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Mitochondrial-targeted aryl hydrocarbon receptor and the impact of 2,3,7,8-tetrachlorodibenzo-p-dioxin on cellular respiration and the mitochondrial proteome

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ABSTRACT

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor within the Per-Arnt-Sim (PAS) domain superfamily. Exposure to the most potent AHR ligand, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), is associated with various pathological effects including metabolic syndrome. While research over the last several years has demonstrated a role for oxidative stress and metabolic dysfunction in AHR-dependent TCDD-induced toxicity, the role of the mitochondria in this process has not been fully explored. Our previous research suggested that a portion of the cellular pool of AHR could be found in the mitochondria (mitoAHR). Using a protease protection assay with digitonin extraction, we have now shown that this mitoAHR is localized to the inter-membrane space (IMS) of the organelle. TCDD exposure induced a degradation of mitoAHR similar to that of cytosolic AHR. Furthermore, siRNA-mediated knockdown revealed that translocase of outer-mitochondrial membrane 20 (TOMM20) was involved in the import of AHR into the mitochondria. In addition, TCDD altered cellular respiration in an AHR-dependent manner to maintain respiratory efficiency as measured by oxygen consumption rate (OCR). Stable isotope labeling by amino acids in cell culture (SILAC) identified a battery of proteins within the mitochondrial proteome influenced by TCDD in an AHR-dependent manner. Among these, 17 proteins with fold changes ≥ 2 are associated with various metabolic pathways, suggesting a role of mitochondrial retrograde signaling in TCDD-mediated pathologies. Collectively, these studies suggest that mitoAHR is localized to the IMS and AHR-dependent TCDD-induced toxicity, including metabolic dysfunction, wasting syndrome, and hepatic steatosis, involves mitochondrial dysfunction.

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1. Introduction

The aryl hydrocarbon receptor (AHR), one of the PAS domain family members, is a ligand-activated transcription factor that mediates the toxic response to several prominent environmental pollutants, such as halogenated aromatic hydrocarbons and polycyclic aromatic hydrocarbons (McIntosh et al., 2010). Among these toxicants, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is the most potent AHR ligand (Mandal, 2005). TCDD exposure alters multiple signaling pathways in a species- and tissue-specific manner, showing various deleterious physiological effects such as tumor promotion, endocrine disruption, chloracne, wasting syndrome, and hepatic steatosis (Mandal, 2005). In the absence of a ligand, the AHR resides in cytoplasm in complex with a homodimer of the heat shock protein of 90 kDa (HSP90) and an immunophilin-like protein called the AHR-interacting protein (AIP,

Abbreviations: ACOT2, acyl-CoA thioesterase 2; AHR, aryl hydrocarbon receptor; AIP, AHR-interacting protein; ARNT, AHR nuclear translocator; COX4, cytochrome c oxidase subunit 4; CPOX, coproporphyrinogen-III oxidase; CYB5, cytochrome b5; EGFR, epidermal growth factor receptor; ENTPD2, ectonucleoside triphosphate diphosphohydrolase 2; ETC, electron transport chain; H6PD, hexose-6-phosphate dehydrogenase; HSP, heat shock protein; IMS, inter-membrane space; mitoAHR, mitochondrial AHR; MTS, mitochondrial targeting signal; OCR, oxygen consumption rate; OXPHOS, oxidative phosphorylation; PAS, Per-Arnt-Sim; RCR, respiratory control ratio; ROS, reactive oxygen species; SILAC, stable isotope labeling by amino acids in cell culture; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; TOMM, translocase of the outer-mitochondrial membrane.

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also known as ARA9, or XAP2) (Carver et al., 1998; Heid et al., 2000; LaPres et al., 2000). In the presence of a ligand, the AHR is translocated to the nucleus and forms a heterodimer with the AHR nuclear translocator (ARNT). The AHR:ARNT heterodimer acts as a functional transcription factor by binding to a specific nucleotide sequence, called a dioxin-responsive element or xenobiotic-responsive element, in regulatory regions of DNA and alters target gene expression (e.g., cytochrome P450 monooxygenases, UDP-glucuronosyltransferases, glutathione S-transferases, and TCDD-inducible poly(ADP-ribose) polymerase) (McIntosh et al., 2010).

As many drug-metabolizing and detoxification enzymes induced by TCDD are linked to reactive oxygen species (ROS) generation, oxidative stress is commonly associated with TCDD exposures (Kennedy et al., 2013; Lu et al., 2011; Shen et al., 2005; Shertzer et al., 2006). Mitochondria play an important role in TCDD-induced oxidative stress. TCDD exposure causes AHR-dependent mitochondrial ROS production, a shift in thiol redox state between cytosol and mitochondria, suppression of mitochondrial electron transport chain (ETC) activities, and mitochondrial membrane hyperpolarization in hepatocytes (Aly and Domenech, 2009; Bansal et al., 2014; Senft et al., 2002a, 2002b; Shen et al., 2005). In addition, the AHR has been linked to mitochondria-to-nucleus stress signaling (Biswas et al., 2008). Moreover, the AHR can induce changes in metabolic flux, independent of transcription (Tappenden et al., 2011). Given that mitochondria are critical components of cellular metabolism, the main sites for energy production, and that many pathophysiological effects linked to TCDD exposure (e.g. diabetes, wasting syndrome, hepatic steatosis, and embryonic development) are related to metabolic pathways, researchers have focused on mitochondrial dysfunction as a potential player in TCDD-induced toxicity (Aly and Domenech, 2009; Angrish et al., 2011; Carreira et al., 2015; Diani-Moore et al., 2010, 2013; Forgacs et al., 2010, 2013).

Recently, high-throughput proteomic analysis uncovered an interaction between the AHR and ATP5α1, an ATP synthase subunit, and MRPL40, a mitochondrial ribosomal protein. Interestingly, these interactions were lost upon exposure to TCDD (Tappenden et al., 2011, 2013). In addition, the cytosolic binding partners of the AHR (i.e., AIP and HSP90) can bind the mitochondrial translocase of outer-membrane complex, and facilitate mitochondrial import of proteins lacking classic mitochondrial targeting sequences (MTS), such as the AHR. Therefore, we investigated the putative mitochondrial localization of the AHR (mitoAHR) and AHR-mediated TCDD-induced mitochondrial changes. Here, we demonstrate that TOMM20 is important for mitochondrial import of mitoAHR and that mitochondrial function is negatively impacted by TCDD exposure in an AHR-dependent manner. Furthermore, using stable isotope labeling by amino acids in cell culture (SILAC), we identified a battery of mitochondrial proteins whose expression was influenced by TCDD in an AHR-dependent manner.

2. Materials and methods

2.1. Cell culture

The mouse hepatoma cell line, hepa1c1c7, was grown in Dulbecco's modified Eagle's Medium (DMEM) with L-glutamine (#11965, Gibco, Life Technologies, Grand Island, NY) supplemented with 10% cosmic calf serum (Hyclone, GE, Logan, UT), 1 mM sodium pyruvate (#11360, Gibco), 100 U/mL penicillin and 100 μg/mL streptomycin (#15140, Gibco). The mouse hepatoma cell line, hepac12, was grown in DMEM with L-glutamine (#11965, Gibco) supplemented with 10% cosmic calf serum (Hyclone) and 1 mM sodium pyruvate (#11360, Gibco). All cell culture work was performed under standard cell culture conditions (5% CO_2, 35% humidity and 37 °C) in a NAPCO 7000 incubator (NAPCO, Winchester, VA) unless specified.

2.2. Preparation of intracellular fractions

Nuclear, cytosolic, and mitochondrial fractions were isolated using protocols adapted from previous reports (Frezza et al., 2007; Vengellur and LaPres, 2004; Yang et al., 2009). Cells were washed with cold PBS (4 °C) and removed from the plate surface by being scraped in mitochondrial buffer A (250 mM sucrose, 20 mM HEPES, 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM ethylene glycol-bis(2-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF)). Each sample was then homogenized with 100 strokes in a Dounce homogenizer on ice. A 50 μL aliquot was saved and represents a whole cell lysate. Insoluble material was removed by centrifugation (400 ×*g* for 10 min at 4 °C) and the pellet was collected for further preparation of a nuclear fraction (see below). The supernatant was cleared by centrifugation (10,000 ×*g* for 10 min at 4 °C). The subsequent supernatant was collected as a cytosolic fraction and the pellet was resuspended in mitochondrial buffer A for further preparation of mitochondrial fraction. The resuspended pellet was centrifuged to remove insoluble material (400 ×*g* for 10 min at 4 °C). The supernatant was further cleared by centrifugation (10,000 ×*g* for 10 min at 4 °C) and aspirated and the mitochondrial pellet was obtained for further analysis. The nuclear pellet was suspended with nuclear extraction buffer [20 mM Tris (pH 7.5), 1.5 mM magnesium chloride ($MgCl_2$), 420 mM KCl, 20% glycerol, 2 mM dithiothreitol, 1 mM sodium orthovanadate, and 0.4 mM PMSF and Complete-mini EDTA-free protease inhibitor (Roche Applied Science, Indianapolis, IN)], incubated for 30 min at 4 °C, and, then, cleared by centrifugation (17,000 ×*g* for 30 min at 4 °C). The supernatant was collected and represents the nuclear fraction. Each intracellular fraction was stored at −80 °C until appropriate assays were performed.

2.3. siRNA knockdown of AIP or TOMM20

When cells were 50% confluent, the siRNAs specific for AIP (Ambion s62179 (siAIP1), s62181 (siAIP2), Life Technologies), and the Silencer® Select Negative Control no. 1 siRNA (Ambion) were transfected into hepa1c1c7 cells using Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. For TOMM20 knockdown, the siRNA for TOMM20 (Tomm20 ON-TARGET plus, SMARTpool L-006487-01-0005, Dharmacon, GE) and the nontargeting siRNA (Dharmacon, GE) were transfected into hepa1c1c7 cells. After a 72 h incubation, cells were washed three times in cold (4 °C) phosphate buffered saline (PBS) and harvested with 1 mL of mitochondrial buffer A per 15 cm plate. Cells were stored at −80 °C until purification of the nuclear, cytosolic, and mitochondrial fraction was performed.

2.4. Protein concentration determination

Protein concentrations for samples used for the SILAC experiments were determined using Pierce™ BCA protein assay kit (Thermo Scientific, Waltham, MA). Protein concentrations for samples used for all other experiments were determined using Bio-Rad (Hercules, CA) Bradford assay kit and bovine serum albumin (BSA) standards (Lowry et al., 1951).

2.5. Trypsin treatment of digitonin extracted mitochondria

Protease protection in combination with digitonin extraction of mitochondria was adapted from a previous report (Griparic and van der Bliek, 2005). Briefly, purified mitochondria were washed with mitochondrial buffer A without dithiothreitol and PMSF and diluted to 1 μg/μL. Mitochondrial samples (400 μg) were then treated with trypsin (100 μg/mL) in the presence and absence of varying concentrations of digitonin (final concentration = 0, 0.2, 0.4, 0.6, 1.0, 1.5, 2.0 mg digitonin/mg protein). Samples were rotated at 4 °C for 30 min and digestion was stopped with the addition of cold 20% trichloroacetic acid to a final concentration

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