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Crosstalk Between the Aryl Hydrocarbon Receptor and Hypoxia on the Constitutive Expression of Cytochrome P4501A1 mRNA

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Abstract

Endothelial cells are a target of halogenated aromatic hydrocarbon toxicity following aryl hydrocarbon receptor (AHR) activation. Further, evidence suggests that AHR has a physiological function in endothelial cells in the absence of exogenous ligands. Understanding these “normal” functions of AHR may help to reveal the mechanisms that contribute to the toxicity of xenobiotic ligands. Thus, this study focused on the crosstalk between hypoxia and AHR in the absence of exogenous ligands. Constitutive CYP1A1 mRNA was measured by real time PCR in human pulmonary microvascular endothelial cells exposed to hypoxia (1 or 2.5% O₂), 25 nM AHR siRNA, 25 nM hypoxia-inducible factor (HIF)-2 α siRNA, or their combinations. Hypoxia significantly induced known hypoxia-regulated genes, and this induction was highly attenuated by HIF-2 α siRNA, suggesting that HIF-2 α is a primary mediator of hypoxic responses in these cells. Hypoxia also significantly reduced CYP1A1 mRNA and this reduction was also attenuated by HIF-2 α siRNA. As expected, AHR siRNA significantly reduced constitutive CYP1A1 mRNA. While the combination of hypoxia plus AHR siRNA reduced CYP1A1 mRNA more than either treatment alone, the reduction was less than additive, suggesting that hypoxia and AHR deficiency may share a common pathway in reducing CYP1A1 expression. Finally, hypoxia significantly reduced AHR mRNA and this reduction was completely prevented by HIF-2 α siRNA. In conclusion, constitutive CYP1A1 mRNA expression is dependent on AHR and is reduced by hypoxia via a HIF-2 α -dependent mechanism, which may be mediated by a HIF-2 α -dependent reduction of AHR expression.

Keywords

Aryl hydrocarbon receptor; Hypoxia-inducible factor 2 α ; Cytochrome P4501A1; Endothelial cells

The aryl hydrocarbon receptor (AHR) is a member of the basic helix-loop-helix/PER-ARNT-SIM family of DNA-binding proteins. The AHR resides in the cytosol in a multimeric complex with two molecules of HSP90 and a single molecule of X-associated protein 2 and p23 [1]. Following ligand binding, the AHR translocates into the nucleus, dimerizes with the AHR nuclear translocator (ARNT), and transactivates gene expression by binding dioxin response elements in promoters of target genes, including cytochromes P450 (CYP) 1A1 and CYP1B1.

It is well established that the AHR mediates the induction of metabolic enzymes and toxicity following activation by xenobiotic ligands, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). In addition, a number of lines of evidence suggest that an endogenous ligand(s) exists that activates AHR under physiological conditions. For example, stimulation of AHR activity has been reported in a variety of cell culture systems in the absence of exogenous agonists, including following shear stress of endothelial cells [2,3] and suspension of keratinocytes [4, 5]. Further, *Ahr* null mice exhibit a variety of abnormal phenotypes, including defective hepatic

vascular development, cardiac hypertrophy, and altered blood pressure regulation [6–10]. Thus, understanding the normal physiological function of the AHR may help to reveal the pathways and mechanisms that lead to toxicity following exposure to xenobiotic ligands.

The hypoxia-inducible factors (HIFs) are also members of the basic helix-loop-helix/PER-ARNT-SIM family of DNA-binding proteins. In contrast to AHR, HIFs do not require ligand activation, but rather their signaling is regulated by cellular oxygen. Under normoxia, α subunits of HIFs are hydroxylated by oxygen-dependent prolyl hydroxylases, targeting them for ubiquitination and proteasomal degradation [11]. However, under hypoxia, the α subunits are stabilized and translocate into the nucleus where they dimerize with a β subunit (also known as ARNT) and transactivate gene expression by binding hypoxia response elements (HRE) in targets, such as vascular endothelial growth factor-A (VEGF-A) and endothelin-1 (ET-1).

The AHR and hypoxia signaling pathways share a number of commonalities. Both pathways mediate the responses of organisms to their environment. Both pathways also share a common dimerization partner (ARNT) and molecular chaperones and other transactivating proteins [12]. Thus, it is not surprising that numerous studies have suggested that these pathways may exhibit crosstalk in their regulation of gene expression [13–16]. However, essentially all previous studies have focused on the interaction of hypoxia and AHR signaling when the AHR is activated by exogenous ligands, despite the fact that AHR regulates constitutive CYP1A1 expression in the absence of exogenous ligands [17,18] and hypoxia alone can induce or repress CYP1A1 expression depending on the model studied [19,20].

Thus, we focused this study on the crosstalk between hypoxia and AHR in the absence of exogenous ligands on the regulation of constitutive CYP1A1 mRNA expression. We chose primary human pulmonary microvascular endothelial cells, since the pulmonary microvasculature represents a primary site for responses to inspired oxygen, the lung exhibits relatively high AHR-dependent constitutive CYP1A1 mRNA expression [18], and one potential endogenous AHR ligand has been isolated from the lung [21]. We exposed the cells to hypoxia by altering the percentage of oxygen, using both severe hypoxia (1% O₂) and a more physiologically relevant level of hypoxia for these cells (2.5% O₂). We modulated both the hypoxia and AHR signaling pathways using siRNA, and then assessed the endogenous mRNA expression of CYP1A1. Using these experimental approaches, we tested the hypothesis that hypoxia and AHR, in the absence of exogenous ligands, would exhibit crosstalk in the regulation of constitutive CYP1A1 mRNA expression.

Materials and Methods

Reagents

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), a gift from Dr. Richard E. Peterson (University of Wisconsin-Madison), was dissolved in dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO) to produce a 20 μ M stock solution. Lipofectamine 2000 and Optim-MEM I reduced serum media were purchased from Invitrogen (Carlsbad, CA).

Cell Culture, TCDD and Hypoxia Exposure, and siRNA Transfection

Human microvascular endothelial cells isolated from the lung (HMVEC-L) were purchased from Lonza (Walkersville, MD). Cells were cultured in microvascular endothelial cell media-2 (EGM-2 MV, Lonza), which contained 5% fetal bovine serum, 0.1% antibiotics (GA-1000) and other supplements, at 37°C in 5% CO₂ according to manufacturer's directions. Cells at passage 4 or 5 were used in all experiments. For hypoxia exposure, HMVEC-L were plated at ~80% confluence for 24 h, and then exposed to 2.5% or 1% oxygen, 5% CO₂ for 18 h in a NAPCO 7001H incubator (Fisher Scientific, Pittsburgh, PA), using N₂ infusion. HMVEC-L

cultured at 21% oxygen, 5% CO₂ in NAPCO 6001 incubator (Fisher Scientific) served as the normoxic control. For siRNA transfection, HMVEC-L were plated at ~80% confluence for 24 h in EGM-2MV without antibiotics. On day 2, Lipofectamine 2000 was incubated with Opti-MEM I reduced-serum media, mixed with control, AHR, or HIF-2 α siRNA (Dharmacon, Chicago, IL) at a final concentration of 25 nM, added to the cells, and cells incubated at 21% oxygen, 5% CO₂ for 48 h. For TCDD exposure, HMVEC-L were plated at ~80% confluence for 24 h, and then exposed to 0.01% DMSO or 2 nM TCDD at 21% oxygen for 18 h. When the cells were exposed to both siRNA, and either hypoxia or TCDD, hypoxia and TCDD exposures were performed during the last 18 h of the 48-h siRNA treatment period.

RNA Isolation and PCR Analysis

RNA was isolated from HMVEC-L using RNeasy mini kit (QIAGEN, Valencia, CA). Total RNA (250 ng) was reverse transcribed using iScript Select cDNA Synthesis Kit (BioRad, Hercules, CA) with random primers at 42°C for 30 min. PCR primers (Sigma-Genosys, Woodlands, TX; Table 1) were used at 500 nM, except for aldehyde dehydrogenase 1A3, which was used at 250 nM, and all exhibited amplification efficiencies > 90%. Quantitative real time PCR analysis with SYBR green was performed using a BioRad Icyler optical system: initial denaturation at 95°C for 1 min followed by 40 cycles of 30 s at 95°C and 30 s at 60°C. After cycling, a melt curve was generated to confirm the synthesis of a single PCR product. 18s rRNA did not vary with any treatment and was used as an internal normalization control.

Statistics

The data were expressed as mean $\pm$ SEM. Statistical analysis was performed with Student's *t*-test, Welch unpaired *t*-test, one-way analysis of variance (ANOVA), or two-way ANOVA as noted. Statistical analysis on log₁₀-transformed values was performed when either the normality test, or equal variance test, or both failed when analyzing untransformed values. A *p* < 0.05 was considered significant in all cases.

Results

Human Pulmonary Endothelial Cells were Highly Responsive to Hypoxia-induced Gene Expression

To confirm the degree, to which HMVEC-L were responsive to hypoxia, we first assessed the mRNA expression of four known hypoxia-inducible genes, prepro-endothelin-1 (ET-1), prepro-adrenomedullin (ADM), vascular endothelial growth factor A (VEGF-A), and phosphoglycerate kinase (PGK1), following 18 h exposure to 2.5 or 1% oxygen. Hypoxia induced the mRNA expression of three of the four genes, including ET-1, ADM, and VEGF-A (Fig. 1). In these three cases, expression was significantly induced as the hypoxia became more severe. ET-1 and VEGF-A were the least and most responsive with 1% oxygen inducing a 2- and 12-fold increase in mRNA expression, respectively. Notably, however, PGK1 mRNA expression was not induced following 18 h of exposure to either 2.5 or 1% oxygen.

HIF-2 α siRNA Significantly Inhibited Hypoxia-mediated Gene Induction in Pulmonary Endothelial Cells

Previous studies have shown that HIF-2 α is more highly expressed in endothelial cells than HIF-1 α and that hypoxic-induction of glycolytic enzymes, such as PGK1, is mediated exclusively by HIF-1 α [22,23]. Thus, we investigated the degree, to which HIF-2 α mediated the hypoxic induction of PGK1 and ADM by transfecting HMVEC-L with HIF-2 α siRNA for 48 h and exposing the cells to 1% oxygen for the final 18 h. HIF-2 α mRNA expression was reduced to 1% of basal levels, compared to cells treated with control siRNA (Fig. 2). In addition, while 1% oxygen significantly induced ADM by 5.9-fold in the presence of control siRNA, it

increased ADM only 1.8-fold in the presence of HIF-2 α siRNA, suggesting that hypoxic induction of ADM is primarily mediated by HIF-2 α . Interestingly, in this experiment, 1% oxygen induced a small, but significant increase in PGK1 mRNA (1.4-fold) in the presence of control siRNA and HIF-2 α knockdown resulted in a further induction (2.1-fold), suggesting that HIF-1 α is present in these cells, albeit at low levels, but may have increased in activity to compensate for the loss of HIF-2 α .

Human Pulmonary Endothelial Cells were Highly Responsive to TCDD-induced Gene Expression

Previous studies have shown that endothelial cells express AHR and are highly responsive to AHR ligands, like TCDD [24]. We confirmed the responsiveness of the AHR signaling pathway by treating HMVEC-L for 18 h with DMSO or 2 nM TCDD. As expected, HMVEC-L were highly responsive to TCDD-mediated induction of known AHR-regulated genes. CYP1A1 and CYP1B1 mRNA were induced 10- and 35- fold, respectively (Fig. 3), while two other genes identified in the AHR gene battery, TCDD-inducible poly(ADP)ribose polymerase (TiPARP) [25] and aldehyde dehydrogenase 1A3 (ALDH1A3), were induced 2- to 3-fold by TCDD exposure.

AHR siRNA Effectively Suppressed AHR Signaling in Pulmonary Endothelial Cells

To evaluate the activity of AHR in constitutive CYP1A1 regulation in the absence of exogenous ligands, we knocked down AHR expression using siRNA, and first confirmed the effectiveness of the knockdown by analyzing AHR mRNA expression and TCDD-mediated induction of CYP1A1 and CYP1B1 mRNA. AHR mRNA expression was reduced to ~40% of basal levels after AHR siRNA, compared to untreated cells and those treated with control siRNA (Fig. 4a). In addition, while TCDD induced CYP1A1 and CYP1B1 by 17- and 26- fold, respectively; however, in the presence of AHR siRNA, it increased their expression by 2- and 1.4-fold, respectively, with only the induction of CYP1A1 mRNA remaining statistically increased (Fig. 4b).

Both AHR siRNA and Hypoxia Significantly Reduced Constitutive CYP1A1 mRNA Expression in Pulmonary Endothelial Cells

We next determined the degree, to which AHR was required for constitutive CYP1A1 mRNA expression and the manner, in which hypoxia altered this constitutive expression. HMVEC-L were transfected with AHR siRNA for 48 h or were exposed to graded levels of hypoxia for 18 h and CYP1A1 mRNA measured by real time PCR. AHR siRNA significantly reduced CYP1A1 mRNA expression by 50% (Fig. 5a), suggesting that the high level of constitutive CYP1A1 mRNA expression in pulmonary endothelium is mediated, in part, by AHR. In addition, hypoxia significantly reduced constitutive CYP1A1 mRNA expression in a graded manner with 1% oxygen reducing CYP1A1 by nearly 80% (Fig. 5b). Since it has been shown that hyperoxia induces CYP1A1 expression [26], we also cultured the HMVEC-L at 10% oxygen and did not observe any changes in CYP1A1 mRNA expression, compared to 21% O₂ (data not shown), suggesting that the hypoxia-mediated reduction was not a result of induced levels at 21% oxygen.

Simultaneous Exposure to AHR siRNA and Hypoxia Resulted in a Greater Suppression of CYP1A1 mRNA Expression than Either Treatment Alone

To determine if hypoxia could further suppress constitutive CYP1A1 mRNA expression in AHR deficient cells, HMVEC-L were transfected with AHR siRNA for 48 h and exposed to either 2.5 or 1% oxygen for the final 18 h. The combination of hypoxia and AHR siRNA reduced CYP1A1 mRNA to a greater degree than either treatment alone, although the combined level of reduction was less than additive at both levels of hypoxia (two-way ANOVA, AHR

siRNA-oxygen interaction, $p < 0.001$) (Fig. 6). These data suggest that the pathways reducing constitutive CYP1A1 mRNA expression via hypoxia or AHR knockdown may not be mutually exclusive.

HIF-2 α siRNA Partially Prevents Hypoxia-mediated Reduction in CYP1A1 mRNA Expression

Since the hypoxia-mediated induction of genes in HMVEC-L was primarily dependent on HIF-2 α , we sought to determine the degree, to which HIF-2 α mediated the reduction of CYP1A1 mRNA by hypoxia. HMVEC-L were transfected with control or HIF-2 α siRNA for 48 h and exposed to 1% for the final 18 h. Hypoxia significantly reduced CYP1A1 mRNA by 50% in the presence of control siRNA, but only by 30% in the presence of HIF-2 α siRNA (Fig. 7), suggesting that the hypoxia-mediated reduction in CYP1A1 mRNA is dependent, in part, on HIF-2 α signaling.

HIF-2 α siRNA Completely Prevents Hypoxia-mediated Reduction in AHR mRNA Expression

To determine if hypoxia, via HIF-2 α , reduces AHR mRNA expression, HMVEC-L were transfected with control or HIF-2 α siRNA, for 48 h and exposed to 1% oxygen for the final 18 h. Hypoxia significantly reduced AHR mRNA expression by 30% in the presence of control siRNA, but this reduction was completely abrogated in cells transfected with HIF-2 α siRNA (Fig. 8a). To determine if hypoxia could further reduce AHR mRNA expression in the presence of AHR siRNA, and whether this reduction was mediated by HIF-2 α , HMVEC-L were transfected with AHR siRNA or the combination of AHR and HIF-2 α siRNA for 48 h and exposed to 1% oxygen for the final 18 h. The combination of hypoxia and AHR siRNA reduced AHR mRNA to a greater degree than AHR siRNA alone, and this reduction was effectively prevented by HIF-2 α siRNA (Fig. 8b). Taken together, these data suggest that hypoxia regulates AHR mRNA expression via a HIF-2 α -dependent pathway.

Discussion

Considerable research has been conducted to elucidate the mechanism(s), by which xenobiotic ligands of the AHR, such as TCDD, mediate toxicity. While these studies have revealed a number of key pathways that are disrupted by sustained AHR activation, such as inhibition of cell cycle progression and promotion of differentiation, the specific changes leading to toxicity remain unclear. As noted by a number of investigators, understanding how xenobiotic ligands of the AHR cause toxicity may be more clearly illuminated by understanding the physiological role of the AHR in the absence of exogenous ligands [18,27]. Furthermore, since AHR and HIFs are members of the same transcription factor family, share common signaling proteins, and can exhibit crosstalk in the presence of AHR xenobiotic ligands, investigating the interaction of hypoxia and AHR signaling in the absence of these ligands represents one approach to study the physiological role of the AHR.

One obvious tissue to investigate the interaction of hypoxia and AHR in the absence of exogenous ligands is the lung. The lung exhibits some of highest levels of AHR-dependent constitutive CYP1A1 mRNA expression of any tissue [18], is the source from which a high affinity endogenous AHR ligand has been isolated [21], and is highly responsive to changes in inspired oxygen. Thus, we used human primary pulmonary microvasculature endothelial cells to probe the crosstalk between hypoxia and AHR in the regulation of constitutive CYP1A1 mRNA expression.

AHR is Required for the Constitutive Expression of CYP1A1 mRNA in Pulmonary Microvascular Endothelial Cells

We found that AHR deficiency reduces basal expression of CYP1A1 mRNA. While it has been established that the AHR is required for the induction of CYP1A1 by exogenous ligands, only

a few studies have investigated the requirement for AHR in constitutive CYP1A1 expression, and this is likely because basal CYP1A1 expression is very low in most tissues [18]. Nonetheless, our results, which demonstrate that the AHR is required for CYP1A1 mRNA constitutive expression in pulmonary microvascular endothelial cells, are consistent with reports that AHR is required for basal CYP1A1 expression in MCF-7 cells [28], a neuroblastoma cell line [29], and the heart, liver, lung, and kidney [18]. Further, this result supports the idea that an endogenous AHR ligand may be present in the lung. Organic extracts of the lung from *Ahr* null mice can stimulate a 4-fold increase in AHR transcriptional activity, suggesting that an AHR ligand accumulates in the lung in the absence of constitutive CYP1A1 expression [18].

Hypoxia Suppresses CYP1A1 mRNA Expression in Pulmonary Microvascular Endothelial Cells

We also found that hypoxia reduces basal expression of CYP1A1 mRNA. While it has been established that hypoxia inhibits AHR-dependent CYP1A1 induction by exogenous ligands, the effects of hypoxia alone on basal CYP1A1 expression have been reported in only a limited number of studies. Hypoxia reduces CYP1A1 mRNA expression in hepatocytes [30], but induces it in MCF-7 cells [20], and in the enterocytic Caco-2 cell line [31]. In vivo, exposure to acute moderate hypoxia reduces CYP1A1 expression. Rabbits exposed to 10% O₂ for 24 h exhibit reduced expression of hepatic CYP1A1 activity, protein, and mRNA [19,32]. Thus, our results demonstrating that hypoxia reduces constitutive expression of CYP1A1 mRNA in pulmonary endothelial cells are consistent with in vitro and in vivo studies showing hypoxia down regulates CYP1A1 expression in the liver, suggesting that a common mechanism may regulate CYP1A1 mRNA in the liver and lung.

It is noteworthy that hyperoxia induces CYP1A1 in pulmonary endothelial cells in vivo [26]. Further, studies have shown that this induction is required to protect the lung from hyperoxia-induced injury and the AHR is required for hyperoxia-mediated induction [33,34]. Treatment of wildtype mice with a CYP inhibitor or exposure of *Ahr* null mice to hyperoxia results in a significant exacerbation of the hyperoxic injury. Thus, these data, combined with our observations showing that hypoxia reduces CYP1A1 expression, suggest that AHR may play a role in regulating pulmonary responses to changes in oxygen by regulating CYP1A1 expression. It is not known if the *Ahr* or *Cyp1a1* null mice have a pulmonary or respiratory phenotype, but given that hyperoxia and hypoxia both regulate pulmonary CYP1A1 expression, future studies of respiratory responses to oxygen in these mice may help to reveal the physiological role of AHR and CYP1A1 in the lung.

Hypoxia Mediates Induction of Gene Expression and Reduction in CYP1A1 mRNA Expression, in part, via HIF-2 α

We found that hypoxia results in limited-to-no induction of the mRNA of the glycolytic enzyme, PGK1, and that HIF-2 α deficiency significantly attenuates hypoxia-induced expression of ADM. These results suggest that HIF-2 α is a primary mediator of hypoxia-induced gene expression in human pulmonary microvascular endothelial cells. These data are consistent with the fact that HIF-2 α is expressed more highly than HIF-1 α in endothelial cells, and that hypoxic-induction of glycolytic enzymes, such as PGK1, is mediated by HIF-1 α [22,23]. Further, the importance of HIF-2 α in pulmonary responses to hypoxia is demonstrated by HIF-2 α heterozygote mice, which are completely protected from pulmonary hypertension and right ventricular hypertrophy following exposure to chronic hypoxia [35]. In contrast, HIF-1 α heterozygote mice exhibit these pulmonary-related pathological responses to chronic hypoxia, although the rate of their development is delayed [36].

In addition to mediating hypoxia-induced gene expression, HIF-2 α also partially mediated the hypoxia-induced reduction of CYP1A1 mRNA expression. The specific mechanism that mediates this reduction is not known. Putative HREs are present in the CYP1A1 promoter, although it has not been determined whether they are functional [37]. Since HIF-2 α deficiency did not fully restore expression of CYP1A1 mRNA following hypoxia, it would suggest that other factors are involved and future studies of the role of HIF-1 α in this response are warranted. However, it has been shown that the mechanism, by which hypoxia inhibits AHR-stimulated increase in CYP1A1 expression in mouse fibroblasts does not require HIF-1 α [38], suggesting that other mediators are involved.

The Signaling Pathways Mediating Reduction of CYP1A1 mRNA Expression by Hypoxia and AHR Deficiency do not Appear to be Mutually Exclusive

We found that the combined exposure of pulmonary microvascular endothelial cells to hypoxia and AHR siR-NA reduces CYP1A1 mRNA expression more than either treatment alone, although it results in a less than additive reduction. These data demonstrate that crosstalk between hypoxia and AHR deficiency exists and suggests that these two signaling pathways may regulate CYP1A1 mRNA expression partially through a common mechanism. One possible explanation may be that hypoxia reduces CYP1A1 mRNA expression, in part, by reducing AHR expression. Our data are consistent with this idea showing that hypoxia reduces AHR mRNA expression both in AHR sufficient and deficient cells and this reduction is mediated by HIF-2 α . However, more experiments are needed to determine whether the hypoxia-dependent reduction in AHR mRNA is reflected by a decrease in AHR protein, and whether artificially sustaining AHR expression would prevent the hypoxia-mediated reduction in CYP1A1 mRNA.

In conclusion, constitutive CYP1A1 mRNA expression in pulmonary microvascular endothelial cells is dependent on AHR and is reduced by hypoxia via a HIF-2 α -dependent mechanism. Further, AHR and hypoxia exhibit crosstalk in their regulation of CYP1A1 mRNA expression in the absence of exogenous ligands, and part of this crosstalk may include regulation of AHR expression by hypoxia, although this needs further investigation. These results, taken together with other published studies, suggest that AHR may play a physiological role in the pulmonary responses to oxygen.

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References

1. Chen HS, Perdeu GH. Subunit composition of the heteromeric cytosolic aryl hydrocarbon receptor complex. *The Journal of Biological Chemistry* 1994;269:27554–27558. [PubMed: 7961671]
2. Eskin SG, Turner NA, McIntire LV. Endothelial cell cytochrome P450 1A1 and 1B1: Up-regulation by shear stress. *Endothelium* 2004;11:1–10. [PubMed: 15203874]
3. McMillan BJ, Bradfield CA. The aryl hydrocarbon receptor is activated by modified low-density lipoprotein. *Proceedings of the National Academy of Sciences of the United States of America* 2007;104:1412–1417. [PubMed: 17227852]
4. Sadek CM, Allen-Hoffmann BL. Suspension-mediated induction of Hepa 1c1c7 Cyp1a-1 expression is dependent on the Ah receptor signal transduction pathway. *The Journal of Biological Chemistry* 1994;269:31505–31509. [PubMed: 7989317]
5. Sadek CM, Allen-Hoffmann BL. Cytochrome P4501A1 is rapidly induced in normal human keratinocytes in the absence of xenobiotics. *The Journal of Biological Chemistry* 1994;269:16067–16074. [PubMed: 8206906]

6. Lahvis GP, Lindell SL, Thomas RS, McCuskey RS, Murphy C, Glover E, Bentz M, Southard J, Bradfield CA. Portosystemic shunting and persistent fetal vascular structures in aryl hydrocarbon receptor-deficient mice. *Proceedings of the National Academy of Sciences of the United States of America* 2000;97:10442–10447. [PubMed: 10973493]
7. Lund AK, Goens MB, Kanagy NL, Walker MK. Cardiac hypertrophy in aryl hydrocarbon receptor (AhR) null mice is correlated with elevated angiotensin II, endothelin-1 and mean arterial blood pressure. *Toxicology and Applied Pharmacology* 2003;193:177–187. [PubMed: 14644620]
8. Thackaberry EA, Gabaldon D, Walker MK, Smith SM. Aryl hydrocarbon receptor null mice exhibit cardiac hypertrophy and increased cardiac hypoxia-inducible factor 1- α in the absence of cardiac hypoxia. *Cardiovascular Toxicology* 2002;4:263–273. [PubMed: 12665660]
9. Vasquez A, Atallah-Yunes N, Smith FC, You X, Chase SE, Silverstone AE, Vikstrom KL. A role for the aryl hydrocarbon receptor in cardiac physiology and function as demonstrated by AhR knockout mice. *Cardiovascular Toxicology* 2003;3:153–163. [PubMed: 14501033]
10. Fernandez-Salguero PM, Ward JM, Sundberg JP, Gonzalez FJ. Lesions of aryl-hydrocarbon receptor-deficient mice. *Veterinary Pathology* 1997;34:605–614. [PubMed: 9396142]
11. Wenger RH. Cellular adaptation to hypoxia: O₂-sensing protein hydroxylases, hypoxia-inducible transcription factors, and O₂-regulated gene expression. *FASEB Journal* 2002;16:1151–1162. [PubMed: 12153983]
12. Kewley RJ, Whitelaw ML, Chapman-Smith A. The mammalian basic helix-loop-helix/PAS family of transcriptional regulators. *The International Journal of Biochemistry & Cell Biology* 2004;36:189–204.
13. Chan WK, Yao G, Gu YZ, Bradfield CA. Cross-talk between the aryl hydrocarbon receptor and hypoxia inducible factor signaling pathways. Demonstration of competition and compensation. *The Journal of Biological Chemistry* 1999;274:12115–12123. [PubMed: 10207038]
14. Gradin K, McGuire J, Wenger RH, Kvietikova I, Whitelaw ML, Toftgard R, Tora L, Gassmann M, Poellinger L. Functional interference between hypoxia and dioxin signal transduction pathways: Competition for recruitment of the Arnt transcription factor. *Molecular and Cellular Biology* 1996;16:5221–5231. [PubMed: 8816435]
15. Pollenz RS, Davarinos NA, Shearer TP. Analysis of aryl hydrocarbon receptor-mediated signaling during physiological hypoxia reveals lack of competition for the aryl hydrocarbon nuclear translocator transcription factor. *Molecular Pharmacology* 1999;56:1127–1137. [PubMed: 10570039]
16. Nie M, Blankenship AL, Giesy JP. Interactions between aryl hydrocarbon receptor (AhR) and hypoxia signaling pathways. *Environmental Toxicology and Pharmacology* 2001;10:17–27. [PubMed: 11382553]
17. Roblin S, Okey AB, Harper PA. AH receptor antagonist inhibits constitutive CYP1A1 and CYP1B1 expression in rat BP8 cells. *Biochemical and Biophysical Research Communications* 2004;317:142–148. [PubMed: 15047159]
18. Chiaro CR, Patel RD, Marcus CB, Perdew GH. Evidence for an Ah receptor-mediated cytochrome P450 auto-regulatory pathway. *Molecular Pharmacology*. 200710.1124/mol.107.038968Published on-line: August 24, 2007
19. Fradette C, Du SP. Effect of hypoxia on cytochrome P450 activity and expression. *Current Drug Metabolism* 2004;5:257–271. [PubMed: 15180495]
20. Elvidge GP, Glenny L, Appelhoff RJ, Ratcliffe PJ, Ragoussis J, Gleadle JM. Concordant regulation of gene expression by hypoxia and 2-oxoglutarate-dependent dioxygenase inhibition: The role of HIF-1 α , HIF-2 α , and other pathways. *The Journal of Biological Chemistry* 2006;281:15215–15226. [PubMed: 16565084]
21. Song J, Claget-Dame M, Peterson RE, Hahn ME, Westler WM, Sicinski RR, DeLuca H. A ligand for the aryl hydrocarbon receptor isolated from lung. *Proceedings of the National Academy of Sciences of the United States of America* 2002;99:14694–14699. [PubMed: 12409613]
22. Hu CJ, Sataur A, Wang L, Chen H, Simon MC. The N-terminal transactivation domain confers target gene specificity of hypoxia inducible factors HIF-1{ α } and HIF-2{ α }. *Molecular Pharmacology*. 200710.1091/mbc.E06–05–0419Cell Published on-line: September 5, 2007

23. Hu CJ, Wang LY, Chodosh LA, Keith B, Simon MC. Differential roles of hypoxia-inducible factor 1alpha (HIF-1alpha) and HIF-2alpha in hypoxic gene regulation. *Molecular and Cellular Biology* 2003;23:9361–9374. [PubMed: 14645546]
24. Stegeman JJ, Hahn ME, Weisbrod R, Woodin BR, Joy JS, Najibi S, Cohen RA. Induction of cytochrome P4501A1 by aryl hydrocarbon receptor agonists in porcine aorta endothelial cells in culture and cytochrome P4501A1 activity in intact cells. *Molecular Pharmacology* 1995;47:296–306. [PubMed: 7870038]
25. Ma Q, Baldwin KT, Renzelli AJ, McDaniel A, Dong L. TCDD-inducible poly(ADP-ribose) polymerase: A novel response to 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Biochemical and Biophysical Research Communications* 2001;289:499–506. [PubMed: 11716501]
26. Couroucli XI, Welty SE, Geske RS, Moorthy B. Regulation of pulmonary and hepatic cytochrome P4501A expression in the rat by hyperoxia: Implications for hyperoxic lung injury. *Molecular Pharmacology* 2002;61:507–515. [PubMed: 11854430]
27. McMillan BJ, Bradfield CA. The Aryl Hydrocarbon Receptor sans Xenobiotics: Endogenous Function in Genetic Model Systems. *Molecular Pharmacology* 2007;27:487–498. [PubMed: 17535977]
28. Abdelrahim M, Smith R III, Safe S. Aryl hydrocarbon receptor gene silencing with small inhibitory RNA differentially modulates Ah-responsiveness in MCF-7 and HepG2 cancer cells. *Molecular Pharmacology* 2003;63:1373–1381. [PubMed: 12761348]
29. Akahoshi E, Yoshimura S, Ishihara-Sugano M. Over-expression of AhR (aryl hydrocarbon receptor) induces neural differentiation of Neuro2a cells: Neurotoxicology study. *Environmental Health* 2006;5:24. [PubMed: 16956419]
30. Fradette C, Bleau AM, Pichette V, Charet N, Du SP. Hypoxia-induced down-regulation of CYP1A1/1A2 and up-regulation of CYP3A6 involves serum mediators. *British Journal of Clinical Pharmacology* 2002;137:881–891.
31. Carriere V, Dussault E, Rousset M, Zweibaum A, Cambier D. Decreased expression of gamma-glutamyl-transpeptidase in the intestinal cell line Caco-2 by inducers of cytochrome P450 1A1. *Biochemical Pharmacology* 1998;56:817–823. [PubMed: 9774143]
32. Fradette C, Batonga J, Teng S, Piquette-Miller M, Du SP. Animal models of acute moderate hypoxia are associated with a down-regulation of CYP1A1, 1A2, 2B4, 2C5, and 2C16 and up-regulation of CYP3A6 and P-glycoprotein in liver. *Drug Metabolism and Disposition* 2007;35:765–771. [PubMed: 17303624]
33. Jiang W, Welty SE, Couroucli XI, Barrios R, Kondraganti SR, Muthiah K, Yu L, Avery SE, Moorthy B. Disruption of the Ah receptor gene alters the susceptibility of mice to oxygen-mediated regulation of pulmonary and hepatic cytochromes P4501A expression and exacerbates hyperoxic lung injury. *The Journal of Pharmacology and Experimental Therapeutics* 2004;310:512–519. [PubMed: 15123765]
34. Moorthy B, Parker KM, Smith CV, Bend JR, Welty SE. Potentiation of oxygen-induced lung injury in rats by the mechanism-based cytochrome P-450 inhibitor, 1-aminobenzotriazole. *The Journal of Pharmacology and Experimental Therapeutics* 2000;292:553–560. [PubMed: 10640292]
35. Brusselmans K, Compennolle V, Tjwa M, Wiesener MS, Maxwell PH, Collen D, Carmeliet P. Heterozygous deficiency of hypoxia-inducible factor-2alpha protects mice against pulmonary hypertension and right ventricular dysfunction during prolonged hypoxia. *The Journal of Clinical Investigation* 2003;111:1519–1527. [PubMed: 12750401]
36. Yu AY, Shimoda LA, Iyer NV, Huso DL, Sun X, McWilliams R, Beaty T, Sham JSK, Wiener CM, et al. Impaired physiological responses to chronic hypoxia in mice partially deficient for hypoxia-inducible factor 1alpha. *The Journal of Clinical Investigation* 1999;103:691–696. [PubMed: 10074486]
37. Lee K, Burgoon LD, Lamb L, Dere E, Zacharewski TR, Hogenesch JB, LaPres JJ. Identification and characterization of genes susceptible to transcriptional cross-talk between the hypoxia and dioxin signaling cascades. *Chemical Research in Toxicology* 2006;19:1284–1293. [PubMed: 17040097]
38. Allen JW, Johnson RS, Bhatia SN. Hypoxic inhibition of 3-methylcholanthrene-induced CYP1A1 expression is independent of HIF-1alpha. *Toxicology Letters* 2005;155:151–159. [PubMed: 15585370]

Fig. 1.

Effect of graded levels of oxygen on hypoxia-regulated gene expression. HMVEC-L were exposed to 21%, 2.5%, or 1% oxygen for 18 h and ADM, VEGF-A, ET-1, and PGK1 mRNA expression was analyzed by real time PCR. The data represent the mean $\pm$ SEM of three experiments and were analyzed by one-way ANOVA, using post hoc Holm-Sidak comparisons; * $p < 0.05$, compared to 21% O₂

Fig. 2.

Effect of HIF-2 α siRNA on hypoxia-regulated gene expression. HMVEC-L were transfected with HIF-2 α siRNA for 48 h, and then exposed to 1% oxygen for the final 18 h when HIF-2 α , PGK1, and ADM mRNA expression was analyzed by real time PCR. The data represent the mean $\pm$ SEM of three experiments and were analyzed by one-way ANOVA, using post hoc Holm-Sidak comparisons; * $p < 0.05$ compared to control siRNA at 21% O₂; # $p < 0.05$, compared to control siRNA at 1% O₂

Fig. 3. Effect of AHR activation by TCDD on AHR-regulated gene expression. HMVEC-L were exposed to 0.01% DMSO or 2 nM TCDD for 18 h and CYP1A1, CYP1B1, TiPARP, and ALDH1A3 mRNA expression was analyzed by real time PCR. The data represent the mean $\pm$ SEM and were analyzed by Student's t-test or Welch unpaired t-test (CYP1B1); * $p < 0.05$

Fig. 4. Effect of AHR siRNA on AHR mRNA and TCDD-induced CYP1A1 and CYP1B1 mRNA expression. HMVEC-L were transfected with 25 nM control or AHR siRNA for 48 h, and some cells were also exposed to 2 nM TCDD for the final 18 h. (a) AHR, and (b) CYP1A1 and CYP1B1 mRNA expression was analyzed by real time PCR. The data represent the mean $\pm$ SE of three experiments and were analyzed by one-way ANOVA, using post hoc Holm-Sidak comparisons; * $p < 0.05$, compared to control siRNA; # $p < 0.05$, compared to control siRNA plus TCDD

Fig. 5.

Effect of AHR knockdown or hypoxia on constitutive CYP1A1 mRNA expression. CYP1A1 mRNA expression was analyzed by real PCR following exposure of HMVEC-L to (a) 25 nM control or AHR siRNA for 48 h or (b) 21, 2.5, or 1% oxygen for 18 h. The data represent the mean $\pm$ SEM of three experiments and were analyzed by Student's test and one-way ANOVA, using post hoc Holm-Sidak comparisons, respectively; * $p < 0.05$

Fig. 6.

Effect of simultaneous exposure to AHR siRNA and hypoxia on constitutive CYP1A1 mRNA expression. CYP1A1 mRNA expression was analyzed by real PCR following exposure of HMVEC-L to 25 nM control or AHR siRNA for 48 h, and exposed to 2.5% or 1% oxygen for the final 18 h. The data represent the mean $\pm$ SEM of three experiments and were analyzed by two-way ANOVA, using post hoc Holm–Sidak comparisons. Two-way ANOVA of CYP1A1 mRNA expression demonstrated significant differences based on oxygen ($p < 0.001$), AHR siRNA ($p < 0.001$), and an oxygen-AHR siRNA interaction ($p < 0.001$); * $p < 0.05$ compared to control siRNA at 21% O₂; # $p < 0.05$ compared to both AHR siRNA at 21% O₂, and control siRNA at the comparable level of hypoxia

Fig. 7.

Effect of HIF-2 α siRNA on hypoxia-mediated reduction of constitutive CYP1A1 mRNA expression. CYP1A1 mRNA expression was analyzed by real PCR following exposure of HMVEC-L to 25 nM control or HIF-2 α siRNA for 48 h, and exposed to 1% oxygen for the final 18 h. The data represent the mean $\pm$ SEM of three experiments and were analyzed by one-way ANOVA, using post hoc Holm–Sidak comparisons. * $p < 0.05$ compared to control siRNA at 21% O₂; # $p < 0.05$ compared to control siRNA at 1% O₂

Fig. 8.

Effect of HIF-2 α siRNA on hypoxia-mediated reduction of AHR mRNA expression. AHR mRNA expression was analyzed by real PCR following exposure of HMVEC-L to (a) 25 nM control or HIF-2 α siRNA, or (b) 25 nM control, AHR, or AHR plus HIF-2 α siRNA for 48 h, and exposed to 1% oxygen for the final 18 h. The data represent the mean $\pm$ SEM of three experiments and were analyzed by one-way (panel A) or two-way (panel B) ANOVA, using post hoc Holm-Sidak comparisons. * $p < 0.05$ compared to control siRNA at 21% O₂; Bars with different letters are significantly different, $p < 0.05$

Table 1

Primer sequences used for real time PCR analysis of mRNA expression

Gene	Forward primer (5' to 3')	Reverse primer (5' to 3')
18s	CGGAGGTTCTGAAGACGATCAGATA	TTGGTTTCCCGGAAGCTGCC
AHR	GGCCGTGTCGATGTATCAGTG	GTAAGTGGATTGTACTGCATCTGAC
Adrenomedullin (ADM)	GGTGCAGAAGAATCCGAGTG	ACGCCGTGAGAAATCAGTTT
Vascular endothelial growth factor - A (VEGF-A)	CTGCTGTCTTGGGTGCATTG	CGCTGATAGACATCCATGAACTTC
Endothelin-1 (ET-1)	CATTGGTGACAGACCTTCGGG	GATGCTCCTGCTCTGATCCCA
Phosphoglycerate kinase 1 (PGK1)	TGAAGATTACCTTGCCTGTTGACT	TCTTGCTGCTTTCAGGACCAC
CYP1A1	CACATGCTGACCCTGGGAAAG	GGTGTGGAGCCAATTCGGATC
CYP1B1	CGTCATGAGTGCCGTGTGTT	GCCCGAACTCTTCGTTGTGG