The Aryl Hydrocarbon Receptor Regulates Distinct Ligand-Dependent and Ligand-Independent Gene Batteries

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Introduction

Initial studies of the aryl hydrocarbon receptor (AHR) focused on its roles in regulating induction of CYP1 enzymes[1] and mediating toxicity of dioxin-like chemicals[2]. More recently, the creation of *Ahr*-null mice revealed phenotypic changes which implicate the AHR in multiple aspects of growth, development, differentiation and physiology, irrespective of exposure to toxic environmental chemicals[3, 4].

The AHR is a ligand-dependent transcription factor but the spectrum of genes regulated by the AHR is not completely defined. Several previous microarray studies examined responses of various cells or tissues to TCDD or to other xenobiotic chemicals that act as AHR ligands[5-7]. Very few of the expression changes in previous studies have been shown to be direct AHR-mediated responses, either by promoter analysis or by determination of AHR-dependence. As a result, these studies are useful to toxicology but are relatively uninformative regarding potential physiologic roles of the AHR. One avenue towards understanding basic biological roles of the AHR is to contrast gene expression profiles in *Ahr*-null mice with profiles in mice that have wildtype AHR. Prior expression array studies of *Ahr*-null models have been confined to primary cell cultures derived from $Ahr^{-/-}$ mice vs. $Ahr^{+/+}$ mice[5, 6].

We used expression arrays to identify the batteries of genes whose hepatic expression *in vivo* is affected by AHR status, by TCDD or by the AHR:TCDD interaction. This is the first transcriptomic analysis of tissue from $Ahr^{-/-}$ animals.

Materials and Methods

Animal Treatment and Total RNA Isolation—Ahr-null ($Ahr^{-/-}$) mice (10 weeks old) in a C57BL/6J background and wildtype ($Ahr^{+/+}$) C57BL/6J mice (15 weeks old) were obtained from **The Jackson Laboratory,** Bar Harbor, ME. Mice were given a single dose of 1000 mg/kg TCDD or corn oil vehicle by gavage at age six weeks. Liver was harvested 19 hours after treatment. There were 3 TCDD-treated and 3 control mice in the $Ahr^{-/-}$ groups and 6 TCDD-treated and 5 control mice in the $Ahr^{+/+}$ groups. Total RNA was extracted using RNeasy kits (Qiagen) according to the manufacturer's instructions.

Expression Array Studies—10 µg of total RNA was assayed on Affymetrix MOE430-2 arrays at The Centre for Applied Genomics (The Hospital for Sick Children, Toronto, Canada) following standard manufacturer's protocols. Array data were loaded into the R statistical package (v2.0.0) using the affy package (v1.5.8)[8] of the BioConductor open-source project[9]. Data were investigated for spatial and distributional homogeneity, pre-processed with a sequence-specific version of the RMA algorithm[10] termed GCRMA, as implemented in BioConductor (v1.1.3). The data then were written to disk and parsed with Perl scripts into a custom-built Oracle database partially derived from the MAGE-OM-compliant RAD schema[11]. Normalized, annotated data were written out from the Oracle database and significance-tested with a general-linear model (GLM) using the limma package (v1.8.14) in BioConductor. The following linear model was fit to each individual ProbeSet on the MOE430-2 array:

Y= Basal + AHR +TCDD + AHR:TCDD + Batch

Here Y refers to the expression-level of a single ProbeSet; Basal refers to the underlying basal expression level across all animals; AHR captures "AHR-dependent, TCDD-independent" expression changes; TCDD captures "TCDD-dependent, AHR-independent" expression changes; AHR:TCDD captures "AHR-dependent, TCDD-dependent" changes; and Batch captures the effects of the hybridization batch. After fitting the linear-model, we employed empirical Bayes moderation of the standard error[12], followed by a false-discovery rate correction for multiple testing[13]. To identify differentially expressed genes we used a nested F-test as implemented in limma on the AHR, TCDD, and AHR:TCDD effects. ProbeSets were deemed differentially expressed at the $p<10^{-3}$ significance level.

Functional Analysis of Differentially-Expressed Genes—Ontological analysis employed build 140 of the GO-Miner software package[14], which utilizes the Fisher's Exact Test to identify significantly enriched functional categories (GO terms) in the lists of differentially expressed genes.

Real-Time Quantitative RT-PCR— mRNA levels for selected genes were quantitated by real-time qRT-PCR using specific primers and probes and Brillant® QPCR Master mix (Stratagene). The real-time PCR was performed on a Stratagene MX4000 real-time PCR system.

Results and Discussion

Our array experiments in *Ahr*$^{-/-}$ vs. *Ahr*$^{+/+}$ mice are intended to comprehensively define the separate sets of genes whose expression is significantly affected by AHR status *per se* or by the AHR:TCDD interaction in mouse liver. This is a prelude to the longer-term goal of determining which AHR-regulated genes are central to normal physiology/development and which genes are dysregulated by dioxins in a manner that evokes toxicity.

We characterized three distinct sets of genes: **(1)** those affected by *Ahr* genotype independent of TCDD; **(2)** those responsive to TCDD in an AHR-dependent manner; **(3)** those responsive to TCDD in an AHR-independent manner (Fig 1).

(1) *AHR Effects Independent of TCDD*—Most studies of the AHR's role in gene expression have focused on AHR-mediated transcriptional enhancement in response to foreign agonists such as TCDD. Our array experiment reveal a total of 392 ProbeSets whose expression levels differ significantly between *Ahr*$^{-/-}$ and *Ahr*$^{+/+}$ mice independent of treatment (Fig 1). Basal expression of some genes is dramatically affected by the presence or absence of the AHR. For example, our array study (confirmed by real-time qRT-PCR) reveals that in untreated mice the mRNA for Serpina12, a proteinase inhibitor, was 220-fold more abundant in liver from *Ahr*$^{+/+}$ mice than from *Ahr*$^{-/-}$ mice. Proteinase inhibitors play major roles in cell growth and differentiation by reducing degradation of the extracellular matrix by enzymes such as matrix metalloproteinases (MMPs) and also aid in protecting tissues from inflammatory stress. Our functional analysis identified also broad changes in expression of genes coding for proteins involved in electron transport, drug metabolism and defense against xenobiotic toxicants. This impact of AHR status on constitutive expression of numerous genes suggests multiple and diverse roles for the AHR in normal physiology in addition to the AHR's ability to mediate gene expression in response to xenobiotic ligands.

(2) *AHR-dependent Effects of TCDD*—A total of 456 ProbeSets had their expression significantly altered by the AHR:TCDD interaction (Fig 1). The AHR:TCDD interaction contains approximately 3-fold more ProbeSets that are induced than are repressed. As expected, classical AHR-regulated genes such as *Cyp1a1*, *Cyp1b1*, *Nqo1*, and *Tiparp* displayed robust AHR-dependent induction by TCDD, confirming that the experimental protocol was sound. Several novel dioxin response genes were identified. The flavin-containing monooxygenases Fmo2 and Fmo3 mRNAs are highly induced by TCDD (30-fold and 80-fold) in *Ahr*$^{+/+}$ mice. For Fmo3, real-time qRT-PCR confirms array results. FMOs play an important role in detoxification of many foreign chemical, including psychoactive drugs, pesticides and dietary-derived compounds. FMO induction may be a further adaptive mechanism by which the AHR fosters protection from xenobiotic chemicals. The ontological analysis for the AHR:TCDD interaction show a profound upregulation of genes involved in electron transport, detoxification and ribosome assembly and structure and a strong down-regulation of genes involved in amino acid metabolism.

(3) *TCDD Has Few AHR-Independent Effects*—One of the most striking findings of our study is that very few genes respond to TCDD in absence of the AHR. Only 32 genes showed statistically significant AHR-independent

responses to TCDD (Fig 1) and the maximum difference between TCDD-treated and untreated mice in absence of the AHR was approximately 2-fold. Our data suggest that almost all transcriptomic effects of TCDD do indeed require the AHR and the majority of the biological effects of dioxins are mediated by AHR.

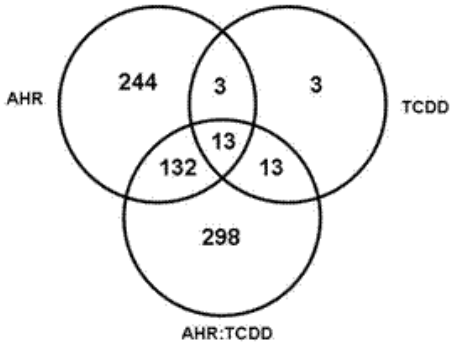

Figure 1: Venn Diagram presenting the overlap between effects in terms of the number of Probesets

A group of 11 genes that showed significant gene expression changes related to AHR status and/or TCDD from the arrays results were selected for the real-time qRT-PCR. The results reveal a strong correlation between array and real-time qRT-PCR data.

To sum up, our key findings are that virtually all effects of TCDD on gene expression require the AHR. Since TCDD toxicity is inextricably linked to altered gene expression via a functional AHR, the novel TCDD-responsive genes that we identified are worthy of evaluation for their possible role in dioxin toxicity, at least in mouse liver.

The fact that almost as many genes are responsive to AHR status *per se* as are responsive to the AHR:TCDD interaction supports the concept that the AHR plays important roles in normal development and physiology, not just in response to toxic environmental chemicals. Array studies provide menus of genes that are plausible candidates to be involved in particular biological or toxicological outcomes. The challenge now is to link AHR-mediated expression of specific candidate genes (or gene sets) to those outcomes.

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