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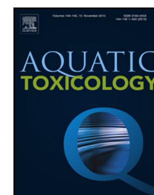

17α-Ethinylestradiol *(EE2)* effect on global gene expression in primary rainbow trout (*Oncorhynchus mykiss*) hepatocytes

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ABSTRACT

The potential impact of endocrine disrupting chemicals (EDCs) in the aquatic environment has driven the development of screening assays to evaluate the estrogenic properties of chemicals and their effects on aquatic organisms such as fish. However, obtaining full concentration–response relationships in animal (*in vivo*) exposure studies are laborious, costly and unethical, hence a need for developing feasible alternative (non-animal) methods. Use of *in vitro* bioassays such as primary fish hepatocytes, which retain many of the native properties of the liver, has been proposed for *in vitro* screening of estrogen receptor (ER) agonists and antagonists. The aim of present study was to characterize the molecular mode of action (MoA) of the ER agonist 17α-ethinylestradiol (EE2) in primary rainbow trout (*Oncorhynchus mykiss*) hepatocytes. A custom designed salmonid 60,000-feature (60k) oligonucleotide microarray was used to characterize the potential MoAs after 48 h exposure to EE2. The microarray analysis revealed several concentration-dependent gene expression alterations including classical estrogen sensitive biomarker gene expression (*e.g. estrogen receptor α, vitellogenin, zona radiata*). Gene Ontology (GO) analysis displayed transcriptional changes suggesting interference of cellular growth, fatty acid and lipid metabolism potentially mediated through the estrogen receptor (ER), which were proposed to be associated with modulation of genes involved in endocrine function and reproduction. Pathway analysis supported the identified GOs and revealed modulation of additional genes associated with apoptosis and cholesterol biosynthesis. Differentially expressed genes (DEGs) related to impaired lipid metabolism (e.g. *peroxisome proliferator-activated receptor α and γ*), growth (*e.g. insulin growth factor protein 1*), phase I and II biotransformation (*e.g. cytochrome P450 1A, sulfotransferase, UDP-glucuronosyltransferase and glutathione S-transferase*) provided additional insight into the MoA of EE2 in primary fish hepatocytes. Results from the present study suggest that biotransformation, estrogen receptor-mediated responses, lipid homeostasis, growth and cancer/apoptosis in primary fish hepatocytes may be altered after short-term exposure to ER-agonists such as EE2. In many cases the observed changes were similar to those reported for estrogen-exposed fish *in vivo*. In conclusion, global transcriptional analysis demonstrated that EE2 affected a number of toxicologically relevant pathways associated with an estrogenic MoA in the rainbow trout hepatocytes.

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1. Background

Compounds that modulate the endocrine system and cause adverse effects causally related to these changes are known as endocrine disrupting chemicals (EDCs). These chemicals may enter the environment through anthropogenic activities such as effluents of sewage treatment plants, industrial processes and agricultural run-off (Sumpter, 2005). During the past decade, increasing awareness of the adverse effects of EDCs in wildlife and human has given rise to the implementation of stricter legislations in international regulatory organizations worldwide (Hecker and Hollert, 2011). Adverse effects such as impaired reproduction in fish, reproductive disorders and various cancer types (*e.g.* breast and ovary cancer) in human and other mammals have been associated with EDCs such as 17α-ethinylestradiol (EE2), dichlorodiphenyltrichloroethane (DDT) and bisphenol A (BPA) (Benninghoff and Williams, 2008; Purdom et al., 1994; Soto and Sonnenschein, 2010; Vom Saal et al., 2007). Characterization of a chemical's mode of action (MoA) involving interference with specific molecular, cellular and biochemical changes, behavioral alterations and adverse effects are often laborious and expensive due to extensive use of

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animals (Aardema and MacGregor, 2002). Although the use of single biomarker screening approaches has facilitated the understanding of MoAs of EDCs, the knowledge obtained from such biomarker studies is still limited, as the response of a single endpoint may not always represent complex biological responses at higher levels of organization.

In recent years, the development of broad-content screening approaches such as transcriptomics has made it possible to characterize the global gene expression changes after exposure to single EDCs and mixture of these in different *in vitro* and *in vivo* experimental models (Finne et al., 2007; McHale et al., 2010; Wang et al., 2010; Yang et al., 2007). Deciphering complex molecular interactions of chemicals using transcriptomic studies has enabled detailed studies on the *in vivo* responses of (xeno) estrogens in common laboratory species such as zebrafish (*Danio rerio*), fatheaded minnow (*Pimephales promelas*), rainbow trout (*Oncorhynchus mykiss*), but also non-model species such as coho salmon (*Oncorhynchus kisutch*) (Harding et al., 2013; Hook et al., 2008; Levi et al., 2009; Villeneuve et al., 2011; Wang et al., 2010). Many of these studies involve the analysis of estrogenic responses, which typically involve the binding and activation of the estrogen receptor (ER) and of genes containing estrogen response elements (ERE) to initiate multi-organ endocrine responses in fish. The ER signaling pathway may further regulate the expression of several classical estrogenic biomarker genes such as the egg-yolk precursor protein *vitellogenin* (*vtg*), egg envelope proteins *zona pellucida* and *zona radiata protein* (*zrp*) (Arukwe et al., 1997; Sumpter and Jobling, 1995). The endocrine modulatory effects of EDCs have been extensively studied *in vivo,* including the characterization of MoA associated with hormone binding, lipid and cholesterol metabolism and steroidogenesis, immune function and ion homeostasis in fish (Colli-Dula et al., 2014; Flores-Valverde et al., 2010; Hook et al., 2007; Kausch et al., 2008; Levi et al., 2009; Wit et al., 2010). Various estrogen-mediated responses have also been observed in biomarker studies in various piscine *in vitro* (non-animal) bioassays, and consequently led to the proposal of using these experimental models as screening assays for environmental estrogens and antiestrogens (Björkblom et al., 2008; Hultman et al., 2015; Kordes et al., 2002; Navas and Segner, 2006; Rankouhi et al., 2004; Tollefsen et al., 2003).

Use of alternative approaches provided by *in vitro* methods have offered rapid screening methods and facilitated better understanding of the MoA of chemicals whilst implementing the 3R's (refinement, reduction and replacement) into toxicological testing (National research council (NRC), 2007). *In vitro* hepatic models such as primary hepatocytes have demonstrated to be advantageous proxies for the assessment of *in vivo* bioactivity as the cells retain many of the native hepatic functions including biotransformation, detoxification and ER-mediated responses (Flouriot et al., 1993; Pedersen and Hill, 2000; Pesonen and Andersson, 1997; Segner and Cravedi, 2000). The hepatocytes have previously demonstrated their potential for toxicological screening of cellular toxicity, endocrine disruption (ED) and bioaccumulation in various assay formats including suspension, monolayer and 3-dimensional spheroid cultures (Baron et al., 2012; Hultman et al., 2015; Mingoia et al., 2010; Smeets et al., 1999; Tollefsen et al., 2008a). Despite the broad applicability of such assays, thorough characterization of the MoAs and concentration-dependent global changes of gene and protein expression are generally lacking, and effort to provide this for EDCs is highly warranted.

The objectives of this study were (1) to characterize the molecular MoAs of the ER-agonist 17α-Ethinylestradiol (EE2) in primary rainbow trout (*O. mykiss*) hepatocytes after a short-term (48 h) *in vitro* exposure; (2) to determine the concentration-dependent transcriptional changes occurring, and (3) to evaluate the potential of primary hepatocytes to predict *in vivo* hepatic responses in fish.

2. Material and methods

2.1. Chemicals

17α-Ethinylestradiol (EE2, ≥98%, CAS 57-63-6) and sodium bicarbonate (CAS 144-55-8) were purchased from Sigma–Aldrich (St. Louis, MI, US). The test chemical was dissolved in dimethylsulfoxide (DMSO) and stored in the dark at −20 °C until use.

2.2. Fish

Sexually immature rainbow trout (200–500 g) from the same fish stock were obtained from the Valdres rakfisk AB hatchery (Valdres, Norway) and reared at the Department of Biosciences, University of Oslo (Norway) for a minimum of 4 weeks prior to the start of the studies. The fish were maintained in tap water at 6 ± 2 °C, pH 6.6, 100% air saturation and light regime of 12 h light/12 h dark. Rainbow trout were fed daily with commercial pellets (Skretting, Stavanger, Norway) corresponding to approximately 0.5% of total body weight.

2.3. Cell culture and exposure

A total of four donor fish were collected (January–July, 2012) and terminated by cephalic concussion, followed by immediate dissection to expose the abdominal cavity. Only juvenile fish (with no visual gonads) were used in a 2-step hepatic cell isolation procedure as described by Tollefsen et al. (2003) and modified for studies on gene expression by Hultman et al. (2015). The viability of primary hepatocytes (>80%) was assessed using a Bürker counting chamber and trypan blue:cell suspension (2:1). The cell suspension was diluted to 500,000 cells/ml in serum-free L-15 medium with phenol-red containing amphotericin (0.25 g/ml), L-glutamine (0.29 mg/ml), streptomycin (100 g/l), penicillin (100 Units/ml) and $NaHCO_3$ (4.5 mM), seeded in 24-well Primaria™ microtiter plates (Falcon, Becton Dickinson Labware, Oxnard, CA, USA) with a density of 625,000 cells/well and incubated in ambient atmosphere at 15 °C. After 24 h of acclimation to the test wells, 50% of the medium was removed from the cells and replaced with media spiked with EE2 (0.03, 0.3, 3 and 30 nmol/l (nM)) or solvent control (0.1% DMSO) in triplicate. The chemical exposure concentration in the medium at the start of the experiment was verified by ultra-performance liquid-chromatography tandem mass spectrometer (UPLC-MS) analysis on derivatized EE2 and d3 labeled estradiol (d2-E2) and described in detail by Hultman et al. (2015). The measured concentrations ranged between 75% and 93% of the nominal concentrations (Hultman et al., 2015). After 48 h exposure, the test medium was removed and the cells were sampled and lysed with RNeasy lysis buffer (Qiagen GmbH, Hilden, Germany) and stored at −80 °C for later RNA isolation and gene expression analysis.

2.4. Gene expression analysis

2.4.1. Microarray analysis

A high-density (60,000-feature) custom salmonid oligonucleotide microarray (Agilent Technologies, City, Country; GEO accession number: GPL18864) was used to study the global transcriptional changes in the rainbow trout hepatocytes. The performance of the array was thoroughly evaluated for different salmonid species and pollutants (Song et al., 2012). Prior to microarray hybridization, the frozen primary hepatocytes were subjected to RNA extraction using Qiagen RNeasy Plus mini kit with on-column DNAs treatment (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions with minor modifications to accommodate high RNA purity and yield. The modifications included extended incubation time of RNA membrane-bound

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