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Dose–response relationships in gene expression profiles in rainbow trout, *Oncorhynchus mykiss*, exposed to ethynylestradiol

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Abstract

Determining how gene expression profiles change with toxicant dose will improve the utility of arrays in identifying biomarkers and modes of toxic action. Isogenic rainbow trout, *Oncorhynchus mykiss*, were exposed to 10, 50 or 100 ng/L ethynylestradiol (a xeno-estrogen) for 7 days. Following exposure hepatic RNA was extracted. Fluorescently labeled cDNA were generated and hybridized against a commercially available Atlantic Salmon/Trout array (GRASP project, University of Victoria) spotted with 16,000 cDNAs. Transcript expression in treated vs control fish was analyzed via Genespring (Silicon Genetics) to identify genes with altered expression, as well as to determine gene clustering patterns that can be used as “expression signatures”. Array results were confirmed via qRT PCR. Our analysis indicates that gene expression profiles varied somewhat with dose. Established biomarkers of exposure to estrogenic chemicals, such as vitellogenin, vitelline envelope proteins, and the estrogen receptor alpha, were induced at every dose. Other genes were dose specific, suggesting that different doses induce distinct physiological responses. These findings demonstrate that cDNA microarrays could be used to identify both toxicant class and relative dose.

Keywords

Rainbow trout; cDNA microarray; Estrogens

The field of toxicogenomics holds great promise for environmental and aquatic toxicology. Specific applications of microarrays in ecotoxicological studies include the identification of contaminants through analysis of expression patterns measured via microarrays in test organism exposed to previously uncharacterized contaminant mixtures, the identification of chemical mode of action based on the mRNA expression patterns, and the development of tools to assess endpoints for chronic exposure to mixtures that are tied to population-level consequences (Miracle and Ankley, 2005). However, the baseline transcriptomic response in organisms exposed to model toxicants must be characterized before this technology can be meaningfully applied to contaminated field sites. The objective of this study is to determine the relationship between gene expression profile and toxicant dose for the synthetic estrogen, ethynylestradiol (EE2).

Methods for exposure and EE2 kinetics are described in more detail in Skillman et al. (in press), while RNA preparation and labeling, microarray hybridization and data analysis are described in more detail in Hook et al. (2006). We used isogenic fish in this study to minimize the confounding effects of individual variability. Male isogenic Rainbow Trout (*Oncorhynchus mykiss*) were exposed to EE2 at nominal concentrations of 10, 50 and 100 ng/L EE2 (actual

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concentrations were 8, 33, and 115 ng/L EE2) via methanol drip (methanol concentration was less than 0.02%) for seven days and compared to methanol only controls. Fish were euthanized, then RNA was harvested from the livers of exposed and control trout using TRIzol reagent (Invitrogen) and the manufacturer's protocol. RNA was DNAase treated using TURBO – DNA free clean up procedure (Ambion). Total RNA (2 µg) was labeled with Cy 3 and Cy 5 fluors (Amersham) using Invitrogen's superscript indirect cDNA labeling system following the manufacturer's protocol. Three replicates of RNA from three different individual fish were labeled (3 × 3 design). High density microarrays (16,000 cDNA spots per array) were obtained from GRASP project at the University of Victoria and were prepared as described in Rise et al. (2004a). Fluorescently labeled cDNA was hybridized to the arrays overnight at 45° in a humidified chamber, then washed following Genisphere's protocols. Slides were imaged using a Scan-Array Express (Perki-Elmer) and data were extracted using the instrument manufacturers software. Data were imported into Genespring (Silicon Dynamics), LOWESS normalized, and screened for significance using a *t*-test ($p < 0.05$). Microarray results were validated by qRT PCR of selected genes using fluorescently-labelled primers (Table 1) using an ABS 7300 realtime system as described in Hook et al. (2006).

We found significant changes in gene expression at all three EE2 doses tested. As shown in Fig. 1, many genes have consistent patterns of expression at the three different doses. For instance, the genes that are recognized as biomarkers of exposure to estrogenic compounds (such as vitellogenin and the vitelline envelope protein; Larkin et al., 2003) have increased expression at all three doses. Other genes, such as hemopexin and pentraxin are consistently downregulated at all three doses while others exhibited altered expression patterns depending on dose. Many of the genes that are up regulated to a greater extent with increasing dose do not have an established function. In contrast, several of the genes that are downregulated with increasing dose are metabolic genes, such as phosphofructokinases (involved in glycolysis), NADH dehydrogenase, and cytochrome *c* oxidase. No generalizations as to the abundance of unique genes at any specific dose can be made, although many more genes were downregulated than were upregulated following exposure to EE2 at all doses (Fig. 1; Hook et al., 2006; Skillman et al., in press).

The qPCR validation shows good agreement with the array measurements as to the direction of change, even if the magnitude of change is not consistent between the two doses (Fig. 2). For instance, vitellogenin was found to be upregulated about 10-fold at the highest exposure level as measured by the arrays, yet upregulated greater than 10,000-fold as measured via qPCR. Also, changes in gene expression can be measured with greater sensitivity with qPCR, as haptoglobin is significantly upregulated at all doses as measured with qPCR, but only at the highest dose as measured using the arrays.

Arrays are known to be semiquantitative, and are known to saturate at high levels (Wu, 2001). One measure that differed sharply between the two techniques was the estrogen receptor. Rainbow trout have two forms of the estrogen receptor, one of which is inducible following estrogen exposure (Pakdel et al., 2000). The sequence used to measure expression of the estrogen via qPCR did not align to the sequence on the microarray, and was likely a different isoform (Hook et al., 2006).

The findings of this study suggest that microarrays could be used to determine relative toxicant dose in environmental samples. A gene expression profile typical of exposure to estrogenic compounds was seen at all doses. However, some of the changes in gene expression were unique to dose, suggesting that arrays could be used to determine both the class of compound and the relative dose to which an organism has been exposed.

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Fig. 1.

Gene tree generated using Pearson's correlation using those genes with expression levels that change at one or more EE2 dose. The color scale in the figure depicts the fold change for each gene at each treatment level. Yellow coloration is indicative of no change in expression, green coloration is indicative of downregulation, red coloration shows upregulation (For interpretation of the references to the color in this figure legend, the reader is referred to the web version of this article.).

Fig. 2. Fold change in gene expression as measured by qPCR (black circles) or microarrays (white circles). A fold change of 1 indicates no change.

Table 1

Primers and Probes used for qPCR analysis

Gene	Forward	Reverse	Fluorescently labeled
Beta actin	ACGGCCAGAGGCGTACAG	TTCAACCCCTGCCATGT	ACAACACGGCCTGGATGGCCA
Estrogen receptor	GCAGGACCAAACTCCGTAGTG	TGGCCAAACGCGAGGTA	TACCCAGAGGCAAAAGTCGCTGCAGA
GnRH-1	AATGGTCTCTCAGTACGCCCTATGTT	GCATCCCCCTATCCACATCA	ATATTCCATAACAAATCTGCCATTTCACAGCTACAA
Haptoglobin	CACGGCACAGGACTTATCGA	CAGTCCAGGACCCCAAAGAC	ACAGGATCCCTGCAGCATACACTCT
Pentraxin	GCTGGGTCAGACCCGTTTGA	CAGTTGGCCAGATTCAAAAATGA	AGTCCCAGGTCCAGCAGATGGAGATCC
Serum albumin 2	TCTTCCCAGAGAGCAGCAGATC	GATGCCAGCAGCTTCCACAT	TTGCTGTCTTGTGGTGCAGAGCTCG
Vitellogenin	CTTCTGAACCCCTGAGATC	GCAGCTGGGACGAAAGG	TTGAGTACAGTGGTGTGTGGCCCAAAGA
Vitelline envelope	GCCGGTTCTCTCTCCAAAT	TCCGCTGCCAGTCTGA	CTGATATAGCTCTGGGGCCCTCATAGTTG

All primers are listed from 5' to 3'. Fluorescently labeled primers have a 6-FAM fluor on the 5' end and a TAMRA fluor on the 3' end.