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Estrogenic and anti-estrogenic influences in cultured brown trout hepatocytes: Focus on the expression of some estrogen and peroxisomal related genes and linked phenotypic anchors

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ABSTRACT

Estrogens, estrogenic mimics and anti-estrogenic compounds are known to target estrogen receptors (ER) that can modulate other nuclear receptor signaling pathways, such as those controlled by the peroxisome proliferator-activated receptor (PPAR), and alter organelle (inc. peroxisome) morphodynamics. By using primary isolated brown trout (*Salmo trutta* f. *fario*) hepatocytes after 72 and 96 h of exposure we evaluated some effects in selected molecular targets and in peroxisomal morphological features caused by: (1) an ER agonist (ethinylestradiol—EE2) at 1, 10 and 50 μM; (2) an ER antagonist (ICI 182,780) at 10 and 50 μM; and (3) mixtures of both (Mix I—10 μM EE2 and 50 μM ICI; Mix II—1 μM EE2 and 10 μM ICI and Mix III—1 μM EE2 and 50 μM ICI). The mRNA levels of the estrogenic targets (*ERα*, *ERβ-1* and *vitellogenin A—VtgA*) and the peroxisome structure/function related genes (*catalase, urate oxidase—Uox, 17β-hydroxysteroid dehydrogenase 4—17β-HSD4, peroxin 11α—Pex11α* and *PPARα*) were analyzed by real-time polymerase chain reaction (RT-PCR).

Stereology combined with catalase immunofluorescence revealed a significant reduction in peroxisome volume densities at 50 μM of EE2 exposure. Concomitantly, at the same concentration, electron microscopy showed smaller peroxisome profiles, exacerbated proliferation of rough endoplasmic reticulum, and a generalized cytoplasmic vacuolization of hepatocytes. *Catalase* and *Uox* mRNA levels decreased in all estrogenic stimuli conditions. *VtgA* and *ERα* mRNA increased after all EE2 treatments, while *ERβ-1* had an inverse pattern. The EE2 action was reversed by ICI 182,780 in a concentration-dependent manner, for *VtgA*, *ERα* and *Uox*. Overall, our data show the great value of primary brown trout hepatocytes to study the effects of estrogenic/anti-estrogenic inputs in peroxisome kinetics and in ER and PPARα signaling, backing the still open hypothesis of crosstalk interactions between these pathways and calling for more mechanistic experiments.

1. Introduction

Synthetic hormonally active compounds, such as xenoestrogenic substances, have been recognized as prime stressors acting on the endocrine reproductive axis in fish. Numerous studies have documented reproductive impairment, *e.g.*, (Nash et al., 2004; Panter et al., 1998), with still unknown ultimate consequences at the population level (Harris et al., 2011; Kidd et al., 2007). From a classical point of view, exogenous estrogens primarily act *via* estrogen nuclear receptors (ER) on their transcriptionally regulated pathway in the liver, brain and gonads (key estrogen target organs) in fish and other vertebrates (Nelson and Habibi, 2013), despite the distribution of ER in other organs (Menuet et al., 2002). Nonetheless, evidence exists that estrogens can interfere with pathways regulated by other nuclear receptors, such as those controlled by the peroxisome proliferator-activated receptor (PPAR). This

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particular aspect has been addressed in mammalian model systems, with strong evidence of interfering interaction between ER and PPAR signaling (*e.g.*, Chu et al., 2014; Zhang et al., 2015; Wang and Kilgore, 2002). Many of these data describe an inhibitory effect of PPAR agonists on estrogen-dependent pathways, *e.g.*, in the uterus (Gunin et al., 2004; Houston et al., 2003). Cancer cell lines have been widely used to study specific interactions between PPARγ and ERα/ERβ (Chu et al., 2014; Wang and Kilgore, 2002), although regulatory effects between PPARα and estrogens have also been suggested (Jeong and Yoon, 2007; Kim et al., 2009).

We have previously hypothesized an interference scenario between estrogens and PPARs in teleost fish (Batista-Pinto et al., 2009). Such crosstalk would lead to an array of peroxisomal and lipid profiling impacts that, if disrupted, could compromise hepatic function, ovary differentiation and normal egg development. A set of morphological experiments using brown trout (*Salmo trutta* f. *fario*) as a model revealed a negative correlation between estrogens titers and peroxisome size during gonadal maturation (Rocha et al., 1999). Further, the activities of target peroxisomal enzymes were reduced under similar conditions (Resende et al., 2005; Rocha et al., 2004). In a second set of tests, lower *PPARα* expression in vitellogenic females was noted in parallel to higher estradiol levels (Batista-Pinto et al., 2009). With this background, a crosstalk between estrogens and PPAR-dependent pathways in brown trout was proposed (Batista-Pinto et al., 2009).

To further clarify these questions we established an *in vitro* assay using primary hepatocytes isolated from brown trout, which may be used in future applications as a tool to understand *in vivo* endocrine disruption processes in this species. As metabolically active cells, primary fish hepatocytes have been successfully used to test distinct xenobiotic inputs, including estrogenic ones, within various research purposes, *e.g.*, (Hultman et al., 2015; Maradonna et al., 2013; Sovadinová et al., 2014). Our goal was to address the impacts of a model ER agonist (ethinylestradiol—EE2), at 1, 10 and 50 μM, an antagonist of ER (ICI 182,780—ICI), at 10 and 50 μM, and their combined action (Mix I—10 μM EE2 and 50 μM ICI; Mix II—1 μM EE2 and 10 μM ICI and Mix III—1 μM EE2 and 50 μM ICI), in brown trout hepatocytic peroxisomes, regarding morphological qualitative and quantitative aspects. In addition, we were also interested in measuring the mRNA expression levels of genes involved in biological pathways potentially related to estrogen signaling and peroxisomal activity and function. For estrogenic response, we selected *ERα, ERβ-1* and the gene encoding female egg yolk protein—*vitellogenin A* (*VtgA*). As peroxisomal endpoints, we analyzed the following targets: *catalase*, the product of which is a peroxisomal enzyme used as an environmental indicator of stress (Orbea et al., 2002); *urate oxidase—Uox*, the product of which degrades urate to allantoin (Hayashi et al., 2000); *PPARα*, mainly expressed in the liver, with a product that plays a major role in governing fatty acid oxidation (Grygiel-Gorniak, 2014); *peroxin 11α—Pex11α*, the product of which is involved in peroxisome division (Fagarasanu et al., 2007); and *17β-hydroxysteroid dehydrogenase 4—17β-HSD4*, with a product which is as an active participant on peroxisomal fatty acid β-oxidation and in the conversion of estradiol to estrone (Breitling et al., 2001).

The aim of the study was to elucidate estrogenic/anti-estrogenic effects, not only in direct estrogenic signaling, but also on PPARα-mediated pathways, some of them directly influencing the hepatocytic structure and function. Our investigation aims to shed new light on the regulatory processes involved in PPAR and ER interactions in fish, which will be crucial to decode crosstalk aspects between the two nuclear pathways.

2. Materials and methods

2.1. Animals

One-year-old juveniles of brown trout (*S. trutta* f. *fario*) were obtained from a state facility for repopulation purposes (Aquaculture Station of Torno, Portugal), and maintained under controlled conditions with a natural photoperiod (13–15 h daylight hours), at approximately 16 °C. Animals were allowed to acclimate to laboratory conditions for 4 weeks before the experiments. All the animal procedures were performed according to the Portuguese Decree-Law No. 113/2013 implementing EU Directive No. 2010/63 on animal protection for scientific purposes. Two trout batches were used in two independent experiments. In experiment I and II, individuals had a mean (standard deviation) weight of 79.0 (±2.5) g and 158.8 (±41.2) g and a total length of 19.0 (±0.0) cm and 23.3 (±1.1) cm, respectively. Water quality parameters (temperature, nitrite, ammonium, pH, oxygen) were checked periodically during acclimatization, and registered values were consistently within recommended ranges (MacIntyre et al., 2008). Animals were fed every day (except on the day before the experiments) with dry granules for salmonids (T-4 Optiline, Skretting).

2.2. Hepatocyte isolation

First, fish were irreversibly anesthetized with an aqueous solution of ethylene glycol monophenyl ether (0.6 mL/L) (Merck) and then exsanguinated through caudal vein. The primary hepatocyte culture was generated with the two-step collagenase perfusion technique. Briefly, after abdominal cavity exposure, the liver was initially perfused with a Hank's Buffered Salt Solution (HBSS), supplemented with 10 mM EDTA (Merck), to remove as much blood as possible. The cells were dislodged with HBSS with 0.05% collagenase type IV (Sigma–Aldrich) and 1.3 mM $CaCl_2$ (Merck). Hepatocytes were gently dissociated using the HBSS buffer, but without collagenase, and after nylon membrane filtration (200 μm and 50 μm), cells were centrifuged for 3 × 5 min at 160 × *g*. Pellets were resuspended in Leibovitz's L-15 medium without phenol red (Invitrogen) – to avoid estrogenic stimulation from the media – supplemented with 5% charcoal-stripped fetal bovine serum (FBS) (Sigma–Aldrich), 100 μg/mL of streptomycin and 100 U/mL of penicillin (PAA Laboratories GmbH). Cell number was counted in a Neubauer chamber and the viability was estimated using the trypan blue exclusion assay. At the end, hepatocytes were plated at 1×10^6 cells/mL in 500 μL of L-15 medium and maintained at 19 °C without additional O_2/CO_2 in 24-well plates (SPL Life Sciences), previously coated with poly-L-lysine (300 μg/mL) (Sigma–Aldrich).

2.3. In vitro *exposure conditions*

For each experiment, primary hepatocytes were isolated from distinct fish and plated according to a defined experimental design in order to obtain representative material from each animal, at each treatment condition, for the distinct outputs (as detailed below). Cultures were maintained for 24 h under the standard conditions already described. After this period of cell adhesion, hepatocytes were exposed for 72 h and 96 h to different treatments, with a daily schedule for changing solutions, since it has earlier been shown in a similar experimental assay with rainbow trout hepatocytes that EE2 decreases to less than 50% in the wells after 24 h of exposure (Hultman et al., 2015). Exposure times are in accordance with other reported data obtained from isolated hepatocytes (Hultman et al., 2015). In experiment I, four treatments were done: 0.1% of ethanol p.a (Merck) – solvent control, 1, 10 and 50 μM of EE2 (CAS 57-63-6 , Sigma–Aldrich). In experiment II, the exposure groups were: 0.1% ethanol – solvent control, 1 μM of EE2, 10 and 50 μM of ICI 182,780