

**Role of aryl hydrocarbon receptor in modulation
of the expression of the hypoxia marker carbonic anhydrase IX.**

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Abstract

Tumor-associated expression of carbonic anhydrase IX (CA IX) is to a major extent regulated by hypoxia inducible factor-1 (HIF-1) which is important for transcriptional activation and consists of the oxygen-regulated subunit HIF-1 α and the partner factor ARNT. We have previously observed that HIF-1 α competes with the aryl hydrocarbon receptor (AhR) for interaction with aryl hydrocarbon nuclear translocator (ARNT) under conditions when both conditionally regulated factors are activated. We have therefore investigated whether 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-induced activation of the AhR pathway might interfere with CA IX expression. Our data suggest that TCDD treatment reduces hypoxic induction of both CA IX mRNA and protein expression. Moreover, the transcriptional activity of the CA9 promoter was significantly reduced by expression of constitutively active AhR (CAAhR), which activates transcription in a ligand-independent manner. Finally, we found that ARNT is critical for both hypoxic induction and the TCDD-mediated inhibition of CA9 expression.

Short (page heading) title: Dioxin-mediated inhibition of carbonic anhydrase IX expression.

Keywords: carbonic anhydrase IX, hypoxia, Ah receptor, dioxin, transcriptional regulation, nuclear translocator of AhR

Abbreviations: CA IX, carbonic anhydrase IX protein; CA9, carbonic anhydrase IX gene; HIF, hypoxia inducible factor; AhR, aryl hydrocarbon receptor; ARNT, nuclear translocator of AhR; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin

Introduction

Carbonic anhydrase IX (CA IX) belongs to the family of Zn²⁺ binding metalloenzymes (carbonic anhydrases) which catalyze the reversible conversion of carbon dioxide to bicarbonate and proton and thus are involved in gas exchange, ion transport and the acid-base balance across cell membranes and between different intracellular compartments [1]. Fifteen human carbonic anhydrases identified to date participate in a variety of physiological processes like respiration, bone resorption, gluconeogenesis, renal acidification, formation of cerebrospinal fluid and gastric acid [2-4].

Besides physiological expression, several carbonic anhydrase isoforms have been described to be deregulated in various types of malignancies [5-8]. So far, the most studied carbonic anhydrase related to oncogenesis is CA IX [9, 10]. The strong association of CA IX expression with tumor development is mainly related to diminished oxygen supply, hypoxia [11, 12]. In general, the primary cellular response to hypoxia is the stabilization of HIF-1 transcriptional complex composed of a constitutively expressed ARNT (aryl hydrocarbon receptor nuclear translocator, also known as HIF-1 β) and the rate-limiting subunit HIF-1 α , which is regulated by oxygen availability [13]. The resulting HIF-1 complex activates transcription of a variety of genes the promoters of which contain hypoxia response elements (HRE, 5'-RCGTG-3'). CA9 belongs to genes with a very strong hypoxia response mainly due to the unique localization of its HRE element just -3/-10 nucleotides from the transcription start site [12]. Moreover, efficient hypoxia response is achieved through HIF-1 and SP1 cooperation on CA9 promoter [11]. The basal promoter of the CA9 gene is localized within a sequence of approximately 200 nucleotides upstream of the transcription start site.

In addition to hypoxia, expression of CA IX is also enhanced by high cell density in cell culture models [14] and modulated by the phosphatidylinositol-3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways [14-16]. Moreover, extracellular acidosis, which is characteristic for most solid tumors, increases CA IX at both protein and mRNA levels in certain cell types [17]. However, it is clear that CA IX expression could be modulated by an interplay between several stimuli and pathways with HIF-1 and SP1 representing the crucial targets of these pathways.

During the past decades, an increasing attention has started to be paid to the role of various environmental contaminants in the process of cancer development. Direct cellular response to the action of these toxic compounds is the induction of xenobiotic-metabolizing enzymes among which cytochrome P450 (CYP1A1) is best characterized (reviewed in [18]). The environmental toxin 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) induces a broad spectrum of biochemical and toxic effects such as teratogenesis, immunosuppression and tumor development [19]. Its effects are at the molecular level primarily mediated through activation of the aryl hydrocarbon receptor (AhR) [20]. Under conditions when physiological ligand or TCDD are absent, AhR is present in the cytoplasm and forms a complex stably associated with the heat shock protein 90 (Hsp90) and additional co-chaperone proteins [21]. After ligand binding, the activated AhR translocates into the nucleus [22], where it dimerizes with ARNT allowing both proteins to recognize the asymmetrical E-box motif known as xenobiotic response element (XRE, 5'-GCGTG-3'), which is involved in regulation of dioxin-responsive genes [23].

AhR, HIF-1 α and ARNT belong to a group of structurally-related transcription factors known as basic helix/loop/helix (bHLH) – Per/ARNT/Sim (PAS) family of proteins [24]. The main characteristic of PAS proteins is their ability to form various homo- and heterodimeric complexes, which regulate expression of target genes. The ability of ARNT to act as a heterodimeric partner for both HIF-1 α and AhR raises the possibility of cross-talk between the hypoxia and dioxin-signaling cascades. The studies published to date seem to indicate that the degree of interaction among ARNT-dependent pathways might be cell-, tissue- or species-specific [25, 26] and it may depend on cellular abundance of ARNT [27]. Consequently, a possible competition for ARNT may lead to regulation of target gene expression in a positive or negative manner. Therefore, the aim of our study was to elucidate whether dioxin activation of the AhR pathway could interfere with the expression of the hypoxia marker CA IX.

Materials and methods

Cell culture and dioxin treatment

Mouse hepatoma cell lines, wild-type Hepa-1c1c7 and the ARNT-deficient subline Hepa-1c4, as well as HeLa cells derived from human cervical carcinoma, were cultured in DMEM supplemented with 10% FCS (BioWhittaker, Verviers, Belgium) and 40 µg/ml gentamicin (Lek, Ljubljana, Slovenia) in a humidified atmosphere with 5% CO₂ at 37°C. Hypoxic treatments were performed in an anaerobic workstation (Ruskin Technologies, Bridgend, UK) in 2% O₂, 5% CO₂, 10% H₂ and 83% N₂ at 37°C.

TCDD was diluted to working concentrations of 1 nM or 10 nM. The final concentration of DMSO was less than 0.1% including controls. Cells were pre-treated with TCDD or DMSO for 1 h prior to being placed into hypoxia (2% O₂), for additional 24 h. Parallel control dishes were pre-treated and maintained in normoxia (21% O₂) for the same time period.

Antibodies and plasmids

Mouse monoclonal antibody M75, specific for the human MN/CA IX protein, was characterized earlier [28]. Anti-actin goat polyclonal antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Secondary anti-mouse peroxidase-conjugated antibody was from Sevapharma (Prague, Czech Republic) and anti-goat peroxidase-conjugated antibody was from Dako (Glostrup, Denmark). For chromatin immunoprecipitation, rabbit polyclonal antibody against ARNT was used as described in [29]. Anti-human IgG antibodies (rabbit polyclonal, Abcam, Cambridge, UK) were used as a negative control and for chromatin pre-clearing.

Human promoter constructs were generated by an insertion of PCR-amplified -50/+37 and -174/+37 CA9 genomic fragments upstream of the firefly luciferase gene in pGL3-Basic luciferase reporter vector (Promega, Madison, WI) [16]. pRL-TK renilla vector (Promega) served as a transfection efficiency control. Plasmids containing full-length ARNT (pARNT/GEM7), XRE-driven reporter gene construct pTX.DIR, and dioxin receptor deletion mutant lacking the minimal PAS B motif (DRΔPASB) that functions as a constitutively active AhR (CAAHR) have been described previously [30-32]. HIF-1α cDNA in pcDNA1 expression plasmid [33] was kindly provided by Prof. Patrick Maxwell (Imperial College, London, UK). Plasmid containing SP1 cDNA was generated by Dr. Robert Tjian and purchased from Addgene (plasmid no.12097, Cambridge, MA). CBP/p300 expression plasmid was kindly provided by Dr. Jiri Vachtenheim (Third Faculty of Medicine, Prague, Czech Republic).

Transfection and luciferase assay

Cells were plated into 35-mm Petri dishes to reach ~70% density on the following day. Transient transfection was performed with 1 µg of promoter-containing luciferase construct and 50 ng of pRL-TK plasmid DNA using GenePorterII reagent (Genlantis, San Diego, CA) according to the manufacturer's recommendations. To analyze the effect of CAAHR either with or without transcription factors (ARNT, HIF-1α, SP1 or p300), the plasmids containing corresponding cDNAs were co-transfected. Cells transfected with control empty vectors (pCMV) served as negative controls. The day after transfection, cells were trypsinized and plated in triplicates into 24-well plates. Cells were allowed to attach for 24 h, and then they were either transferred to hypoxia or maintained in normoxia for additional 24 h. Reporter gene expression was assessed 72 h after transfection using Dual-Luciferase Reporter Assay System (Promega), and luciferase activity was normalized against renilla activity.

Western blotting

HeLa cells were plated in 35-mm Petri dishes to reach ~90% density on the following day. Pre-treatment with TCDD or DMSO was done 1 h before the induction of hypoxia in the anaerobic workstation. Parallel control dishes were pre-treated and maintained in normoxia for the same time period. 24 h later, the cells were lysed in ice-cold RIPA buffer (1% Triton X-100, 1% deoxycholate in PBS) containing inhibitors of proteases (Roche Applied Science, Mannheim, Germany) for 30 min on ice. Cell lysates were scraped off and centrifuged at 10000 g for 10 min at 4°C. Protein concentration was determined using the BCA protein assay reagent (Pierce, Rockford, IL).

Total protein extracts (50 µg/lane) were separated in 10% SDS-PAGE and transferred onto PVDF membrane (ImmobilonTM-P, Millipore, Billerica, MA). For CA IX detection, the membrane was incubated with M75 primary antibody diluted 1:2 in blocking buffer for 1 h. Secondary anti-mouse peroxidase-conjugated antibody (Sevapharma) was diluted 1:7500 in blocking buffer. For loading control, the membrane was probed with anti-actin antibody (Santa Cruz) and the polyclonal rabbit anti-goat IgG-HRP (Dako). The membrane was developed by enhanced chemiluminescence using ECL kit (Amersham Pharmacia Biotech, Buckinghamshire, UK).

The intensity of bands corresponding to individual proteins was evaluated with ImageJ 1.38x software (Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, Maryland, USA, <http://rsb.info.nih.gov/ij/>, 1997-2007). ImageJ can create density histograms and calculate area and pixel value statistics of user-defined selections. Amount of proteins was expressed as the ratio of the intensity of each band to the intensity of the related actin internal standard.

Reverse transcription PCR

Total RNA was isolated from cells using InstaPure reagent (Eurogentec, Seraing, Belgium) according to the manufacturer's instructions. Reverse transcription was performed with M-MuLV reverse transcriptase (Finnzymes, Espoo, Finland) using random heptameric primers. The mixture of 3 µg of total RNA and random primers (400 ng/µl) was heated for 10 min at 70°C, cooled quickly on ice and supplemented with 0.5 mM dNTPs (Finnzymes), M-MuLV reverse transcriptase buffer, and 200 U of reverse transcriptase M-MuLV, incubated for 1 h at 42°C, heated for 15 min at 70°C and stored at -80°C until further use.

Real-time PCR

Real-time PCR was performed with POWER SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA) with gene-specific primers (*CA9* and *CYP1A1*) and primers for β-actin that served as internal standards. The primers were as follows (S=sense, A=antisense): *CA9* S: 5'-CCGAGCGACGCAGCCTTTGA-3' and *CA9* A: 5'-GGCTCCAGTCTCGGCTACCT-3' (252 bp product); *CYP1A1* S: 5'-TCTTTGGAGCTGGGTTTGACA-3' and *CYP1A1* A: 5'-GCTGTGGGGGATGGTGAA-3' (227 bp product); β-actin S: 5'-CCAACCGCGAGAAGATGACC-3' and β-actin A: 5'-GATCTTCATGAGGTAGTCAGT-3' (236 bp product).

The amplification of the samples was carried out in a final volume of 20 µl on StepOneTM Real-Time PCR System (Applied Biosystems, Foster City, CA), using the following program: initial denaturation at 95°C for 10 min followed by 40 cycles of: denaturation at 95°C for 15 s and annealing at 60°C for 1min. All PCR reactions were performed in triplicates and repeated three times.

Chromatin immunoprecipitation (ChIP) assay

HeLa cells were plated into 500 cm² plates at a ~90% density, left to attach overnight and incubated in hypoxia in absence or presence of 10 nM TCDD for 2 h. The cells were fixed in 1% formaldehyde in PBS for 10 min at room temperature. Chromatin isolation, shearing to size of ~600-800 bp and its immunoprecipitation with rabbit polyclonal antibodies against ARNT [29] was performed as described in [34]. Anti-human IgG antibodies (rabbit polyclonal, Abcam) were used

as a negative control and for chromatin preclearing.

Purified DNA was subjected to 35 cycles of PCR with primers flanking the putative hypoxia response element (HRE) (*hCA9-HRE-S*: 5'-TCCTAGCTTTGGTATGGGGGAGAG-3' and *hCA9-HRE-A*: 5'-AGTGACAGCAGCAGTTGCACAGT-3'; 277 bp product) within the *CA9* promoter. Primers flanking the xenobiotic response element (XRE) of the *CYP1A1* promoter (*hCYP1A1-XRE-S*: 5'-CACCTTCGACAGTTCCTCTCCCT-3' and *hCYP1A1-XRE-A*: 5'-CTCCCGGGTGGCTAGTGCTTTGA-3'; 391 bp product) were used as a positive control.

Statistical analysis

Results were analysed by two-tailed unpaired *t* test (Student's test), and $P < 0.05$ was considered significant.

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Results and discussion

TCDD inhibits hypoxia-induced CA IX expression

Our initial aim was to determine whether TCDD treatment influences expression of CA IX. For this purpose, HeLa cells grown in sparse culture were treated under both normoxic and hypoxic conditions with TCDD and DMSO as a control, and CA9 mRNA expression was analyzed using real-time PCR (Fig.1A). As expected, the highest level of CA9 expression was observed in control HeLa cells exposed to hypoxia for 24 h, whereas TCDD treatment significantly decreased CA9 mRNA levels under hypoxic conditions. Due to sparse culture conditions of the treated cells, the expression of CA9 in normoxia was very low and statistically insignificant. Subsequently, results obtained by real-time PCR were confirmed by Western blotting of corresponding protein level (Fig.1B). Treatment of HeLa cells with both concentrations of TCDD under hypoxic conditions resulted in a decrease in CA IX protein.

Expression of *CYP1A1* was also determined by real-time PCR as a control for induction of the dioxin-responsive pathway (Fig.1A). In accord with previous observation [30], hypoxia decreased expression of TCDD-induced *CYP1A1* suggesting reciprocal influence between the hypoxia and dioxin signaling pathways in the cell culture model used in our experiments.

These experiments suggested that TCDD treatment inhibited CA9 expression at the transcriptional level. Therefore, in the next step, we decided to analyze this mode of inhibition utilizing CA9 promoter constructs in reporter gene experiments.

HIF-1 binding site within CA9 promoter is critical for dioxin mediated inhibition

Previous studies have determined the region -174 nt upstream from the transcription start site as the basal CA9 promoter. Moreover, the -50 nt proximal region of the CA9 promoter containing SP1 binding site and HRE was found as crucial for responses to cell density and hypoxia [11, 35].

In order to determine the region critical for CA9 promoter inhibition caused by the dioxin-induced AhR pathway, we used HeLa cells transiently transfected with the -174/+37 luciferase reporter construct [16] and treated with TCDD for 24 h in normoxia or hypoxia. Activation of AhR by TCDD treatment led to reduced activity of the -174/+37 luciferase reporter construct. Simultaneously, in order to examine direct effects of AhR on CA9 transcription, HeLa cells were co-transfected with 1 µg of constitutively active AhR (CAAhR), which is known to activate transcription in a ligand-independent manner. This construct is not subject to repression by the ligand binding domain in the absence of dioxin and may, in fact, under certain conditions show a more potent effect as transactivator than the ligand-occupied wild-type receptor [32]. As shown in Fig.2A, both TCDD treatment and CAAhR co-transfection significantly reduced promoter activities, with CAAhR being a more potent inhibitor.

To confirm functionality of the CAAhR in our assay, CAAhR-expressing HeLa cells were also co-transfected with the XRE-containing reporter plasmid pTX.DIR ([30], Fig.2B). As expected, CAAhR dramatically elevated pTX.DIR reporter activity under normoxia and in the absence of AhR ligand. On the other hand, this activity was markedly diminished under hypoxic conditions to approximately one half of the CAAhR-induced value, thus providing additional evidence of a reciprocal impact between the hypoxia and dioxin pathways under these assay conditions.

CAAhR was also transiently expressed in HeLa cells in the presence of the minimal CA9 promoter (-50/+37) (Fig.2A). In a manner similar to above, overexpression of CAAhR resulted in significant inhibition of the activity of the minimal reporter construct. The minimal CA9 promoter only contains SP1 and HIF-1 binding sites, therefore it seems plausible that the TCDD- and CAAhR-mediated CA9 repression may affect these transcription factors.

In the case of the CA9 promoter, both HIF-1 α and SP1 are indispensable in responsiveness to hypoxic- and cell density-dependent induction. To determine the significance of these factors in CA9 repression, HeLa cells were co-transfected with equal amounts of CAAhR and expression vectors of either HIF-1 α , SP1 or co-factors, and with the minimal CA9 reporter plasmid. Co-

transfection with the empty vector did not have any effect on CAAhR mediated inhibition of reporter gene activity. In contrast, the expression of HIF-1 α completely abrogated CAAhR-mediated inhibition of promoter activity. On the other hand, introduction of SP1 did not have this effect of counteracting CAAhR-mediated repression of CA9 expression (Fig.2C). Moreover, transient co-expression of p300 and CAAhR did not have any effect on CAAhR-mediated inhibition of the hypoxic induction of the CA9 promoter (Fig.2C). It is well known that the co-activator CBP/p300 functionally and physically interacts with HIF-1 α /ARNT as well as with the AhR/ARNT complex and supports their functional activity in a conditionally regulated manner [36].

We have to keep in mind, that AhR could disrupt a HIF-1 α -SP1 complex and by this mechanism reduce CA9 promoter activity. In this situation HIF-1 α , due to its lower abundance comparing to SP1, could be a limiting factor.

TCDD mediated inhibition of CA9 expression is strictly dependent on the presence of ARNT

In order to form a transcriptionally functional complex, both HIF-1 α and AhR must dimerize with their common partner ARNT. It has been reported that these pathways can mutually interfere with one another by competition for recruitment of ARNT [25, 27, 30, 37]. To elucidate whether AhR-mediated inhibition of CA9 expression is dependent on ARNT, we performed transfection experiments using both wild-type and ARNT-deficient mouse hepatoma cell lines. As shown in Fig.3A, hypoxic induction of CA9 reporter activity was observed in wild-type Hepa-1c1c7 cells, but was not detected in Hepa-1c4 cells expressing a functionally deficient form of ARNT [38]. In the presence of TCDD, hypoxic induction of CA9 promoter activity was decreased in a dioxin dose-dependent manner (Fig.3B).

Transient expression of CAAhR did not have any effect on the CA9 promoter activity in Hepa-1c4 cells either under normoxic or hypoxic conditions. On the other hand, the inhibitory effect of CAAhR was restored following co-expression of wild-type ARNT (Fig.3C). Most notably, this inhibition became significant only after hypoxic treatment of Hepa-1c4 cells. The data obtained from these experiments demonstrate that AhR/CAAhR mediated inhibition of CA9 expression is critically dependent on a functional ARNT.

AhR activation directly affects binding of ARNT to CA9 promoter

The fact that both HIF-1 α and AhR require dimerization with ARNT to form functional transcription complexes prompted us to consider the possible rivalry between both transcription factors for ARNT binding. To get a more direct proof about the possibility of cross-talk between hypoxia and dioxin pathways in the regulation of CA9 expression, we performed chromatin immunoprecipitation assay (ChIP). In order to activate CA9 expression, HeLa cells were grown in hypoxia and subsequently treated with TCDD to ensure the activation of AhR. DMSO treated cells were used as a negative control. The cells were then fixed with formaldehyde and shared chromatin was immunoprecipitated with either ARNT specific or IgG control antibodies. Finally, ARNT-specific immunoprecipitation was investigated by PCR reaction. As shown in Fig.4, PCR performed with the primers flanking HRE within the CA9 promoter clearly demonstrates a weaker signal detected after ARNT-specific DNA precipitation in the TCDD treated cells when compared to control cells. The proper functionality and activation of AhR in our assay conditions was confirmed by PCR performed on the same precipitated DNA utilizing primers flanking XRE within the CYP1A1 promoter (Fig.4). This result directly implicates possible inhibition of CA9 expression by decreasing number of ARNT molecules accessible for binding to HRE element within the CA9 promoter.

It has already been postulated by several research groups that activation of one pathway would inhibit the other due to a competition for ARNT or other limiting cellular factors. In accordance with our results obtained from chromatin immunoprecipitation, Chan and colleagues observed in gel mobility shift experiments that AhR inhibited formation of the HIF-1 α /ARNT complex with HREs, and, conversely, that HIF-1 α inhibited formation of the AhR/ARNT complex with an XRE motif [25]. In an earlier report, it was demonstrated *in vitro* that HIF-1 α exhibits a very high affinity for ARNT

resulting in a competition with the activated AhR for the recruitment of ARNT [30]. A similar observation was made following overexpression of HIF-1 α , resulting in an apparent inhibition of the DNA-binding activity of dioxin-activated AhR [30]. In those experiments, however, activation of both hypoxia- and dioxin-signaling pathways was not mutually exclusive because activation of the AhR pathway did not result in inhibition of hypoxia- dependent expression of target genes. On the other hand, there is a conflicting report stating that the DNA-binding activity of AhR/ARNT heterodimers was only slightly reduced under hypoxic conditions [26]. Regardless of the identification of a cross-talk between hypoxia and dioxin pathways, even Pollenz and colleagues failed to detect competition between HIF-1 α and AhR for ARNT binding [39].

Taken together these seemingly conflicting reports would argue that the interplay between both pathways would obviously be dependent on the architecture of the target promoter/enhancer and the battery of cognate transcription factors and co-factors in close connection with the extent of chromatin remodeling but, importantly, competition for ARNT might be a valid scenario for negative regulation. In particular, this model is supported by the limiting ARNT concentrations observed in most cell types.

Very recently it has been reported that the environmentally abundant polycyclic aromatic hydrocarbon benzo[a]pyrene downregulates vascular endothelial growth factor (VEGF) expression through disruption of HIF-1 α and Hsp90 association, which promotes ubiquitination and subsequent degradation of HIF-1 α by the proteasome [40]. This is most probably not the mechanism underlying CAAhR-mediated downregulation of CA9 expression, because, in contrast to the wild-type dioxin receptor, CAAhR lacks the minimal ligand binding domain (PASB domain) and is therefore active in both a ligand- and Hsp90-independent manner [32].

A large scale microarray analysis has recently been employed to determine the extent of cross-talk between the hypoxia- and dioxin-signaling pathways. Interestingly, CA IX was identified among 33 unique transcripts, the expression of which was affected by both pathways [41]. Computational analysis of these genes identified some common DNA motifs present in their regulatory regions. This observation might help to distinguish between results obtained from different experiments done on different cell culture models and with distinct target genes and their regulatory regions. Nevertheless, these results should be interpreted with caution as Lee and colleagues [41] used cobalt chloride as a mimic of hypoxic conditions.

In our work we have demonstrated that the activated AhR can impair hypoxic induction of both CA IX mRNA and protein expression. We have thus observed a cross-talk between hypoxia and AhR pathways in regulation of CA9 expression. This mode of regulation seems to occur primarily at the transcriptional level. We have further provided evidence for competition between HIF-1 α and AhR for limited intracellular levels of ARNT. This mode of competition for recruitment of ARNT may play a role in CA IX regulation in tumors that are exposed to xenobiotics.

From the pathophysiological point of view it is quite conceivable that exposure to dioxin could have a negative influence on normal cells and tissues that rely on protective mechanisms activated by HIF-regulated pathways. However, situation might be more complicated in tumors due to a high plasticity of malignant cells which could react to acute exposure to dioxin or other xenobiotics by a transiently reduced hypoxic response. On the other hand, chronic exposure might induce either a switch to alternative pathways, or to selection of those tumor cells, which are able to return back to the hypoxic phenotype that provides a significant survival advantage in hostile tumor microenvironment. These issues were not addressed in any of the studies published so far and remain to be resolved in different experimental settings that are currently underway.

In conclusion, our observations support the model that the existence of limiting concentrations of bHLH/PAS proteins is involved in the cross-talk among members of this protein family. The expression of CA IX is strongly induced by hypoxia and its distribution in tumor tissues significantly correlates with poor prognosis of many tumor types. Our results extend data about factors and signaling pathways involved in the regulation of CA IX expression.

Acknowledgements

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Figure legends

Fig.1. Effect of TCDD on CA IX expression at mRNA and protein level. (A) Real-time PCR results from HeLa cells treated with 10 nM TCDD or DMSO (0.1%) under normoxic (21% O₂) or hypoxic (2% O₂) conditions demonstrate the levels of CA9 expression. As a control for dioxin-responsive pathway induction, expression of *CYP1A1* was determined. The results are expressed as the ratio of the mRNA of interest to that of the β -actin control and are the means \pm SE of three independent experiments. Means from different conditions/treatments were compared using Student's *t* test ($P < 0.05$). (B) Expression of CA IX protein in TCDD-treated HeLa cells, grown in normoxia or hypoxia for 24 h, was analyzed by Western blotting using specific monoclonal antibody M75. Parallel control dishes were treated with DMSO and maintained in normoxia or hypoxia for the same time period. Anti-actin antibody was used for a loading control. Relative levels of CA IX protein calculated to actin are shown below the blots (ND-not determined).

Fig.2. Transcriptional activity of CA9 promoter constructs after TCDD treatment or co-transfection with CAAhR. (A) HeLa cells transfected with 1 μ g of the promoter-containing luciferase construct (full-length -174/+37 or minimal -50/+37) were either pre-treated with TCDD or co-transfected with 1 μ g of CAAhR expressing plasmid or empty vector (pCMV). Transfected cells were maintained in normoxia or hypoxia for 24 h. CA9 promoter activity was measured 72 h after transfection and calculated as the ratio of luciferase to renilla activity. Results are expressed as the mean percentage of activity of the -174/+37 promoter construct observed in hypoxia \pm SE of 3 independent experiments. (B) Co-transfection of 1 μ g of XRE-driven reporter construct pTX.DIR confirmed the functionality of CAAhR in our assay. Results are expressed as the mean percentage of activity obtained in normoxic culture co-transfected with CAAhR \pm SE of 3 independent experiments. (C) Effect of co-transfection of the same amount of HIF-1 α , SP1 or p300 together with 1 μ g of CAAhR on the activity of the -174/+37 luciferase construct. Co-transfection of an empty vector served as a control. Results are expressed as the mean percentage of activity of the -174/+37 promoter construct observed in hypoxia \pm SE of three independent experiments.

Fig.3. Inhibition of CA9 expression mediated by TCDD or CAAhR depends on the presence of ARNT. (A) Wild-type mouse hepatoma cells Hepa-1c1c7 and the ARNT-deficient subline Hepa-1c4 cells were transfected with the -174/+37 luciferase construct and exposed to normoxia or hypoxia for 24 h. CA9 promoter activity was determined as described in Fig.2. Results are expressed as the mean percentage of activity obtained from the wild-type cells exposed to hypoxia \pm SE of 3 independent experiments. (B) Pre-treatment with TCDD (1 nM or 10 nM) in normoxia or hypoxia of Hepa-1c1c7 cells transfected with 1 μ g of the -174/+37 promoter construct. Results are expressed as the mean percentage of activity obtained from the DMSO-treated cells exposed to hypoxia \pm SE of 3 independent experiments. (C) Effect of 1 μ g of CAAhR co-transfected in Hepa-1c4 cells with or without functional ARNT maintained for 24 h in normoxia and hypoxia. Results are expressed as the mean percentage of activity obtained from the cells with restored ARNT that were co-transfected with an empty vector \pm SE of three independent experiments.

Fig.4. Chromatin immunoprecipitation assay. HeLa cells were treated with 10 nM TCDD in hypoxia. DMSO treated cells were used as a negative control. DNA was immunoprecipitated with antibody specific for ARNT or anti-human IgG antibodies to visualise background signal. Recovered DNA was used in PCR with primers flanking ARNT-binding sites within CA9 (HRE) and *CYP1A1* (XRE) promoters. DNA samples from non-immunoprecipitated extracts were amplified as input. Numbers given below the figures represent relative levels of PCR products (ND-not determined). A representative of three experiments is shown.

Figure 1.

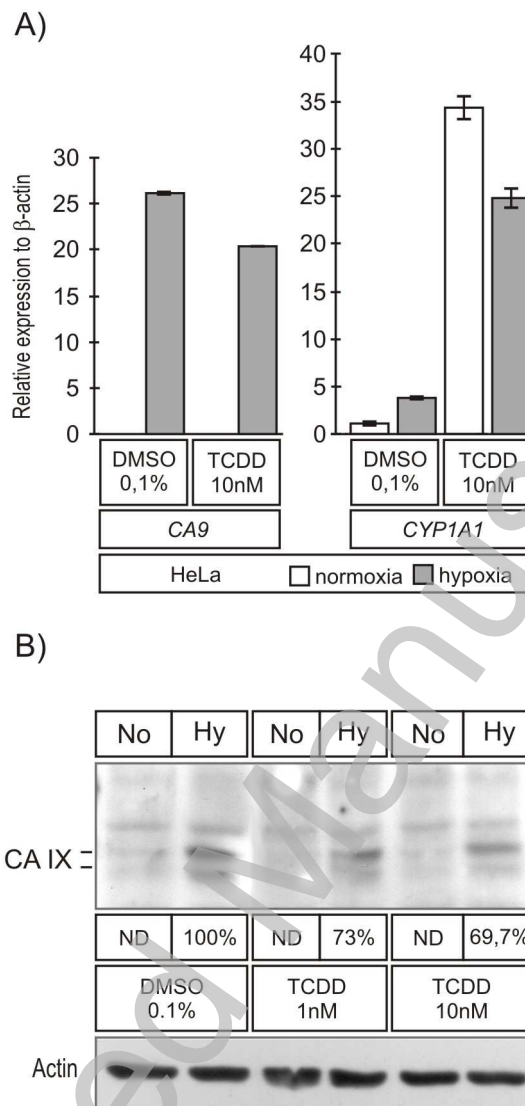


Figure 2.

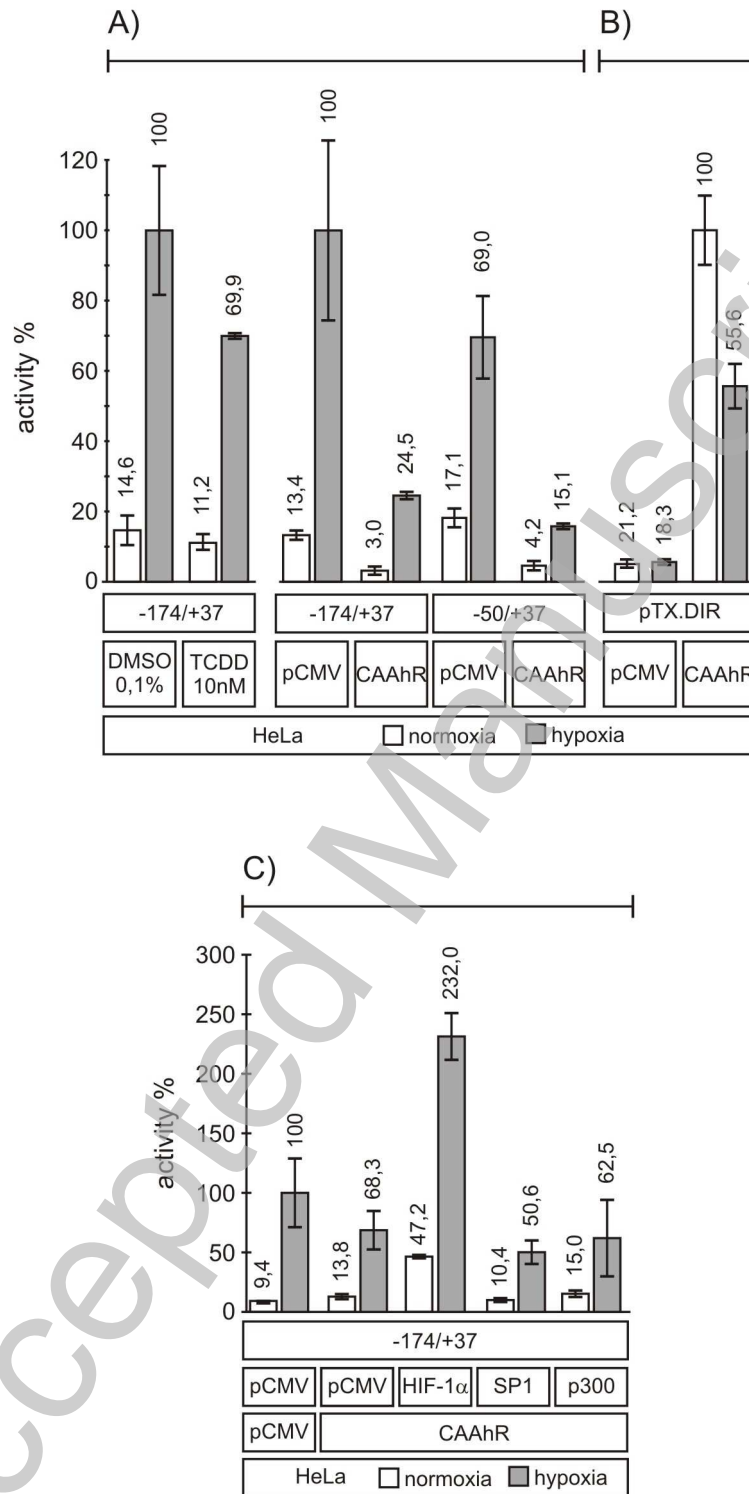
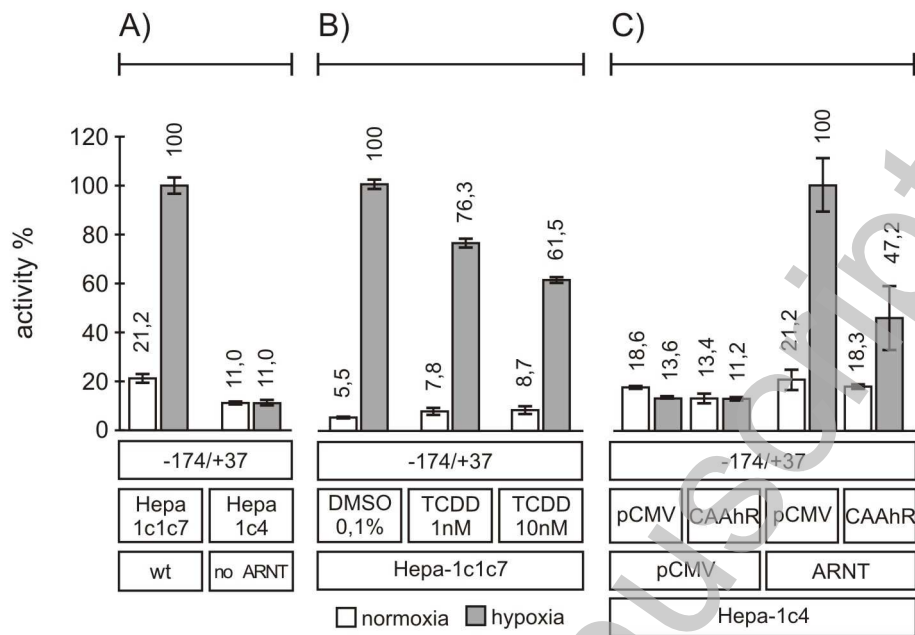
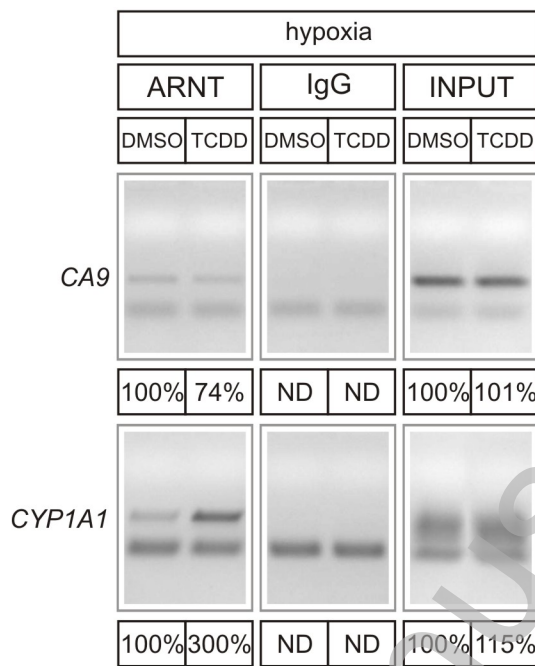


Figure 3.



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Figure 4.



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References

- 1 Pastorekova, S., Parkkila, S., Pastorek, J. and Supuran, C. T. (2004) Carbonic anhydrases: current state of the art, therapeutic applications and future prospects. *J Enzyme Inhib Med Chem.* **19**, 199-229
- 2 Fleming, R. E., Parkkila, S., Parkkila, A. K., Rajaniemi, H., Waheed, A. and Sly, W. S. (1995) Carbonic anhydrase IV expression in rat and human gastrointestinal tract regional, cellular, and subcellular localization. *J Clin Invest.* **96**, 2907-2913
- 3 Hewett-Emmett, D. and Tashian, R. E. (1996) Functional diversity, conservation, and convergence in the evolution of the alpha-, beta-, and gamma-carbonic anhydrase gene families. *Mol Phylogenet Evol.* **5**, 50-77
- 4 Sly, W. S. and Hu, P. Y. (1995) Human carbonic anhydrases and carbonic anhydrase deficiencies. *Annu Rev Biochem.* **64**, 375-401
- 5 Ivanov, S. V., Kuzmin, I., Wei, M. H., Pack, S., Geil, L., Johnson, B. E., Stanbridge, E. J. and Lerman, M. I. (1998) Down-regulation of transmembrane carbonic anhydrases in renal cell carcinoma cell lines by wild-type von Hippel-Lindau transgenes. *Proc Natl Acad Sci U S A.* **95**, 12596-12601
- 6 Kivela, A. J., Saarnio, J., Karttunen, T. J., Kivela, J., Parkkila, A. K., Pastorekova, S., Pastorek, J., Waheed, A., Sly, W. S., Parkkila, T. S. and Rajaniemi, H. (2001) Differential expression of cytoplasmic carbonic anhydrases, CA I and II, and membrane-associated isozymes, CA IX and XII, in normal mucosa of large intestine and in colorectal tumors. *Dig Dis Sci.* **46**, 2179-2186
- 7 Parkkila, A. K., Herva, R., Parkkila, S. and Rajaniemi, H. (1995) Immunohistochemical demonstration of human carbonic anhydrase isoenzyme II in brain tumours. *Histochem J.* **27**, 974-982
- 8 Tureci, O., Sahin, U., Vollmar, E., Siemer, S., Gottert, E., Seitz, G., Parkkila, A. K., Shah, G. N., Grubb, J. H., Pfreundschuh, M. and Sly, W. S. (1998) Human carbonic anhydrase XII: cDNA cloning, expression, and chromosomal localization of a carbonic anhydrase gene that is overexpressed in some renal cell cancers. *Proc Natl Acad Sci U S A.* **95**, 7608-7613
- 9 Liao, S. Y., Brewer, C., Zavada, J., Pastorek, J., Pastorekova, S., Manetta, A., Berman, M. L., DiSaia, P. J. and Stanbridge, E. J. (1994) Identification of the MN antigen as a diagnostic biomarker of cervical intraepithelial squamous and glandular neoplasia and cervical carcinomas. *Am J Pathol.* **145**, 598-609
- 10 Pastorek, J., Pastorekova, S., Callebaut, I., Mornon, J. P., Zelnik, V., Opavsky, R., Zat'ovicova, M., Liao, S., Portetelle, D., Stanbridge, E. J. and et al. (1994) Cloning and characterization of MN, a human tumor-associated protein with a domain homologous to carbonic anhydrase and a putative helix-loop-helix DNA binding segment. *Oncogene.* **9**, 2877-2888
- 11 Kaluz, S., Kaluzova, M. and Stanbridge, E. J. (2003) Expression of the hypoxia marker carbonic anhydrase IX is critically dependent on SP1 activity. Identification of a novel type of hypoxia-responsive enhancer. *Cancer Res.* **63**, 917-922
- 12 Wykoff, C. C., Beasley, N. J., Watson, P. H., Turner, K. J., Pastorek, J., Sibtain, A., Wilson, G. D., Turley, H., Talks, K. L., Maxwell, P. H., Pugh, C. W., Ratcliffe, P. J. and Harris, A. L. (2000) Hypoxia-inducible expression of tumor-associated carbonic anhydrases. *Cancer Res.* **60**, 7075-7083
- 13 Wang, G. L., Jiang, B. H., Rue, E. A. and Semenza, G. L. (1995) Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O₂ tension. *Proc Natl Acad Sci U S A.* **92**, 5510-5514
- 14 Kaluz, S., Kaluzova, M., Chrastina, A., Olive, P. L., Pastorekova, S., Pastorek, J., Lerman, M. I. and Stanbridge, E. J. (2002) Lowered oxygen tension induces expression of the hypoxia marker MN/carbonic anhydrase IX in the absence of hypoxia-inducible factor 1 alpha stabilization: a role for phosphatidylinositol 3'-kinase. *Cancer Res.* **62**, 4469-4477
- 15 Kaluz, S., Kaluzova, M. and Stanbridge, E. J. (2006) The role of extracellular signal-regulated protein kinase in transcriptional regulation of the hypoxia marker carbonic anhydrase IX. *J Cell Biochem.* **97**, 207-216
- 16 Kopacek, J., Barathova, M., Dequiedt, F., Sepelakova, J., Kettmann, R., Pastorek, J. and Pastorekova, S. (2005) MAPK pathway contributes to density- and hypoxia-induced expression of

- the tumor-associated carbonic anhydrase IX. *Biochim Biophys Acta*. **1729**, 41-49
- 17 Ihnatko, R., Kubes, M., Takacova, M., Sedlakova, O., Sedlak, J., Pastorek, J., Kopacek, J. and Pastorekova, S. (2006) Extracellular acidosis elevates carbonic anhydrase IX in human glioblastoma cells via transcriptional modulation that does not depend on hypoxia. *Int J Oncol*. **29**, 1025-1033
- 18 Shimada, T. and Fujii-Kuriyama, Y. (2004) Metabolic activation of polycyclic aromatic hydrocarbons to carcinogens by cytochromes P450 1A1 and 1B1. *Cancer Sci*. **95**, 1-6
- 19 Nebert, D. W., Dalton, T. P., Okey, A. B. and Gonzalez, F. J. (2004) Role of aryl hydrocarbon receptor-mediated induction of the CYP1 enzymes in environmental toxicity and cancer. *J Biol Chem*. **279**, 23847-23850
- 20 Marlowe, J. L. and Puga, A. (2005) Aryl hydrocarbon receptor, cell cycle regulation, toxicity, and tumorigenesis. *J Cell Biochem*. **96**, 1174-1184
- 21 Wilhelmsson, A., Cuthill, S., Denis, M., Wikstrom, A. C., Gustafsson, J. A. and Poellinger, L. (1990) The specific DNA binding activity of the dioxin receptor is modulated by the 90 kd heat shock protein. *EMBO J*. **9**, 69-76
- 22 Jain, S., Dolwick, K. M., Schmidt, J. V. and Bradfield, C. A. (1994) Potent transactivation domains of the Ah receptor and the Ah receptor nuclear translocator map to their carboxyl termini. *J Biol Chem*. **269**, 31518-31524
- 23 Sun, Y. V., Boverhof, D. R., Burgoon, L. D., Fielden, M. R. and Zacharewski, T. R. (2004) Comparative analysis of dioxin response elements in human, mouse and rat genomic sequences. *Nucleic Acids Res*. **32**, 4512-4523
- 24 Kewley, R. J., Whitelaw, M. L. and Chapman-Smith, A. (2004) The mammalian basic helix-loop-helix/PAS family of transcriptional regulators. *Int J Biochem Cell Biol*. **36**, 189-204
- 25 Chan, W. K., Yao, G., Gu, Y. Z. and Bradfield, C. A. (1999) Cross-talk between the aryl hydrocarbon receptor and hypoxia inducible factor signaling pathways. Demonstration of competition and compensation. *J Biol Chem*. **274**, 12115-12123
- 26 Gassmann, M., Kvietikova, I., Rolfs, A. and Wenger, R. H. (1997) Oxygen- and dioxin-regulated gene expression in mouse hepatoma cells. *Kidney Int*. **51**, 567-574
- 27 Nie, M., Blankenship, A. L. and Giesy, J. P. (2001) Interactions between aryl hydrocarbon receptor (AhR) and hypoxia signaling pathways. *Environ Toxicol Pharmacol*. **10**, 17-27
- 28 Pastorekova, S., Zavadova, Z., Kostal, M., Babusikova, O. and Zavada, J. (1992) A novel quasi-viral agent, MaTu, is a two-component system. *Virology*. **187**, 620-626
- 29 Mason, G. G., Witte, A. M., Whitelaw, M. L., Antonsson, C., McGuire, J., Wilhelmsson, A., Poellinger, L. and Gustafsson, J. A. (1994) Purification of the DNA binding form of dioxin receptor. Role of the Arnt cofactor in regulation of dioxin receptor function. *J Biol Chem*. **269**, 4438-4449
- 30 Gradin, K., McGuire, J., Wenger, R. H., Kvietikova, I., Whitelaw, M. L., Toftgard, R., Tora, L., Gassmann, M. and Poellinger, L. (1996) Functional interference between hypoxia and dioxin signal transduction pathways: competition for recruitment of the Arnt transcription factor. *Mol Cell Biol*. **16**, 5221-5231
- 31 Lindebro, M. C., Poellinger, L. and Whitelaw, M. L. (1995) Protein-protein interaction via PAS domains: role of the PAS domain in positive and negative regulation of the bHLH/PAS dioxin receptor-Arnt transcription factor complex. *EMBO J*. **14**, 3528-3539
- 32 McGuire, J., Okamoto, K., Whitelaw, M. L., Tanaka, H. and Poellinger, L. (2001) Definition of a dioxin receptor mutant that is a constitutive activator of transcription: delineation of overlapping repression and ligand binding functions within the PAS domain. *J Biol Chem*. **276**, 41841-41849
- 33 Wood, S. M., Wiesener, M. S., Yeates, K. M., Okada, N., Pugh, C. W., Maxwell, P. H. and Ratcliffe, P. J. (1998) Selection and analysis of a mutant cell line defective in the hypoxia-inducible factor-1 alpha-subunit (HIF-1alpha). Characterization of hif-1alpha-dependent and -independent hypoxia-inducible gene expression. *J Biol Chem*. **273**, 8360-8368
- 34 Lofstedt, T., Jogi, A., Sigvardsson, M., Gradin, K., Poellinger, L., Pahlman, S. and Axelson, H. (2004) Induction of ID2 expression by hypoxia-inducible factor-1: a role in dedifferentiation of hypoxic neuroblastoma cells. *J Biol Chem*. **279**, 39223-39231
- 35 Kaluz, S., Kaluzova, M., Opavsky, R., Pastorekova, S., Gibadulinova, A., Dequiedt, F., Kettmann, R. and Pastorek, J. (1999) Transcriptional regulation of the MN/CA 9 gene coding for the tumor-associated carbonic anhydrase IX. Identification and characterization of a proximal silencer element. *J Biol Chem*. **274**, 32588-32595
- 36 Kobayashi, A., Numayama-Tsuruta, K., Sogawa, K. and Fujii-Kuriyama, Y. (1997) CBP/p300

functions as a possible transcriptional coactivator of Ah receptor nuclear translocator (Arnt). *J Biochem.* **122**, 703-710

37 Kim, J. E. and Sheen, Y. Y. (2000) Inhibition of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-stimulated Cyp1a1 promoter activity by hypoxic agents. *Biochem Pharmacol.* **59**, 1549-1556

38 Hoffman, E. C., Reyes, H., Chu, F. F., Sander, F., Conley, L. H., Brooks, B. A. and Hankinson, O. (1991) Cloning of a factor required for activity of the Ah (dioxin) receptor. *Science.* **252**, 954-958

39 Pollenz, R. S., Davarinos, N. A. and Shearer, T. P. (1999) Analysis of aryl hydrocarbon receptor-mediated signaling during physiological hypoxia reveals lack of competition for the aryl hydrocarbon nuclear translocator transcription factor. *Mol Pharmacol.* **56**, 1127-1137

40 Li, Z. D., Liu, L. Z., Shi, X., Fang, J. and Jiang, B. H. (2007) Benzo[a]pyrene-3,6-dione inhibited VEGF expression through inducing HIF-1alpha degradation. *Biochem Biophys Res Commun.* **357**, 517-523

41 Lee, K., Burgoon, L. D., Lamb, L., Dere, E., Zacharewski, T. R., Hogenesch, J. B. and LaPres, J. J. (2006) Identification and characterization of genes susceptible to transcriptional cross-talk between the hypoxia and dioxin signaling cascades. *Chem Res Toxicol.* **19**, 1284-1293

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