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

DEMONSTRATION OF COMPETITION AND COMPENSATION

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The arylation hydrocarbon receptor (AHR) and the α-class hypoxia inducible factors (HIF1α, HIF2α, and HIF3α) 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 HIF1α: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 AHR regulates a variety of biological responses to environmenally 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 excretible 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 HIF1α: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; βNF, β-naphthoflavone; bHLH, basic helix-loop-helix; PAS, Per, ARNT, AHIM homology domain; ARNT, Ah receptor nuclear translocator; HIF1α, hypoxia inducible factor 1α; 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.
Experiments explain 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. For example, the DRE core sequence is 5'-GGGGCCAGC; OL73: 5'-GGAGATCTGGTACCGGTGGCCCAGGGACTCTGCG; OL1205: 5'-GGGCCCATTGCGTGATCTAC; OL524; 5'-GGGCCACTCGAGATCCTGGTACCGGTGGCCCAGGGACTCTGCG; OL73: 5'-GGGGCCAGC; OL74: 5'-GGAGATCTGGTACCGGTGGCCCAGGGACTCTGCG; OL1205: 5'-GGGCCCATTGCGTGATCTAC; OL524; 5'-GGGCCACTCGAGATCCTGGTACCGGTGGCCCAGGGACTCTGCG; OL1205: 5'-GGGCCCATTGCGTGATCTAC; OL524; 5'-GGGCCACTCGAGATCCTGGTACCGGTGGCCCAGGGACTCTGCG. 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 (Promega, Madison, WI). The plasmid PL449 derived from the SV40 early promoter was obtained from Pharmacia Biotech Inc. (Fair Lawn, NJ). The plasmid phuAHR1267 harbors the first 1267 nucleotides of the human AHR. This cDNA was amplified from phuAHR1267 that had been linearized with BglII. A 265-nucleotide CYP1A1 riboprobe was generated with T7 RNA polymerase using phuAHR1267 that had been linearized with EcoRI. Analysis was performed on lysates from cells grown in 75-cm² flasks at 37 °C at 5% CO₂. 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 × g for 5 min at 4 °C. The supernatant was resuspended in 20 mM HEPES-KOH, pH 7.8, containing 250 mM sucrose. Nuclear extracts were prepared by resuspending the nuclei-enriched fraction (1,000 × g supernatant from above) in 50 μl of "nuclear extract buffer" (20 mM HEPES-KOH, pH 7.8, containing 0.42 mM KCl, 1.5 mM MgCl₂, 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 × g for 30 min at 4 °C. The supernatant was used directly and defined as nuclear extract.

**Western Blot Analysis**—Anti-AHR monoclonal antibody (V99) 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-1α polyclonal antibodies, R5372B, were raised in rabbits against a protein fragment corresponding to amino acids 328–527 of the human HIF1α isoform. 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 phosphate-coupled secondary antibodies and developed by a colorimetric assay, as described previously (19). The HIF-1α 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 CoCl₂ 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 550-nucleotide riboprobe was generated using T7 RNA polymerase using phuAHR1267 that had been linearized with BglII. A 390-nucleotide CYP1A1 riboprobe was generated with T7 polymerase using phuAHR1267 that had been linearized with EcoRI. Analysis was performed on lysates from cells grown in 75-cm² flasks at 37 °C at 5% CO₂. 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 × g for 5 min at 4 °C. The pellet was resuspended into 1 ml of "Direct Protect Lysate Solution" and stored at −80 °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 × 10⁶ cpm/mg RNA and a 60–90% incorporation efficiency. The riboprobes (1 × 10⁶ 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 °C) and proteinase K (20 °C) were then added, followed by another 37 °C incubation for 30 min. Samples were precipitated by the addition of 0.5 ml of isopropanol alcohol, 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 × 10⁶ cpm/mg RNA and a 60–90% incorporation efficiency. The riboprobes (1 × 10⁶ 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 °C) and proteinase K (20 μg/ml, 10 °C) were then added, followed by another 37 °C incubation for 30 min. Samples were precipitated by the addition of 0.5 ml of isopropanol alcohol, stored at −20 °C for 30 min, and subjected to centrifugation at 16,000 × g for 30 min at 4 °C. The pellets were dried and resuspended into 10 μl of buffer, before being loaded onto an 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.
A transfection solution of 1.0 ml of serum-free minimal essential medium containing plasmid (0.5 μg of PL1A1N, 1.0 μg of PL1018 or 1.0 μg of PL1018 or 1.0 μg of pβEP-luciferase) and 5 μl of LipofectAMINE reagent was added to each well. Each plasmid transfection was accompanied by 0.2 μg of the LacZ reporter pCH110, which acted as an efficiency control. Transfection was allowed to occur by incubation at 37 °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 cell lysis buffer (Promega, Madison, WI). Following 5 min at room temperature, the lysed cells were collected and cleared by centrifugation at 12,000 × g for 1 min. The supernatants were assayed for luciferase activity (20 μl/ reaction) and β-galactosidase activity (6–10 μl/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 βNF. Using this system, we observed that the addition of increasing amounts of HIF-1α protein inhibited the amount of AHR and ARNT complexes measured. All the counts are normalized with the control (HIF1α + ARNT in the absence of βNF). The data shows the mean from three separate experiments and the error bars show the standard error of the mean.

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respectively), but was unaffected by exposure to CoCl2 (Fig. 2). After 5, 10, and 23 h of dioxin exposure (14-, 16-, and 27-fold, respectively), luciferase activity was markedly up-regulated by exposure to CoCl2 and CoCl2 plus dioxin (10 nM) (Fig. 3). In keeping with the expected pharmacology of this reporter we observed induction of luciferase expression (Fig. 3). In keeping with the expected pharmacology of this reporter we observed induction of luciferase expression (Fig. 3).

**Cross-talk between AHR and HIF Pathways**

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 μM), dioxin (10 nM), or 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).

The 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 HIF1α, ARNT, AHR, and CYP1A1 protein that was expressed under the various treatment conditions (Fig. 4A). As expected, the amount of HIF1α 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 observed 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.

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 ~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 μM 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-
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., $5'\text{-CACGC-3'}$ (25). We found five such sequences within 600 nucleotides upstream of the Epo trans-

containing 40 μg of proteins were used to detect the protein levels of HIF-1α, AHR, and ARNT, whereas microsomes containing 20 μg 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-1α, 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 μM CoCl₂, 10 nM dioxin, and 75 μM 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 replicate 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 ($p < 0.05$).

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FIG. 4. A, Western blot analysis of HIF-1α, 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 μM CoCl₂, 10 nM dioxin, or 75 μM 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 μM CoCl₂, 10 nM dioxin, and 75 μM 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 replicate 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 ($p < 0.05$).
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 CoCl₂ 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, CoCl₂ (100 μM), dioxin (10 nM), or 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 the text. For each time point, those determinations not sharing a superscript (a, b, or c) are significantly different (p < 0.05).

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 enhancer (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-
The 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. 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. 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 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.

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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 CoCl₂ induction of an HRE driven promoter and CoCl₂ inhibited the dioxin-induction of a DRE driven promoter (Figs. 2 and 3). Finally, experiments where we monitored the impact of CoCl₂ 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 CoCl₂ 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 CoCl₂ 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 βNF. We found that the Epo promoter and the CYP1A1 enhancer region displayed the same relative transcriptional responses to βNF 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 crosstalk between these two pathways. At the present time, it is not known whether this phenomenon extends to other HRE driven genes like Vegf (27), Pfk, (28), or if this specificity 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 HIF1α 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 HIF1α has a greater affinity for ARNT than the AHR. At first inspection, our gel shift experiments are in agreement with the idea that HIF1α 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 HIF1α for ARNT may be simply due to the fact that more HIF1α 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-1α 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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Cross-talk between AHR and HIF Pathways

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