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Functional analysis of six human aryl hydrocarbon receptor variants in human breast cancer and mouse hepatoma cell lines

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ABSTRACT

The aryl hydrocarbon receptor (AHR) is a ligand-dependent transcription factor that mediates the toxic responses of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). The identification and functional analysis of AHR single nucleotide polymorphisms is important in understanding the functional diversity of this receptor as it might give rise to individuals with differing sensitivities to TCDD. In this study, the functional properties of six (I277V, P517S, R554K, V570I, Q666K, and R554K/V570I) human AHR variants were examined in human breast cancer cells (MCF-7 AHR100) and mouse hepatoma cells (Hepa c12) deficient in AHR. *CYP1A1*- or *CYP1B1*-regulated reporter gene assays in AHR100 or Hepa c12 cells exposed to TCDD revealed no significant differences in reporter gene activity among the different AHR variants. In contrast to previous findings describing the AHR-R554K/V570I variant to be transcriptionally deficient, no differences in TCDD-dependent increases in CYP1A1 and CYP1B1 mRNA levels were observed between AHR and AHR-R554K/V570I after 24 h or 48 h of exposure. Chromatin immunoprecipitation assays also revealed similar recruitment patterns of AHR and AHR-R554K/V570I to the 5′-regulatory regions of *CYP1A1* and *CYP1B1*. Collectively, our findings show that none of the AHR variants examined exhibited an altered ability to regulate CYP1A1- or CYP1B1-driven transcription in AHR100 or Hepa c12 cell lines, and that AHR and AHR-R554K/V570I are functionally equivalent in the two cell lines examined.

1. Introduction

The aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor and a member of the basic-helix–loop–helix Per (Period) ARNT (aryl hydrocarbon receptor nuclear translocator) SIM (single-minded) (bHLH/PAS) family. The AHR mediates most, if not all, of the toxic effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (Fernandez-Salguero et al., 1996). TCDD binding to AHR causes its translocation to the nucleus where it dimerizes with ARNT. The AHR/ARNT complex binds to its cognate DNA recognition sequence, termed AHR response element (AHRE) or dioxin response element (DRE), which are located in regulatory regions of genes involved in xenobiotic metabolism and potentially toxic responses, including *cytochrome P4501A1* (*CYP1A1*) and *CYP1B1* (Hankinson, 1995). Although the precise biological role of AHR is unknown, studies in model organisms suggest that orthologs of AHR are involved in growth and development (Hahn, 2002). Moreover, *Ahr*-knockout mice are resistant to TCDD toxicities, but also exhibit a number of diverse phenotypes including decreased liver size and lower fecundity, supporting a role for AHR beyond xenobiotic metabolism (Fernandez-Salguero et al., 1995).

The human *AHR* gene is located on chromosome 7p15 and includes 12 exons, 11 of which give rise to a protein with a theoretical mass of ~96 kDa (Micka et al., 1997). Genetic variations in AHR among laboratory animals result in altered ligand binding and transcriptional regulation by the receptor leading to significant differences in sensitivity to the toxic effects of TCDD (Hahn, 1998). One of the earliest examples of this diversity is the difference in TCDD responsiveness of the C57BL/6J mice relative to the non-responsive DBA/2 mice (Birnbaum et al., 1990). The differences in sensitivity are due to an alanine to valine change at amino acid 375 with the ligand binding cavity that results in an approximate 10-fold decrease in binding affinity for TCDD (Poland et al., 1994). Altered binding affinity cannot solely explain the vast difference in dioxin sensitivity among or within species. The TCDD resistant Han/Wister strain of rat has an intronic mutation that causes the deletion of 38 or 43 amino acids from the transactivation domain (Pohjanvirta et al., 1998). The resulting change influences the regulation of key genes involved in the toxic response to TCDD (Moffat et al., 2010). However, the TCDD-dependent induction of CYP1A1 mRNA and affinity for TCDD are virtually identical between the Han/Wistar and the TCDD-sensitive Sprague–Dawley strains (Pohjanvirta et al., 1998).

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Studies have shown that humans are less sensitive to TCDD exposure compared to other mammals (Connor and Aylward, 2006). A number of single nucleotide polymorphisms (SNPs) in the human *AHR* have been identified and some of them have been associated with reduced receptor function (Harper et al., 2002). The most widely studied polymorphism in the human *AHR* is the R554K; however, the interpretation of the phenotypic effects of this polymorphism is complicated and confusing (Harper et al., 2002). This SNP has been reported to segregate with V570I and *in vitro* experiments suggest that the R554K/V570I variant exhibits a compromised ability to induce TCDD-dependent induction of Cyp1a1 mRNA levels (Wong et al., 2001a). Since these studies were performed in mouse hepatoma cells, it is unknown if the same effects will be observed in human cell lines.

Recently the sequencing of 108 human AHR genes representing six different ethnic groups was reported (Rowlands et al., 2010). The study identified six exonic SNPs, four of which led to changes in amino acid sequence (I277V, R554K, V570I, Q666K). In agreement with previous reports, the R554K was the most abundant SNP and one individual containing AHR-R554K/V570I was also identified (Rowlands et al., 2010). Two newly reported SNPs, I277V and Q666R, reside in the ligand binding domain and in proximity to the transactivation domain, respectively (Rowlands et al., 2010). The functional activities of these newly identified AHR variants have not been evaluated.

The aim of the present study was to characterize the ability of six (I277V, P517S, R554K, V570I, Q666K, and R554K/V570I) human AHR variants, including two newly identified variants (I277V and Q666R) to mediate TCDD-dependent signal transduction using AHR-deficient human breast cancer cells, AHR100 or mouse hepatoma, Hepa c12 cells. The AHR100 cell line displays resistance to the cytotoxic effects of several polycyclic hydrocarbons and has very low levels of AHR but has normal levels of ARNT (Ciolino et al., 2002). Hepa c12 cells also express low levels of AHR and have been previously used to evaluate AHR variant function (Wong et al., 2001a; Ciolino et al., 2002). Our findings show that none of the AHR variants examined exhibited an altered ability to regulate CYP1A1- or CYP1B1-driven reporter gene activity or mRNA expression levels in AHR100 or mouse Hepa c12 cell lines and that AHR and AHR-R554K/V570I are functionally equivalent in the two cell lines examined.

2. Materials and methods

2.1. Materials

TCDD was purchased from Wellington Laboratories (Guelph, ON, Canada). Cell culture media, foetal bovine serum (FBS), and trypsin were all purchased from Wisent (St. Bruno, QC) and from Invitrogen (Carlsbad, CA). SYBR Green PCR Master Mix and 96-well real-time PCR plates were from KapaBiosystems (Woburn, MA). All other reagents were of the highest quality available from commercial sources.

2.2. Cell culture

Benzo[*a*]pyrene resistant MCF-7 human breast cancer cells (referred to as AHR100 cells) were obtained from Dr. Grace Chao Yeh, Centre for Cancer Research, National Institutes of Health (Frederick, MD, US). AHR100 cells were maintained in DMEM (low glucose) containing 10% FBS and penicillin/streptomycin (PEST). Mouse Hepa1c1c7 variant B cells (Hepa c12) were purchased from American Type Culture Collection (ATCC; Manassas, VA) and maintained in α-MEM medium containing FBS and PEST. Cell lines were kept at 37 °C and 5% CO_2/air incubator with 90% humidity, medium was changed three times a week and cells were sub-cultured twice a week.

2.3. Site-directed mutagenesis

All mutants were prepared using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The desired human AHR point mutations were introduced using the following PCR primer pairs; I277V (5′-GCCACCATCCGTACTTGAAATCC-3′ and 5′-GGATTTCAAGTACGGATGGTGGC-3′); P517S (5′-AATTGACCAG TCTCAGGATGTGA-3′ and 5′-TCACATCCTGAGACTGGTCAATT-3′); R554K (5′-TGAAGACATAAACACATGCAGA-3′ and 5′-TCTGCATGTGTTTGATGTC TTCA-3′); V570I (5′-TTCTGGTGAGATTGACTTCAGAG-3′ and 5′-CTCTGAAGTC AATCTCACCAGAA-3′); and Q666K (5′-CAATTGTCCAAAGCAAGACCCAC-3′ and 5′-GTGGGTCTTGCTTTGGACAATTG-3′). The mutated residues are underlined. The following PCR conditions were used for the mutagenesis: 95 °C for 5 min followed by 25 cycles of 95 °C for 1 min, 50 °C for 1 min and 72 °C for 10 min. The plasmid Rc-AHR (Roblin et al., 2004) provided by Dr. Patricia Harper (Hospital for Sick Children, Toronto, Canada) was used as a template in the PCR reactions. The sequence of each construct was confirmed by DNA sequencing.

2.4. Transient transfection and reporter gene assays

AHR100 or Hepa c12 cells were plated (150,000 cells/well) in 12-well plates. The following day, AHR100 cells were transfected with 500 ng of CYP1A1- or CYP1B1-regulated luciferase plasmid, 10 ng AHR variant, 10 ng of ARNT and 100 ng β-galactosidase plasmid to normalize for transfection efficiency. CYP1A1- and CYP1B1-regulated reported gene plasmids have been previously described (MacPherson et al., 2009). Hepa c12 cells were transfected with 500 ng of CYP1A1-, or CYP1B1-regulated luciferase plasmid, 25 ng AHR variant, 25 ng ARNT, 100 ng β-galactosidase plasmid. All transfections were done using Lipofectamine 2000 according to the manufacturer's recommendations (Invitrogen). The next day the medium was changed and the cells were exposed to 10 nM TCDD or DMSO for 24 h. Luciferase activity was measured using the ONE-glo reagent following the manufacturer's instructions (Promega, Madison WI).

2.5. Reverse-transcription PCR

AHR100 cells or Hepa c12 cells were plated in 6-well plates (300,000 cells/well) 1 day prior to transfection. Cell lines were then transfected with 500 ng of AHR expression plasmid (empty vector, wt AHR, or AHR variants) using Lipofectamine 2000. Following transfection cells were exposed to either a single dose of TCDD (10 nM) for 24 h or 48 h or to increasing amounts of TCDD (0.001–10 nM) for 24 h. Total RNA was isolated using RNeasy mini-kits (Qiagen, Mississauga, ON) and 500 ng of total RNA was reverse transcribed using random hexamers and Superscript II according to the manufacturer's instructions (Invitrogen). One microliter of reverse-transcription reaction was used for each qPCR reaction (10 μl). The primers used to amplify human CYP1A1 mRNA were 5′-TGGTCTCCCTTCTCTACACTCTTGT-3′ and 5′-ATTTTCCCTATTACAT TAAATCAATGGTTCT-3′; to amplify human CYP1B1 mRNA were 5′-GGCGACCTCTTCAGATGGATTA-3′ and 5′-TGGCGCTTGATTTCCTTTAAA-3′; to amplify mouse CYP1A1 were 5′-CGTTATGACCATGATGACCAAGA-3′ and 5′-TCCCCAAACTCATTGCTCAGAT-3′; and to amplify mouse CYP1B1 mRNA were 5′-CCAGATCCCGCTGCTCTACA-3′ and 5′-TGGACTGTCTGCACTAAGGCTG-3′. The expression levels of target mRNAs were normalized to those of 18S rRNA and analyzed using the comparative C_T ($\Delta\Delta C_T$) method.

2.6. Chromatin immunoprecipitation (ChIP) assay

For ChIP assays AHR100 cells were plated in 6-well plates (300,000 cells/well) and exposed to either 10 nM TCDD or DMSO for 1.5 h. Three wells of a 6-well dish were pooled and ChIP assays were performed as previously described (Matthews et al., 2007). Immunoprecipitated DNA (1 μl) was PCR amplified using primers 5′-AGGCGTGGACCGAAAATG-3′ and 5′-CTAGGTCTGCGTGTGGCTTCT-3′ for the human *CYP1A1* enhancer region, or 5′-ATATGACTGGAGCCGACTTTCC-3′ and 5′-GGCGAACTTTATCGGGTTGA-3′ for the human *CYP1B1* enhancer region.

2.7. Western blot

AHR100 cells were plated in 6-well plates (300,000 cells/well), transfected with 500 ng of receptor plasmid (empty vector, wt AHR, or AHR-R554K/V570I variant) and exposed to either 10 nM TCDD or DMSO for 24 h. Whole cell extracts were prepared by sonicating cells in 400 μl of hepes-buffer (25 mM hepes pH 7.4, 20 mM sodium molybdate 5 mM EGTA, 10% glycerol, 3 mM magnesium chloride) containing protease inhibitors, 1 mM DTT, 0.5% NP-40 and centrifuged for 10 min at 20,000 × *g* at 4 °C. Twenty micrograms of protein was separated by 8% SDS-PAGE and transferred to nitrocellulose membrane. Membranes were blocked for 4 h before overnight incubation with rabbit anti-human AHR antibodies. The membranes were washed the following day and incubated with anti-rabbit IgG conjugated to horseradish peroxidase. Protein bands were visualized using ECL Advance Western blotting detection kit.

2.8. Statistical analysis

All data are presented as mean and standard error of the mean of at least two independent experiments and were statistically analyzed using the Student's two-tailed *t*-test.

3. Results

The recent DNA sequencing of 108 human AHR genes from six different ethnic populations identified six exonic SNPs, four of