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Stability of the aryl hydrocarbon receptor and its regulated genes in the low activity variant of Hepa-1 cell line

Andria Humphrey-Johnson, Rawia Abukalam, Sakina E. Eltom *

Department of Biochemistry and Cancer Biology, Meharry Medical College, 1005 D.B. Todd Blvd., Nashville, TN 37208, USA

HIGHLIGHTS

- LA1 variant of Hepa-1 mouse hepatoma cell line and WT are compare for TCDD induction and AhR stability.
- Turnover of CYP1A1 and other related genes are much slower in LA1 compared to WT.
- Actinomycin D reversed TCDD-induced depletion of AhR protein in WT but not in LA1.
- Actinomycin D stabilized the constitutive depletion of AhR in LA1 but not in WT.

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ABSTRACT

We examined the expression kinetics of some of the aryl hydrocarbon receptor (AhR)-regulated genes in LA1 variant cells compared to wild type (WT) Hepa-1 mouse hepatoma cell lines, and we investigated the stability of AhR protein as a key step in the function of this receptor. Treatment of both cell types with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) resulted in increased CYP1A1 and CYP1B1 mRNA with a subsequent down regulation of AhR. We show here that co-treatment with transcription inhibitor actinomycin D (ActD) has reversed the TCDD-induced depletion of AhR protein in WT. However, the proteolytic degradation of AhR in absence of TCDD was significantly higher in LA1 cells than in WT, and ActD treatment reduced this loss. Induction of CYP1A1 and CYP1B1 mRNA by TCDD in WT cells each exhibited bursts of activity in the initial hour which were about 3-fold greater than in LAI cells. The induced mRNA levels in LA1 exhibited a slow and sustained increase approximating the WT levels by 20 h. The induction of two other AhR-regulated genes also showed comparable turnover differences between the two types of cell. Thus, altered regulation of the AhR responsive genes in LA1 may result from a difference in AhR stability.

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1. Introduction

The aryl hydrocarbon receptor (AhR) which is a ligand-activated basic helix–loop–helix (bHLH) transcriptional factor (Burbach et al., 1992), binds poly aromatic hydrocarbons (PAHs), including 2,3,7,8 tetrachloro-dibenzo-*p*-dioxin (TCDD), and mediates their toxic responses (Poland and Knutson, 1982). Binding of PAHs to the cytosolic AhR triggers a sequence of events which include the dissociation of AhR from chaperone proteins, including heat shock protein 90 (hsp90) and immunophilin-type chaperon termed ARA9, AIP or XAP2 (Carver and Bradfield 1997; Ma and Whitlock 1997; Meyer et al., 1998; Meyer and Perdew, 1999; LaPres et al., 2000; Kazlauskas et al., 2001). The AhR is then transformed into a form that readily translocates to the nucleus where it forms a heterodimer with the related bHLH, Ah receptor nuclear translocator (ARNT) protein (Hoffman et al. 1991). Binding of this heterodimer to DNA recognition motifs designated as xenobiotic-responsive elements (XREs), results in enhanced transcription of multiple genes (Jones and Galeazzi, 1985; Denison et al. 1989). These genes known as the Ah-responsive genes include *CYP1A1*,

Abbreviations: PAH, polycyclic aromatic hydrocarbon; AhR, aryl hydrocarbon receptor; ARNT, aryl hydrocarbon receptor nuclear translocating protein; AHH, aryl hydrocarbon hydroxylase; Hepa-1, mouse hepatoma cell line; WT, wild type; LA1, low AHH-activity, class I variant; LA2, low AHH-activity, class II variant; CYP1B1, cytochrome P4501B1; CYP1A1, cytochrome P4501A1; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; RT-PCR, reverse transcription-polymerase chain reaction; cDNA, complementary DNA; UGT, UDP-glucuronyltransferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ECL, enhanced chemiluminescence; DRB, 5,6-dichloro-ribofuranosyl benzimidazole.

* Corresponding author. Tel.: +1 615 327 5713; fax: +1 615 327 6442.
E-mail address: seltom@mmc.edu (S.E. Eltom).

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CYP1A2 (Gonzalez and Mackenzie, 1984) and *CYP1B1* (Savas et al. 1994; Bhattacharyya and Brake, 1995). The protein products of these *CYPs* are catalytically active in metabolizing not only many endogenous compounds, such as β-estradiol, but also many drugs, dietary components, mutagens, carcinogens and environmental pollutants (Conney, 1982).

Subsequent to transcriptional activation, the AhR undergoes a rapid depletion leading to substantially decreased cellular levels within hours (Prokipcak and Okey, 1991; Reick et al. 1994; Pollenz, 1996). This ligand-induced down-regulation of the receptor was shown to be blocked by inhibitors to calpain, proteasomes and nuclear export, suggesting a role for calpain and proteasome-dependent degradation and the subcellular localization (Davarinos and Pollenz, 1999; Ma and Baldwin, 2000; Ma et al., 2000; Dale and Eltom, 2006; Dale and Eltom, 2006a).

The mouse hepatoma cell line Hepa1c1c7 (Hepa-1), in which CYP1A1 is highly inducible, is commonly used as a model system to study the regulation of CYP1A1 and other AhR-regulated genes (Miller et al., 1983; Whitlock and Galeazzi 1984). Multiple clones of Hepa-1 were isolated by selection for resistance to benzo[a] pyrene toxicity (Hankinson 1979; Miller et al., 1983). Two of these mutant clones, the low-activity class I (LAI) and the low-activity class II (LA2) variants were identified by their failure to induce *CYP1A1*-dependent aryl hydrocarbon hydroxylase in response to PAHs treatment (Miller et al., 1983; Whitlock and Galeazzi, 1984). The LA1 variant defect was attributed to a decreased transcriptional level of AhR compared to WT (Miller et al., 1983), while LA2 cells express normal level of cytosolic AhR but is defective in nuclear localization due to mutation in *ARNT* gene (Hoffman et al. 1991). Even though the LA1 cells were originally isolated as multiple clones with AhR levels ranging from 5 to 40% of WT and paralleled by equivalent low TCDD-induced CYP1A1 protein levels, these cells have been invariably reported to express only 10% of the wild type *CYP1A1* mRNA level (Miller et al., 1983). However, our analysis of TCDD-induced *CYP1A1* expression in these cells has shown its level to be only slightly lower than that of WT (Eltom et al., 1999), which is consistent with a finding by other investigators using these cells (Sadek and Allen-Hoffmann, 1994). In order to discern the difference of TCDD-inducibility between these two cell lines, in this report we examine the expression kinetics of some AhR-regulated genes in LA1 variant cells compared to WT Hepa-1 cells, and we investigate the kinetics of AhR nuclear-translocation and turnover, key steps in the function of this receptor, as a possible coupled-regulatory mechanism.

2. Materials and methods

2.1. Tissue culture and treatment

Mouse hepatoma cell lines, Hepa-1 WT and mutants (LAI or LA2) were the kind gift of Dr. James Whitlock, Jr. (Stanford University, Stanford, CA). Cells were maintained in Dulbecco's minimum essential medium eagle (DMEM) with high glucose (Sigma) and 5% heat inactivated fetal bovine serum (Gibco), 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin B as fungizone® (Sigma). All cultures were maintained in a humidified atmosphere containing 5% CO_2 and 95% air, at 37 °C. Typically, cells were treated at ~85% confluence with 10 nM TCDD or equivalent volume of DMSO (not to exceed 0.1%) for the indicated times. Cells which were used for RNA isolation and analysis were lyzed in Trizol® reagent immediately following the removal of treatment media. Alternatively, cells were harvested by mechanical scraping in cold PBS, and cell pellets were washed in PBS, lysed and used in fractionation experiments.

2.2. RNA isolation and northern analysis

Northern analysis was done as described (Eltom et al., 1998), using the following probes: mouse *CYP1A1* cDNA (Gonzalez and Mackenzie, 1984), human *CYP1A2* cDNA (Parikh et al. 1997) *GAPDH* cDNA (Fort et al. 1985) and *UDP-glucuronsyl transferase*6* (Vasiliou et al. 1995). Probes were labeled non-radioactively using digoxigenin-dUTP random primed DNA labeling kit (Roche Diagnostics), following the supplier's instructions. For quantification of *CYP1B1* mRNA, a semi-quantitative RT-PCR assay was developed as described previously (Eltom et al., 1999), to quantify the very low levels of CYP1B1 mRNA expressed by Hepa-1 cells.

2.3. Isolation of total cellular proteins

Cells were harvested under denaturing condition by lysis in Trizol. Total RNA was first isolated from the Trizol lysates, subsequently total cellular proteins were isolated from the remaining lysate, as described previously (Eltom et al., 1999).

2.4. Cell fractionation and nuclear translocation experiments

In these experiments, cells were harvested by mechanical scraping in cold PBS, washed two times in cold PBS and suspended and lysed for 30 min at 4 °C in lysis buffer: (1% NP-40, 0.025% SDS in 25 mM Mops buffer pH 7.4, containing 0.02% Na azide, 1 mM EDTA, 10% glycerol, 5 mM EGTA and 20 mM Na molybdate), supplemented with protease inhibitors cocktail: (5 μg/ml leupeptin, 0.15 units/ml aprotinin, 10 μg/ml TLCK, 1 mM PMSF, 5 μg/ml soy bean trypsin inhibitor), and phosphatase inhibitors (1 mM Na orthovanadate and 1 mM Na fluoride). Cell lysates were centrifuged at 2000 rpm for 5 min in microcentrifuge at 4 °C to pellet nuclei. Supernatants were saved at −20 °C until analyzed, and nuclei were washed four times in lysis buffer to remove cytosolic contamination. Nuclear pellets were then homogenized in lysis buffer at 4 °C by sonication on ice bath.

2.5. Protein electrophoresis and immunoblotting

Gel electrophoresis and immunoblotting was done as described (Eltom et al., 1999).

2.6. Measurement of mRNA stability

Hepa-1 WT cells and LA1 cell variant (at passage 9) growing in DMEM medium containing 5% heat inactivated-FBS were treated with 10 nM TCDD or equivalent amount of DMSO (0.1%) for 20 h. Actinomycin D (Sigma) was dissolved at 10 mg/ml in 100% ethanol, and was added to the treatment media at 10 μg/ml final concentration (0.1% ethanol final concentration in medium). At the indicated times, plates were removed and cells were lyzed in Trizol for RNA and protein isolation. No toxic effect of actinomycin D was observed on the cell viability up to 6 h. To assess the validity of GAPDH for loading normalization, and that actinomycin D did not affect GAPDH expression within the experimental time, ribosomal RNA was checked by staining gels with ethidium bromide and were found to match GAPDH signal.

2.7. Reverse transcriptase – polymerase chain reaction (RT-PCR)

Total RNA isolation and semi-quantitative RT-PCR was done as described previously (Eltom et al., 1999).

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