time, minimal essential medium plus 10% fetal bovine serum was added prior to addition of dioxin or CoCl₂. Dioxin and/or CoCl₂ treatments were started at this time, or after 20 additional hours of incubation. Cells were then washed with 2.0 ml of phosphate-buffered saline per well, followed by addition of 150 μ l of cell lysis buffer (Promega, Madison, WI). Following 5 min at room temperature, the lysed cells were collected and cleared by centrifugation at 12,000 \times *g* for 1 min. The supernatants were assayed for luciferase activity (20 μ l/reaction) and β -galactosidase activity (6–10 μ l/reaction) as described previously (15).

Statistics—Differences between treatment groups were identified by the Bonferroni Multiple Comparison Test (24). Statistical significance was set at $p < 0.05$.

RESULTS

Gel Shift Assays—In the early stages of this work, we asked whether the activation of either the dioxin or hypoxia pathway

would inhibit signaling by the other. As an initial test of this idea, we employed gel shift assays where the readout of pathway activity was the interaction of each heterodimer with their corresponding response element. In gel shift assays with fixed amounts of radiolabeled DREs, AHR, and ARNT, we first identified the specific complex using the well characterized inducibility of AHR agonist β NF. Using this system, we observed that the addition of increasing amounts of HIF-1 α protein inhibited the amount of AHR-ARNT-DRE complex in a dose-dependent fashion (Fig. 1, *A* and *C*). In gel shift assays using fixed amounts of radiolabeled HREs, HIF1 α , and ARNT, we first identified the specific complex by demonstrating its formation only in the presence of both bHLH-PAS proteins. In a manner similar to that described above, we observed that increasing the amount of AHR in the system inhibited the formation of the HIF-1 α -ARNT-HRE complex (Fig. 1, *B* and *D*). Interestingly, this inhibition of HIF1 α -ARNT-HRE binding by AHR was observed in both the presence and absence of the AHR ligand β NF. Although, in the presence of the ligand, the competition

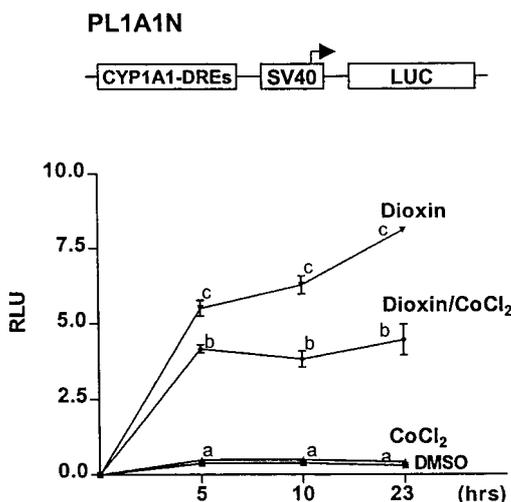


FIG. 2. Cross-talk between dioxin and CoCl₂ characterized using a DRE driven reporter. Above, schematic of the PL1A1N plasmid. CYP1A1-DRE is the 1612-bp region from the *Cyp1A1* gene that harbors three DREs. SV40 is the minimal early promoter from the SV40 genome. Arrow indicates the predicted transcriptional start site. LUC is the luciferase open reading frame. Below, results from transient transfection assays with PL1A1N under various chemical treatments at different time points. RLU, relative light units. All transfections were normalized to a β -galactosidase internal control (see “Experimental Procedures”). The four treatments are dimethyl sulfoxide (DMSO) control, CoCl₂ (100 μ M), dioxin (10 nM), or dioxin (10 nM) plus CoCl₂ (100 μ M). Each time point represents the mean of triplicates and the error bars represent the standard error of the mean. Statistical analysis is described in text. For each time point, those determinations not sharing a superscript (a, b, or c) are significantly different ($p < 0.05$).

was increased compared with lack of agonist (compare $4 \times$ plus or minus β NF, Fig. 1, B and D). Analysis from three separate experiments revealed strong reproducibility of these conclusions (Fig. 1, C and D).

Cross-talk with Synthetic Reporters—We also examined the cross-talk phenomenon in Hep3B cells using transient transfection of reporter constructs that were specific for activation of either the dioxin or hypoxia pathway. To characterize the AHR/ARNT mediated response, a time course was performed in cells transiently transfected with the dioxin inducible reporter PL1A1N (13). In keeping with the expected pharmacology of this reporter, luciferase activity was markedly up-regulated after 5, 10, and 23 h of dioxin exposure (14-, 16-, and 27-fold, respectively), but was unaffected by exposure to CoCl₂ (Fig. 2). Importantly, co-exposure to both dioxin and CoCl₂ led to a decrease in the luciferase response at all time points, as compared with dioxin alone (Fig. 2). To characterize the HIF1 α /ARNT response, a similar experiment was performed using a reporter plasmid, PL412, with a single HRE element derived from the *Epo* gene upstream of an SV40 promoter driving luciferase expression (Fig. 3). In keeping with the expected pharmacology of this reporter we observed induction of luciferase activity at all time points (4-, 9-, and 10-fold for 5, 10, and 23 h, respectively). Importantly, co-exposure to both CoCl₂ and dioxin led to a decrease in the luciferase response at all time points, as compared with CoCl₂ alone (Fig. 3).

CoCl₂ and Dioxin Effects on Endogenous CYP1A1 Expression in Hepatoma Cells—To minimize the possibility that cross-talk was a phenomenon specific to cellular conditions observed only *in vitro* or in transient transfection assays, we performed additional experiments to demonstrate that cross-talk between hypoxia and dioxin signaling could occur using the endogenous *Cyp1a1* and *Epo* loci as reporters. Using Hep3B cells, we first analyzed the amount of HIF1 α , ARNT, AHR, and CYP1A1 protein that was expressed under the various treatment con-

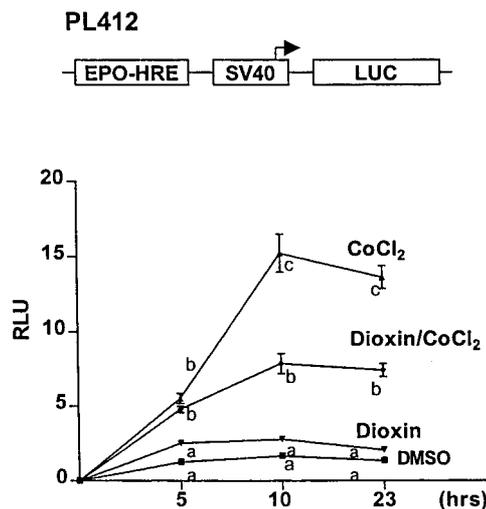


FIG. 3. Cross-talk between dioxin and CoCl₂ characterized using an HRE driven reporter. Above, schematic of the PL412 plasmid. EPO-HRE is the 193-bp region derived from the *Epo* gene that harbors a single HRE. SV40 is the minimal early promoter from the SV40 genome. Arrow indicates the predicted transcriptional start site. LUC is the luciferase open reading frame. Below, results from transient transfection assays with PL412 under various chemical treatments at different time points. RLU, relative light units. All transfections were normalized to a β -galactosidase internal control (see “Experimental Procedures”). The four treatments are dimethyl sulfoxide (DMSO) control, CoCl₂ (100 μ M), dioxin (10 nM), or dioxin (10 nM) plus CoCl₂ (100 μ M). Each time point represents the mean of triplicates and the error bars represent the standard error of the mean. Statistical analysis is described in text. For each time point, those determinations not sharing a superscript (a, b, or c) are significantly different ($p < 0.05$).

ditions (Fig. 4A). As expected, the amount of HIF1 α protein was markedly up-regulated by exposure to CoCl₂ and CoCl₂ plus dioxin. Although expression of the AHR protein was not affected by any of the treatments, we did observe a small but reproducible CoCl₂ related up-regulation of the ARNT protein over basal expression levels. Finally, the microsomal CYP1A1 protein was up-regulated by dioxin exposure and this up-regulation was markedly inhibited by co-exposure to CoCl₂.

To control for the possibility that CoCl₂ might be acting by influencing the stability of CYP1A1 protein, we monitored the response by directly measuring the levels of CYP1A1 mRNA (Fig. 4B). Using an RPA assay at a 5-h time point, we observed that the level of CYP1A1 transcript was up-regulated by dioxin, ~ 4 -fold, and unaffected by CoCl₂ exposure. Importantly, co-administration of CoCl₂ inhibited the dioxin induced levels of the CYP1A1 mRNA by $\sim 40\%$. As a loading control, we analyzed the levels of the AHR mRNA, which was unaffected by any treatment. To demonstrate that CoCl₂ inhibition of CYP1A1 induction was mediated by genomic regulatory elements, we employed the HepG2-101L cell line with an integrated reporter constructed from a fusion of the DREs from the CYP1A1 gene to an SV40 promoter driven luciferase reporter (Fig. 4C). Using this reporter cell line, we observed pharmacological results that were essentially identical to those observed using the RPA. That is, after 23 h of exposure, 10 nM dioxin induced luciferase 73-fold and this induction was reduced approximately 70% in the presence of 75 μ M CoCl₂. CoCl₂ alone did not have any effect on the luciferase activity.

CoCl₂ and Dioxin Effects on Endogenous EPO Expression in Hepatoma Cells—Using Hep3B cells, we used an RPA assay to examine the effects of CoCl₂ and/or dioxin exposure on expression of the endogenous *Epo* gene (Fig. 5). In agreement with the known regulation of this gene, we observed that *Epo* mRNA was up-regulated more than 5-fold over basal levels by exposure to CoCl₂. To our surprise, we also found that dioxin up-

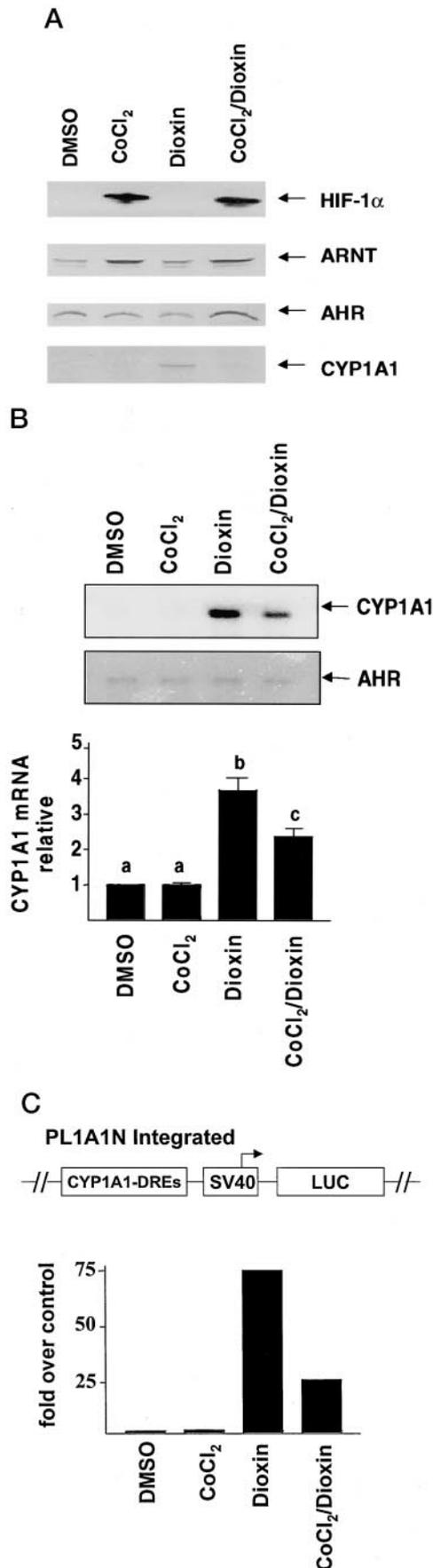


FIG. 4. A, Western blot analysis of HIF-1 α , ARNT, AHR, and CYP1A1 protein levels in Hep3B cells after various treatments. Cells were treated for 23 h at 37 °C with dimethyl sulfoxide (DMSO), 75 μ M CoCl₂, 10 nM dioxin, or 75 μ M CoCl₂ plus 10 nM dioxin. Nuclear extracts

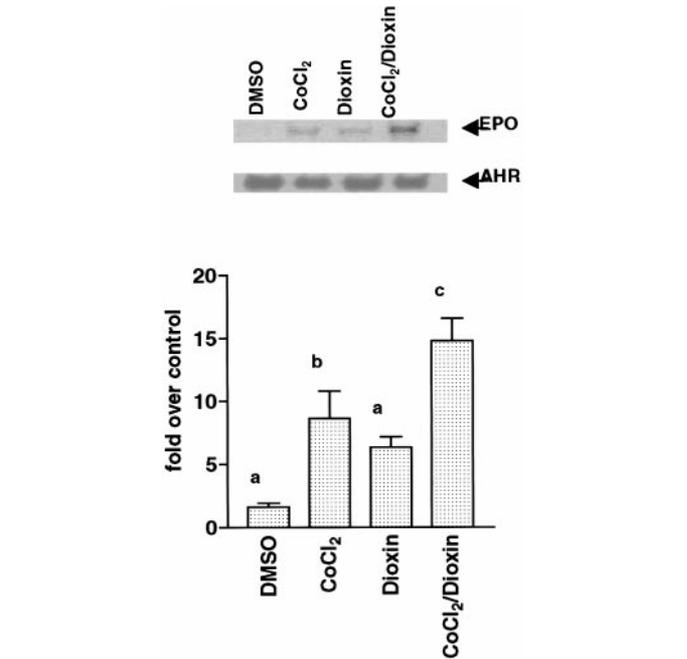


FIG. 5. Quantitation of EPO mRNA levels by RPA following various treatments. Above, an example of an RPA result from Hep3B cells exposed to dimethyl sulfoxide (DMSO), 75 μ M CoCl₂, 10 nM dioxin, and 75 μ M CoCl₂ plus 10 nM dioxin after 18 h. EPO mRNA level was normalized to AHR mRNA as the internal standard. The sizes of the protected EPO and AHR fragments were 312 and 225 bp, respectively. Below, data from triplicate samples were quantitated on a Fuji PhosphorImager and the mRNA levels were presented as fold induction compared with the dimethyl sulfoxide control. Statistical analysis is described in text. For each time point, those determinations not sharing a superscript (a, b, or c) are significantly different ($p < 0.05$).

regulated the *Epo* mRNA expression greater than 3-fold. Co-exposure to both CoCl₂ and dioxin led to an additive effect, with an 8-fold *Epo* mRNA induction over basal levels.

The *Epo* Promoter Contains a Functional Dioxin Responsive Enhancer—We performed a number of experiments to test the idea that additive induction by dioxin and CoCl₂ at the endogenous *Epo* locus was due to the presence of dioxin responsive elements within the *Epo* gene. First, we examined the available DNA sequence of the human *Epo* gene (GenBank accession number M11319) for sequences that conformed to the core sequence of the DRE, i.e. 5'-CACGC-3' (25). We found five such sequences within 600 nucleotides upstream of the *Epo* trans-

containing 40 μ g of proteins were used to detect the protein levels of HIF-1 α , AHR, and ARNT, whereas microsomes containing 20 μ g of proteins were used to detect CYP1A1 protein levels. Antibodies are described under “Experimental Procedures.” The migration of each band corresponds to molecular masses of 120, 86, 104, and 58 kDa, respectively, for HIF-1 α , ARNT, AHR, and CYP1A1. B, quantitation of the CYP1A1 mRNA levels by RPA upon various treatments. Above, an example of the RPA result from Hep3B cells exposed to dimethyl sulfoxide (DMSO), 75 μ M CoCl₂, 10 nM dioxin, or 75 μ M CoCl₂ plus 10 nM dioxin for 4 h. An AHR probe was used as the internal standard. The size of the protected fragments of CYP1A1 and AHR were 340 and 539 bp, respectively. Below, data from five replicates were quantitated on a Fuji PhosphorImager and the mRNA levels were presented as fold induction compared with the dimethyl sulfoxide control. C, characterization of cross-talk using a stable cell line (HepG2-101L) expressing a DRE driven luciferase reporter. Above, schematic of the reporter. CYP1A1-DREs is the 1612 bp of the 5'-flanking region derived from the human *Cyp1a1* locus that harbors three bona fide DREs. SV40 is the minimal promoter regions of the SV40 early promoter. LUC defines the luciferase reporter open reading frame. Below, cells were exposed to dimethyl sulfoxide, 75 μ M CoCl₂, 10 nM dioxin, or 75 μ M CoCl₂ plus 10 nM dioxin for 23 h. The luciferase activity was then assayed as described under “Experimental Procedures.” The data are normalized to total protein in the cellular extracts.

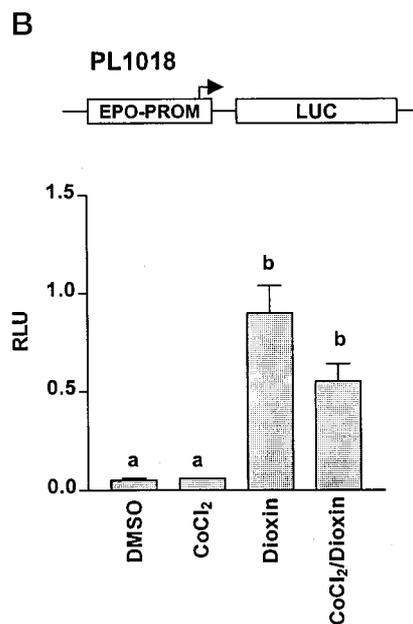
FIG. 6. Analysis of the consensus DREs within the *Epo* gene promoter.

A, sequence of the *Epo* gene promoter region. The sequence was obtained from GenBank data base (accession number M11319). The five consensus DREs are underlined and in **bold**. The nucleotide "A" in the translational start site (ATG) is defined as position number +1. The *arrows* indicate the boundaries of the element used in the construction of PL1018. B, above, schematic of the *Epo* promoter reporter plasmid PL1018. EPO-PROM represents the 327-bp *Epo* promoter region that harbors five putative DREs. The *arrow* represents the predicted transcriptional start site. LUC is the luciferase open reading frame. *Below*, the response of the *Epo* promoter to CoCl_2 and/or dioxin. Results from transient transfection assays with PL1018 under various chemical treatments for 18 h. RLU, relative light units. All transfections were normalized to a β -galactosidase internal control (see "Experimental Procedures"). The four treatments are dimethyl sulfoxide control, CoCl_2 (100 μM), dioxin (10 nM), or dioxin (10 nM) plus CoCl_2 (100 μM). Each time point represents the mean of triplicates and the *error bars* represent the standard error of the mean. Statistical analysis is described in the text. For each time point, those determinations not sharing a superscript (*a*, *b*, or *c*) are significantly different ($p < 0.05$).

A

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AAGCTTCTGGGCTTCCAGACCCAGCTACTTTGCGGAAGCTCAGCAACCCAGGCATCTCTGA -565
      ↓
GTCTCCGCCCAAGACCGGGATGCCCCAGGGGAGGTGTCGGGAGCCCAGCCTTTCCCA -505
GATAGCACGCTCCGCCAGTCCCAAGGGTGCGCAACCGGCTGCACTCCCCTCCCGCACCC -445
AGGGCCCGGGAGCAGCCCCATGACCCACACGCACGTCTGCAGCAGCCCCGCTCACGCCC -385
CGGCGAGCCTCAACCCAGGCGTCTGCCCTGCTCTGACCCGGGTGCCCTACCCCTG -325
GCGACCCCTCACGCACCAGCCTCTCCCCACCCACCCGCGCACGCACACATGCAGAT -265
      ↓
AACAGCCCCGACCCCGCCAGAGCCGAGAGTCCCTGGGCCACCCGGCCGCTCGTGCT -205
GCTGCGCCGACCCGCGTCTCTCCCGAGCCGACCGGGGCCACCCGCGCCGCTCTGCT -145
CCGACACCGCGCCCTGGACAGCCGCTCTCTCTAGGCCCGTGGGGCTGGCCCTGCA -85
CCGCCGAGCTTCCCGGGATGAGGGCCCGGTGTGGTACCCGGCGCCCCAGGTCGCT -25
      +1
GAGGGACCCCGCCAGGCGCGGAGATGGGGGTGCACGGTG
  
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lational start site (Fig. 6A). All five sequences within the upstream promoter region were in the same orientation and had a consensus of **CACGCNC**. To determine if this region could act as a functional dioxin responsive enhancer, we assayed luciferase reporter constructs that were regulated by the 330-bp promoter derived from the *Epo* gene, which harbored the putative DREs. We observed that this reporter was unaffected by exposure to CoCl_2 , but was up-regulated by exposure to dioxin and that the dioxin effects were inhibited by co-exposure to CoCl_2 (Fig. 6B).

In an effort to demonstrate that we could recapitulate the additive cross-talk observed using the endogenous *Epo* locus, we repeated these experiments using the pbEP-luciferase reporter which was regulated by both the *Epo* HRE and the *Epo* promoter (Fig. 7). We observed that this construct displayed pharmacology identical to the endogenous *Epo* reporter and that additive cross-talk could be observed with two distinct AHR agonists, βNF and dioxin (compare Fig. 5 to Fig. 7, A and B). As an additional proof that region -499 to -275 of the *Epo* promoter is a functional dioxin responsive enhancer, we compared the structure-activity relationship of this response to that observed using the CYP1A1 dioxin responsive enhancer.

In this experiment, we examined the dose-response curves for the two AHR agonists βNF and dioxin. We observed that the relative potencies and efficacies of these two agonists were essentially identical when using a reporter plasmid driven by the CYP1A1 dioxin responsive enhancers (PL1A1N) or driven by an *Epo* promoter harboring the putative dioxin responsive enhancers (PL1018) (Fig. 8).

DISCUSSION

Ah receptor agonists can initiate pathways that mediate metabolic adaptation to environmental pollutants in addition to a number of toxic responses (1-3). In the well characterized "adaptive pathway," the ligand-induced AHR-ARNT heterodimers bind to DREs resulting in the transcriptional activation of genes encoding a variety of XMEs. Unfortunately, an understanding of the molecular details that underlie dioxin's "toxic pathway" have yet to be developed. We postulate that the high binding affinity of dioxins may translate into a high fractional activation of the receptor, and a corresponding depletion of limiting factors within a cell that are required not only for AHR signaling, but also for parallel signal transduction pathways. The discovery that ARNT was also a heterodimeric part-

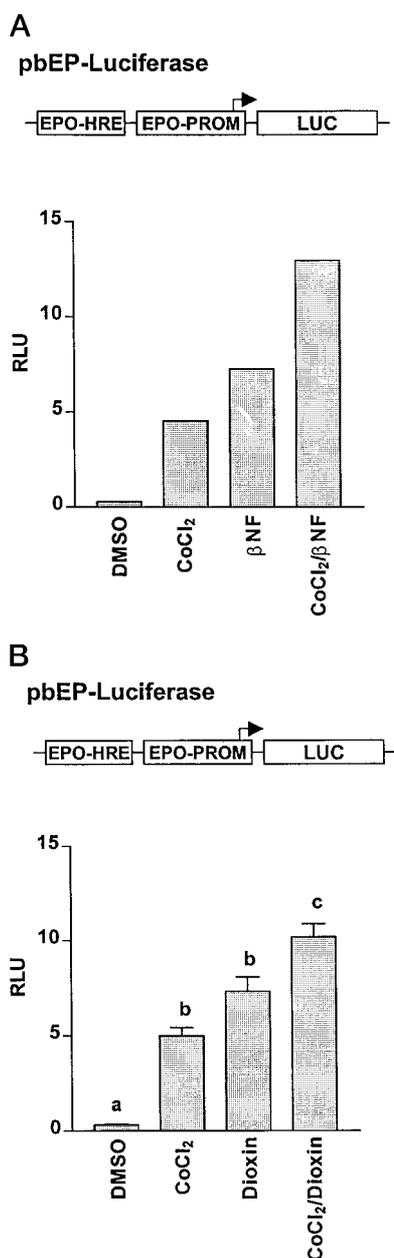


FIG. 7. A synthetic reporter construct mimics the cross-talk and pharmacology of the endogenous *Epo* gene. *A*, response of the *Epo* enhancer and promoter to CoCl₂ and/or βNF. Above, schematic of the luciferase reporter. EPO-PROM represents the 327-bp *Epo* promoter region that harbors five putative DREs. EPO-HRE is a 193-bp region derived from the *Epo* gene enhancer that harbors a single HRE. LUC is the luciferase open reading frame. Below, results from transient transfection assays with pbEP-luciferase under various chemical treatments for 18 h. RLU, relative light units. All transfections were normalized to a β-galactosidase internal control (see “Experimental Procedures”). The four treatments are dimethyl sulfoxide (DMSO) control, CoCl₂ (100 μM), βNF (1 μM), and βNF (1 μM) plus CoCl₂ (100 μM). Each time point represents the average of duplicates. *B*, response of the *Epo* enhancer and promoter to CoCl₂ and/or dioxin. Above, schematic of the luciferase reporter, same as in *A*. Below, same as in *A* except that the four treatments are dimethyl sulfoxide control, CoCl₂ (100 μM), dioxin (10 nM), and dioxin (10 nM) plus CoCl₂ (100 μM). Each time point represents the mean of triplicates and the error bars represent the standard error of the mean. Statistical analysis is described in text. For each time point, those determinations not sharing a superscript (*a*, *b*, or *c*) are significantly different ($p < 0.05$).

ner of HIF1α and that this complex bound to HREs to activate transcriptional responses to hypoxia, CoCl₂, and certain iron chelators provided a system to test this idea. In its simplest

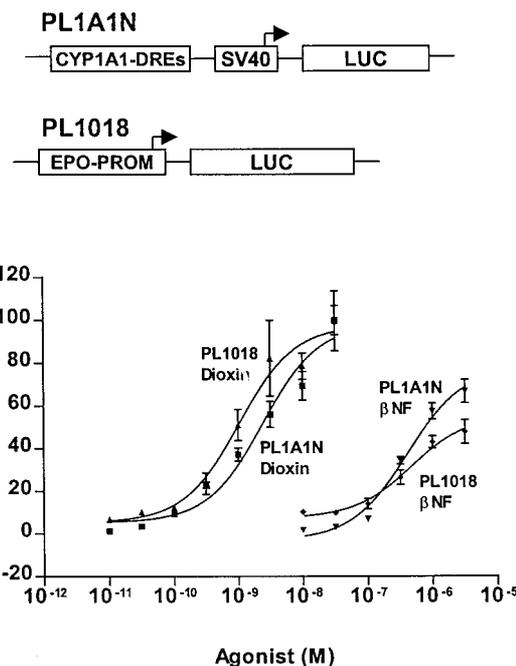


FIG. 8. Pharmacological comparison of a bona fide DRE reporter (PL1A1N) and a putative one (PL1018). Above, schematics, see figure legends 2 and 6. Below, dose-response curves for dioxin or βNF using PL1A1N or PL1018 as reporters. The ordinate is “% Maximal Response” with responses for each reporter normalized to the greatest response achieved. RLU, relative light units. All transfections were normalized to a β-galactosidase internal control (see “Experimental Procedures”). Each point represents the mean of triplicates and the error bars represent the standard error of the mean.

form, this model predicts that a reciprocal relationship would exist between the dioxin and hypoxia signaling pathways. For example, if ARNT was the putative limiting factor, agonists like dioxin would up-regulate DRE driven genes through the formation of AHR-ARNT heterodimers, while at the same time inhibiting the hypoxia response due to the decreased availability of ARNT for HIF1α dimerization. In a similar manner, this model also predicts that activation of HIF1α would up-regulate the levels of hypoxia responsive genes, while at the same time decreasing the availability of ARNT and inhibiting the dioxin response.

In an effort to test this model, we first examined a number of simple systems to see if we could find evidence that the AHR and HIF1α were in competition for limiting cellular factors *in vitro*. In gel shift experiments we observed that the AHR inhibited HIF1α-ARNT interactions with an HRE and that HIF1α inhibited AHR-ARNT interactions with a DRE (Fig. 1). These experiments were performed in a preliminary phase of our investigation and led us to perform additional tests of the cross-talk model. The observation that the AHR was capable of inhibiting HIF1α-ARNT interactions in the absence of agonist is consistent with a high level of constitutive activity of the human AHR *in vitro*.² The human AHR was chosen for these studies so that only human proteins would be used. The high level of constitutive human AHR would also explain why the addition of βNF to these reactions only modestly increased the inhibition of the HIF1α-ARNT complex. Given that the *in vitro* constitutive activity of the human AHR is a peripheral issue to this report, it has not been pursued further by using a more tightly controlled AHR form (e.g. murine b-1 allele) or by optimizing incubation conditions. We can only note that the recip-

² W. K. Chan, G. Yao, Y-Z. Gu, and C. A. Bradfield, unpublished observations.

rocal nature of the inhibition is reproducible, and that these observations led us to test this model *in vivo*, where compelling evidence was also obtained.

Transient transfection assays using a luciferase reporter that was linked to either the HRE derived from the *Epo* gene or the multiple DREs derived from the *Cyp1A1* gene also supported this model. That is, dioxin inhibited the CoCl_2 induction of an HRE driven promoter and CoCl_2 inhibited the dioxin-induction of a DRE driven promoter (Figs. 2 and 3). Finally, experiments where we monitored the impact of CoCl_2 on dioxin's induction of the endogenous *CYP1A1* gene provided additional evidence that hypoxia signaling inhibited the dioxin pathway (Fig. 4). These experiments also indicated that this effect was occurring at the level of *Cyp1A1* transcription and via DRE enhancer elements.

Given the supportive data generated in early experiments, we were surprised when we observed that dioxin exposure did not inhibit the induction of EPO mRNA by CoCl_2 in Hep3B cells (Fig. 5). Fortunately a clue was found in the observations that dioxin alone up-regulated EPO mRNA and that co-administration of CoCl_2 and dioxin displayed an additive effect in this system. This led us to investigate the possibility that the *Epo* gene may be a dioxin inducible gene and harbor DREs within its regulatory regions. The identification of five sequences within the proximal *Epo* promoter that closely matched the functional consensus sequence found in bona fide DREs, supported this idea (Fig. 6A) (26). To support the functional identity of this region as a composite dioxin response enhancer, we performed a pharmacological comparison with dioxin and β NF. We found that the *Epo* promoter and the *CYP1A1* enhancer region displayed the same relative transcriptional responses to β NF and dioxin (Fig. 8). Two important conclusions arise from these observations. First, that *Epo* is a dioxin responsive gene. Second, that the DREs within the promoter region of the *Epo* gene can compensate for the inhibitory effects that dioxin has on HRE mediated transcription at this locus. The net result of these multiple elements is a dampening of the inhibitory/reciprocal cross-talk between these two pathways. At the present time, it is not known whether this phenomenon extends to other HRE driven genes like *Vegf* (27), *Pgk* (28), or if this scenario is specific to *Epo* expression.

Our experiments also support the idea that the hypoxia and dioxin response pathways can compete for limiting cellular factor(s) and that reciprocal cross-talk is probably occurring at certain loci within mammalian cells. Although we have used ARNT as an example of this limiting factor in model development, it is important to point out that we have not proven this to be the case. Although our attempts have been limited, our preliminary experiments to reduce this cross-talk by transfection of ARNT, yielded ambiguous results. Although we observed some degree of reduction, we also observed that the basal activities of each promoter increased, thus clouding any potential conclusions. An argument for the importance of limiting factors other than ARNT can be found in the recent observation that demonstrates that dioxin can also inhibit progesterone receptor signaling (29). This may be an indication that the limiting factor(s) may also be shared with nuclear receptor signal transduction pathways. Although the candidates are many, it is tempting to speculate that competition for shared coactivators like SRC-1 may be important (30). Finally, this "limiting cellular factor" may not be a single protein, but rather may be a composite of limiting levels of multiple factors.

Interestingly, data from other investigators has demonstrated that dioxin signaling can be inhibited by activation of

HIF1 α even though inhibition of hypoxia signaling was not observed by dioxin (31, 32). Data from these earlier papers suggested that this unidirectional cross-talk might be related to the fact that HIF1 α has a greater affinity for ARNT than the AHR. At first inspection, our gel shift experiments are in agreement with the idea that HIF1 α has a greater affinity for ARNT than does the AHR. Nevertheless, it may be premature to compare the relative affinities of these two proteins since we do not know the fraction of the AHR that is activated in gel shift assays (or in any *in vitro* assays), nor do we know how long this species remains active. Thus, the increased binding of HIF1 α for ARNT may be simply due to the fact that more HIF1 α per unit time is in a receptive form to bind to ARNT, as compared with AHR in *in vitro* assays. Moreover, these earlier studies did not reveal that dioxin up-regulated EPO mRNA. This may be due to the use of a weaker agonist than dioxin, or to the fact that readily metabolized agonists do not exhibit their activity for the prolonged time periods as compared with dioxin (33, 34). Thus the differences in EPO response may simply be due to the different time points examined in these studies.

In summary, we have shown that reciprocal cross-talk between the AHR and HIF-1 α signaling pathways can occur *in vitro* and *in vivo* and that this cross-talk occurs at their cognate response elements. These experiments also support the idea that a limiting cellular factor is shared by these two pathways, the obvious candidate being ARNT. Our data also point to the complexity of cross-talk. Although the activation of the hypoxia pathway inhibited the up-regulation of the *Cyp1a1* gene, the effect of dioxin on the up-regulation of the *Epo* gene is more complex. Most importantly, we examined the *Epo* gene and found that its promoter functions as a dioxin responsive enhancer and thus, *Epo* transcription can be up-regulated by both hypoxia and dioxin. Studies are now in progress to determine the importance of these novel responses and whether they are essential steps in the toxic pathway of dioxins.

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