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Effects of 17β-estradiol, 4-nonylphenol, and β-sitosterol on the growth hormone-insulin-like growth factor system and seawater adaptation of rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

Previous studies show that successful adaptation of euryhaline teleost fish to seawater (SW) involves the GH-IGF system. The increasing occurrence, distribution, and concentration of environmental contaminants, including environmental estrogens (EE), in aquatic habit over recent time may compromise the hypoosmoreegulatory ability of fish. In this study, we used rainbow trout (*Oncorhynchus mykiss*) to assess the effects of EE on the GH-IGF system and adaptation to increased salinity. Juvenile trout (ca. 30 g) were exposed to either low (10 μg/l) or high (100 μg/l) concentrations of β-sitosterol, 4-n-nonylphenol (NP), or 17β-estradiol (E2) for 28 days in fresh water (FW); after which, fish were exposed to 20‰ SW. Plasma chloride levels in control fish rose initially, and then declined to initial levels after 48 h. By contrast, plasma chloride levels in all EE-treated groups except β-sitosterol low increased and remained elevated over initial levels after 48 h. Levels GH receptor 1 (GHR 1), GHR 2, insulin-like growth factor-1 (IGF-1), and IGF-2mRNAs in liver of control fish increased 6–12 h after SW exposure. In gill, levels of GHR 1, GHR 2, IGF-1, IGF-2, IGF receptor 1A (IGFR1A), and IGFR1B mRNAs increased in control fish 6–12 h after 20‰ SW exposure. Levels of IGFR1A and IGFR1B mRNAs in white muscle and of IGFR1A mRNA in red muscle increased in control fish 6–12 h after 20‰ SW exposure. Expression of all mRNAs in liver, gill, and red and white muscle declined from peak levels in control fish by 48 h after transfer. Exposure of fish to β-sitosterol, NP, and E2 abolished or attenuated normal salinity-induced changes in the expression of GHR, IGF, and IGFR1 mRNAs in all tissues. These results indicate that EE reduces salinity adaptation by inhibiting components of the GH-IGF system.

1. Introduction

Hypoosmotic regulation of euryhaline fish, including salmonids, is accompanied by an increase in the number and size of seawater (SW)-type chloride cells and by a corresponding increase in the expression and function of Na^+, K^+ ATPases (NKA), Na^+, K^+, $2Cl^-$ cotransporters (NKCC), and Cl^- channels in chloride cells (Evans and Claiborne, 2009). Considerable research has shown that SW adaptability of euryhaline fish is influenced by numerous hormones, including growth hormone (GH), insulin-like growth factor (IGF-1), cortisol, and thyroid hormones (Mancera and McCormick, 1998; McCormick, 2001; Evans, 2002).

Increasing attention has been given to the GH-IGF system and its role in SW readiness and hypoosmoregulatory ability, which appear to be distinct from the actions of the GH-IGF system on growth (Sakamoto and McCormick, 2006; Klein and Sheridan, 2008). Plasma levels of GH and IGF-1 increase following exposure to SW accompanied by increased mRNA expression of the hormones (Sakamoto et al., 1993; McCormick et al., 2000; Agustsson et al., 2001; Shepherd et al., 2005; Nilsen et al., 2008). Heightened sensitivity to GH and IGFs also appears to accompany SW exposure, as expression of GHR and type 1 IGF receptors (IGFR1) increase (Poppinga et al., 2007). In addition, GH and IGF-1 treatment increase salinity tolerance and chloride cell number as well as NKA and NKCC activity/biosynthesis (McCormick et al., 1991; Seidelin et al., 1999; Pelis et al., 2001). The GH-IGF system also appears important for the SW preparatory changes associated with salmonid smoltification. Levels of GH and IGF-1 increase during smoltification in association with increases in chloride cell size/number, NKA, and NKCC activity (Young et al., 1989; McCormick et al., 2002).

The increasing production, use, and disposal of an expanding array of chemicals that enter the environment pose a serious threat to terrestrial and aquatic animals as well as to humans. Of particular concern is a broad spectrum of natural and synthetic compounds that mimic estrogen. Environmental estrogens (EE) include endogenous and synthetic animal estrogens (e.g., 17β-estradiol), phytoestrogens (e.g., β-sitosterol), mycotoxins (e.g., zearalenone), organochlorine pesticides

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(e.g., DDT), polychlorinated biphenyls (PCBs), and alkylphenol polyethoxylates (APEs; e.g., 4-nonylphenol). The impacts of EE are magnified because they accumulate in tissues, have epigenetic effects and affect progeny, and are rapidly transferred through the food web (Hester and Harrison, 1999). Environmental estrogens have been found to disrupt a wide variety of reproductive processes in fish, amphibians, reptiles, and birds, including inhibited testicular growth, reduced sperm production, intersex gonads, reduced egg production, and altered reproductive timing and behavior (Tyler et al., 1998). Notably, 4-nonylphenol (NP) and β-sitosterol increase vitellogenin in the liver of male trout, an action that appears mediated via the estrogen receptor (Jobling and Sumpter, 1993; Tremblay and Van Der Kraak, 1998, 1999). β-sitosterol also decreases plasma levels of sex steroids by reducing gonadal steroidogenesis in goldfish and rainbow trout (MacLatchy and Van Der Kraak, 1995).

Knowledge of the effects of EE on processes other than reproduction is just emerging. For example, 17β-estradiol (E2) and 4-nonylphenol (NP) have been found to reduce salinity tolerance in Atlantic salmon smolts and reduce plasma levels of IGF-1 (Madsen et al., 2004, McCormick et al., 2005; Lerner et al., 2007a). The aims of the present study were to further elucidate the role of the GH-IGF system in adaptation to increased salinity and to determine if the osmodisrupting effects of EE are mediated by alterations in the GH-IGF system.

2. Materials and methods

2.1. Animals

Juvenile rainbow trout of both sexes were obtained from Dakota Trout Ranch near Carrington, ND, and transported to North Dakota State University where they were maintained in 800-l circular tanks supplied with recirculated (100% replacement volume per day) dechlorinated municipal water at 14 °C under a 12L:12D photoperiod. Fish were fed to satiation twice daily with AquaMax Grower (PMI Nutrition International, Inc., Brentwood, MO), except 24–36 h before initiating experimental manipulations.

2.2. Experimental conditions

Fish (ca. 30 g) were anesthetized, measured, weighed, and transferred to 40-l glass aquaria (15 fish per tank) containing fresh water (FW) with or without an EE. Three estrogenic compounds were used: 17β-estradiol (Sigma, St. Louis, MO), 4-*n*-nonylphenol (AlfaAesar, Ward Hill, MA), and β-sitosterol (Calbiochem, San Diego, CA); the treatments groups were as follows: two doses of E2 (10 μg/l and 100 μg/l), two doses of NP (10 μg/l and 100 μg/l), and two doses of β-sitosterol (10 μg/l and 100 μg/l). All compounds were dissolved in ethanol; the final concentration of ethanol did not exceed 0.007%. The vehicle control group received ethanol only. The water was well aerated and the tanks were kept at 14 °C under a 12L: 12D photoperiod. Twenty-four hours after transfer to aquaria, feeding recommenced (1% body weight once per day) and continued throughout the FW exposure period; however, feeding was suspended 24 h prior to salinity challenge. One-half the volume of each tank was removed and replaced with FW containing the appropriate treatment (added so as to maintain the desired final concentration of each test agent) every other day, in a manner similar to that described by Tremblay and Van Der Kraak (1999). Under these conditions, dissolved oxygen ranged from 8–10 mg/l, and ammonia did not exceed 0.25 ppm. Discarded water was filtered through activated charcoal before disposal.

After 28 days, fish were exposed to a salinity challenge. Water was removed from treatment tanks and replaced with Instant Ocean (Aquarium Systems Inc., Mentor, OH) to achieve a final concentration of 20‰ (w/v). The replacement 20‰ SW did not contain EE treatment or vehicle, and the fish were not fed during the challenge period. Fish were sampled 0 h, 6 h, 12 h, and 48 h following 20 ‰ SW exposure. At sampling, fish were anesthetized with 0.05% (v/v) 2-phenoxyethanol, measured, and weighed. Blood was collected with heparinized glass capillary tubes from the severed caudal vessels and centrifuged (5000 *g* for 5 min). Plasma was collected and stored at −80 °C for later analysis. Liver, gill filaments, red muscle, and white muscle samples were taken for mRNA analysis. All samples were immediately placed on dry ice and stored at −80 °C.

2.3. Plasma chloride

Plasma chloride was measured by silver titration with a Buchler–Cotlove Chloridometer and using external standards.

2.4. Quantitative real-time PCR

Frozen tissues were homogenized and total RNA was extracted using TRI reagent® (Molecular Research Center, Cincinnati, OH, USA) as specified by the manufacturer's protocol. RNA pellets were dissolved in 40–100 μl RNase-free deionized water and total RNA was quantified by UV (A_{260}) spectrophotometry. Total RNA was diluted with RNase-free deionized water to 100 ng/μl. RNA was reverse transcribed according to the manufacturer's protocol in a 10-μl reaction using 200 ng total RNA with an AffinityScript QPCR cDNA Synthesis Kit (Stratagene, La Jolla, CA).

Steady-state mRNA levels of GHR 1, GHR 2, IGF-1, IGF-2, IGFR1A, and IGFR1B were determined by quantitative real-time PCR using a Stratagene Mx 3000p detection system (Stratagene, La Jolla, CA) as previously described (Very et al., 2005; Poppinga et al., 2007; Malkuch et al., 2008). Briefly, real-time PCR reactions were carried out for controls, standards, and samples in a 10 μl total volume (1 μl cDNA from reverse transcriptase reactions; 5 μl 2× Brilliant II Master Mix; 1.0 μl of each gene-specific probe, forward primer, and reverse primer at concentrations optimized for each RNA species; 1 μl RNase-free deionized water). Cycling parameters were set as follows: 95 ° C for 10 min, and 50 cycles of 92 ° C for 15 s plus 58 ° C for 1 min. Sample copy number was calculated from the threshold cycle number (C_T), and the C_T was related to a gene-specific standard curve followed by normalization to β-actin. No difference ($p>0.05$) was observed in β-actin among the treatment groups.

2.5. Statistics

Quantitative data are expressed as means ± S.E.M. Statistical differences were analyzed by ANOVA followed by Duncan's multiple range test using SigmaStat (SPSS, Chicago, IL). A probability of 0.05 was used to indicate significance.

3. Results

3.1. Body characteristics

The effects of EE on the body characteristics of rainbow trout following a 28-day exposure in FW are shown in Table 1. Over the course of 28 days, juvenile trout in the control group grew significantly in terms of body length, and although body weight also increased, this change was not significant. Exposure to estrogenic compounds retarded growth to some extent. Although none of the estrogenic compounds affected body length, there was a trend ($p=0.26$ E2 low; $p=0.34$ E2 high) for E2 to repress growth in terms of body weight compared to control-treated fish and a significant ($p<0.05$) depression of body weight observed in fish treated with the high dose of NP. Estrogenic compounds also tended to reduce the condition of fish. This was evidenced by the trend toward reduced condition observed in E2 ($p=0.18$ E2 low; $p=0.30$ E2 high) and low β-sitosterol ($p=0.09$)-