Comparative Biochemistry and Physiology, Part D 19 (2016) 112–119

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part D

journal homepage: www.elsevier.com/locate/cbpd

Time course of hepatic gene expression and plasma vitellogenin protein concentrations in estrone-exposed juvenile rainbow trout (*Oncorhynchus mykiss*)☆

Heather L. Osachoff [a,b], Lorraine L.Y. Brown [b,c], Leena Tirrul [b], Graham C. van Aggelen [b], Fiona S.L. Brinkman [c], Christopher J. Kennedy [a,*]

[a] Department of Biological Sciences, Simon Fraser University, 8888 University Drive, Burnaby, British Columbia V5A 1S6, Canada
[b] Environment Canada, Pacific Environmental Science Centre, 2645 Dollarton Hwy., North Vancouver, British Columbia V7H 1B1, Canada
[c] Department of Molecular Biology and Biochemistry, Simon Fraser University, 8888 University Drive, Burnaby, British Columbia V5A 1S6, Canada

ARTICLE INFO

Article history:
Received 31 August 2015
Received in revised form 8 February 2016
Accepted 21 February 2016
Available online 26 February 2016

Keywords:
Estrone
QPCR
RNA-Seq
Rainbow trout
Vitellogenin
Vitelline envelope proteins
Estrogen receptor
Transcriptome

ABSTRACT

Estrone (E1), a natural estrogen hormone found in sewage effluents and surface waters, has known endocrine disrupting effects in fish, thus, it is a contaminant of emerging concern. Juvenile rainbow trout (*Oncorhynchus mykiss*) were exposed to an environmentally-relevant concentration of E1 (24 ng/L E1 [0.1 nM]) for 7 d and then placed in clean water for a 9 d recovery period. RNA sequencing showed transcripts from numerous affected biological processes (e.g. immune, metabolic, apoptosis, clotting, and endocrine) were altered by E1 after 4 d of treatment. The time course of E1-inducible responses relating to vitellogenesis was examined daily during the two phases of exposure. Hepatic gene expression alterations evaluated by quantitative polymerase chain reaction (QPCR) were found during the treatment period for vitellogenin (*VTG*), vitelline envelope proteins (*VEP*s) α, β and γ, and estrogen receptor α1 (*ERα1*) transcripts. *ERα1* was the only transcript induced each day during the treatment phase, thus it was a good indicator of E1 exposure. Gradual increases occurred in *VEPβ* and *VEPγ* transcripts, peaking at d7. *VTG* transcript was only elevated at d4, making it less sensitive than *VEP*s to this low-level E1 treatment. Inductions of *ERα1*, *VEPα*, *VEPβ* and *VEPγ* transcripts ceased 1 d into the recovery phase. Plasma VTG protein concentrations were not immediately elevated but peaked 7 d into the recovery phase. Thus, elevated vitellogenesis-related gene expression and protein production occurred slowly but steadily at this concentration of E1, confirming the sequence of events for transcripts and VTG protein responses to xenoestrogen exposure.

1. Introduction

Contaminants are released into surface waters from anthropogenic activities and may cause harmful effects in exposed organisms. Exogenous exposure to estrogen hormones (e.g. 17β-estradiol [E2], the main vertebrate estrogen hormone) and chemicals that act like estrogens (xenoestrogens) in aquatic environments are of particular concern because of their bioactive nature and ability to cause feminization of male and juvenile organisms (Kime, 1998). The estrogen hormone estrone (E1) is the oxidized form of E2 and is commonly found in aquatic environments, with reported environmental concentrations ranging from 0.1–40 ng/L (Belfroid et al., 1999; Ternes et al., 1999; Kolpin et al., 2002; Aerni et al., 2004; Kim et al., 2007; Jeffries et al., 2010; Sousa et al., 2010; Lee et al., 2011; Furtula et al., 2012a; Williams et al., 2012; Barber et al., 2013). E1 is equipotent to, or slightly less potent (up to 2.5-fold), than E2 (Metcalfe et al., 2001; Thorpe et al., 2003a; Aerni et al., 2004; Van den Belt et al., 2004; Dammann et al., 2011). Since E1 is excreted in the urine and feces of vertebrates (Khanal et al., 2006), it is found associated with agricultural operations (Lange et al., 2002; Kolodziej et al., 2004; Soto et al., 2004; Jeffries et al., 2010; Furtula et al., 2012a) and sewage treatment plant (STP) discharges (Osachoff et al., 2013a). Reported concentrations of E1 in STP effluents range from 0.2–50 ng/L (Johnson et al., 2005; Lishman et al., 2006; Vajda et al., 2011; Furtula et al., 2012b; Williams et al., 2012). STP effluents are mixtures of estrogenic chemicals including surfactants (e.g. alkylphenol ethoxylates), plastic-related compounds (e.g. phthalates), and natural and synthetic estrogen hormones. Research has focused mainly on surfactants and 17α-ethinylestradiol (EE2) as contributors to the feminization of fish downstream of STP discharges (Jobling et al., 1998; Vajda et al., 2008), and, only recently, E1 has been recognized as being an important contributor to these effects (Dammann et al., 2011).

E1 can feminize fish and have subsequent developmental and reproductive repercussions, which makes it an endocrine disrupting compound (EDC). E1 can inhibit testicular growth (Panter et al., 1998), induce intersex, skew female:male sex ratios (Metcalfe et al.,

☆ This paper is a contribution to the Special Issue on Environmental Omics and Toxicology.

* Corresponding author.
E-mail address: ckennedy@sfu.ca (C.J. Kennedy).

http://dx.doi.org/10.1016/j.cbd.2016.02.002
1744-117X/

2001), reduce spawning frequency, increase male mortality (Thorpe et al., 2003b), inhibit egg production (Thorpe et al., 2007), and decrease reproductive success (Dammann et al., 2011). To provide insights into the mechanisms behind xenoestrogen actions, several studies examined the alterations in gene expression caused by xenoestrogen exposures (Gunnarsson et al., 2007; Larkin et al., 2007; Moens et al., 2007; Garcia-Reyero et al., 2008; Levi et al., 2009; Shelley et al., 2012). To date, however, there is a distinct lack of research regarding the effects of E1 on gene expression in fish. The present study evaluated the effects of E1 on the rainbow trout hepatic transcriptome using RNA sequencing (RNA-Seq), a technique used to discover novel gene targets, identify biological processes affected, and potentially provide insights into mechanisms of action.

Vitellogenin (VTG) gene transcription and protein induction have been commonly used as biomarkers of xenoestrogen exposure since VTG is a biomarker of vitellogenesis, a process that normally occurs in maturing females when producing eggs (Herman and Kincaid, 1988; Sumpter and Jobling, 1995). Vitellogenesis can be initiated in hepatocytes by xenoestrogens binding to estrogen receptors (ERs) resulting in the transcription of estrogen-responsive genes and the production of egg proteins: VTG, a precursor to egg yolk, and vitelline envelope proteins (VEPs), structural parts of the egg envelope (Mommsen and Walsh, 1988; Arukwe and Goksoyr, 2003; Darie et al., 2004). In adult females, the egg proteins are transported to the ovary for inclusion as components in egg formation (Mommsen and Walsh, 1988; Arukwe and Goksoyr, 2003); however, in male or juvenile fish, these egg proteins remain in circulation longer than in females until they are cleared by the kidney (Hemmer et al., 2002). Since xenoestrogens induce vitellogenesis in both male and juvenile fish, a model system has been developed for evaluating potentially estrogenic chemicals by measuring estrogen-responsive gene transcripts or proteins associated with egg production (Sumpter and Jobling, 1995; Arukwe and Goksoyr, 2003; Moura Costa et al., 2010; Lei et al., 2014). This model system of vitellogenesis was evaluated in the present study using rainbow trout (*Oncorhynchus mykiss*) exposed to an environmentally-relevant concentration of E1, to specifically characterize the time course of these responses. The time course included a recovery period to evaluate the return of altered parameters to baseline levels. This knowledge may aid with selecting transcripts that could be biomarkers of E1 exposure and will further characterize the use of VTG protein as a biomarker so that temporal trends are identified and can indicate appropriate sampling timeframes.

2. Material and methods

2.1. Estrone exposures and sampling

Exposures were conducted as static renewal in 38 L glass aquaria maintained at 15 ± 1 °C under a 16:8 h light:dark photoperiod. Water quality parameters (dissolved oxygen, pH, conductivity and temperature) were measured daily, as well as before and after each water change. Juvenile rainbow trout (*O. mykiss*) (mass 3.8 ± 1.1 g, mean ± SEM) were obtained from Sun Valley Trout Hatchery (Mission, BC, Canada) and housed according to Environment Canada method EPS1/RM/09 (Environment Canada, 1990). Experimental protocols and fish use were in adherence with the Canadian Council on Animal Care guidelines. On day 0 (d0), 6 fish were randomly placed into each of 64 aquaria, with 32 containing solvent control (CON) and 32 containing E1 treatments. In the solvent control, fish were exposed to well water containing 0.001% (v/v) anhydrous 100% ethanol (Commercial Alcohols, Brampton, ON, Canada). In the E1 exposure, fish were exposed to well water containing 27 ng/L E1 (CAS = 53-16-7; Sigma-Aldrich, Oakville, ON, Canada) in anhydrous 100% ethanol (final solvent concentration of 0.001% (v/v)). On d0, 1 L each of prepared aqueous CON and E1 treatments was collected from 2 aquaria (i.e. duplicates), prior to the introduction of fish. These samples were analyzed in duplicate for E1 using the gas chromatography mass spectrometry method of Furtula et al. (2012b). Fish were exposed to CON or E1 treatments for up to 7 days (d), with 100% water renewal every second day. After 7 d, fish were transferred to clean aquaria containing well water only for a depuration period of 9 d. During this period, a 100% water renewal occurred every second day. Fish were fed 2.5% of their body mass of commercial feed pellet (Skretting, Vancouver, BC, Canada) 2 h prior to a water renewal.

Each day for 16 d, fish in 2 aquaria per treatment (n = 6 per aquarium, 12 fish total per treatment) were euthanized in 150 mg/L buffered MS-222 (tricaine methanesulfonate; Syndel Labs, Vancouver, BC, Canada), blotted dry, and then measured for mass and fork length. Whole blood was collected from caudal sever into heparinized capillary tubes (Chase Scientific Glass, Inc., Rockwood, TN, USA) and spun for 3 min in a microcapillary centrifuge (International Equipment Company, Chattanooga, TN, USA). Plasma from each fish was separated from red blood cells and stored in a 1.5 mL tube (VWR, Mississauga, ON, Canada) at −80 °C until analyzed for VTG protein. The liver was collected, weighed, and stored in a 1.5 mL tube containing 1.2 mL of RNALater® (Ambion, Life Technologies Inc., Burlington, ON, Canada) at −80 °C for total RNA extraction.

2.2. Plasma analysis

VTG protein concentrations in plasma were determined using a rainbow trout VTG ELISA kit (Biosense® Laboratories, Abraxis, Warminster, PA, USA). Samples in which VTG was not detected were set to the detection limit of the kit for statistical purposes. Plates were read on a Bio-tek® PowerWave Reader (Bio-Tek, Winooski, VT, USA) using parameters specified in the manufacturer's protocol.

2.3. Transcriptomics evaluation

2.3.1. RNA isolation

Hepatic total RNA was extracted from all liver samples (30 mg) for QPCR and RNA-Seq analysis using RNeasy mini extraction kits (Qiagen, Mississauga, ON, Canada). The manufacturer's protocol was followed except two 35 μL elutions were used in the final step, and the optional on-column DNase step was included. Total RNA concentration was determined via A_{260}/A_{280} concentration analysis on a Nanodrop 1000 (Thermo Fisher Scientific, Mississauga, ON, Canada). RNA quality was evaluated using the Bio-Rad Experion Electrophoresis Station and the RNA StdSens Analysis Kit as per manufacturer's protocols (Bio-Rad, Mississauga, ON, Canada).

2.3.2. QPCR

Preparation of cDNA from 1 μg of total RNA occurred using a QuantiTect Reverse Transcription kit (Qiagen), with a 60 min synthesis time. A working dilution of cDNA was prepared for each sample by diluting stock cDNA 15-fold in nuclease-free water (Ambion). QPCR analysis was performed on both treatments from each day (for 16 d). Samples were set-up in triplicate on ice in 96-well Axygen chimney plates (VWR), sealed with MicroAmp® optical adhesive film (Applied Biosystems, Life Technologies, Burlington, ON, Canada), and run on Stratagene Mx3000P instruments (Agilent, Mississauga, ON, Canada) as described in Osachoff et al. (2013b) and Shelley et al. (2013). In brief, each well contained 50% iTaq SYBR green Supermix (Bio-Rad, Mississauga, ON, Canada), nuclease-free water (Ambion), and 20 pmol of each primer (primer sequences and gene transcript details in Table 1). *VTG*, *VEPα*, *VEPβ*, and *VEPγ* were used to evaluate egg-protein related induction by E1. All four known isoforms of rainbow trout ER transcripts were evaluated (*ERα1*, *ERα2*, *ERβ1*, *ERβ2*; Nagler, 2007). Each amplicon was verified as the correct product by agarose gel electrophoresis (band size), cloning and sequencing in TopoTA kits (Invitrogen, Life Technologies, Burlington, ON, Canada), and confirmation of a unique, characteristic temperature for a single peak on the