Characterizing TCDD-Dependent and TCDD-Independent Modes of AHR Transactivation: Enhancer Analysis in an AHR-Null System

Paul Boutros[1], Nathalie Tijet[1], Ivy D Moffat[1], Allan B Okey[1], Juoko Tuomisto[2], Raimo Pohjanvirta[3]

[1]University Of Toronto
[2]National Public Health Institute
[3]University of Helsinki

Introduction

Dioxin toxicity is an intensely studied but poorly understood phenomena. Dioxins can induce a wide range of toxic outcomes, ranging from chloracne and wasting syndrome to hepatic lesions and acute lethality. Surprisingly, none of these phenotypic end-points can yet be described in terms of a complete series of events molecular events[1].
Nevertheless, substantial evidence suggests that many of these toxic endpoints are mediated through the transcriptional activities of the Aryl Hydrocarbon Receptor (AHR) and its heterodimerization partner, ARNT. For example, mice with a mutated ligand-binding domain[2], with hypomorphic ARNT expression[3], with a aberrant AHR localization[4], or lacking AHR entirely[5] are all highly resistant to dioxins.
Unfortunately the transcriptional role of the AHR has itself become startling complex over the last several years. Extensive cross-talk between the AHR and estrogen receptor (ER) signaling pathways has been identified, culminating in the detection of direction interactions between both ARNT and the ER[6] and of AHR-dependent and dioxin-dependent recruitment of the ER to AHR-responsive promoters[7,8]. Further, direct interactions have been detected between the AHR and SP1 transcription-factors[9], and the expression of several transcription-factors are modulated in a TCDD-dependent fashion[10-12].
From these data, it appears likely that the AHR is at the heart of a tight transcriptional- regulatory network, and its perturbation by TCDD leads to a cascade of molecular changes that culminate in toxicity. Thus, a major question in the field is the elucidation of these regulatory networks: what transcription-factors cooperate with or antagonize the effects of the AHR in both its endogenous and ligand-activated states?
We provide an initial answer to this key question here, by combining microarray data on AHR knockout mice with searches for transcription-factor binding-sites.

Materials & Methods

Expression Profiling—Ahr-null (Ahr-/-) mice (10 weeks old) in a C57BL/6J background were obtained from The Jackson Laboratory, Bar Harbor, ME. Wildtype (Ahr+/+) C57BL/6J mice (15 weeks old) were bred at the National Public Health Institute, Kuopio, Finland from stock originally obtained from The Jackson Laboratory. Mice were given a single dose of 1000 µg/kg TCDD or corn oil vehicle by gavage at age six weeks and sacrificed 19 hours after treatment. RNA was extracted using RNeasy kits (Qiagen), then hybridized to Affymetrix MOE430-2 arrays.
Analysis of Array Data—Array data were pre-processed with a sequence-specific version of the RMA algorithm[13] termed GC-RMA. The normalized dataset was significance-tested with a general-linear model (GLM) using the limma package (v1.8.14) of BioConductor. The linear model captured two major effects: the ligand-dependent effects of the AHR and the ligand-independent effects of the AHR . After fitting the linear-model, we employed empirical Bayes moderation of the standard error[14], followed by a false-discovery rate correction for multiple testing. To identify differentially expressed genes we used a nested F-test and deemed ProbeSets differentially expressed at the $p<10^{-3}$ significance level.
Transcription-Factor Binding Site Analysis—To understand the regulatory networks underlying AHR-dependent expression we performed two separate transcription-factor binding-site (TFBS) analyses. Both analyses employed UCSC build mm5 of the mouse genome from which regulatory sequences were extracted using BioPerl-based scripts[15]. Transcriptional start sites (TSSs) were taken from the UCSC Genome Browser Database[16].
First, in order to identify known AHR-binding sites, we scanned the region from -5000 bp to +1000 bp relative to each TSS for AHRE-I and AHRE-II binding sites, as described previously[12].

Second, to characterize patterns of transcription-factor binding sites across the set of putative AHR-regulated genes we employed a library-based TFBS search. These library-based searches compare a set of known TFBSs to the promoter regions of a set of co-regulated genes, then apply statistical tests to identify TFBSs that are found more often (enriched) or less often (depleted) than expected by chance in the set of co-regulated genes. Using the Clover software[17], we tested separately the sets of genes up- and down-regulated in both the AHR and AHR:TCDD contrasts. Statistical testing employed both mononucleotide and dinucleotide randomizations of the parental sequences (1000 iterations), randomization of scoring matrices, and two separate background sequence sets (murine CpG islands and human chromosome 21). Two separate libraries of TFBSs were scanned: the JASPAR database[18] and a custom-designed database that included three variants of the AHRE-I consensus sequence, the AHRE-II sequence, and the AnoC element as (all described previously[12]) as well as the Cyp1a1 negative regulatory element[19]. This search scanned the region from -4000 bp to +1000 bp relative to the annotated TSS for each gene.

Results & Discussion

Our expression profiling identified profound changes in expression. Of the 31,099 ProbeSets on the RAE230-2 array, 392 (1.26%) showed AHR-dependence in the absence of TCDD and 456 showed AHR-dependence in the presence of TCDD (1.47%).
To rationalize these changes, we searched for AHRE-I and AHRE-II motifs in the 5'-flanking regions of all genes whose expression showed AHR-dependence. Surprisingly, neither motif was significantly enriched and minimal overlap was found with two earlier phylogenetic footprinting studies[12,20]. This is consistent with the observation that our gene-lists show only moderate overlap with previous published array studies (data not shown). We hypothesize that this is partially due to large differences basal expression between AHR knockout and wild-type mice, and partially due to secondary effects not directly attributable to the AHR.
To further characterize the transcriptional-regulatory networks underlying the vast expression changes observed we next employed a library-based transcription-factor binding-site (TFBS) search. We searched the lists of up- and down-regulated genes separately, leading to four separate sets of transcription-factor enrichment. Table 1, below, highlights TFBSs enriched or depleted in all four lists while Table 2, below, highlights some of the condition-specific results.

ID	Name	AHR(↓)	AHR(↑)	AHR:TCDD (↓)	AHR:TCDD (↑)
MA0041	HFH-2 FORKHEAD	$<10^{-3}$	$<10^{-3}$	$<10^{-3}$	$<10^{-3}$
MA0042	HFH-3 FORKHEAD	$<10^{-3}$	$<10^{-3}$	$<10^{-3}$	$<10^{-3}$
MA0047	HNF-3beta FORKHEAD	$<10^{-3}$	$<10^{-3}$	$<10^{-3}$	$<10^{-3}$
MA0088	Staf ZN-FINGER, C2H2	$<10^{-3}$	0.010	$<10^{-3}$	$<10^{-3}$
MA0086	Snail ZN-FINGER, C2H2	0.035	$<10^{-3}$	0.116	0.003
Custom	AHRE-II	0.957	0.897	0.892	0.999
MA0024	E2F Unknown	1	1	1	1

Table 1: Selected TFBSs enriched or depleted in all differentially-expressed genes from the microarray screen. P-Values for enrichment are shown; values close to 0 represent enrichment, values close to one represent depletion.

Effect	Matrix	TF Name	P
AHR (↑)	MA0068	Pax-4	$<10^{-3}$
AHR (↑)	MA0065	PPAR-gamma	$<10^{-3}$
AHR:TCDD(↓)	MA0106	p53	0.001
AHR:TCDD(↑)	MA0079	SP1	$<10^{-3}$

Table 2: selected TFBSs enriched or depleted in specific sets of differentially expressed genes. P-values for enrichment are shown and have the same meaning as in Table 1.
Many of these results are well-supported by literature evidence. For example, Snail has been shown to play a critical role in the progression of hepatocellular carcinomas[21]. Most intriguingly, these results suggest that the AHRE-II is

depleted in the full gene-set. This strongly suggests that the AHRE-II is broadly depleted in hepatically-expressed genes, and hence induction of genes through AHRE-II may lead to ectopic-expression of genes related to toxic endpoints. Similarly, the enrichment of p53 binding-sites in TCDD-repressed genes (Table 2) is interesting, since p53 regulates many tumour-suppressors and the repression of such genes may contribute to downstream toxicities.
In summary, these results help provide an initial characterization of the transcriptional-regulatory networks regulated by the AHR in both its endogenous and TCDD-activated states. Further characterization of these networks may help elucidate the molecular mechanisms of dioxin toxicity.

Acknowledgments

We thank the Bioconductor user-support group for outstanding support, Dr. Gordon Smyth for his assistance in fitting linear models, and Dr. Lyle Burgoon for helpful discussions.

References

1. Okey, A.B., Riddick, D.S. and Harper, P.A. (1994) Trends Pharmacol Sci 15, 226-32.

2. Huang, Y., Harper, P.A. and Okey, A.B. (1995) Can J Physiol Pharmacol 73, 18-26.

3. Walisser, J.A., Bunger, M.K., Glover, E., Harstad, E.B. and Bradfield, C.A. (2004) J Biol Chem 279, 16326-31.

4. Bunger, M.K., Moran, S.M., Glover, E., Thomae, T.L., Lahvis, G.P., Lin, B.C. and Bradfield, C.A. (2003) J Biol Chem 278, 17767-74.

5. Schmidt, J.V., Su, G.H., Reddy, J.K., Simon, M.C. and Bradfield, C.A. (1996) Proc Natl Acad Sci U S A 93, 6731-6.

6. Brunnberg, S., Pettersson, K., Rydin, E., Matthews, J., Hanberg, A. and Pongratz, I. (2003) Proc Natl Acad Sci U S A 100, 6517-22.

7. Ohtake, F., Takeyama, K., Matsumoto, T., Kitagawa, H., Yamamoto, Y., Nohara, K., Tohyama, C., Krust, A., Mimura, J., Chambon, P., Yanagisawa, J., Fujii-Kuriyama, Y. and Kato, S. (2003) Nature 423, 545-50.

8. Matthews, J., Wihlen, B., Thomsen, J. and Gustafsson, J.A. (2005) Mol Cell Biol 25, 5317-28.

9. Kobayashi, A., Sogawa, K. and Fujii-Kuriyama, Y. (1996) J Biol Chem 271, 12310-6.

10. Karyala, S., Guo, J., Sartor, M., Medvedovic, M., Kann, S., Puga, A., Ryan, P. and Tomlinson, C.R. (2004) Cardiovasc Toxicol 4, 47-73.

11. Moennikes, O., Loeppen, S., Buchmann, A., Andersson, P., Ittrich, C., Poellinger, L. and Schwarz, M. (2004) Cancer Res 64, 4707-10.

12. Boutros, P.C., Moffat, I.D., Franc, M.A., Tijet, N., Tuomisto, J., Pohjanvirta, R. and Okey, A.B. (2004) Biochem Biophys Res Commun 321, 707-15.

13. Irizarry, R.A., Bolstad, B.M., Collin, F., Cope, L.M., Hobbs, B. and Speed, T.P. (2003) Nucleic Acids Res 31, e15.

14. Smyth, G.K. (2003) Statistical Applications in Genetics and Molecular Biology 3, 1-26.

15. Stajich, J.E., Block, D., Boulez, K., Brenner, S.E., Chervitz, S.A., Dagdigian, C., Fuellen, G., Gilbert, J.G., Korf, I., Lapp, H., Lehvaslaiho, H., Matsalla, C., Mungall, C.J., Osborne, B.I., Pocock, M.R., Schattner, P., Senger, M., Stein, L.D., Stupka, E., Wilkinson, M.D. and Birney, E. (2002) Genome Res 12, 1611-8.

16. Karolchik, D., Baertsch, R., Diekhans, M., Furey, T.S., Hinrichs, A., Lu, Y.T., Roskin, K.M., Schwartz, M., Sugnet, C.W., Thomas, D.J., Weber, R.J., Haussler, D. and Kent, W.J. (2003) Nucleic Acids Res 31, 51-4.

17. Frith, M.C., Fu, Y., Yu, L., Chen, J.F., Hansen, U. and Weng, Z. (2004) Nucleic Acids Res 32, 1372-81.

18. Sandelin, A., Alkema, W., Engstrom, P., Wasserman, W.W. and Lenhard, B. (2004) Nucleic Acids Res 32 Database issue, D91-4.

19. Boucher, P.D., Ruch, R.J. and Hines, R.N. (1993) J Biol Chem 268, 17384-91.

20. Sun, Y.V., Boverhof, D.R., Burgoon, L.D., Fielden, M.R. and Zacharewski, T.R. (2004) Nucleic Acids Res 32, 4512-23.

21. Sugimachi, K., Tanaka, S., Kameyama, T., Taguchi, K., Aishima, S., Shimada, M. and Tsuneyoshi, M. (2003) Clin Cancer Res 9, 2657-64.