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The aryl hydrocarbon receptor interacts with ATP5α1, a subunit of the ATP synthase complex, and modulates mitochondrial function

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ABSTRACT

Dioxins, including 2,3,7,8 tetrachlorodibenzo-*p*-dioxin (TCDD), produce a wide range of toxic effects in mammals. Most, if not all, of these toxic effects are regulated by the aryl hydrocarbon receptor (AHR). The AHR is a ligand activated transcription factor that has been shown to interact with numerous proteins capable of influencing the receptor's function. The ability of secondary proteins to alter AHR-mediated transcriptional events, a necessary step for toxicity, led us to determine whether additional interacting proteins could be identified. To this end, we have employed tandem affinity purification (TAP) of the AHR in Hepa1c1c7 cells. TAP of the AHR, followed by mass spectrometry (MS) identified ATP5α1, a subunit of the ATP synthase complex, as a strong AHR interactor in the absence of ligand. The interaction was lost upon exposure to TCDD. The association was confirmed by co-immunoprecipitation in multiple cell lines. In addition, cell fractionation experiments showed that a fraction of the AHR is found in the mitochondria. To ascribe a potential functional role to the AHR:ATP5α1 interaction, TCDD was shown to induce a hyperpolarization of the mitochondrial membrane in an AHR-dependent and transcription-independent manner. These results suggest that a fraction of the total cellular AHR pool is localized to the mitochondria and contributes to the organelle's homeostasis.

Introduction

2,3,7,8 tetrachlordibenzo-*p*-dioxin (TCDD) is a prototypical dioxin and a member of the halogenated aromatic hydrocarbons (HAH) (Poland and Glover, 1980; Bradfield and Poland, 1988; Mandal, 2005). Dioxins are pervasive, being found in soil and at every level of the food chain (Kearney et al., 1973; Poland and Knutson, 1982). These compounds are highly stable, lipophilic molecules that induce a battery of toxic endpoints in mammals (Poland et al., 1979). Among the adverse effects attributed to dioxin exposure are compromised immune system responses, wasting disease, hyperplasia of the liver, birth defects, metabolic syndrome, and an increased risk of diabetes and cancer (Schmidt and Bradfield, 1996; Mandal, 2005). The cellular mechanisms that govern these toxic endpoints have not yet been fully elucidated; however; most, if not all, involve the aryl hydrocarbon receptor (AHR).

TCDD and other planar HAHs have been characterized as exogenous ligands for the AHR (Poland et al., 1976; Poland and Glover, 1977). In the absence of ligand, the AHR is found in the cytosol, complexed with the aryl-hydrocarbon receptor associated protein nine (ARA9, also known as XAP2 and AIP), and a homodimer of heat shock protein 90 (Hsp90) (Perdew, 1988; Carver et al., 1998). Upon ligand binding, the AHR translocates into the nucleus, and becomes an active transcription factor via heterodimerization with the aryl hydrocarbon receptor nuclear translocator (ARNT) (Prokipcak et al., 1990). The AHR/ARNT dimer binds to xenobiotic response element (XRE) regions of DNA to regulate gene expression (Fujisawa-Sehara et al., 1987; Denison et al., 1988). XREs are found in a wide battery of genes, including those that encode cytochrome P450s, such as *CYP1A1*, the canonical marker of dioxin exposure (Poland and Kende, 1977). Though AHR-regulated gene expression is necessary for toxicity, a complete understanding of the relationship between AHR cellular signaling, gene regulation, and the pleiotropic toxicity observed in dioxin exposure has not been achieved.

The AHR cytosolic complex has been previously reported to influence a host of proteins that are intimately involved with cell cycle, cellular homeostasis, mitochondrial function, disease, and cell death (Schmidt and Bradfield, 1996; Tian et al., 2002; Barhoover et al., 2009). The objective of the present investigation was to identify novel protein interactions with the AHR using tandem affinity purification (TAP) and mass spectrometry (MS). Using this approach, a physical

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interaction between the AHR and ATP5α1, a subunit of the ATP synthase complex, was demonstrated. The AHR/ATP5α1 interaction was further verified by co-immunoprecipitation followed by Western blotting and cellular fractionation studies which also identified the AHR in the mitochondria. Interestingly, TCDD treatment of several different cell lines that vary in their level of AHR expression demonstrated a hyperpolarization of the mitochondrial inner membrane that was only observed in AHR expressing cells. Although the physiological role of the AHR within the mitochondria compartment is not fully understood, it is tempting to speculate that the metabolic disorders, including the enigmatic wasting syndrome, produced in some animal species by TCDD may be, in part, related to disruption of AHR function within the mitochondria. Collectively our findings suggest that the AHR plays a direct role in mitochondrial function and cellular energetics through protein–protein interactions with ATP5α1.

Materials and methods

Materials. Oligonucleotide synthesis was performed at Macromolecule Synthesis Facility at Michigan State University. pTarget and pGEM-T Easy vectors were obtained from Promega (Madison, WI). The pZome1C vector was obtained from Cellzome (Cambridge, UK). The Phoenix-eco cell line was a generous gift from Dr. Garry Nolan (Stanford University, Palo Alto, CA) and the AHR−/− mouse embryonic fibroblast cell line and rabbit polyclonal anti-AHR BEAR3 were generous gifts from Dr. Christopher Bradfield (University of Wisconsin-Madison). The other antibodies used were obtained from the following sources: mouse monoclonal anti-ATP5α1 (cat #ab14748) and rabbit polyclonal anti-Hsp90 (cat #ab19021) were obtained from Abcam (Cambridge, MA), mouse monoclonal anti-COXIV (cat #A21349) was obtained from Invitrogen, and rabbit anti-LDH was a generous gift from Dr. John Wang (Michigan State University). Goat polyclonal anti-SODII (cat #sc18503), donkey anti-goat (cat #sc2033), goat-anti rabbit (cat # sc2004) and mouse (cat #sc2005), normal mouse IgG (cat #sc2025) and protein G resin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal mouse anti-rabbit IgG, light chain specific, DyLight 488 conjugated (cat #211-482-171) and goat anti-mouse IgG, light chain specific, DyLight 594 conjugated (cat#151-515-174) were obtained from Jackson Immunoresearch (West Grove, PA). Protease inhibitor tablets (cat #11836170001) were purchased from Roche (Indianapolis, IN). All other chemicals were reagent grade and purchased from Sigma Aldrich (St. Louis, MO).

Plasmids. The cDNAs for the murine AHR and green fluorescent protein (GFP) were amplified using the following primers: AHR: 5′-ggatccccaccatgagcagcggcgccaacatcacc-3′ and 5′-ggatcctgcactctgcaccttgcttagg-3′ GFP: 5′-gggggatccaccatggtgagcaagggcgac-3′ and 5′-gtggatccccgggcccgcggtaccgtcgactgc-3′ The amplicons were cloned into p-Target and pGEM-T Easy, respectively. Each cDNA was subcloned into pZome1C vector. Use of the pZome1C vector places the TAP-tag at the C terminal of both the AHR and GFP gene. Clones were isolated with ampicillin selection and sequence verified.

Cell culture and dosing regime. The Phoenix-eco and Hepa1c1c7 cells were maintained in DMEM with L-glutamine and supplemented with 10% cosmic calf serum, 100 units/mL penicillin, 100 μg/mL streptomycin, and 1 mM sodium pyruvate. Hepa C12 cells were maintained in DMEM with L-glutamine and supplemented with 10% cosmic calf serum and 1 mM sodium pyruvate. Tissue culture media and supplements were purchased from Invitrogen (Carlsbad, CA) and cosmic calf serum was purchased from HyClone (Waltman, MA). Hepa1c1c7 TAP-AHR and TAP-GFP were grown in the above media, supplemented with puromycin (2 μg/mL) (US Biological, Swampscott, MA). The AHR−/− mouse embryonic fibroblast (MEF) cell line was maintained in DMEM with L-glutamine and supplemented with 10% fetal calf serum, 100 units/mL penicillin, 100 μg/mL streptomycin, 1 mM sodium pyruvate, and 1× non-essential amino acids. AHR−/− MEF cell lines with TAP-AHR or TAP-GFP were grown in the above media and supplemented with puromycin (3 μg/mL). The CH12.LX B cell line derived from the murine CH12 B-cell lymphoma (Arnold et al., 1983), which arose in B10.H-2^{a}H-4^{b}p/Wts mice (B10.A×B10.129), has been previously characterized by Bishop and Haughton (1986) and was a generous gift from Dr. Geoffrey Haughton (University of North Carolina). The BCL-1 B-cell line was derived from a murine B-cell lymphoma that spontaneously arose in a BALB/c mouse (Slavin and Strober, 1978) and was generously provided by Dr. Kathryn H. Brooks (Michigan State University). CH12.LX and BCL-1 cells are mature B cells (surface Ig^{+}) and were grown in RPMI-1640 (Gibco BRL, Grand Island, NY) supplemented with heat-inactivated 10% bovine calf serum (Hyclone, Logan, UT), 13.5 mM HEPES, 23.8 mM sodium bicarbonate, 100 units/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1.0 mM sodium pyruvate, and 50 μM b-mercaptoethanol. Cells were maintained at 37 °C in an atmosphere of 5% CO_2.

Transfection/stable cell line infection. The TAP-AHR and TAP-GFP retroviral vectors were transfected into Phoenix-eco (P-eco) cells for packaging using Lipofectamine (Invitrogen) via manufacturer's instructions. After the initial incubation (5 to 8 h, 37 °C) in the presence of DNA, the media was removed and replaced with Phoenix cell growth media containing chloroquine (25 μM). Cells were incubated for 24 h (37 °C) and media was replaced with fresh Phoenix cell growth media and incubated for an additional 24 h (32 °C). Cellular debris was removed from the media by centrifugation (3 min, 67*g*, in RT7, Sorvall, Rockford, IL), and purified by filtration through a 0.45 μm membrane filter (Millipore, Billerica, MA). Hepa1c1c7 wild type and MEF AHR−/− target cell lines were exposed to this virus containing media for 3 h at 32 °C and 5% CO_2. After this incubation, Hepa media containing 15 μg/mL polybrene was added and target cells were incubated for 24 h (32 °C). Media was replaced and the target cells were cultured at 37 °C to approximately 80% confluence. Cells were then passaged and selected using Hepa media containing 2 μg/mL puromycin.

Western blot analysis. Total protein samples were prepared and concentrations determined as previously described (Lowry et al., 1951; Lee et al., 2006). Proteins were separated on 4–12% Nu-Page Bis-Tris gels (Invitrogen), transferred to nitrocellulose membranes, and probed with various antibodies as previously described (Vengellur and LaPres, 2004). Westerns blots were visualized using ECL Western blotting substrate (Pierce, Rockford, IL)

Quantitative real-time PCR analysis. The AHR-TAP and GFP-TAP MEF AHR−/− cell lines were used for functional analysis of the TAP-AHR. *CYP1A1* gene expression was examined after exposure to vehicle (0.01% dimethyl sulfoxide, DMSO) or TCDD (10 nM) for 6 h. Total RNA was extracted from the cells using TRIzol (Invitrogen) per manufacturer's protocol. RNA concentrations were determined by UV spectrophotometry (Nanodrop, Wilminton, DE). Total RNA (1 μg) was reverse-transcribed using anchored oligo $(dT)_{18}$ primers and M-MLV reverse transcriptase (Invitrogen). Reactions were carried out according to manufacturer's protocol. CYP1A1 gene expression was measured by quantitative real-time PCR (qRT-PCR) using the SYBR-Green system and an ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA) (Vengellur and LaPres, 2004). The primers used were: CYP1A1, 5′-AAGTGCAGATGCGGTCTTCT-3′ and 5′-AAAGTAGGAGGCAGGCACAA-3′; Hypoxanthine-guanine phosphoribosyltransferase (HGPRT) 5′-AAGCCTAAGATGAGCGCAAG-3′ and 3′-TTACTAGGCAGATGGCCACA-5′.