Mol Cell Toxicol (2019) 15:459-467
DOI 10.1007/s13273-019-0050-8

ORIGINAL PAPER

Negative regulation of aryl hydrocarbon receptor by its lysine mutations and exposure to nickel

Xun Che[1] **& Wei Dai**[1,2]

Received: 11 February 2019 / Accepted: 22 May 2019

Abstract

Backgrounds: Aryl Hydrocarbon Receptor (AhR) is a nuclear receptor for many environmental toxicants including TCDD. It mediates the induction of many metabolic enzymes of the cytochrome P450 family after engaging with its ligands. Meanwhile, nickel, also an environmental toxicant, induces a hypoxic response, leading to stabilization of HIF-1α. Lysine residues known for posttranslational modifications are important for subcellular localization, stability, and/or activity of gene products.
Methods: In this study, we have determined nickel's suppressive effect on TCDD-induced expression of CYP1A1 and CYP1A2, two members of the cytochrome P450 super-family. We have also analyzed CYP1A1 and CYP1A2 levels after expression of AhR with various lysine mutations.
Results: We have shown that induction of CYP1A1 and CYP1A2 by TCDD and other AhR ligands is significantly suppressed by hypoxia-mimetics including nickel. This effect is likely due to a competition for β-subunit shared by AhR and HIF-1 transcription factors. We have also found that substitutions of lysines 14, 17, and 21 with arginines suppress the ability of AhR to transactivate P450 genes after treatment with TCDD.
Conclusion: Ligand-mediated AhR activation can be significantly modulated by co-exposure to other environmental toxicant(s) or by its lysine mutations.

Keywords: Aryl hydrocarbon receptor, HIF-1α, TCDD, Nickel chloride, Cytochrome P450, Posttranslational modification, Lysine

Introduction

Persistent industrial organic pollutants including polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs), and dioxins are persistently present in the environment, posing a serious threat to the well-being of human health and ecosystem[1]. These chemical compounds compromise cellular and molecular functions of living organisms through directly or indirectly interaction with nucleic acids and/or key proteins[2]. Aryl hydrocarbon receptor (AhR), as the receptor for dioxins, PCBs and PAHs, plays the major role in cellular responses to the exposure to these compounds. The classical AhR ligand is 2,3,7,8-Tetrachlorodibenzodioxin (TCDD), which is categorized as a Group I carcinogen as classified by International Agency for Research on Cancer (IARC). Upon ligand binding, AhR protein is translocated into the nucleus where it dimerizes with AhR nuclear translocator (ARNT), acting as a transcription factor[3]. NQO1, GST-Ya, and several genes including CYP1A1, CYP1A2 and CYP1B1 from the cytochrome P450 (CYP450) family are among the transcription targets of AhR[4]. Many of these gene products are enzymes involved in the metabolism of xenobiotic compounds, thus functioning as key factors in both the detoxification and carcinogenic transformation/activation in certain cases[5]. CYP1A1 and CYP1A2 are fre-

Electronic supplementary material The online version of this article (doi: 10.1007/s13273-019-0050-8) contains supplementary material, which is available to authorized users.

[1]Department of Environmental Medicine, New York University Langone Medical Center
[2]Department of Biochemistry and Molecular Pharmacology, New York University Langone Medical Center
Correspondence and requests for materials should be addressed to W. Dai (✉wei.dai@nyumc.org)

quently used as markers of AhR induction in both clinical and laboratory studies[5,6]. AhR is also involved in the inflammatory response as an endogenous receptor for kynurenine[7] and appears to possess a transcription-independent function acting as an E3 ligase component[8].

Extensive studies in the past have elucidated major signaling pathways that mediate AhR function *in vivo*. In the classical AhR signaling axis, AhR is released from a protein complex consisting of src, hsp90, and AH receptor-interacting protein (AIP) upon binding with a cognate ligand. This process exposes the nuclear localization signal of AhR, leading to its nuclear translocation. In the nucleus, AhR dimerizes with ARNT, which in turn binds to certain DNA sequences, primarily xenobiotic response elements (XREs), in gene promoters; the binding initiates the transcription of these targeting genes[9]. While ligand binding initiates the AhR activation process, full activation of the receptor molecule requires other regulatory mechanisms including post-translational modifications (PTM). For example, phosphorylation of AhR is closely linked to its subcellular translocation by influencing AhR's binding to its partners through Nuclear Localization Signal (NLS) and Nuclear Export Signal (NES)[10,11]. Moreover, AhR ubiquitination has been characterized to be closely linked to the ligand-dependent activation of AhR[12]. As lysines are distributed in all domains of AhR protein and are potentially subject to many PTMs, it is worthwhile to investigate whether AhR is regulated by lysine modifications besides a few discovered ones.

Nickel, also an environmental toxicant, is widely used in industry. A major route of human exposure to nickel occurs through inhalation, which is believed to have a carcinogenic effect[13]. Recent studies show that nickel induces malignant transformation via perturbing various signaling pathways of inflammation, hypoxia, apoptosis, and autophagy[14-16]. One significant impact of nickel on the cell after treatment is the stabilization of HIF-1α[17], which in turns mediates transcriptional responses to hypoxia after its association with HIF-1β (ARNT) in the nucleus[18]. Interestingly, HIF-1β/ARNT also functions as β-subunit of AhR. The fact that ARNT is a shared β-subunit between HIF-1α and AhR suggests a possible functional interaction between two signaling pathways in response to exposure to two distinct classes of environmental toxicants.

In the current study, we have studied factors that may modulate AhR activities after ligand exposure and found that AhR function can be significantly affected by co-exposure of an environmental toxicant(s) and perturbations of its lysine residues.

Materials & Methods

Cell culture and treatments

Human HepG2, HEK293T and BEAS-2B cell lines were cultured in Dulbecco's Modified Eagle Media (DMEM) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 μg/mL of penicillin and 50 μg/mL of streptomycin sulfate, Invitrogen) at 37°C in a 5% CO_2 incubator.

Antibodies and reagents

Antibodies to AhR, CYP1A1 and CYP1A2 were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA) Antibodies to DYKDDDDK (flag-tag), PARP, Actin, and tubulin were purchased from Cell Signaling Technology (Danvers, MA). HIF-1α antibody was purchased from Bethyl Laboratory (Montgomery, TX). TCDD was purchased from Cambridge Isotope Laboratories (Tewksbury, MA). Nickel chloride, cobalt chloride, benzo[α]pyrene and kynurenine was purchased from Sigma Aldrich (St. Louis, MO).

Plasmids and transfection

Flag-tagged AhR plasmid was purchased from Sinobiological (Wayne, PA). AhR mutants were obtained using Quikchange Site-directed mutagenesis kit by Agilent (Santa Clara, CA) following the company protocol. Briefly, 20-40 nucleotide long mutation primers covers several adjacent mutations were incubated with the wild type AhR plasmid template, and mixture of Quikchange enzymes, buffer, dNTP in the synthesis thermal cycling. Then template was digested by Dpn I enzyme. The obtained DNA were transformed into competent cells for selection and plasmid purification. All obtained plasmids were sequenced by GENEWIZ (South Plainfield, NJ). For transfection, plasmids and polyJet (SignaGen, Rockville, MD) were diluted by serum free DMEM medium and then mixed, dropped into cell culture medium, following protocol of the transfection reagent. Transfection efficiency was estimated to be above 90%.

Western blotting

After indicated treatment, cells were washed once with cold phosphate-buffered saline (PBS) and collected in SDS sample buffer (10 mM Tris-HCl, pH 7.4, 1% SDS, 1 mM Na_3VO_4, and 1x protease inhibitor). Samples were then boiled in heat block for 5 minutes and sonicated for 30 seconds. Protein concentration were measured using Nanodrop 2000 (ThermoFisher, Waltham, MA) and diluted to same concentration for SDS-PAGE analysis followed by immunoblotting. Pri-

mary antibody-bound proteins were detected using an alkaline phosphatase-linked secondary antibody with an ECF Western blotting system (Amersham, Piscataway, NJ). All Western blotting results are representative of at least three independent experiments.

Results

Nickel suppresses CYP1A induction by AhR ligands

To confirm the CYP1A2 induction by AhR ligands in HepG2, we exposed the cells to different concentrations of TCDD for 24 h. Western blotting revealed that that 0.1 nM TCDD was capable of inducing CYP1A2 expression and that the induction was more pronounced when the concentration was further increased (Figure 1A). For comparison, significant induction of CYP1A1 occurred only when concentration of TCDD reached 1 nM. We also noticed that AhR was somewhat reduced when cells were treated with high concentrations of TCDD. We then exposed HepG2 cells to 10 nM TCDD for various times and equal amounts of cell lysates were blotted for both CYP1A1 and CYP1A2. We noticed that CYP1A2 induction occurred as early as 3 h after treatment with TCDD and that CYP1A2 protein levels kept increasing up to at least 48 h (Figure 1B). CYP1A1 exhibited a similar induction pattern except that a significant induction was only observed at 6 h post treatment. This could also be caused by different antibody sensitivity to detect the proteins.

Given that TCDD is frequently mixed with other chemical toxicants including metal nickel in the environment, we asked whether nickel had any effect on AhR-induced gene expression. HepG2 cells treated with either TCDD alone, or together with different concentrations of $NiCl_2$ for 24 h were analyzed. Western blotting revealed that both CYP1A1 and CYP1A2 protein levels were suppressed by co-treatment with nickel in a concentration-dependent manner (Figure 2A). The decrease in CYP1A1 and CYP1A2 was associated with an increase in HIF-1α levels, consistent with the fact that HIF-1α is stabilized by the hypoxia mimetic[19]. Moreover, the decrease was not due to enhanced cell death as PARP-1 cleavage was not observed after treatment with 400 μM of $NiCl_2$. Again, TCDD caused reduction of AhR, which appeared to be rescued by inclusion of $NiCl_2$ at a high concentration (400 μM, Figure 2A).

To confirm that nickel is capable of suppressing TCDD-induced CYP1A2 expression, we also performed the time course experiment in which TCDD-treated cells were cultured in the presence or absence of $NiCl_2$ for various times. We observed that nickel suppressed the levels of both CYP1A1 and CYP1A2 in a time-dependent manner. Specifically, induced expression of both CYP1A1 and CYP1A2 became detectable by 6 h and that the increase in their protein levels lasted at least up to 48 h post TCDD treatment (Figure 1B). However, nickel suppressed the magnitude of increase in both CYP1A1 and CYP1A2 upon TCDD exposure (Figure 2B). By 48 h post TCDD treatment, CYP1A1 decreased to almost the pretreatment level in the presence of nickel, again indicating the negative effect of nickel on TCDD-induced cytochrome P450 gene expression. HIF-1α induction by nickel was observed in the co-treated samples.

To eliminate the possibility that nickel-mediated suppression of TCDD-induced gene expression was cell-type specific, we also treated BEAS-2B cells with TCDD in the presence of various concentrations of $NiCl_2$ for 24 h. Immunoblotting showed that $NiCl_2$ at a concentration of 200 μM already greatly suppressed CYP1A2 induction by TCDD (Figure 3A). HIF-1α induction by $NiCl_2$ was intact in a concentration-dependent manner.

As AhR functions as a cellular receptor for a large family of xenobiotics, we then determined whether this suppressive effect by nickel is exclusive to TCDD.

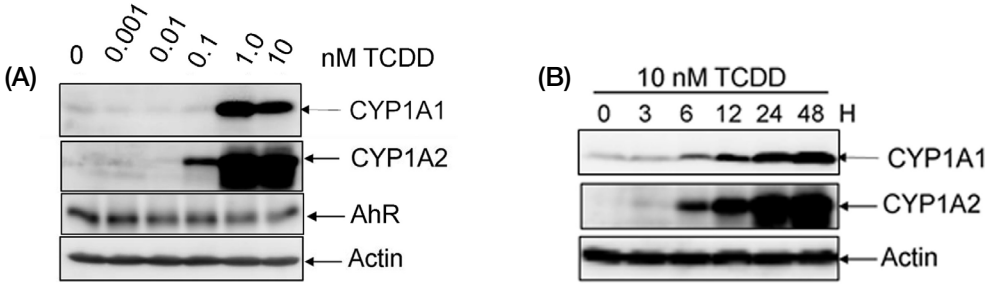

Figure 1. TCDD, an AhR ligand, induces CYP1A1/2 expression. (A) HepG2 cells were treated with indicated concentrations of TCDD for 24 h and then harvested and lysed. Equal amounts of cell lysates from various treatments were blotted for CYP1A1, CYP1A2, AhR, and β-actin. (B) HepG2 cells were treated with 10 nM TCDD for various times as indicated. At the end of treatment, cells were harvested and lysed and equal amounts of cell lysates were blotted for CYP1A1, CYP1A2 and β-actin. Each experiment was performed at least three times and the representative results are included.

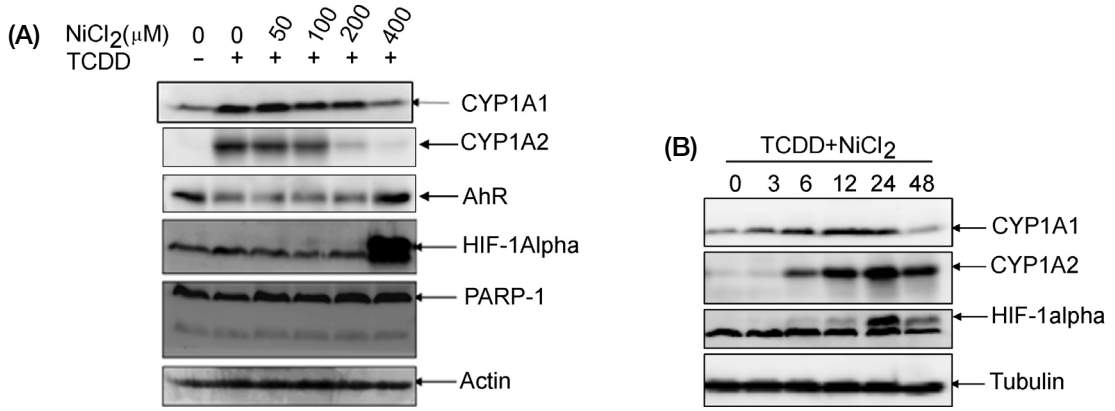

Figure 2. Nickel suppresses CYP1A induction by TCDD. (A) HepG2 cells were treated with either medium control or 10 nM TCDD, together with different concentrations of $NiCl_2$ as indicated. Cells were harvested after 24 h treatment. Equal amounts of cell lysates were blotted for CYP1A1, CYP1A2, AhR, HIF-1α, PARP-1, and β-actin. (B) HepG2 cells were incubated in 10 nM TCDD together with 400 nM $NiCl_2$ for indicated times before cells were harvested and lysed. Equal amounts of cell lysates were blotted for CYP1A1, CYP1A2, HIF-1α, and α-Tubulin. Each experiment was performed at least three times and the representative results are included.

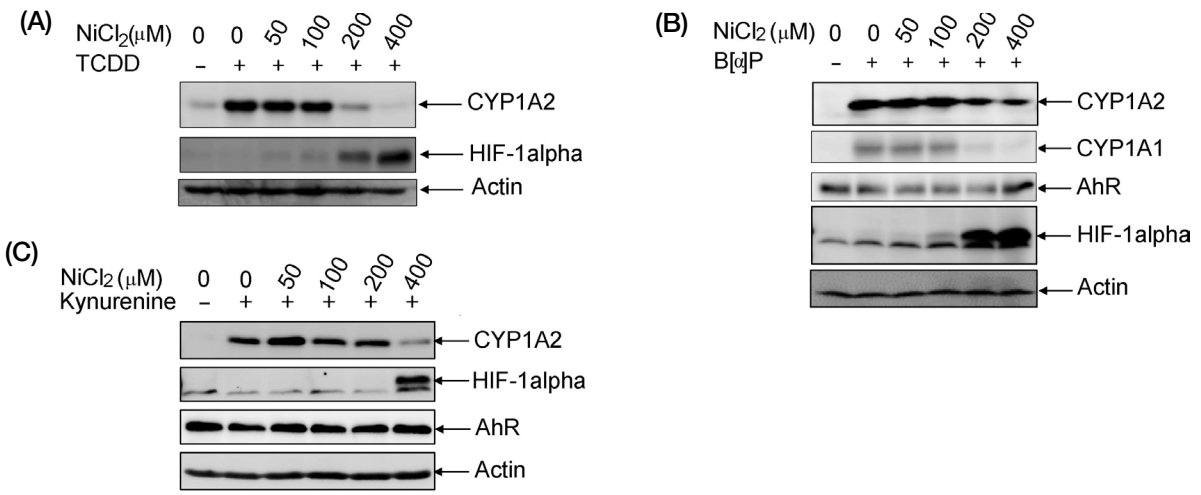

Figure 3. Nickel suppresses CYP1A induction on a dose-dependent manner. (A) BEAS-2B cells were treated with either medium control or 10 nM TCDD, together with different concentrations of $NiCl_2$ as indicated. Cells were harvested after 24 h treatment. Equal amounts of cell lysates were blotted for CYP1A1, HIF-1α and β-actin. (B) HepG2 cells were treated with either medium control or 1 μM B[α]P, together with different concentration of $NiCl_2$ as indicated. Cells were harvested and lysed after 24 h treatment. Equal amounts of cell lysates were blotted for CYP1A1, CYP1A2, HIF-1α, AhR, and β-actin. (C) HepG2 cells were treated with either medium control or different concentrations of $NiCl_2$ as indicated for 6 h and then incubated in 100 nM kynurenine for another 6 h. Cells were harvested and lysed at the end of treatment. Equal amounts of lysate were blotted for CYP1A2, HIF-1α, AhR and β-actin. Each experiment was performed at least three times and the representative results are included.

HepG2 cells treated with Benzo[α]pyrene (B[α]P), an AhR ligand and Group I carcinogen as classified by IARC, in the presence or absence of $NiCl_2$ for 24 h, after which equal amounts of cell lysates were analyzed for gene expression of various genes. As predicted, CYP1A1 and CYP1A2 expression was effectively induced by B[α]P (Figure 3B). Consistent with our early observations, nickel significantly suppressed CYP1A2 protein level at 200 μM or higher.

We then determined whether nickel suppressed CYP1A2 expression induced by kynurenine, which is an endogenous AhR ligands and involved in tryptophan metabolism[20]. Kynurenine induced CYP1A2 expression, peaking at 6 h post-treatment (Figure S1). Again, kynurenine-induced CYP1A2 was significantly suppressed by nickel (Figure 3C). Combined, these observations

strongly suggest that nickel inhibits AhR activation/function after exposure to various categories of ligands.

Suppression of CYP1A by nickel is at the transcription level

Functioning as a hypoxia mimetic, nickel is capable of significantly modulating certain protein levels at post-transcriptional levels. To confirm our hypothesis that CYP1A2 decrease by nickel was at the transcription level rather than at the level of protein degradation, we determined AhR levels in cells treated with MG132, together with co-exposure to $NiCl_2$ and an AhR ligand. We observed that although TCDD or B[α]P caused a decrease of AhR, Ni^{2+} treatment did cause its further decrease (Figure 4). MG132 greatly enhanced the AhR level, indicating that the transcription factor also has a short half-life. Significantly, AhR levels had no effect on its transactivtion activity because CYP1A2 induced by either TCDD or B[α]P remained suppressed due to Ni^{2+} despite an elevated level of AhR in the presence of MG132. As expected, HIF-1α was stabilized by nickel, which was enhanced by MG132.

CYP1A induction by AhR ligands is suppressed by cobalt

Similar to nickel, cobalt also functions as hypoxia-mimetic, stabilizing HIF-1α. We then exposed HepG2 cells to TCDD alone or together with different concentrations of cobalt chloride. Immunoblotting revealed that $CoCl_2$ was also capable of suppressing the TCDD-induced CYP1A1 and CYP1A2 in a concentration-dependent manner (Figure 5). The suppression of CYP1A2 expression was inversely correlated with HIF-1α induction. Moreover, TCDD-induced reduction of AhR levels was almost blocked by cobalt at a high concentration (400 μM).

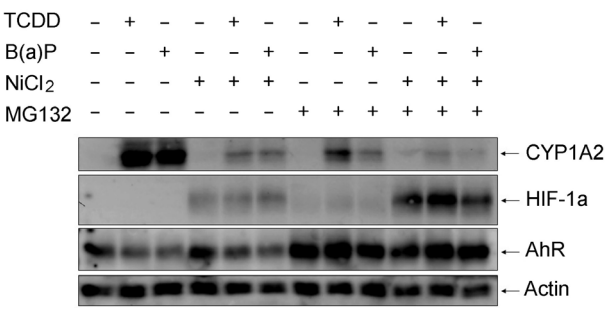

Figure 4. Suppression of CYP1A by nickel is at the transcription level. HepG2 cells were treated with either medium control or 10 μM MG132, together with 10 nM TCDD, 1 μM B[α]P and/or 400 μM $NiCl_2$ as indicated. Cells were harvested and lysed 6 h post treatment. Equal amounts of cell lysates were blotted for CYP1A2, HIF-1α, AhR, and β-actin. Each experiment was performed at least three times and the representative results are included.

AhR lysine mutation suppresses CYP1A induction by AhR ligands

AhR level and/or activity can be significantly regulated by post-translational modifications. Given that lysine residues are subjected to many types of posttranslational modifications including ubiquitination and sumoylation[21], we hypothesized that lysine mutations could alter AhR's responses to xenobiotic exposure. To investigate the function of individual lysines within human AhR protein, we substituted lysine residues with arginines, the latter of which is not subjected to commonly known posttranslational modifications. AhR cDNAs with a cluster of mutated lysines are illustrated in Table 1. To compare the response to treatment between wild-type and single nucleotide mutant, the dose of treatment can be important too. Based on the rational that the most discrepancy of response is more likely to show up when the dose is around the EC50, we plotted a dose-response

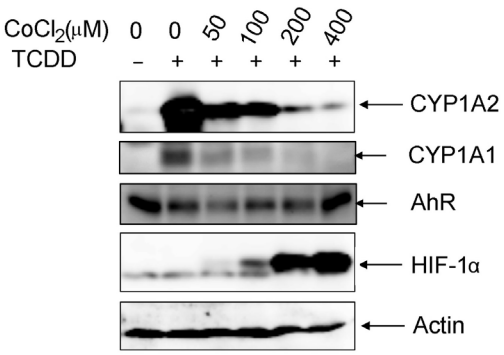

Figure 5. CYP1A induction by AhR ligands is suppressed by cobalt. HepG2 cells were treated with either medium control or 10 nM TCDD, together with different concentrations of $CoCl_2$ as indicated. Cells were harvested and lysed after 24 h treatment. Equal amounts of cell lysates were blotted for CYP1A1, CYP1A2, AhR, HIF-1α, and β-actin as indicated. Each experiment was performed at least three times and the representative results are included.

Table 1. By using QuikChange Lightning Multi Site-Directed Mutagenesis Kit, several mutant AhR plasmids were generated and the mutation sites are illustrated in this table. All indicated mutations are lysine to arginine mutations.

Cluster	Lysine	Domains
#1	14,17,21,24,32,37	bHLH
#2	63,66,80,88	bHLH
#3	242,244,250,251,252,254	PAS
#4	283,290,292,303	PAS
#5	342,356,397,401	PAS, TAD
#6	432,438,448,510,544,560	TAD
#7a	591,628,713,801	TAD
#7b	591,688,801	TAD
#8	372,535,544,749	PAS, TAD

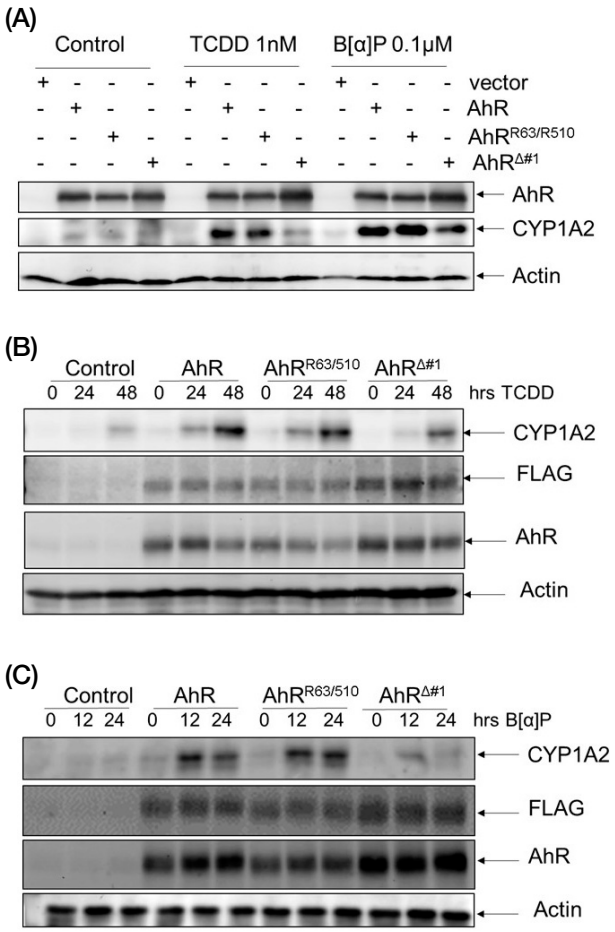

Figure 6. AhR lysine mutation suppresses CYP1A induction by AhR ligands. (A) Flag-tagged wild-type AhR, $AhR^{R63/R510}$, $AhR^{\Delta\#1}$ plasmids were transfected into HEK293T cells. Twenty-four h after transfection, cells were exposed to 1 nM TCDD or vehicle control for 24 h. AhR and CYP1A2 expression was determined by Western Blotting. β-Actin was used as loading control. (B, C) Flag-tagged wild-type AhR, $AhR^{R63/R510}$, $AhR^{\Delta\#1}$ plasmids were transfected into BEAS-2B cells. Twenty-four h after transfection, cells were exposed to 10 nM TCDD, 1 μM B[α]P or vehicle control for indicated times. AhR and CYP1A2 expression was determined by Western blotting. β-Actin was used as loading control. Each experiment was performed at least three times and the representative results are included.

curve for determination of the EC50s in terms of CYP1A2 induction by TCDD or B[α]P (Figure S2). One nanomole per liter for TCDD or 0.1 μM for B[α]P 0.1 were estimated to be EC50s for either chemicals and are used in the following comparison. Expression plasmids of AhR (wild-type and its mutated counterparts) are transfected into HEK293T cells for 24 h, after which transfected cells were treated with 1 nM TCDD or 0.1 μM B[α]P. Plasmids of sumoylation-deficient mutants of AhR were also transfected into cells as control. Sumoylation-deficient mutant (K63R/K510R) exhibited a similar ability to wild-type AhR in transactivating CYP1A2 expression after treatment with either TCDD and/or B[α]P (Figure 6A). On the other hand, AhR mutant-1 ($AhR^{\Delta\#1}$), which carries mutations at Lysines 14, 17, 21, 24, 32 and 37 suppressed CYP1A2 induction after treatment with either TCDD or B[α]P. The observation with $AhR^{\Delta\#1}$ was also confirmed in BEAS-2B cells. We noticed that transfected AhR was expressed at a much higher level than endogenous one, leading to much higher levels of induction of CYP1A2. However, at 24 h and 48 h for TCDD or at 12 h and 24 h for B[α]P, expression of $AhR^{\Delta\#1}$ consistently led to less induction of CYP1A2 as compared with cells transfected with either wild-type or sumoylation-deficient AhR (Figure 6B & 6C). Other tested lysine mutants did not display significant alterations in mediating CYP expression in HEK293T cells (Figure S3).

Table 2. The mutation sites of mutant #1, #1a and #1b AhRs are illustrated in this table and compared to the sild type AhR. Amino acids not shown in this table are the same among all these plasmids.

	14	17	21	24	32	37
WT	K	K	K	K	K	K
Δ#1	R	R	R	R	R	R
Δ#1a	R	R	R	K	K	K
Δ#1b	K	K	K	K	R	R

$AhR^{\Delta\#1}$ contains totally six lysine mutations (Table 1). It is conceivable that these mutations could cause conformational changes, thus affecting its functions. To eliminate the possibility, two AhR cDNAs, each of which carries a subset of those mutations in $AhR^{\Delta\#1}$, were made and tested for their responses to ligand (s) in activating downstream gene expression. Mutant $AhR^{\Delta\#1a}$ contains lysine mutations 14, 17, and 21 whereas $AhR^{\Delta\#1b}$ contains lysine mutations at 32 and 37 (Table 2). These two mutants, along with wild-type AhR and mutant $AhR^{\Delta\#1}$, were tested for their responses to TCDD or B[α]P treatment in BEAS-2B cells. We observed that CYP1A2 induction mediated by $AhR^{\Delta\#1b}$ was quite comparable to wild-type AhR (Figure 7A and 7B). However, $AhR^{\Delta\#1a}$, similar to $AhR^{\Delta\#1}$, was much less responsive to either TCDD or B[α]P treatment in induction of CYP1A2 expression. These observations thus suggest that the inhibitory effect of $AhR^{\Delta\#1}$ mutant is likely due to lysine mutations but not major alterations in its 3-D structures and that Lys14, Lys17 and Lys21 may be crucial for AhR functions.

Discussion

It has been well documented that AhR mounts a strong cellular response to environmental xenobiotics or

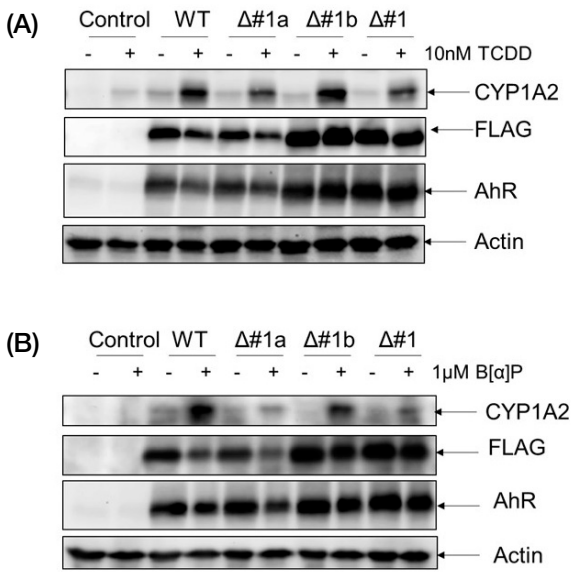

Figure 7. AhR Lys14, Lys17, and Lys21 mutation suppresses CYP1A induction by AhR ligands. Flag-tagged wild-type AhR, $AhR^{\Delta\#1}$, $AhR^{\Delta\#1a}$, and $AhR^{\Delta\#1b}$ (as WT, Δ#1, Δ#1a, Δ#1b in the figure, respectively) expression plasmids were transfected into BEAS-2B cells. Twenty-four h after transfection, cells were exposed to (A) 10 nM TCDD, (B) 1 μM B[α]P or vehicle control for additional 24 h. AhR and CYP1A2 expression was determined by Western Blotting. β-Actin was used as loading control. Each experiment was performed at least three times and the representative results are included.

toxicants[22]. CYP1A1 and CYP1A2 are two of the major downstream targets of AhR, mediating such response as Phase I enzymes for metabolic conversion of TCDD in the cell. There are some differences in the metabolism of TCDD by these two isoenzymes. CYP1A1 causes the formation of more 8-hydroxylated TCDD whereas CYP1A2 preferentially catalyzes the formation of 6-hydroxylated counterpart[23]. CYP1A1 and CYP1A2 also catalyze the conversion of B[α]P into a more potent DNA adduct[24]. However, cytochrome P450 enzymes exhibit some major differences in the metabolism of B[α]P. For example, CYP1A2 is the major enzyme responsible for 7,8-epoxidation, a predominantly toxic form of B[α]P; on the other hand, other CYP enzymes including CYP1A1 and CYP3A4 are believed to catalyze both bio-activation and detoxification reactions at the same time[25]. Overall, the modes of action of metabolites of both TCDD and B[α]P remain poorly understood. The effect of non-metabolized TCDD *in vivo* remains unclear as well.

As a consequence of the interaction between AhR and its ligands, cytochrome P450 genes are trans-activated by AhR. Our current study found that CYP1A enzyme induction can be altered by two factors: (1) lysine mutation on AhR protein; (2) co-exposure to a non-AhR-ligand metal, nickel. Although AhR's downstream targets and functions can vary among different cell types and environments, CYP1A enzyme is consistently sensitive to AhR activation. Thus the revelation of the fact that CYP enzyme induction can be altered by AhR mutation or co-exposure to nickel opens the door for further functional studies on these two AhR regulators.

Our studies identify three lysine residues (Lys14, Lys17, and Lys21) that are important for AhR activity as their mutations cause compromised induction of CYP enzymes by AhR ligands. The mechanism by which these lysine residues affect AhR functions remains unclear. It is likely that post-translational modifications play an important role as lysines can be modified by methylation, acetylation, sumoylation and ubiquitination. Consistent with the notion, it has been shown that nuclear translocation of AhR requires signal recognition of a specific motif(s) and protein-protein interaction, both of which are subjected to post-translational modifications[26,27]. In fact, Lys14, Lys17, and Lys21 are located in a close proximity to the Nuclear Localization Signal. It is reasonable to speculate that modification(s) of these lysine residues may significantly affect the interaction of AhR with importin-α and subsequent nuclear translocation.

Many environmental toxicants to human often co-exist in nature. Extensive research in the past has revealed that many signaling pathways are involved in response to the exposure to various xenobiotics. These responses are either beneficial for chemical elimination or having toxic side effects[28,29]. In addition, signaling pathways in response to different environmental toxicants may have crosstalk or functional interactions, leading to more complicated consequences. AhR can be activated by several categories of xenobiotics such as PCBs, PAHs, and dioxins, which is responsible for both of their elimination and toxic side-effects. HIF-1 functions as a major transcription factor in response to hypoxic stress[30]. It is well known that HIF-1α is stabilized by hypoxia-mimetics including nickel and cobalt[31,32]. HIF-1α also plays important roles in tumor development and progression[31] and it is also believed to contribute to nickel induced cell transformation[33,34]. In the current study, we provide evidence that nickel greatly inhibits AhR-mediated induction of cytochrome P450 enzymes. At the molecular level, functional AhR and HIF-1 transcription factors share a common β subunit ARNT[35]. Consistent with our study, TCDD interferes with the hypoxia response induced by desferrioxamine or cobalt in independent studies[36,37].

Early studies on TCDD toxicity and cytochrome P450 enzymes mostly rely on rats and mice models. However due to the interspecies variation, these cell models

may not be the best for describing the effect of co-exposure in human. In our current study, human HepG2, a well-established liver disease model, is used to examine the effect of co-exposure to toxicants of different categories. We observed that the inhibitory effect of nickel on TCDD-induced cytochrome P450 enzymes is in contrast to an earlier study that used rat liver cells[38]. In this rodent model, only ionic lead, but not nickel, cobalt or cadmium, inhibits CYP1A2 induction. As inhalation is the major route of exposure for both TCDD and nickel, lung epithelial cells are a reasonable target for responding to co-exposure. As anticipated, we demonstrate that BEAS-2B cells, derived from the human bronchial epithelium, display a similar response to that described in HepG2 cells, suggesting that our observed phenomenon may represent the general responses of human cells to co-exposure to AhR ligands and hypoxia-mimetics.

Alterations in expression of CYP enzymes are usually consistent with changes of their corresponding metabolisms. It has been shown that both arsenic and ascorbic acid display an inhibitory effect on CYP1A1 induction by AhR ligand(s)[39,40]. Interestingly, one study suggests the suppressive effect of ascorbic acid can be used as a preventive and/or treatment agent for TCDD intoxication whereas the other study took the same effect of arsenic as an example of the arsenic toxicity. Generally, it is agreed that ligand-induced CYP functions can vary, depending on tissues/organs. Based on CYP expression in HepG2 cells and Beas-2B cells we examined, it is reasonable to state that in the first-exposed organ(s), such as lung, less CYP enzymes can be beneficial after exposure to certain xenobiotics, thus, minimizing the formation of DNA adducts. Therefore, our study provided valuable information of AhR regulation by different factors.

Conclusion

In this study, we have identified two factors that negatively regulate AhR functions: 1) Nickel, a metal toxicant, is capable of contributing to toxicity of environmental AhR ligands such as TCDD; 2) Lysine mutations of AhR can dysregulate its activation due to disrupting normal post-translational modifications. Our findings help elucidate molecular regulation of AhR activation in response to exposure to exogenous ligands including TCDD and B[α]P.

Acknowledgements We thank co-workers in the laboratory for valuable discussions during the course of the study. This work was supported in part by National Institutes of Health (ES020591 and CA150512) and National Institute of Environmental Health Sciences Center grant (ES000260).

Conflict of Interest Xun Che and Wei Dai declare that they have no conflicts of interest.

Human and animal rights The article does not contain any studies with human and animal and this study was performed following institutional and national guidelines.

References

1. Abel, J. & Haarmann-Stemmann, T. An introduction to the molecular basics of aryl hydrocarbon receptor biology. *Biol Chem* **391**, 1235-1248 (2010).
2. Jones, K. C. & de Voogt, P. Persistent organic pollutants (POPs): state of the science. *Environ Pollut* **100**, 209-221 (1999).
3. Probst, M. R. *et al*. Role of the aryl hydrocarbon receptor nuclear translocator protein in aryl hydrocarbon (dioxin) receptor action. *Mol Pharmacol* **44**, 511-518 (1993).
4. Hao, N. & Whitelaw, M. L. The emerging roles of AhR in physiology and immunity. *Biochem Pharmacol* **86**, 561-570 (2013).
5. Pavanello, S. *et al*. Role of CYP1A2 polymorphisms on lung cancer risk in a prospective study. *Cancer Genetics* **205**, 278-284 (2012).
6. Okino, S. T. *et al*. Toxic and Chemopreventive Ligands Preferentially Activate Distinct Aryl Hydrocarbon Receptor Pathways: Implications for Cancer Prevention. *Cancer Prevention Research* **2**, 251-256 (2009).
7. Mezrich, J. D. *et al*. An interaction between kynurenine and the aryl hydrocarbon receptor can generate regulatory T cells. *J Immunol* **185**, 3190-3198 (2010).
8. Ohtake, F. *et al*. Dioxin receptor is a ligand-dependent E3 ubiquitin ligase. *Nature* **446**, 562-566 (2007).
9. Swanson, H. I. & Bradfield, C. A. The Ah-Receptor - Genetics, Structure and Function. *Pharmacogenetics* **3**, 213-230 (1993).
10. Ikuta, T., Kobayashi, Y. & Kawajiri, K. Phosphorylation of nuclear localization signal inhibits the ligand-dependent nuclear import of aryl hydrocarbon receptor. *Biochem Biophys Res Commun* **317**, 545-550 (2004).
11. Kido, S. *et al*. Molecular Mechanisms of Cadmium-Induced Fibroblast Growth Factor 23 Upregulation in Osteoblast-Like Cells. *Toxicol Sci* **139**, 301-316 (2014).
12. Ma, Q. & Baldwin, K. T. 2,3,7,8-tetrachlorodibenzo-p-dioxin-induced degradation of aryl hydrocarbon receptor (AhR) by the ubiquitin-proteasome pathway - Role of the transcription activaton and DNA binding of AhR. *J Biol Chem* **275**, 8432-8438 (2000).
13. Salnikow, K. & Zhitkovich, A. Genetic and epigenetic mechanisms in metal carcinogenesis and cocarcinogenesis: Nickel, arsenic, and chromium. *Chem Res Toxicol* **21**, 28-44 (2008).
14. Son, Y. O. *et al*. Nuclear factor erythroid 2-related factor 2 enhances carcinogenesis by suppressing apoptosis and promoting autophagy in nickel-transformed cells. *J Biol*

Chem **292**, 8315-8330 (2017).
15. Yao, Y. X. *et al*. Cobalt and Nickel Stabilize Stem Cell Transcription Factor OCT4 through Modulating Its Sumoylation and Ubiquitination. *Plos One* **9**, e86620 (2014).
16. Huang, H. S. *et al*. Upregulation of SQSTM1/p62 contributes to nickel-induced malignant transformation of human bronchial epithelial cells. *Autophagy* **12**, 1687-1703 (2016).
17. Maxwell, P. & Salnikow, K. HIF-1: An oxygen and metal responsive transcription factor. *Cancer Biology & Therapy* **3**, 29-35 (2004).
18. Wang, G. L. *et al*. Hypoxia-Inducible Factor-1 is a Basic-Helix-Loop-Helix-Pas Heterodimer Regulated by Cellular O_2 Tension. *Proc Natl Acad Sci USA* **92**, 5510-5514 (1995).
19. Salceda, S. & Caro, J. Hypoxia-inducible factor 1 alpha (HIF-1 alpha) protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions - Its stabilization by hypoxia depends on redox-induced changes. *J Biol Chem* **272**, 22642-22647 (1997).
20. Platten, M., Wick, W. & Van den Eynde, B. J. Tryptophan Catabolism in Cancer: Beyond IDO and Tryptophan Depletion. *Cancer Res* **72**, 5435-5440 (2012).
21. Wang, S. *et al*. Computational method for distinguishing lysine acetylation, sumoylation, and ubiquitination using the random forest algorithm with a feature selection procedure. *Comb Chem High Throughput Screen* **20**, 886-895 (2017).
22. Nebert, D. W. *et al*. Role of aryl hydrocarbon receptor-mediated induction of the CYP1 enzymes in environmental toxicity and cancer. *J Biol Chem* **279**, 23847-23850 (2004).
23. Zhang, Z. Y. *et al*. Preferential inducibility of CYP1A1 and CYP1A2 by TCDD: Differential regulation in primary human hepatocytes versus transformed human cells. *Biochem Biophy Res Commun* **341**, 399-407 (2006).
24. Sattar, A. *et al*. Metabolic proficiency and benzo[a] pyrene DNA adduct formation in APC (Min) mouse adenomas and uninvolved mucosa. *Carcinogenesis* **20**, 1097-1101 (1999).
25. Bauer, E. *et al*. Oxidation of Benzo[a]Pyrene by Recombinant Human Cytochrome-P450 Enzymes. *Chem Res Toxicol* **8**, 136-142 (1995).
26. Perkins, N. D. Post-translational modifications regulating the activity and function of the nuclear factor kappa B pathway. *Oncogene* **25**, 6717-6730 (2006).
27. Faus, H. & Haendler, B. Post-translational modifications of steroid receptors. *Biomed Pharmacother* **60**, 520-528 (2006).
28. Che, X. *et al*. p27 suppresses cyclooxygenase-2 expression by inhibiting p38 beta and p38 delta-mediated CREB phosphorylation upon arsenite exposure. *Biochimica Et Biophysica Acta-Molecular Cell Research* **1833**, 2083-2091 (2013).
29. Hu, L. Y. *et al*. Arsenic-induced sumoylation of Mus81 is involved in regulating genomic stability. *Cell Cycle* **16**, 802-811 (2017).
30. Xu, D. Z. *et al*. Plk3 Functions as an Essential Component of the Hypoxia Regulatory Pathway by Direct Phosphorylation of HIF-1 alpha. *J Biol Chem* **285**, 38944-38950 (2010).
31. Yang, Y. L. *et al*. Polo-like kinase 3 functions as a tumor suppressor and is a negative regulator of hypoxia-inducible factor-1 alpha under hypoxic conditions. *Cancer Res* **68**, 4077-4085 (2008).
32. Li, C. *et al*. Mutual regulation between Polo-like kinase 3 and SIAH2 E3 ubiquitin ligase defines a regulatory network that fine-tunes the cellular response to hypoxia and nickel. *J Biol Chem* **292**, 11431-11444 (2017).
33. Salnikow, K. *et al*. The involvement of hypoxia-inducible transcription factor-1-dependent pathway in nickel carcinogenesis. *Cancer Res* **63**, 3524-3530 (2003).
34. Balamurugan, K. HIF-1 at the crossroads of hypoxia, inflammation, and cancer. *Int J Cancer* **138**, 1058-1066 (2016).
35. Hirose, K. *et al*. cDNA cloning and tissue-specific expression of a novel basic helix-loop-helix/PAS factor (Arnt2) with close sequence similarity to the aryl hydrocarbon receptor nuclear translocator (Arnt). *Mol Cell Biol* **16**, 1706-1713 (1996).
36. Seifert, A. *et al*. Significance of prolyl hydroxylase 2 in the interference of aryl hydrocarbon receptor and hypoxia-inducible factor-1 alpha signaling. *Chem Res Toxicol* **21**, 341-348 (2008).
37. Nie, M. H., Blankenship, A. L. & Giesy, J. P. Interactions between aryl hydrocarbon receptor (AhR) and hypoxia signaling pathways. *Environ Toxicol Pharmacol* **10**, 17-27 (2001).
38. Degawa, M. *et al*. Ionic Lead, a Unique Metal-Ion as an Inhibitor for Cytochrome P450ia2 (Cyp1a2) Expression in the Rat-Liver. *Biochem Biophys Res Commun* **200**, 1086-1092 (1994).
39. Elshenawy, O. H. *et al*. Down-regulation of cytochrome P450 1A1 by monomethylarsonous acid in human HepG2 cells. *Toxicol Lett* **270**, 34-50 (2017).
40. Chang, H. J. *et al*. Ascorbic acid suppresses the 2,3,7,8-tetrachloridibenxo-p-dioxin (TCDD)-induced CYP1A1 expression in human HepG2 cells. *Toxicol In Vitro* **23**, 622-626 (2009).