Loss of hepatic aryl hydrocarbon receptor protein in adrenalectomized rats does not involve altered levels of the receptor's cytoplasmic chaperones

Chunja Lee, Anne K. Mullen Grey, and David S. Riddick

Department of Pharmacology and Toxicology, Medical Sciences Building, University of Toronto, Toronto, Ontario, Canada, M5S 1A8

Abstract

The aryl hydrocarbon receptor (AHR) plays physiological roles and mediates adaptive and toxic responses to environmental pollutants. Adrenalectomized rats display decreased hepatic AHR protein levels, with no change in mRNA, and selectively impaired induction of cytochrome P450 1B1. This was similar to reported phenotypes for mice with hepatocyte-specific conditional deletion of AHR-interacting protein (AIP), a chaperone protein of the cytoplasmic AHR complex. In the current study, we demonstrated that adrenalectomy (ADX) and acute dexamethasone (DEX) treatment do not alter hepatic AIP mRNA or protein levels. Also, hepatic protein levels of the 90-kDa heat shock protein and p23 were not altered by ADX or acute DEX treatment. These results suggest that the loss of rat hepatic AHR protein following ADX cannot be explained by changes in the levels of the receptor's cytoplasmic chaperone proteins.

Keywords

aryl hydrocarbon receptor; adrenalectomy; dexamethasone; aryl hydrocarbon receptor-interacting protein; 90-kDa heat shock protein

Introduction

In addition to playing important roles in development and physiology, the aryl hydrocarbon receptor (AHR) also mediates the adaptive and toxic responses to halogenated aromatic hydrocarbons such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and polycyclic aromatic hydrocarbons such as 3-methylcholanthrene (MC). Factors that regulate the expression and function of the AHR remain poorly characterized and we are particularly interested in the modulation of the hepatic AHR pathway by pituitary-dependent hormones (Harper et al. 2006). We showed previously that four days following bilateral adrenalectomy (ADX) rats displayed a ~50% decrease in hepatic AHR protein levels, with no accompanying change in AHR mRNA levels, and a selectively impaired induction of cytochrome P450 1B1 (*CYP1B1*), as opposed to *CYP1A1* and *CYP1A2*, in response to MC treatment (Mullen Grey and Riddick 2009; Mullen Grey and Riddick 2011). These findings were intriguingly

similar to phenotypes previously reported (Nukaya et al. 2010) for mice with hepatocyte-specific conditional deletion of the AHR-interacting protein (*Aip*) gene, which encodes a key chaperone protein of the cytoplasmic AHR complex. These mice displayed a 62% decrease in liver cytosolic AHR protein levels and selectively impaired induction of *Cyp1b1* and the AHR repressor (*Ahr*), as opposed to *Cyp1a1* and *Cyp1a2*, in response to TCDD treatment (Nukaya et al. 2010). This prompted us to investigate whether altered hepatic levels of AIP and other chaperone protein components of the cytoplasmic AHR complex might be implicated in the loss of hepatic AHR protein levels following ADX.

The cytoplasmic AHR exists in complex with multiple chaperones including a dimer of the 90-kDa heat shock protein (hsp90), p23, and AIP (Murray and Perdew 2012). A component of the cytoplasmic AHR complex with structural similarity to the FK506-binding protein class of immunophilins was originally identified independently by three laboratories as AIP (Ma and Whitlock 1997), AHR-associated protein 9 (ARA9) (Carver and Bradfield 1997), and hepatitis B virus X-associated protein 2 (XAP2) (Meyer et al. 1998). AIP appears to contribute to the proper folding of the cytoplasmic AHR, with influences on the subcellular localization, stability and ligand binding of the receptor (Meyer et al. 1998; Meyer and Perdew 1999; LaPres et al. 2000; Petrusis et al. 2000). Hsp90 is also important for the proper folding and stability of the cytoplasmic AHR, maintaining the AHR in a conformation required for ligand binding (Pongratz et al. 1992; Coumailleau et al. 1995) and repressing the ability of the AHR to heterodimerize with the AHR nuclear translocator (ARNT) (Pongratz et al. 1992; McGuire et al. 1994). Finally, p23, which is also known as prostaglandin E synthase 3 (PTGES3) or telomerase-binding protein (TEBP), seems to work along with hsp90 to maintain the cytoplasmic AHR in a ligand-responsive conformation (Kazlauskas et al. 1999; Shetty et al. 2003). Although p23 is an established component of the cytoplasmic AHR complex, it does not appear to be essential for AHR stability and function (Flaveny et al. 2009).

The objective of the present study was to determine if the profound decrease in rat liver cytosolic AHR protein levels seen following ADX is accompanied by changes in levels of the receptor's three key cytoplasmic chaperones: AIP, hsp90, and p23. Our experimental approach involved ADX to remove the endogenous source of glucocorticoids (and other adrenal factors), combined with acute treatment with the potent synthetic glucocorticoid, dexamethasone (DEX).

Materials and Methods

Animals and treatment

Animals were cared for in accordance with the principles of the Canadian Council on Animal Care and experimentation was approved by the University of Toronto Animal Care Committee. Details of the study on ADX rats were described previously (Mullen Grey and Riddick 2009) and a brief summary of the animal work is given here for context. Adult male Fischer 344 rats were purchased from Charles River Canada (St. Constant, QC) and bilateral ADX and SHAM surgeries were performed at 8 weeks of age by an animal surgical technician in the Division of Comparative Medicine, University of Toronto. Rats recovered for 4 days after surgery and then received an intraperitoneal injection of DEX (1.5 mg/kg) or

corn oil vehicle. At 6 h after injection, rats were euthanized and harvested liver tissue was frozen and stored. Total RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform extraction method using Tri-Reagent (Sigma-Aldrich, St. Louis, MO) and cytosol was prepared by differential centrifugation.

Analysis of mRNA levels by real-time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR)

Hepatic levels of mRNA encoding AIP, normalized to β -actin as the internal reference standard, were determined by the comparative threshold cycle relative quantitation method on the ABI Prism 7500 Sequence Detection System (Applied Biosystems, Foster City, CA). β -actin primers were detailed previously (Mullen Grey and Riddick 2009), whereas newly designed AIP primers were synthesized by Integrated DNA Technologies, Inc. (Coralville, IA) with the following sequences: forward, 5'-TGAGGACAGGAGAAGGGATAG-3' and reverse, 5'-AGG GTCATAGAGTAGGGCAAC-3'. The AIP product size was 115 bp. Each 10- μ l PCR reaction contained input cDNA derived from 5 ng of RNA, optimized primer concentrations (AIP, 500 nM; β -actin, 300 nM), and Power SYBR Green Master Mix (Applied Biosystems). Cycling conditions were an initial cycle of 2 min at 50°C and 10 min at 95°C, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min.

Immunoblot analysis

General procedures for electrophoresis, transfer, antibody incubations, chemiluminescence detection, and film scanning and quantitation were described previously (Mullen Grey and Riddick 2009). Blots derived from gels originally loaded with 20 or 25 μ g of hepatic cytosolic protein were probed with the following primary antibodies: mouse monoclonal against human AIP, dilution 1:10,000 (Dr. Christopher A. Bradfield, McArdle Laboratory for Cancer Research, Madison, WI); mouse monoclonal against a human hsp90 β peptide, dilution 1:50,000 (Santa Cruz Biotechnology, Santa Cruz, CA); mouse monoclonal against a human p23 peptide, dilution 1:2,000 (Santa Cruz Biotechnology); and mouse monoclonal against pig β -tubulin, dilution 1:10,000 (Novus Biologicals, Littleton, CO). The immunoreactive signal intensity for each target protein was normalized to the β -tubulin signal intensity for each sample.

Statistical analysis

All data are expressed as mean $\pm$ SD. All statistical analyses were performed on the original raw data and not on the percent control data presented in the figures. Data were analyzed initially using a randomized-design two-way ANOVA to identify significant influences of the two independent variables and their interaction (treatment, surgery, treatment x surgery interaction). Post-test analyses for the planned comparisons (treatment effect, surgery effect) were performed to assess whether there were significant differences between particular groups. Post-tests were Bonferroni-corrected for multiple comparisons and used the mean square residual (pooled variance) and corresponding degrees of freedom from the two-way ANOVA. A result was considered statistically significant if $P < 0.05$.

Results and Discussion

Since AIP plays an important role in the folding and stability of the AHR protein as a component of the receptor's cytoplasmic complex, we assessed hepatic AIP expression at the mRNA and protein levels using stored samples from the same rats shown previously to display a ~50% decrease in liver cytosolic AHR protein levels following ADX (Mullen Grey and Riddick 2009). Neither ADX nor acute DEX treatment altered hepatic AIP mRNA levels (Fig. 1A) or cytosolic protein immunoreactivity levels (Fig. 1B). Similarly, neither ADX nor acute DEX treatment altered liver cytosolic protein immunoreactivity levels for hsp90 (Fig. 2A) and p23 (Fig. 2B), two additional chaperone components of the cytoplasmic AHR complex.

The mechanistic basis for the loss of liver cytosolic AHR protein seen at four days following ADX (Mullen Grey and Riddick 2009) remains unknown. Since there is no accompanying change in the levels of AHR mRNA, it seems most likely that the decrease in AHR protein levels is due to lowered protein stability, perhaps because of accelerated proteolytic degradation. Following ligand activation and translocation of the AHR from the cytoplasm to nucleus where it functions as a transcription factor, an important aspect of signal transduction termination is AHR nuclear export, ubiquitination, and degradation via the 26S proteasome (Pollenz 2002). Future studies should examine whether post-translational modifications (e.g. phosphorylation and SUMOylation) of the AHR or ubiquitin-dependent proteasomal degradation of the AHR are modified by ADX. Although primarily cytoplasmic, the unliganded AHR undergoes dynamic nucleocytoplasmic shuttling; thus, it may be important to study ADX effects on the interaction of the AHR with proteins involved in transport across the nuclear envelope, such as chromosome region maintenance protein-1 (CRM-1) (Berg and Pongratz 2002).

Our working hypothesis is that the loss of hepatic AHR protein seen following ADX is due to the removal of a secreted adrenal factor(s) that plays a physiological role in the maintenance of AHR protein levels in liver. The identity of this factor remains elusive but glucocorticoids have received most attention to date as candidates. We have shown that DEX causes a glucocorticoid receptor (GR)-mediated increase in AHR mRNA, protein, ligand-binding and transactivation in mouse hepatoma cells (Bielefeld et al. 2008). However, acute in vivo treatment with a single dose of DEX shown to selectively activate the GR did not alter expression of AHR (Mullen Grey and Riddick 2009) or its cytoplasmic chaperones, although hepatic ARNT mRNA levels were strongly induced (Mullen Grey and Riddick 2009). A reliable cell culture model of endocrine influences on AHR protein turnover would facilitate the characterization of candidate adrenal factors; however, it remains challenging to study the impact of the removal (as opposed to addition) of putative physiological regulators in cell culture.

A limitation of the current study is that immunoblot-based measurements of total cytosolic levels of chaperone proteins may not be able to detect more subtle changes in the interactions of a chaperone with its client protein, the AHR in this case. Future studies should attempt to use co-immunoprecipitation approaches to determine if the fraction of AHR protein complexed with a given chaperone is altered following ADX. Based on the

work of Nukaya et al. (2010), AIP appears to be a key AHR chaperone for further investigation. Mice with hepatocyte-specific conditional deletion of the *Aip* gene showed decreased liver cytosolic AHR protein levels, were highly resistant to TCDD-induced hepatotoxicity, and regulation of AHR target genes displayed differential dependence on AIP expression (Nukaya et al. 2010). This raises the interesting possibility that different classes of AHR-responsive genes exist and that a subset of such genes showing AIP-dependence may be implicated in TCDD hepatotoxicity.

In summary, we demonstrated that ADX and acute DEX treatment do not alter hepatic levels of AIP, hsp90 and p23, key chaperone proteins of the cytoplasmic AHR complex. Therefore, the loss of rat hepatic AHR protein following ADX cannot be explained by changes in the levels of the receptor's cytoplasmic chaperone proteins.

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Fig. 1.

Effect of acute DEX treatment on hepatic AIP mRNA (A) and protein immunoreactivity (B) in SHAM and ADX male rats. (B) Immunoblot of cytosolic protein (20 μ g) using monoclonal antibodies against human AIP and pig β -tubulin, showing results for two vehicle (V)- and DEX (D)-treated rats per surgical category. Quantitative analysis of AIP mRNA levels, relative to β -actin (A), and AIP protein immunoreactivity levels, relative to β -tubulin (B). Data represent the mean $\pm$ SD of determinations from four rats per group, expressed as a percentage of the mean for the vehicle-treated SHAM rats. The *P* values for the two-way ANOVA main effects were: AIP mRNA (treatment, *P* = 0.4437; surgery, *P* = 0.0323; interaction, *P* = 0.6646) and AIP protein (treatment, *P* = 0.3947; surgery, *P* = 0.5607; interaction, *P* = 0.8734). Planned comparisons via Bonferroni-corrected post-tests identified no treatment or surgery effects.

Fig. 2.

Effect of acute DEX treatment on hepatic hsp90 (A) and p23 (B) protein immuno-reactivity in SHAM and ADX male rats. Immunoblot of cytosolic protein (A, 20 μ g; B, 25 μ g) using monoclonal antibodies against a human hsp90 β peptide (A), a human p23 peptide (B), and pig β -tubulin, showing results for two vehicle (V)- and DEX (D)-treated rats per surgical category. Quantitative analysis of hsp90 (A) and p23 (B) protein immunoreactivity levels, relative to β -tubulin. Data represent the mean $\pm$ SD of determinations from four rats per group, expressed as a percentage of the mean for the vehicle-treated SHAM rats. The *P* values for the two-way ANOVA main effects were: hsp90 protein (treatment, *P* = 0.9117; surgery, *P* = 0.4990; interaction, *P* = 0.8325) and p23 protein (treatment, *P* = 0.4593; surgery, *P* = 0.3993; interaction, *P* = 0.3439). Planned comparisons via Bonferroni-corrected post-tests identified no treatment or surgery effects.