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Activation of AHR mediates the ubiquitination and proteasome degradation of c-Fos through the induction of *Ubcm4* gene expression

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ABSTRACT

The ubiquitin-proteasome system (UPS) is a specific, non-lysosomal pathway responsible for the controlled degradation of abnormal and short-half-life proteins. Despite its relevance in cell homeostasis, information regarding control of the UPS component gene expression is lacking. Data from a recent study suggest that the aryl hydrocarbon receptor (AHR), a ligand-dependent transcription factor, might control the expression of several genes encoding for UPS proteins. Here, we showed that activation of AHR by TCDD and β-naphthoflavone (β-NF) results in *Ubcm4* gene induction accompanied by an increase in protein levels. UbcM4 is an ubiquitin-conjugating enzyme or E2 protein that in association with ubiquitin ligase enzymes or E3 ligases promotes the ubiquitination and 26S proteasome-mediated degradation of different proteins, including p53, c-Myc, and c-Fos. We also present data demonstrating increased c-Fos ubiquitination and proteasomal degradation through the AHR-mediated induction of UbcM4 expression. The present study shows that AHR modulates the degradation of proteins involved in cell cycle control, consistent with previous reports demonstrating an essential role of the AHR in cell cycle regulation.

1. Introduction

The aryl hydrocarbon receptor (AHR) is a ligand-dependent transcription factor that is a member of the bHLH-PAS (basic-helix-loop-helix-Per-ARNT-Sim) superfamily. Upon binding to ligands, AHR translocates from the cytoplasm to the nucleus, heterodimerizes with the AHR nuclear translocator (ARNT) and binds to xenobiotic response elements (XREs) located in the promoter of its target genes. The result is an up-regulation of the expression of a battery of genes encoding xenobiotic-metabolizing enzymes, such as cytochrome P450s (CYP1A1, CYP1A2, CYP1B1), NAD(P)H quinone oxidoreductase, and UDP-glucuronosyl-transferase 6 (Gonzalez and Fernandez-Salguero 1998). AHR also modulates the expression of such genes as those involved in heart and liver development (Fernandez-Salguero et al., 1995; Fernandez-Salguero et al., 1997), homeostasis of the immune system (Rodriguez-Sosa et al., 2005), neurogenesis (Latchney et al., 2013), cholesterol and glucose metabolism (Reyes-Hernandez et al., 2009; Sato et al., 2008), and cell proliferation and apoptosis (Elizondo et al., 2000). Data from a recent study in which the liver gene profile between *AhR-null* and wild-type (WT) mice were compared suggest that AHR might control the expression of several genes coding for the ubiquitin-proteasome system (UPS) (Reyes-Hernandez et al., 2010).

UPS is a specific, non-lysosomal pathway responsible for the controlled degradation of abnormal and short-half-life proteins residing in the nucleus, cytosol and endoplasmic reticulum (Salomons et al., 2010; Sorokin et al., 2009). Because a wide range of proteins are UPS substrates, including transcription factors, regulators of cell cycle, and signal transductions effectors (Scheffner et al., 1998), alterations in the UPS degradation pathway are associated with the development of various diseases (Salomons et al., 2010).

Protein ubiquitination is a process involving three steps. First, an ATP-dependent ubiquitin-activating enzyme (E1) activates ubiquitin (Ub) via C-terminus adenylation, followed by the formation of a high-energy bond thioester. Second, the activated Ub is transferred from E1 to the active-site cysteine of an ubiquitin-conjugating enzyme (E2), preserving the thioester bond. Then, an

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ubiquitin-protein ligase (E3) that recognizes the target protein promotes Ub transfer from E2 to the protein substrate resulting in poly-ubiquitination. Finally, the poly-ubiquitinated target protein is recognized and degraded by the 26S proteasome (Chen et al., 2013).

Mouse UbcM4, also known as UbcH7 or Ube2L3 (human) and E2-F1 (rabbit) (Harbers et al., 1996), is an 18-kDa ubiquitin-conjugating enzyme or E2 protein that mainly consists of the catalytic conserved domain UBC. UbcM4 is classified as an E2 class I enzyme (Harbers et al., 1996) and is necessary for normal mouse development; its inactivation results in abnormal placental development, causing a lethal phenotype (Martinez-Noel et al., 1999). UbcM4 also acts as a cell cycle regulator, delaying entry into S phase (Whitcomb and Taylor 2009), and as a co-activator for steroid receptors, modulating the transcriptional activity of the progesterone receptor, androgen receptor and retinoic acid receptor (Verma et al., 2004). Increased expression of UbcM4 reduces the ability of the glucocorticoid receptor to regulate the expression of its target genes (Garside et al., 2006). As an E2 enzyme, UbcM4 participates in the ubiquitination of different proteins, including p53, Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), the NF-κB precursor (p105), c-Myc, and c-Fos (Moynihan et al., 1999).

The immediate-early gene c-Fos is a transcription factor that heterodimerizes with members of the Jun family to form the AP-1 transcription factor, which activates target genes via CRE domains, thereby regulating several cellular processes including cell proliferation and differentiation, apoptosis and oncogenic transformation (Bossis et al., 2003; Gonzalez et al., 2008; Güller et al., 2008; Tu et al., 2013). c-Fos alone plays an important role in processes such as tumorigenesis, cell transformation, proliferation, angiogenesis, tumor invasion and metastasis. Indeed, dysregulation of c-Fos is linked to several pathological conditions: its overexpression is associated with increased proliferation of human hepatocytes, promoting the development of hepatocarcinoma and osteosarcoma (Güller et al., 2008), whereas its deficiency causes osteoporosis (Shur et al., 2005) and is associated with tumor progression in ovarian carcinoma (Mahner et al., 2008). Inhibition of c-Fos also suppresses human colon carcinoma cell growth (Pandey et al., 2012).

We have previously shown that AHR activation results in an increase in UbcM4 levels together with an increase in p53 ubiquitination and degradation (Reyes-Hernandez et al., 2010). However, several questions arose from this work: (i) Does the induction of UbcM4 through AHR activation occur at the transcriptional level? (ii) Do any other AHR ligands, in addition to TCDD, cause similar effects? (iii) Does AHR-dependent UbcM4 induction also results in the ubiquitination and degradation of other substrates of this E2 enzyme? (iv) Is the increase in ubiquitination and protein degradation after treatment with AHR ligands a direct consequence of increased levels of UbcM4?

Therefore, the aim of the present study was to determine and characterize the mechanism by which AHR regulates *Ubcm4* gene expression and to assess whether AHR activation modifies c-Fos ubiquitination and proteasome-dependent degradation.

2. Materials and methods

2.1. Materials

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) was purchased from AccuStandard (New Haven, CT, USA). β-Naphthoflavone (β-NF), MG132 (Z-Leu-Leu-Leu-al), cycloheximide, and actinomycin-D (Act-D) were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.2. Cell culture

Hepa 1c1c7 (Hepa c7) and c12 B15ECiii2 (Hepa c12, AHR-deficient) cells were obtained from American Type Culture Collection (Manassas, VA, USA). The cells were grown in 100 mm dishes with MEM-α medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA) and 1% antibiotic/antimycotic (Invitrogen, Carlsbad, CA, USA) at 37 °C in humidified incubator with a 5% CO_2 atmosphere.

2.3. Real-time quantitative PCR (qPCR) analysis

Briefly, total RNA was prepared from cells extracts using the TRIzol reagent according to the manufacturer's instructions (Invitrogen, Camarillo, CA, USA). RNA was quantified spectrophotometrically at OD_{260}. Subsequently, RNA integrity was evaluated by electrophoresis through 1% agarose gels. cDNA for the quantitative PCR assay was prepared from 2 μg total RNA using SuperScript First-Strand Synthesis (Invitrogen, Carlsbad, CA, USA) and oligo dT. The PCR reactions were performed using a StepOne Real-Time PCR System (Applied Biosystems, Branchburg, NJ, USA) and analyzed by the comparative threshold cycle (Ct) method. The genes encoding *Ubcm4, Cyp1a1* or *18S* ribosomal RNA (rRNA, endogenous) were amplified in a single PCR reaction to allow for normalization of the mRNA data. The PCR reaction mixture contained 2 μl of cDNA, 1x TaqMan Universal PCR Master Mix (Applied Biosystems, Branchburg, NJ, USA) and 0.9 μM and 0.25 μM primers and probes, respectively. The primers and probes sequences used for *UbcM4* were 5′-TGCCAGTCATTAGTGCTGAAAACT-3′ (forward), 5′-GGGTCATTCACCAGTGCTATGAG-3′ (reverse), and probe (FAM) AAGACTGACCAAGTAATCC. The probes used for *Cyp1a1* and *18S* were obtained from Applied Biosystems (Branchburg, NJ, USA) with identification numbers ID Mm00487218_m1 and ID Mm00507222_s1, respectively.

2.4. Western blot analysis

Cells cultures were lysed in 500 μl of buffer containing 50 mM HEPES, 0.5 M NaCl, 1.5 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 1% Triton-X100 and minicomplete protease inhibitor cocktail. Protein concentrations were determined using the Bradford reaction (Bio-Rad, Hercules, CA, USA). Aliquots (40 μg) were solubilized in sample buffer [60 mM Tris–HCl, pH 6.8; 2% sodium dodecylsulfate (SDS); 20% glycerol; 2% mercaptoethanol; 0.001% bromophenol blue] and subjected to 10% SDS-polyacrylamide gel electrophoresis. The proteins were transferred to a nitrocellulose membrane using a mini trans-blot device (Bio-Rad, Hercules, CA, USA) at a constant voltage of 100 V for 3 h in transfer buffer (48 mM Tris–HCl, 39 mM glycine, pH 8.3; 20% methanol). Following transfer, the membranes were blocked overnight at 4 °C in the presence of 2% nonfat dry milk and 0.5% bovine serum albumin (BSA) in blocking buffer (25 mM Tris–HCl, pH 7.5; 150 mM NaCl) and subsequently incubated at 4 °C for 3 h with goat polyclonal anti-UbcM4 (1:3000; Abcam, Cambridge, MA, USA), anti-c-Fos (1:500; Abcam, Cambridge, MA, USA) or anti-actin (1:1000; Zymed, San Francisco, CA, USA) antibodies diluted in buffer (25 mM Tris–HCl, pH 7.5; 150 mM NaCl; 0.1% Tween 20; 0.05% nonfat dry milk; 0.05% BSA). After washing, the membranes were incubated with corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies: HRP-goat anti-rabbit IgG (Zymed, San Francisco, CA, USA) or HRP-rabbit anti-goat IgG (Pierce, Rockford, IL, USA), for 2 h at 4 °C. The membranes were washed, and the immunoreactive proteins were detected using an ECL western blotting detection kit (Millipore, Billerica, MA, USA). The integrated optical density of the bands was quantified by densitometry using the Image J Software.