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Hypoxic inhibition of 3-methylcholanthrene-induced CYP1A1 expression is independent of HIF-1alpha

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Abstract

Hypoxia-inducible factor-1alpha (HIF-1α) and aryl hydrocarbon receptor (AhR) both require dimerization with AhR nuclear translocator (ARNT) to initiate transcription of their respective target genes. It has been proposed that competition for ARNT results in decreased targeting of AhR to cytochrome P450 1A1 (CYP1A1) under hypoxia. We established primary cultures of HIF-1α null hepatocytes to examine the interaction between HIF-1α and AhR signaling. Gene expression of known HIF targets phosphoglycerate kinase (PGK), vascular endothelial growth factor (VEGF) and glucose transporter-1 (GLUT-1) increased under hypoxia, but was reduced in the HIF null cultures. Concomitant treatment of cultures with hypoxia (1% O_2) and 3-methylcholanthrene (an AhR ligand) did not significantly alter HIF target gene expression. Furthermore, enzymatic activity and transcription of CYP1A1 was inhibited by hypoxia in HIF-1α null cultures, indicating that HIF-1α is not directly involved in negative regulation of AhR signaling.
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1. Introduction

Molecular oxygen is a potent regulator of gene expression and function, in addition to its well-described role in respiration. In particular, systemic adaptations to a low oxygen environment are associated with increased transcriptional activity of a family of hypoxia-inducible factors (HIFs) (Wenger, 2002). Perhaps the most well-characterized of these factors, hypoxia-inducible factor-1alpha (HIF-1α), touted as a key regulator of oxygen homeostasis, plays a significant role in the dynamic processes of development, solid tumor

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growth, angiogenesis, inflammation, and general adaptation to environmental stress (Cramer et al., 2003; Ryan et al., 1998; Semenza, 1999).

From a structural standpoint, HIF-1α belongs to a family of basic helix-loop-helix Per/Arnt/Sim (bHLH-PAS) domain regulatory proteins important in circadian rhythms, xenobiotic metabolism, and hypoxic response (Gu et al., 2000). HIF-1α protein stability is regulated by oxygen-dependent hydroxylation, which results in targeted degradation via the ubiquitination pathway. Under hypoxia, oxygen-dependent hydroxylation of HIF-1α is reduced, stabilizing the protein and allowing dimerization with aryl hydrocarbon receptor nuclear translocator (ARNT), another member of the bHLH-PAS family, forming an active transcriptional complex. Transcriptional upregulation of HIF-1α targets, including erythropoietin (EPO), vascular endothelial growth factor (VEGF), or phosphoglycerate kinase (PGK), provide adaptations that increase oxygen delivery, promote vascularization, and shift to glycolytic metabolism (Semenza, 1999).

ARNT has been identified as a required dimerization partner for both HIF-1α and aryl hydrocarbon receptor (AhR), which is activated by potentially toxic substrates, such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (Tomita et al., 2000). Activation of the AhR/ARNT complex transcriptionally upregulates cytochrome P540 1A1 (CYP1A1), a member of a large family of mono-oxygenases involved in biotransformation of xenobiotics (Denison and Whitlock, 1995). It has been suggested that simultaneous signaling of hypoxia and dioxin pathways results in competition for ARNT dimerization and decreased targeting of both transcriptional complexes (Gradin et al., 1996). However, the role of HIF-1α in the hypoxic inhibition of dioxin pathway via competitive dimerization has not been clearly demonstrated.

Many of the proposed interactions between the HIF-1α and AhR pathways have been elucidated using hepatocyte cell lines, including HepG2, Hep3B, and Hepa1c1c7; however, variability in expression patterns and inducibility amongst these culture systems is significant (Gradin et al., 1996; Kim and Sheen, 2000; Nie et al., 2001; Pollenz et al., 1999). Thus, we established a long-term culture system for primary murine hepatocytes to directly asses the potential for competitive interactions between HIF-1α, AhR, and ARNT. Isolation of transgenic hepatocytes harboring floxed HIF-1α genes allowed generation of HIF null cultures by transduction of adenovirus encoding Cre recombinase. Furthermore, real-time PCR analysis performed on known HIF-1α target and CYP1A1 transcripts from HIF-1α null cultures co-stimulated with hypoxia and 3-methylcholanthrene (3-MC) demonstrated that hypoxic inhibition of CYP1A1 expression is HIF-1α-independent.

2. Methods

2.1. Hepatocyte isolation and culture

Primary mouse hepatocytes were isolated and purified by a modified procedure of Seglen (Seglen, 1976). Mice harboring loxP-flanked HIF-1α alleles, created from a C57BL/6 strain, were described previously (Ryan et al., 1998). Male mice (8–12 weeks) weighing 30–50 g were anesthetized prior to in situ perfusion of the portal vein with Krebs Ringer Buffer followed by collagenase. Dissociated cells were passed through nylon mesh and purified on a Percoll gradient. Cell viability was routinely >90% as assessed by Trypan blue exclusion. Hepatocytes were seeded on 0.1% collagen gel at a density of 300 K cells/60-mm dish and allowed to attach for 2 h, at which point media was replaced. Twenty-four hours post-isolation, cultures were overlaid with another layer of 0.1% collagen gel (Dunn et al., 1991). Cultures were maintained in Dulbecco's Modified Eagle Medium (DMEM, GibcoBRL) with 10% fetal bovine serum, supplemented with insulin, hydrocortisone, and antibiotics. Images were obtained using a Nikon Eclipse TE300 inverted microscope, CCD camera (CoolSnap HQ, Roper Scientific), and Metamorph Image Analysis System (Universal Imaging). Medium was changed every 48 h until day 6 when adenoviral treatment was initiated.

2.2. Adenoviral transduction

The HIF-1α gene on both alleles is floxed by loxP sites which enables gene excision in the presence of Cre recombinase. To generate wild-type (WT) (HIF-1α +/+) or HIF-1α −/− cells, cultures were treated in vitro with recombinant, replication-deficient type 5 adenovirus, containing genes encoding β-galactosidase or Cre, respectively, at multiplicity of infection ranging

from 10 to 200 (gift of Frank Giordano, Yale University, CT). Virus was added to DMEM without serum and incubated at 37 °C for 24 h, after which medium was replaced. Cellular viability was assessed by MTT staining, precipitate extraction, and spectrophotometric measurement at 96 h post-infection. Infection with control adenovirus was determined by fixing cultures win 4% paraformaldehyde and staining with X-gal (Sigma Chemical). Subsequent to viability and gene deletion analysis, 50 multiplicity of infection (MOI) was found to be optimal for further experiments.

2.3. Determination of HIF-1α excision

Hepatocytes were removed from collagen gel sandwich culture by incubating with 0.5 mg/mL collagenase in DMEM without serum for 45 min followed by gentle scraping. Cells were pelleted by centrifugation at 100 × *g* for 3 min and digested in 10 mM Tris–HCl (pH 8.0), 100 mM NaCl, 10 mM EDTA, 0.5% SDS with 0.25 mg/ml proteinase K (Roche) by agitation for 5 h at 37 °C. DNA was extracted using a mixture of phenol/chloroform/isoamylaclohol (25:24:1) and precipitated with ethanol. After pelleting, the DNA was washed in 70% ethanol the resuspended in 10 mM Tris, 1 mM EDTA (pH 8.0) and stored at −80 °C. The frequency of HIF-1α deletion was calculated by comparing the cycle threshold value of HIF-1α genomic DNA to that of a control gene, c-jun (Pfander et al., 2003). The following primers and probes were used to evaluate gene excision:

HIF-1α forward
5′-CTATGGAGGCCAGAAGAGGGTAT-3′,
HIF-1α reverse
5′-CCCACATCAGGTGGCTCATAA-3′,
HIF-1α probe
5′-6FAM-AGATCCCTTGAAGCTAG-BHQ-3′,
c-jun forward
5′-TGCATGCTATCATTGGCTCATAC-3′,
c-jun reverse
5′-CACACCATCTTCTGGTGTACAGTCT-3′,
c-jun probe 5′-VIC-CCCGGCAACACACA-BHQ-3′.

2.4. Hypoxia and 3-methylcholanthrene treatment

Following adenoviral-mediated HIF-1α gene excision, WT or HIF null cultures were subjected to hypoxia (1% O_2) and/or 3-MC treatment (5 μM). Cultures with or without 3-MC were placed in a sealed chamber and purged with a gas mixture of 1% O_2/5% CO_2/94% N_2 for 5 min then incubated at 37 °C. Control cultures with and without 3-MC, were maintained under normoxic conditions, defined as 21% O_2. Cultures were removed after 8 h for PCR analysis and after 24 h for CYP enzymatic assays.

2.5. Quantitative PCR

Gene expression analysis was carried out by real-time analysis of PCR on mRNA transcripts. Total RNA was isolated from cultures using Trizol reagent and purified according to the manufacturer's protocol. Complimentary DNA synthesis was carried using random hexamers with the Superscript II First Strand Synthesis kit (Invitrogen). Real-time PCR was performed using ABI Prism 7700 with the following primer and probe sequences.

Primer	5′–3′ Sequence
VEGF reverse	ATCCGCATGATCTGCATGG
VEGF forward	AGTCCCATGAAGTGATCAAGTTCA
VEGF probe	6FAM-TGCCCACGTCAGAGAGCA-ACATCAC-BHQ
PGK reverse	TTCTTGCTGCTCTCAGTACCACA
PGK forward	CAAATTTGATGAGAATGCCAA-GACT
PGK probe	6FAM-TATACCTGCTGGCTGGATG-GGCTTGGACT-BHQ
GLUT-1 reverse	ACGAGGAGCACCGTGAAGAT
GLUT-1 forward	GGGCATGTGCTTCCAGTATGT
GLUT-1 probe	6FAM-CAACTGTGCGGCCCCTAC-GTCTTC-BHQ
CYP1A1 forward	AAAACACGCCCGCTGTGAA
CYP1A1 reverse	TGAATCACAGGAACAGCCACC
CYP1A1 probe	6FAM-TCAGCATCTTCAGGCTTAG-BHQ
β-Actin forward	AGGCCCAGAGCAAGAGAGG
β-Actin reverse	TACATGGCTGGGGTGTTGAA

Fold change of gene expression was calculated from cycle threshold values relative to the normoxic WT con-

trols. Statistical significance was determined using one-way ANOVA with a Tukey post test ($p < 0.05$, GraphPad Prism).

2.6. EROD assay

Media was collected from sandwich cultures for analysis of Ethoxyresorufin *O*-deethylase (EROD) cleavage, an enzymatic marker of cytochrome P450 activity (CYP). Cultures (300 K hepatocytes/dish) were incubated with EROD for 30 min after which medium was removed to quantify fluorescence of the cleavage product, resorufin (ex 571/em 585). EROD activity is reported as the rate of resorufin formation which is proportional to the amount of CYP enzyme present (Behnia et al., 2000).

3. Results

Isolated hepatocytes from mice with intact, double-floxed HIF-1α gene were cultured in a collagen sandwich configuration for 15 days. Cultures over this period maintained liver-specific function as determined by liver-specific characteristics, including albumin synthesis, urea production (data not shown) and polygonal morphology (Fig. 1A) (Dunn et al., 1991). No significant decrease in viability was observed for adenovirus-treated cultures (either Adβ-gal or AdCre) over 96 h after treatment (Fig. 1C). Verification of control gene delivery, β-galactosidase, and subsequent expression was first carried out in control cultures by X-gal staining, which showed increasing transgene expression with increasing MOI (data not shown). Of primary interest, however, was the efficiency of Cre-mediated HIF-1α excision assessed by real-time PCR of genomic DNA. Results showed a dose-dependent deletion efficiency, with greater than 90% excision at an MOI of 50 and negligible deletion with control Adβ-gal (Fig. 1B).

3.1. Effect of 3-MC on hypoxic gene expression

As further verification that the HIF-1α gene was deleted in vitro, we examined the hypoxia-induced gene expression of several known HIF-1α target genes. PGK mRNA levels, as determined by real-time PCR in Fig. 2A, were moderately increased under hypoxia in WT, but induction was significantly impaired in HIF-

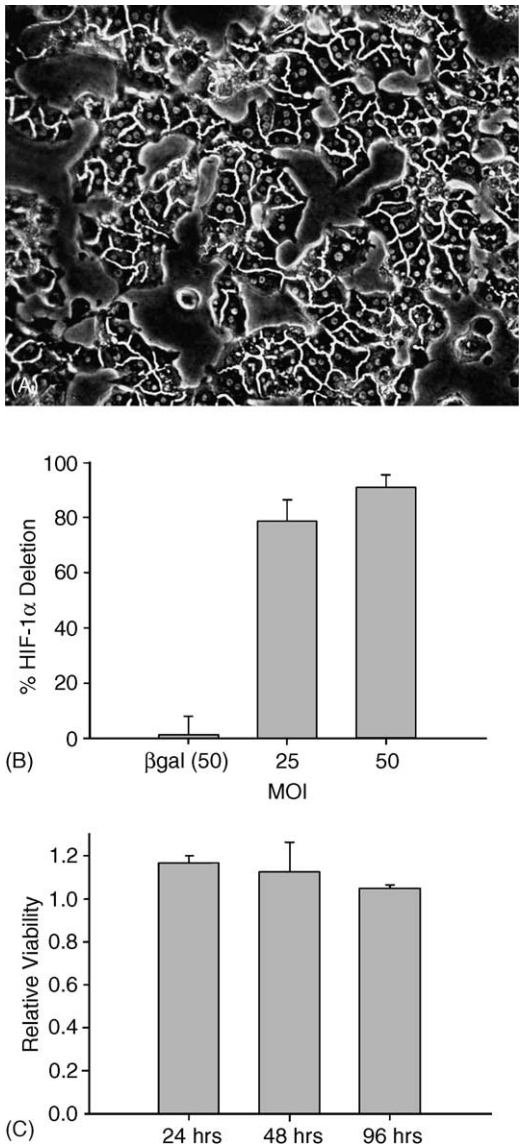

Fig. 1. Characteristics of murine hepatocytes in sandwich culture. (A) Phase contrast micrograph of hepatocytes harboring floxed-HIF-1α on day 8 post-isolation, showing multi-nucleation, polygonal morphology, and bright bile canaliculli at cell boarders. (B) Adenovirus-treated hepatocytes were assayed for HIF-1α deletion. Genomic HIF-1α content as determined by real time PCR, expressed as percent gene deletion by normalizing to the control gene, c-jun. Twenty-five and fifty indicate multiplicity of infection for infection with adenovirus harboring cre-recombinase. Control adenovirus harbored beta-galactosidase reporter at 50 MOI. (C) Viability based on MTT conversion was measured 24, 48, and 96 h after adenovirus treatment (50 MOI) and normalized to untreated controls. Values are the mean and standard error for $n = 3$.

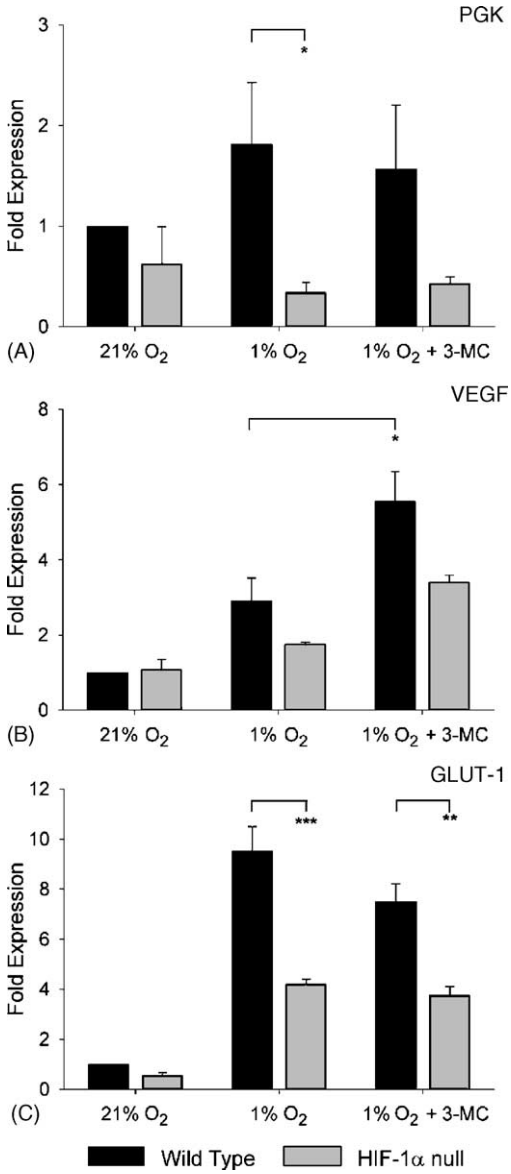

Fig. 2. Hypoxic gene expression of PGK (A), VEGF (B), and GLUT-1 (C). WT (black bars) and HIF-1α null (grey bars) were treated with 1% O_2 with and without 5 μM 3-MC for 8 h. Fold expression values were normalized to normoxic (21% O_2), WT controls. Values are the mean and standard error for $n = 3$: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

1α null ($p < 0.05$). To assess the possible interaction of the AhR pathway on HIF target gene expression, PCR analysis of PGK mRNA was also performed on 3-MC-treated WT and null cultures under normoxia. Trends in PGK expression upon simultaneous exposure hypoxia and 3-MC treatment were similar to those of control cultures.

Hypoxia-induced expression of VEGF (Fig. 2B) was also analyzed and found to be maximal in hypoxia WT (2.9-fold), and half maximal in HIF-1α null (1.7-fold). With 3-MC treatment, hypoxic induction of VEGF was 5.5-fold higher than normoxic WT but decreased to 3.4-fold in HIF-null. The increased hypoxic expression of VEGF with concomitant 3-MC exposure was significant ($p < 0.05$), implicating 3-MC as a possible inducer of VEGF.

Glucose transporter-1 (GLUT-1) expression (Fig. 2C) was similarly evaluated showing 9.5-fold induction in hypoxic WT decreasing to 4.2-fold in HIF-null ($p < 0.001$). Treatment with 3-MC did not significantly alter the hypoxia-induced expression of GLUT-1, showing 7.5- and 3.8-fold increased expression in WT and HIF-null cultures, respectively ($p < 0.01$).

3.2. Hypoxic modulation of CYP1A1 activity and expression

It was observed that the enzymatic activity of CYP1A1 in murine hepatocytes indicated by cleavage of EROD (Fig. 3A), was dramatically decreased (∼50%) under hypoxia ($p < 0.001$). To determine whether HIF-1α was playing a role in the hypoxic decrease in EROD activity, assays were performed in HIF-1α null cultures. Though, EROD activity in HIF-null under 21% O_2 was decreased relative to WT, activity in hypoxic cultures still decreased nearly 50% ($p < 0.01$).

The inhibition of CYP1A1 at the protein level was indicated by assaying EROD activity, but hypoxic regulation may occur at the transcriptional level. To evaluate this possibility, we analyzed mRNA levels of CYP1A1 in response to 3-MC treatment, with or without hypoxia (Fig. 3B). Studies in WT cultures demonstrated that the 3-MC-mediated upregulation of CYP1A1 transcription was inhibited under hypoxic conditions as shown by 16- and 6-fold increased expression under 21% O_2 and 1% O_2, respectively ($p < 0.01$). It was observed that overall fold induction of CYP1A1 was decreased in HIF-null cultures, but that expression was still significantly reduced from 9.4-fold under normoxia to 4.5-fold under hypoxia ($p < 0.01$), implying that HIF-1α activity and gene targeting does not con-

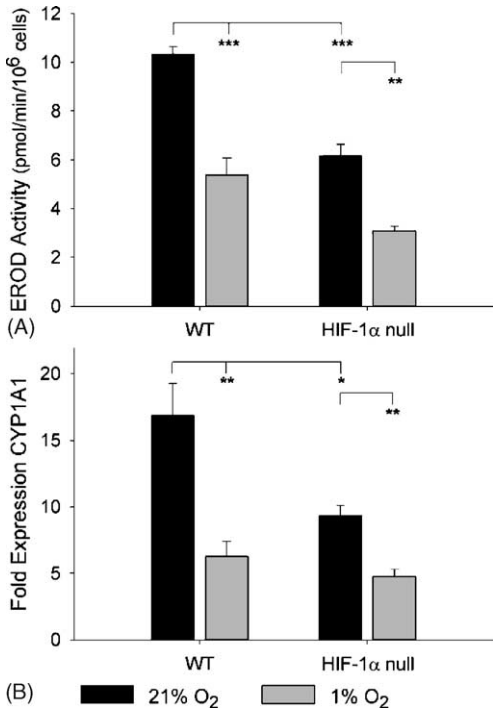

Fig. 3. Enzymatic activity (A) and gene expression (B) of CYP1A1. Rate of conversion of ethoxyresorufin. (A) was assayed in WT and HIF-1α null cultures under normoxia (21% O_2, black bars) or hypoxia (1% O_2, grey bars) with 5 μM 3-MC for 24 h. CYP1A1 mRNA levels (B) were measured by real time PCR after 8 h of normoxia (black) or hypoxia (grey) with 5 μM 3-MC and normalized to untreated, normoxic controls. Values are the mean and standard error for $n = 3$: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$.

tribute to the hypoxic inhibition of CYP1A1 expression.

4. Discussion

We have established a long-term, conditional knockout system for evaluating the interplay between hypoxia and dioxin pathways in primary hepatocyte cultures. Given the rapid loss of viability and function in monolayer hepatocyte culture and the incomplete repertoire of gene expression in immortalized cell lines, sandwich culture of primary hepatocytes and adenoviral Cre-mediated gene excision may provide an ideal platform for a variety of studies. In this study, transduction with adenovirus after 6 days of culture did not result in significant loss in viability at relatively high MOI (50), unlike previous reports in fresh hepatocytes (Prost et al., 2001). Furthermore, adenoviral delivery to hepatocytes resulted in efficient Cre-mediated gene excision of HIF-1α (>90%) as would be expected due to the tropism of adenovirus for the liver and expression of the coxsackievirus and adenovirus receptor (CAR). The efficiency of excision enabled sensitivity to moderate changes in hypoxic gene expression (46 and 50% expression for VEGF and GLUT-1, respectively). For comparison, excision efficiency of only 50% would have required dramatic changes in gene expression (down to 5% of control) to observe the same result. We specifically evaluated the potential competition for ARNT by HIF-1α and AhR by excising HIF-1α and evaluating gene expression of HIF-1α targets (PGK, VEGF, GLUT-1) and a prototypic AhR target (CYPIA1) under simultaneous stimulation of both pathways.

4.1. AhR activation does not diminish HIF-1α gene targeting

Previous studies have suggested that ARNT, the common dimerization partner for HIF-1α and AhR, may be limited during concomitant treatment with hypoxia and AhR agonists, resulting in decreased gene targeting of either pathway. This competition was implied in transfected cell models treated with dioxin and cobalt chloride ($CoCl_2$), a hypoxia mimic, which showed reduced signal of hypoxia response element (HRE)-driven reporters compared to $CoCl_2$ alone (Chan et al., 1999; Nie et al., 2001). In addition, biochemical studies in vitro have shown that both HIF-1α and AhR can compete for and bind ARNT preincubated with AhR or HIF-1α, respectively (Chan et al., 1999; Gradin et al., 1996). However, the magnitude of competition suggests that HIF-1α has a higher affinity for ARNT than does AhR, thus decreasing the likelihood of reduced HIF-1α gene targeting with simultaneous AhR activation.

In this study, gene expression analysis of HIF-1α targets in primary cells showed no significant decrease in the hypoxia-mediated induction of PGK, VEGF, or GLUT-1 in the presence of 3-MC. These results are consistent with HIF-1α having a higher affinity for ARNT, enabling gene targeting despite competing ARNT dimerization. Seemingly, the lack of AhR inhibition of HIF-1α gene targeting contradicts find-

ings of Chan et al. (1999), which demonstrated dioxin-mediated decrease of an EPO-derived HRE reporter under hypoxia. Of note, the same study also implied that the EPO gene may actually contain dioxin response elements, demonstrating the complexity of gene regulation and limitation of partial-promoter reporter systems in evaluating integrated physiologic responses. Interestingly, our data suggest some AhR regulation of VEGF, as demonstrated by increase VEGF expression under hypoxia and 3-MC relative to hypoxia alone, a finding that has been reported in previous gene expression studies (Zeytun et al., 2002).

4.2. Hypoxic inhibition of CYP1A1 is independent of HIF-1α

Among studies of potential cross-talk between hypoxia and dioxin signaling pathways, the most consistent finding is that hypoxia reduces the AhR-mediated induction of CYP1A1. Though competition for ARNT dimerization and shunting to hypoxia pathways is the pervading theory, the inhibitory role of HIF-1α in CYP1A1 upregulation has not been clearly demonstrated.

While several previously mentioned studies present circumstantial evidence of functional interference by HIF-1α, one experiment, which overexpressed HIF-1α in HepG2 cells resulted in inhibition of dioxin-mediated gene expression (Gradin et al., 1996). Whether this inhibition was due to sequestration of ARNT was not clear, but potential limitation of the ARNT pool was addressed in another study. Several cell lines were evaluated for constitutive ARNT expression and fractional nuclear translocation upon dioxin or hypoxic stimuli (Pollenz et al., 1999). Results from quantitative Western blotting indicated that first, basal expression level of ARNT and hypoxia sensitivity among hepatocyte cell lines derived from mouse, rat and human were highly variable and second, hypoxia stimulation may only utilize 15% of the total ARNT pool.

Thus, if HIF-1α indeed limits AhR-mediated CYP1A1 expression by ARNT sequestration, normal CYP1A1 induction would be expected if HIF-1α were removed. However, our results clearly demonstrated that CYP1A1 expression and activity was still attenuated in hypoxia-treated HIF-1α null cultures. Though this contradicts proposed HIF-1α/ARNT/AhR interactions from previous work, this study and model system has several advantages. Primary murine cultures showed long-term maintenance of liver-specific function in contrast to decreased function in many less-differentiated cell lines. Gene expression analysis of HIF-1α targets, in addition to validating the efficient excision of the HIF-1α gene, revealed significant response to physiologic hypoxia. Finally, these studies involve integrated cellular responses as opposed to isolated biochemical assays.

Despite striking evidence of the lack of HIF-1α interference in the dioxin pathway, we did not examine protein dimerization between HIF-1α, AhR, and ARNT, which is the level of the proposed interference. Previous studies have examined these interactions in detail, though semi-quantitative analysis of transcription factor complexes in our primary culture system could yield additional insight. In a broader scope, competitive inhibition among bHLH/PAS domain transcription factors may indeed occur as evidenced by negative regulation of hypoxia signaling via inhibitory PAS domain protein (IPAS) binding (Makino et al., 2001). However, our studies suggest that negative regulation does not occur among, HIF-1α/AhR/ARNT interactions.

With a lack of negative regulation of CYP1A1 expression by HIF-1α, by what mechanism is AhR-dependant gene targeting reduced under hypoxia? Though relatively little is known about redundant or collateral signaling by other HIFs, including HIF-2α and -3α, potential inhibitory interactions by these factors cannot be ruled out (Hu et al., 2003). Alternatively, ligand-bound AhR, even when dimerized with ARNT in the nucleus, may require additional cofactors for efficient CYP1A1 transcription. Inhibition of CYP1A1 transcription has also been observed in tumor necrosis factor-alpha (TNF-α) and lipopolysaccharide (LPS) signaling where downstream activation of NF-κB alters the activity of p300/CBP, a coactivator involved in the AhR/ARNT transcriptional complex (Ke et al., 2001). Given the promiscuity of p300/CBP in many pathways, including hypoxic, inflammatory, proliferative, and apoptotic responses, it is possible that p300/CBP is an important handle for shifting transcriptional activities to critical adaptive pathways (Janknecht, 2002). Based on the present study, further investigation into the potential role of p300/CBP or other cofactors in

altering CYP1A1 expression under hypoxia may be warranted.

Additionally, hypoxia induces oxidative stress and metabolic shift that may divert cellular resources from non-essential pathways. During hypoxic treatment, cellular ATP levels drop significantly while lactate levels increase (Seagroves et al., 2001). Secondary effects of this altered biochemical milieu may shuttle resources to transcriptional and translational pathways required for hypoxic adaptation. The importance of functional redistribution of signaling components is demonstrated by the observed shift of molecular oxygen to alternative oxygen-dependent pathways, including prolyl hydroxylases, from nitric oxide-inhibited mitochondria, thus preventing HIF stabilization (Hagen et al., 2003). It is feasible that similar transients in ATP, pH, or even oxygen, prevent transcription of CYP1A1 as well as many other targets.

In conclusion, we have characterized hypoxia-induced gene expression in HIF-1α null primary hepatocytes. Concomitant treatment with hypoxia and 3-MC did not inhibit HIF-1α targeting to PGK and GLUT-1, and in fact, further induced VEGF expression. Contrary to previous reports implicating competitive inhibition of dioxin signaling by HIF-1α sequestration of ARNT, hypoxic down-regulation of CYP1A1 expression appears to be independent of HIF-1α.

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