Environmental estrogens inhibit mRNA and functional expression of growth hormone receptors as well as growth hormone signaling pathways *in vitro* in rainbow trout (*Oncorhynchus mykiss*)

Andrea M. Hanson, Alicia T. Ickstadt, Dillon J. Marquart, Jeffrey D. Kittilson, and
Mark A. Sheridan

Department of Biological Sciences, North Dakota State University, Fargo 58108, USA

Keywords: environmental estrogens, growth hormone receptor, growth hormone signaling

Address correspondence reprint requests to:

Dr. Mark A. Sheridan
Department of Biological Sciences
North Dakota State University
Box 6050, Dept. 2715
Fargo, ND 58108-6050
Tel: 1-701-231-8110
Fax: 1-701-231-7149
e-mail: mark.sheridan@ndsu.edu

Abstract

Fish in aquatic habitats are exposed to increasing concentrations and types of environmental contaminants, including environmental estrogens (EE). While there is growing evidence to support the observation that endocrine-disrupting compounds (EDCs) possess growth-inhibiting effects, the mechanisms by which these physiological effects occur are poorly understood. In this study, we examined the direct effects of EE, specifically 17 β -estradiol (E2), β -sitosterol (β S), and 4-*n*-nonylphenol (NP), on GH sensitivity as assessed by mRNA expression and functional expression of growth hormone receptor in hepatocytes, gill filaments, and muscle in rainbow trout (*Oncorhynchus mykiss*). Additionally, we examined the effects of EE on signaling cascades related to growth hormone signal transduction (i.e., JAK-STAT, MAPK, and PI3K-Akt). Environmental estrogens directly suppressed the expression of GHRs in a tissue- and compound-related manner. The potency and efficacy varied with EE; effects were most pronounced with E2 in liver. EE treatment deactivated the JAK-STAT, MAPK, and PI3K-Akt pathways in liver a time-, EE- and concentration-dependent manner. Generally, E2 and NP were most effective in deactivating pathway elements; maximum suppression for each pathway was rapid, typically occurring at 10 to 30 min. The observed effects occurred via an estrogen-dependent pathway, as indicated by treatment with an ER antagonist, ICI 182,780. These findings suggest that EEs suppress growth by reducing GH sensitivity in terms of reduced GHR synthesis and reduced surface GHR expression and by repressing GH signaling pathways.

1. Introduction

Endocrine disrupting compounds (EDCs) are exogenous compounds found in an assortment of common materials such as pesticides, personal care products, plastics, and detergents that alter the normal physiology of an organism or its progeny. The increased production, use, and disposal of an expanding array of EDCs has led to increased introduction of these compounds into the environment, which is of concern because of their well-known detrimental effects (e.g., tumorigenesis, reproductive abnormalities, etc.)(Cabaton et al., 2011; Kime et al., 1999).

Environmental estrogens (EE) are a specific class of EDC that include endogenous and synthetic animal estrogens (e.g., 17 β -estradiol), phytoestrogens (β -sitosterol), mycotoxins (e.g., zearalenone), organochlorine pesticides (e.g., DDT), polychlorinated biphenyls (PCBs), and alkylphenol polyethoxylates (APEs; e.g., 4-nonylphenol) (Turner, 1999; Birkett and Lester, 2003).

The growth hormone (GH)-insulin-like growth factor-1 (IGF-1) system modulates a number of physiological processes such as growth, cellular differentiation, metabolism, reproduction, immune function, and behavior (Bjornsson et al., 2004; Duan, 1997; Madsen and Bern, 1993; Mancera and McCormick, 1998; Hull and Harvey, 2000; Norrelund, 2005). The growth-promoting effects of GH are mostly mediated by IGFs. GH, chiefly produced in the pituitary, circulates through the organism bound to binding proteins, where it can then bind to its receptors (GHRs) to activate signaling pathways, including JAK-STAT, MAPK, and PI3K, and subsequently induce the synthesis and secretion of IGF-1, primarily from the liver (Butler and LeRoith, 2001; Reindl et al., 2011; Wood et al., 2005). IGF then binds to its receptors (IGFRs) on various targets to stimulate cellular growth, proliferation, and differentiation (Butler and LeRoith, 2001; Reindl et al., 2011; Wood et al., 2005).

Increasing evidence indicates that growth is retarded in fish exposed to EEs during embryonic and postembryonic development (Arsenault et al., 2004; Ashfield et al., 1998; Hanson et al., 2012, 2014; Lerner et al., 2007; Shved et al., 2008). However, the mechanism(s) by which such effects are exerted remain unknown. Along with 17 β -estradiol (E2), β -sitosterol (β S), and 4-*n*-nonylphenol (NP) have been shown to be capable of binding to the estrogen receptor (ER)(Tremblay and Van der Kraak, 1998), thereby forming an active transcription factor capable of binding to genes possessing an estrogen response element. While steroidal compounds are known to work via classic genomic mechanisms, evidence has also emerged indicating that EE can activate rapid nongenomic responses in the cell at low levels (Bulayeva and Watson, 2004). For example, E2 has been shown to interact with GH signaling pathways (JAK-STAT, PI3K/Akt, ERK) (Leung et al., 2004).

In this study we used rainbow trout as a model to examine the mechanism(s) that underlie the effects of EE on the GH-IGF system. Specifically, the hypothesis was that EE reduce GH sensitivity and repress the GH signaling cascades of JAK-STAT, PI3K/Akt, and ERK. The rationale for this work stemmed from our previous findings that reduced GH sensitivity accompanied periods of reduced growth (Norbeck et al., 2007), growth requires activation of the JAK-STAT, PI3K/Akt, ERK signaling cascades (Bergan et al., 2012; Reindl et al., 2011), and *in vivo* exposure to EE reduced expression of GHR mRNAs (Hanson et al., 2014).

2. Materials and Methods

2.1. Materials

β -sitosterol was purchased from Calbiochem (San Diego, CA), and NP was purchased from AlfaAesar (Ward Hill, MA). Salmonid growth hormone (sGH) was purchased from GroPep, Ltd.

(Adelaide, Australia). ^{125}I -NaI for labeling salmon/trout GH was purchased from MP Biomedicals, Inc. (Irvine, CA). Phospho-specific and total antibodies for Akt, JAK2, STAT5, ERK1/2 horseradish peroxidase-linked anti-rabbit IgG antibody, and cell lysis buffer were obtained from Cell Signaling Technology (Beverly, MA, USA). Molecular weight markers were obtained from Bio-Rad Laboratories (Hercules, CA, USA). All other reagents, including E2 and the ER antagonist, ICI 182, 780, were obtained from Sigma (St. Louis, MO, USA).

2.2. *Experimental Animals*

Juvenile rainbow trout of both sexes were obtained from Dakota Trout Ranch near Carrington, ND, USA and transported to North Dakota State University. Fish 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 before experimental manipulations. All procedures were performed in accordance with the *Guide for the Care and Use of Laboratory Animals* and approved by the North Dakota State University Institutional animal Care and Use Committee.

2.3. *Preparation of tissues for in vitro culture*

Juvenile fish (ca. 50 g) were anesthetized by immersion in 0.05% (v/v) 2-phenoxyethanol, measured, weighed, and bled via the severed caudal vessels. Tissues (gill arches, liver, and white muscle) were removed, perfused *ex vivo* with 0.75% (w/v) saline solution, and stored in ice-cold Hank's Buffered Salt Solution (in mM: 137 NaCl, 5.4 KCl, 4 NaHCO₃, 1.7 CaCl₂, 0.8 MgSO₄, 0.5 KH₂PO₄, 0.3 Na₂HPO₄, 10 HEPES, and 4 glucose; pH 7.6). The tissues were chosen

because of the abundance of GHRs (*cf.* Very et al., 2005), their importance as target of GH action (metabolic, osmoregulatory), and ease of culture. Livers and muscle were cut into 1 mm³ pieces while immersed in Hank's on ice. Individual gill filaments were dissected from the arches while immersed in Hank's on ice. Tissues were washed three times with Hank's Balanced Salt Solution with 0.24% BSA, 2 ml Gibco MEM amino acid mix (50x)/100 mL, and 1 mL Gibco nonessential amino acid mix (100x)/100 mL then plated in 24-well culture plates (8-10 liver pieces, 12-15 gill filaments, 25-30 muscle pieces) containing 1-mL of Hank's medium with 0.24% BSA, 2 ml Gibco MEM amino acid mix (50x)/100 mL, and 1 mL Gibco nonessential amino acid mix (100x)/100 mL and preincubated for 3h at 14 °C under 100% O₂ while being shaken at 100 rpm in a gyratory shaker, then washed prior to further experimental manipulation.

2.4. Experimental Conditions

To examine the direct effects of EE on GH sensitivity in the physiologically important targets of liver, muscle and gill, tissues pieces were subjected to varying concentrations of E2, β S, or NP (all obtained from Sigma). All compounds were dissolved in ethanol; the final concentration of ethanol did not exceed 0.007%. Following incubation, tissues were immediately placed on dry ice and stored at -80° C for later mRNA and binding analyses.

To examine the dependence of the effects of EE on ER, tissues were obtained and treated as described above except following the 3 h preincubation, media was removed and liver and gill filaments were subjected to treatment with or without an ER antagonist, ICI 182,780 (10⁻⁶ M), for 2 h. Following the 2 h incubation period, EE (E2, β S, NP) treatment was administered at concentrations of 10 μ g/L (E2), 75 μ g/L (β S), and 75 μ g/L (NP).

To investigate the signaling pathways disrupted by EE, liver pieces were obtained and treated as described above. A time course of 0 min, 5 min, 10 min, 30 min, 60 min, and 180 min was used to examine rapid signaling effects at concentrations of 10 µg/l (E2) or 75 µg/l (βS, NP) in liver.

2.5. *Quantitative Real-Time PCR*

Frozen tissues were homogenized and total RNA was extracted using RNeasy reagent[®] (Molecular Research Center, Cincinnati, OH) according to the manufacturer's protocol. RNA pellets were dissolved in 75 µL RNase-free deionized water. Total RNA was quantified by UV (A_{260}) spectrophotometry and then diluted with RNase-free deionized water to 200 ng/µl. RNA was reverse transcribed in a 10-µl reaction using 200 ng total RNA with an AffinityScript QPCR cDNA Synthesis Kit (Stratagene, La Jolla, CA) as described by the manufacturer's protocol. Reactions without reverse transcriptase were included as negative controls; no amplification was detected in negative controls.

As previously described (Very et al., 2005), steady-state levels of GHR1 and GHR2 mRNAs were determined by quantitative real-time PCR using a Stratagene Mx 3000p detection system (Stratagene, La Jolla, CA). Real-time PCR reactions were carried out for standards, samples, and no-template controls in a 10 µl total volume (5 µl 2X Brilliant Master Mix; 0.5 µl of each gene-specific probe, forward primer, and reverse primer at concentrations optimized for each RNA species). Cycling parameters were set as follows: 95° C for 10 min, and 40 cycles of 92° C for 15 s plus 57° 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 following normalization to β-actin. No difference ($p > 0.05$) was observed in β-actin expression among treatments.

2.6. Growth hormone binding

Radiolabeling of sGH and binding of ^{125}I -GH to microsomes was conducted as described previously (Gray et al., 1990). Protein concentration was determined with the Bio-Rad, Inc. (Hercules, CA) dye-binding method for microplates using bovine serum albumin as the standard.

2.7. Western Blotting

Tissues were homogenized in 200 μL 1 x cell lysis buffer and the resulting homogenate incubated on ice for 10 min and centrifuged at 16,000 g for 10 min at 4° C. The protein concentration of the supernatant was determined using the dye-binding method for microplates (Bio-Rad Laboratories). Samples containing 50 μg total protein were then separated on a 7.5% SDS-PAGE gel and transferred to a 0.45 μm nitrocellulose membrane (Bio-Rad Laboratories). Membranes were blocked with 5% nonfat milk in TBS-Tween20 (TBS-T) at room temperature for 1 h. Membranes were washed three times with TBS-T and incubated with the indicated rabbit antiserum (1:000) in 5% BSA TBS-T buffer overnight (4° C). Membranes were then washed with TBS-T and incubated at room temperature with goat-anti-rabbit IgG-HRP antiserum (1:2000) for 1 h, washed, and visualized using ECL detection system (GE Healthcare, Buckinghamshire, UK) and bands were quantified with a FluorChem FC2 imager (Alpha Innotech). Membranes were stripped under reducing conditions (100 mM β -mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7 for 30 min at 50° C with agitation), washed twice with a large volume of TBS-T, and the immunodetection protocol was repeated with the control antisera. The use of commercial antisera to detect signaling elements in trout liver was validated previously (Reindl et al., 2011).

2.8. Statistics

Quantitative data are expressed as means $\pm$ 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. Effects of EE on steady-state levels of GHR mRNAs

The direct effects of EE on the expression of the two GHR-encoding mRNAs, GHR1 and GHR2, was studied in liver, gill, and muscle tissues incubated *in vitro*. In liver, EE inhibited the expression of GHR mRNA in a time-, concentration-, and EE-specific manner (Fig. 1). E2 and NP incrementally reduced steady-state levels of GHR1 and GHR2 mRNA over time, with the rate of diminution being similar for both subtypes. Maximum inhibition was observed after 24h, after which time E2- and NP-treated tissue decreased to 61% and 69% (GHR1) and 63% and 68% (GHR2), respectively, of control levels. E2 and NP also inhibited the expression of GHRs in a concentration-related manner; however, the efficacy of E2 to inhibit GHR subtypes (GHR1 IC_{50} ~1.6 ng/ml; GHR2 IC_{50} ~2.8 ng/ml) was significantly greater than that of NP (IC_{50} ~68 ng/ml IC_{50} ~77 ng/ml) and there was no observable difference ($p > 0.05$) in expression between subtypes treated with the same EE (Fig. 1B). Notably, β S had no effect on the expression of GHR mRNAs at any time period on concentration tested.

Exposure of gill filaments to EE did not appear to significantly suppress nor elevate GHR1 or GHR2 steady-state mRNA levels in a time- or concentration-dependent manner for any treatment group (Fig. 2).

In muscle, EE affected the expression of GHR in a concentration-, EE- and GHR subtype-specific manner. E2 and NP inhibited the expression of both GHR1 and GHR2; however, E2 (GHR1 IC_{50} ~0.8 ng/ml; GHR2 IC_{50} ~1.5 ng/ml) displayed significantly greater efficacy than NP (GHR1 IC_{50} ~37 ng/ml; GHR2 IC_{50} ~44 ng/ml) in this regard. Interestingly, β S differently affected GHR expression such that GHR2 was unaffected by β S at any concentration tested, whereas, the abundance of GHR1 was reduced in a concentration-related manner (IC_{50} ~55 ng/ml).

3.2. Effects of EE on functional expression of GHR

Functional expression of GHR was determined by 125 I-GH binding to microsomes (Fig. 4). Binding capacity was greatest in the liver and over twice that observed in gill and muscle. E2 and NP reduced binding capacity by over 40% in hepatic microsomes, whereas β S had no effect on GH binding. Although GH binding capacity in gill was not affected by any EE treatment, binding capacity in microsomes from white muscle decreased to 73% (E2), 77% (β S), and 68% (NP) of control values (Fig. 4). Environmental estrogen treatment had no effect on binding affinity in liver, gill, or white muscle (data not shown).

3.3. ER-dependence of EE action

The effects of EE appeared to be ER dependent, as indicated by the co-incubation of EE in the presence or absence of the ER antagonist, ICI 182,780. In liver, steady-state mRNA expression of GHR1 and GHR2 was significantly reduced by E2 and NP in the absence of the ER antagonist in a manner similar to that described above. Co-incubation with ICI 182,780 completely blocked the E2 and NP-inhibited GHR expression (Fig. 5).

3.4. EE deactivates GH signaling pathways

The activation state of GH signaling pathways was studied in lysates of liver tissue treated *in vitro* with EE. Phospho-JAK2, phospho-STAT5, phospho-Akt, and phospho-ERK were all detected. Environmental estrogens deactivated JAK2, STAT5, Akt, and ERK in a time-related, concentration-related, and compound-specific manner (Fig. 6). Maximum deactivation of JAK2 and STAT5 was rapid and occurred within 10 min (Fig. 6A, 6C). Deactivation of Akt, a downstream target of PI3K, and ERK was less rapid, with the lowest degree of phosphorylation observed at 30 min and with suppressive effects persisting through 180 min (Fig. 6E, 6G). Progressively lower degrees of phosphorylation of all signal elements were observed following treatment with increasing concentrations of EE; however, E2 and NP were significantly more effective than β S. E2 significantly reduced phosphorylation of JAK2, STAT5, Akt, and ERK at concentrations of 1-10 μ g/L and maximally reducing activation to 67-61% of control levels (Fig. 6B, 6D, 6F, 6H). The efficacy and potency of NP in deactivating signal elements was similar to that of E2, with significant inhibitory effects observed at 1-10 μ g/L and maximal inhibition to 63-52% of controls. By contrast, β S only elicited significant inhibitory effects at a concentration 75 μ g/L and maximally reducing phosphorylation to 85-78% of control levels.

4. Discussion

Previous work by us and others has shown that growth is retarded in fish exposed to EEs during embryonic and postembryonic development (Arsenault et al., 2004; Ashfield et al., 1998; Hanson et al., 2012, 2014; Lal et al., 2013; Lerner et al., 2007; Magliulo, et al., 2002; Shved et al., 2008). In this study we used rainbow trout as a model to examine the mechanism(s) that underlie the effects of EE on growth. The findings support our starting hypothesis and indicate

that EEs directly affect peripherally aspects of the GH-IGF system by reducing GH sensitivity and inhibiting GH signaling cascades. Disruption of GH sensitivity and GH signaling were elicited at environmentally (β S and NP) or physiologically (E2) relevant concentrations of EE (Holloway et al., 1999; Blackburn and Wadlock, 1995; Cook et al., 1996; Hale et al., 2000).

The inhibitory actions of EE on GH sensitivity are supported by several observations. First, E2 and NP were able to decrease steady-state levels of GHR1 and GHR2-encoding mRNAs in liver and all three EE tested resulted in decreased steady-state levels of GHR1 mRNA in muscle. Second, functional expression of GHRs, as assessed by 125 I-GH binding, was significantly depressed in liver (following E2 and NP treatment) and muscle (following treatment with all three EE). The reduced efficacy of BS to affect mRNA or functional expression of GHRs in liver and muscle was notable and may be due to the reduced affinity of BS for the estrogen receptor (Tremblay and Van der Kraak, 1998). Moreover, none of the EE affected mRNA or functional expression of GHRs in gill. Taken together, these data indicate that EE reduce peripheral sensitivity to GH by reducing *de novo* synthesis of GHRs and by reducing surface GHR in a compound- and tissue-specific manner. Such peripheral effects are consistent with our previous study in rainbow trout that immersion in E2, β S, NP (Hanson et al., 2014) reduces GH sensitivity and support the notion that EE effects vary by tissue. For example, *in vivo* E2 exposure of mature fish reduced hepatic and pituitary expression of GHR mRNA, but had no effect on GHR expression in gonad, gill, or brain (Filby et al., 2006; Jiao et al., 2006; Davis et al., 2008). Tissue-specific effects of E2 were confirmed *in vitro* as GH binding in trout liver and muscle but not in gill was reduced by hormone treatment (Norbeck et al., 2011). Although the GHR subtypes of teleosts are differentially expressed and may have distinct physiological functions

(Very et al., 2005; Saera-Vila et al., 2007; Reindl and Sheridan, 2012), the current findings suggest that EE generally have equivalent effects on their expression.

The observed effects on the GH-IGF system were ER-dependent, as treatment with each of the EE in combination with the ER antagonist, ICI 182,780, reestablished mRNA expression to levels similar to those observed in control tissues. EE such β S and NP have been found to bind to the estrogen receptor in mammals and teleosts, albeit with a much lower affinity than E2 (Bonefeld-Jorgensen et al., 2001; Tremblay and Van der Kraak, 1998; Knudsen and Pottinger, 1999; Sumpter and Jobling, 1995), which may explain the observed differences in potency and efficacy. ER α mediated E2 and bisphenol A (BPA) attenuated GH signaling as reflected by reduced GH-induced expression of casein in Hepa-T1 cells cotransfected with seabream GHRs (Jiao and Cheng, 2010). ER dependence of EE action appears conserved from fish to mammals as E2-, NP-, BPA-altered GH expression in rat pituitary GH3 cells also could be blocked by ER an antagonist (Dang et al., 2009).

The current findings also indicate that EE represses GH signaling cascades. This is supported by the observed time- and concentration-dependent decrease in the abundance of phosphorylated JAK2, STAT5, Akt, and ERK in trout liver. The JAK-STAT, PI3K-Akt, and ERK signaling pathways are all activated by GH and mediate GH-stimulated IGF-1 expression in hepatocytes (Reindl et al., 2011). Previously, E2 was shown to inhibit JAK-STAT activation in ovariectomized hypophysectomized rats (Murphy and Friesen, 1988) and to suppress GH-induced JAK-STAT activation in human embryonic kidney cells (Leung et al., 2003). In addition, NP and BPA significantly decreased the phosphorylation state of STAT5 hemocytes of mussel (Canesi et al., 2004). However, several studies have found contradictory results. For example, BPA, octylphenol, NP, and E2 rapidly (within 30 minutes) activated ERK1/2 and PI3K

signaling cascades in rat pituitary cells which resulted in increased GH mRNA expression (Dang et al., 2009). Dichlorodiphenyltrichloroethane (DDT) and polychlorinated biphenyls (PCBs) were also shown to be capable of activating the receptor tyrosine kinase pathways such as JAK-STAT (Enan et al., 1998; Shen and Novak, 1997). The basis for the disparate results is not known, but it may be due to cell-specific pathway linkages or the other differences that exist between native cells/tissues and transformed cell lines. It should be noted that multiple subtypes of ER exist--2 subtypes in mammals (Green et al., 1986; Kuiper et al., 1996) and 4 subtypes in teleost fish (Nagler et al., 2007)--that display differential expression (Nagler et al., 2007); therefore, the possibility exists for multiple, tissue-specific ER subtype-effector pathway linkages, some of which may be responsible for the contrasting effects.

The current findings together with previous work help establish a model for the mechanisms through which EE reduce peripheral GH sensitivity. The PI3K-Akt and ERK pathways activate a number of transcription factors (c/βEBP, HNF Sp1, Sp3, MSY-1) that together with STATs bind to GHR promoter regions to stimulate transcription (Schwartzbauer and Menon 1998; Schwartzbauer et al., 1998; Wood et al., 2005; Elango et al., 2006). ER have been shown to directly interact with ERK and PI3K/Akt (Migliaccio, et al., 1996; Simoncini et al., 2000; Hisamoto et al., 2001) as well as STAT5 (Wang and Cheng, 2004). Therefore, the deactivation of JAK-STAT, Akt, and ERK as shown in the current study presents one possible mechanism for EE reducing GH sensitivity. Another possible, but related, mechanism involves SOCS proteins, which are known to block JAK-STAT signaling via inhibition of JAK (Leung et al., 2004). Several studies have linked E2 to increases in SOCS-2 mRNA (Tollet-Egnell et al., 1999; Paul et al., 2000; Leong et al., 2004; Rico-Bautista et al., 2004), which, in turn, could lead to reduced STAT activation and reduced GHR transcription. There also is evidence that ER directly

interacts with ERE in GHR promoter regions to reduce GHR transcription (Leung et al., 2003). Lastly, the existence of membrane-bound ER, to which several EE have been shown to bind (e.g., NP, coumestrol, endosulfan, dichlorodiphenyldichloroethylene, dieldrin)(Bulayeva and Watson, 2004; Watson et al., 2007), offers another means of affecting GH sensitivity.

In summary, we have shown that EE such E2, β S, and NP directly reduce GH sensitivity and disrupt GH signaling pathways and points to at least one means by which EE retard organismal growth. Given that many of the actions of GH are mediated through the JAK-STAT, PI3K/Akt, and ERK signaling pathways, their deactivation also may underlie the disruption of other physiological processes (e.g., development, reproduction, osmoregulation) by EE (Jobling et al., 1996; Filby et al., 2006; Lerner et al., 2012). Moreover, given the paucity of information regarding the negative regulation of JAK-STAT, PI3K-Akt, and MAPK by EDCs, future studies should focus on further elucidating other levels of action on the GH-IGF system (i.e., IGF synthesis, IGF sensitivity), and additional mechanisms (i.e., alternative pathways, transcription factors, epigenetic alterations) by which these compounds inhibit growth and other processes.

Acknowledgements

We would like to thank Heather Bergan, Lincoln Martin, Lindsey Norbeck, and Chad Walock for their technical assistance. This work was supported by grants from the National Science Foundation, USA (IOS 0920116), the US Geological Survey/North Dakota Water Commission, and the NDSU Gerald Larson Agricultural Research Fund to M.A. Sheridan; A. Hanson was supported by fellowships from the North Dakota Water Resources Research Institute and the NDSU Graduate School.

References

- Arsenault, J.T., Fairchild, W.L., MacLachy, D.L., Burridge, L., Haya, K., Brown, S.B., 2004. Effects of water-borne 1-nonylphenol and 17beta-estradiol exposures during parr-smolt transformation on growth and plasma IGF-1 of Atlantic salmon (*Salmo salar* L.). *Aquat. Toxicol.* 66, 255–265.
- Ashfield, L.A., Pottinger, T.G., Sumpter, J.P., 1998. Exposure of female juvenile rainbow trout to alkylphenolic compounds results in modifications to growth and ovosomatic index. *Environ. Toxicol.* 17, 679-686.
- Bergan, H.E., Kittilson, J.D., Sheridan, M.A., 2012. Nutrition-regulated lipolysis in rainbow trout (*Oncorhynchus mykiss*) is associated with alterations in the ERK, PI3K-Akt, JAK-STAT, and PKC signaling pathways. *Gen. Comp. Endocrinol.* 176, 367-376.
- Birkett, J. W., Lester, J.N., 2003. *Endocrine Disrupters in Wastewater and Sludge Treatment Processes*. CRC Press, Boca Raton, FL.
- Bjornsson, B.T, Johansson, V., Benedet, S., Einarsdottir, I.E., Hildahl, J., Agustsson, T., Jonsson, E., 2004. Growth hormone endocrinology of salmonids: regulator mechanisms and mode of action. *Fish Physiol. Biochem.* 27, 227-242.
- Blackburn, M.A., Waldock, M.J, 1995. Concentrations of alkylphenols in rivers and estuaries in England and Wales. *Water Res.* 29, 1623-1629.
- Bonefeld-Jorgensen, E.C., Andersen, H.R., Rasmussen, T.H., Vinggaard, A.M., 2001. Effect of highly bioaccumulated polychlorinated biphenyl congeners on estrogen and androgen receptor activity. *Toxicology* 158, 141-153.

- Bulayeva, N.N., Watson, C.S., 2004. Xenoestrogen-induced ERK-1 and ERK-2 activation via multiple membrane-initiated signaling pathways. *Environ. Health Perspect.* 112, 1481-1487.
- Butler, A.A., LeRoith, D.L., 2001. Control of growth by the somatotrophic axis: growth hormone and the insulin-like growth factors have related and independent roles. *Ann. Rev. Physiol.* 63, 141-164.
- Cabaton, N.J., Wadia, P.R., Rubin, B.S., Zalko, D., Schaeberle, C.M., Askenase, M.H., Gadbois, J.L., Tharp, A.P., Whitt, G.S., Sonnenschein, C., Soto, A.M., 2011. Perinatal exposure to environmentally relevant levels of bisphenol A decreases fertility and fecundity in CD-1 mice. *Environ. Health Perspect.* 119, 547-552.
- Canesi, L., Lorusso, L.C., Ciacci, C., Betti, M., Zampini, M., Gallo, G., 2004. Environmental estrogens can affect the function of mussel hemocytes through rapid modulation of kinase pathways. *Gen. Comp. Endocrinol.* 138, 58-69.
- Cook, D.L., LaFleur, L., Parrish, A., Jones, J., Hoy, D., 1996. Characterization of plant sterols in a select group of US pulp and paper mills. In, Fifth IEAWQ Symposium Preprint of Forest Industry Waste Water, pp. 1-8, Vancouver, Canada.
- Dang, V.H., Nguyen, T.H., Lee, G.S., Choi, K.C., Jeung, E.B., 2009. In vitro exposure to xenoestrogens induces growth hormone transcription and release via estrogen receptor-dependent pathways in rat pituitary GH3 cells. *Steroids* 74, 707-714.
- Davis, L.K., Pierce, A.L., Hiramatsu, N., Sullivan, C.V., Hirano, T., Grau, E.G., 2008. Gender-specific expression of multiple estrogen receptors, growth hormone receptors, insulin-like growth factors and vitellogenins, and effects of 17 β -estradiol in the male tilapia (*Oreochromis mossambicus*). *Gen. Comp. Endocrinol.* 156, 544-551.

- Duan, C., 1997. The insulin-like growth factor system and its biological actions in fish. *Am. Zoo.* 37, 491-503.
- Elango, A., Shepherd, B., Chen T., 2006. Effects of endocrine disruptors on the expression of growth hormone and prolactin mRNA in the rainbow trout pituitary. *Gen. Comp. Endocrinol.* 145, 116-127.
- Enan, E., Matsuura, F., 1998. Activation of c-Neu tyrosine kinase by o,p'-DDT and beta-HCH in cell-free and intact cell preparations from MCF-7 human breast cancer cells. *J. Biochem. Mol. Toxicol.* 12, 83-92.
- Filby, A.L., Thorpe, K.L., Tyler, C.R., 2006. Multiple molecular effect pathways of an environmental oestrogen in fish. *Environ. Mol. Fish Biol.* 37, 121-134.
- Gray, E.S., Young, G., Bern, H.A., 1990. Radioreceptor assay for growth hormone in coho salmon (*Oncorhynchus kitsutch*) and its application to the study of stunting. *J. Exp. Zool.* 256, 290-296.
- Green, S., Walter, P., Kumar, V., Krust, A., Bornet, J.M., Argos, P., Chambon, P., 1986. Human oestrogen receptor cDNA: sequence and homology to v-erb-A. *Nature* 320, 134-139.
- Hale, R.C., Smith, C.L., DeFur, P.O., Harvey, E., Bush, E.O., LaGuardian, M.J., Vadas, G.G. 2000. Nonylphenols in sediments and effluents associated with diverse wastewater outfalls. *Environ. Toxicol. Chem.* 19, 946-952.
- Hanson, A.M., Kittilson, J.D., McCormick, S.D., Sheridan, M.A., 2012 Effects of 17 β -estradiol, 4-nonylphenol, and β -sitosterol on the growth hormone-insulin-like growth factor system and seawater adaptation of rainbow trout (*Oncorhynchus mykiss*). *Aquaculture* 362-363, 241-247.

- Hanson, A.M., Martin, L.E., Kittilson, J.D., Sheridan, M.A., 2014. Environmental estrogens inhibit growth by modulating the growth hormone-insulin-like growth factor system. *Gen. Comp. Endocrinol.* 196, 130-138.
- Hisamoto, K., Ohmichi, M., Kurachi, H., Hayakawa, J., Kanda, Y., Nishio, Y., Adachi, K., Tasaka, K., Miyoshi, E., Fujiwara, N., Taniguchi, N., Murata, Y., 2001. Estrogen induces the Akt-dependent activation of endothelial nitric oxide synthase in vascular endothelial cells. *J. Biol. Chem.* 276, 3459-3467.
- Holloway, A.C., Sheridan, M.A., Van Der Kraak, G., Leatherland, J.F., 1999. Correlations of plasma growth hormone with somatostatin gonadal steroid hormones and thyroid hormones in rainbow trout during sexual recrudescence. *Comp. Biochem. Physiol. Part B* 123, 251-260.
- Hull, K.L., Harvey, S., 2000. Growth hormone: a reproductive endocrine-paracrine regulator? *Rev. Reprod.* 5, 175-182.
- Jiao, B., Huang, X., Chan, C.B., Zhang, L., Wang, D. Cheng, Cheng, C.H.K., 2006. The co-existence of two growth hormone receptors in teleost fish and their differential signal transduction, tissue distribution, and hormonal regulation of expression in seabream. *J. Mol. Endocrinol.* 36, 23-40.
- Jiao, B., Cheng, C.H.K., 2010. Disrupting actions of bisphenol A and malachite green on growth hormone receptor gene expression and signal transduction in seabream. *Fish Physiol. Biochem.* 36, 251-261.
- Jobling, S., Sheahan, D., Osborne, J.A., Matthiessen, P., Sumpter, J.P., 1996. Inhibition of testicular growth in rainbow trout (*Oncorhynchus mykiss*) exposed to estrogenic alkylphenolic chemicals. *Environ. Toxicol. Chem.* 15, 194-202.

- Kime, D.E., Nash, J.P., Scott, A.P., 1999. Vitellogenesis as a biomarker of reproductive disruption by xenobiotics. *Aquaculture* 177, 345-352.
- Knudsen, F.R., Pottinger, T.G., 1999. Interaction of endocrine disrupting chemicals, singly and in combination, with estrogen-, androgen-, and corticosteroid-binding sites in rainbow trout (*Oncorhynchus mykiss*). *Aquat. Toxicol.* 44, 159-170.
- Kuiper, G.C., Enmark, E., Peltö-Huikko, M., Nilsson, S., Gustafsson, J.A., 1996. Cloning of a novel estrogen receptor expressed in rat prostate and ovary. *Proc. Natl. Acad. Sci. (USA)* 93, 5925-5930.
- Lal, B., Sarang, M.K., Kumar, P., 2013. Malathion exposure induces the endocrine disruption and growth retardation in the catfish, *Calarias batrachus* (Linn.). *Gen. Comp. Endocrinol.* 181, 139-145.
- Leong, G.M., Moverare, S., Brce, J., Doyle, N., Sjogren, K., Dahlman-Wright, K., Gustafsson, J.A., Ho, K.K.Y., Ohlsson, C., Leung, K.C., 2004. Estrogen up-regulates hepatic expression of suppressors of cytokine signaling-2 and -3 in vivo and *in vitro*. *Endocrinology* 145, 5525-5531.
- Lerner, D.T., Bjornsson, B.T., McCormick, S.D., 2007. Larval exposure to 4-nonylphenol and 17 β -estradiol affects physiological and behavioral development of seawater adaptation in Atlantic salmon smolts. *Environ. Sci. Technol.* 41, 4479-4485.
- Lerner, D.T., Sheridan, M.A., McCormick, S.D., 2012. Estrogenic compounds decrease growth hormone receptor abundance and alter osmoregulation in Atlantic salmon. *Gen. Comp. Endocrinol.* 179, 196-204.
- Leung, K.C., Doyle, N., Ballesteros, M., Sjogren, K., Watts, C.K.W., Low, T.H., Leong, G.M., Ross, R.J.M., Ho, K.K.Y., 2003. Estrogen inhibits GH signaling by suppressing GH-

- induced JAK2 phosphorylation, an effect mediated by SOCS-2. *Proc. Natl. Acad. Sci. (USA)* 100, 1016-1021.
- Leung, K.C., Johannsson, G., Leong, G.M., Ho, K.Y.Y., 2004. Estrogen regulation of growth hormone action. *Endocr. Reviews.* 25, 693-721.
- Madsen, S.S., Bern, H.A., 1993. In vitro effects of insulin-like growth factor-I on gill Na(+), K(+)-ATPase in coho salmon, *Oncorhynchus kitsutch*. *J. Endocrinol.* 138, 23-30.
- Magliulo, L., Schreiber, M.P., Cepriano, J., Ling, J., 2002. Endocrine disruption caused by two common pollutants at “acceptable” concentrations. *Neurotoxicol. Teratol.* 24, 71-79.
- Mancera, J.M., McCormick, S.D., 1998. Evidence for growth hormone/insulin-like growth factor I axis regulation of seawater acclimation in the euryhaline teleost *Fundulus heteroclitus*. *Gen Comp. Endocrinol.* 111, 103-112.
- Migliaccio, A., Di Domenico, M., Castoria, G., de Falco, A., Bontempo, P., Nola, E., Auricchio, F., 1996. Tyrosine kinase/p21ras/MAP-kinase pathway activation by estradiol-receptor complex in MCF-7 cells. *Eur. Mol. Biol. Org. J.* 15, 1292-1300.
- Mol. Gene. Metab.* 63, 243-253.
- Murphy, L.J., Friesen, H.G., 1988. Differential effects of estrogen and growth hormone on uterine and hepatic insulin-like growth factor 1 expression in the ovariectomized hypophysectomized rat. *Endocrinology* 122, 325-332.
- Nagler, J.J., Cavileer, T., Sullivan, J., Cyr, D.G., Rexroad, C., 2007. The complete nuclear estrogen receptor family in the rainbow trout: discovery of the novel ER α 2 and both ER β isoforms. *Gene* 392, 164-173.

- Norbeck, L.A., Kittilson, J.D., Sheridan, M.A., 2007. Resolving the growth-promoting and metabolic effects of growth hormone: differential regulation of GH-IGF-I system components. *Gen. Comp. Endocrinol.* 151, 332-341.
- Norbeck, L.A., Sheridan, M.A., 2011. An *in vitro* model for evaluating peripheral regulation of growth in fish: Effects of 17 β -estradiol and testosterone on the expression of growth hormone receptors, insulin-like growth factors, and insulin-like growth factor type 1 receptors in rainbow trout (*Oncorhynchus mykiss*). *Gen. Comp. Endocrinol.* 173, 270-280.
- Norrelund, H., 2005. The metabolic role of growth hormone in humans with particular reference to fasting. *Growth Hormone IGF Res.* 15, 95-122.
- Paul, C., Seiliez, I., Thissen, J.P., Le Cam, A., 2000. Regulation of expression of the rat SOCS-3 gene in hepatocytes by growth hormone, interleukin-6, and glucocorticoids mRNA analysis and promoter characterization. *Eur. J. Biochem.* 267, 5849-5857.
- Reindl, K.M., Kittilson, J.D., Bergan, H.E., Sheridan, M.A., 2011. Growth hormone-stimulated insulin-like growth factor-1 expression in rainbow trout (*Oncorhynchus mykiss*) hepatocytes is mediated by ERK, PI3K-AKT, and JAK-STAT. *Am. J. Physiol.* 301:R236-R243.
- Reindl, K.M., Sheridan, M.A., 2012. Peripheral regulation of the growth hormone-insulin-like growth factor system in fish and other vertebrates. *Comp. Biochem. Physiol. Part A.* 163, 231-245.

- Rico-Bautista, E., Negrin-Martinez, C., Novoa-Mogollon, J., Fernandez-Perez, L., Flores-Morales, A., 2004. Downregulation of the growth hormone-induced Janus kinase 2/signal transducer and activator of transcription 5 signaling pathway requires an intact actin cytoskeleton. *Exp. Cell. Res.* 294, 269-280.
- Riley, L.G., Hirano, T., Grau, E.G., 2004. Estradiol-17 β and dihydrotestosterone differentially regulate vitellogenin and insulin-like growth factor-1 in the teleost *Oreochromis mossambicus*. *Comp. Biochem. Physiol. Part C* 138, 177-186.
- Saera-Vila, A., Calduch-Giner, J.A., Perez-Sanchez, J., 2007. Co-expression of IGFs and GH GHR-flanking region. *J. Endocrinol.* 194, 361-372.
- Schwartzbauer, G., Menon, R.K., 1998. Regulation of growth hormone receptor gene expression. *Mol. Gene. Metab.* 63, 243-253.
- Schwartzbauer, G., Yu, J.H., Cheng, H., Menon, R.K., 1998. Transcription factor MSY-1 regulates expression of the murine growth hormone receptor gene. *J. Biol. Chem.* 273, 24760-24769.
- Shen, K., Novak, R.F., 1997. Differential effects of aroclors and DDT on growth factor gene expression and receptor tyrosine kinase activity in human breast epithelial cells. *Adv. Exp. Med. Biol.* 407, 295-302.
- Shved, N., Berishvili, G., Baroiller, J.F., Segner, H., Reinecke, M., 2008. Environmentally relevant concentrations of 17 α -ethinylestradiol (EE2) interfere with the growth hormone (GH)/insulin-like growth factor (IGF)-I system in developing bony fish. *Toxicol. Sci.* 106, 93-102.

- Simoncini, T., Hafezi-Moghadam, A., Brazil, D.P., Ley, K., Chin, W.W., Liao, J.K., 2000. Interaction of oestrogen receptor with the regulatory subunit of phosphatidylinositol-3-OH kinase. *Nature* 407, 538-541.
- Sumpter, J.P., Jobling, S., 1995. Vitellogenesis as a biomarker for estrogenic contamination of the aquatic environment. *Environ. Health Perspect.* 103, 173-178.
- Tollet-Egnell, P., Flores-Morales, A., Stavreus-Evers, A., Sahlin, L., Norstedt, G., 1999. Growth hormone regulation of SOCS-2, SOCS-3, and CIS messenger ribonucleic acid expression in the rat. *Endocrinology* 140, 3693-3704.
- Tremblay, L. Van der Kraak, G., 1998. Use of a series of homologous in vitro and in vivo assays to evaluate the endocrine disrupting action of β -sitosterol in rainbow trout. *Aquat. Toxicol.* 43, 149-162.
- Turner, K.J. 1999. Oestrogens, environmental oestrogens, and male reproduction. In, *Issues in Environmental Science and Technology*, Vol 12: Endocrine Disrupting Chemicals (Hester, R.E. and Harrison, R.M., eds), pp 83-108, The Royal Society of Chemistry, Cambridge, UK.
- Very, N.M., Kittilson, Norbeck, L.A., Sheridan, M.A., 2005. Isolation, characterization, and distribution of two cDNAs encoding for growth hormone receptor in rainbow trout (*Oncorhynchus mykiss*). *Comp. Biochem. Physiol. Part B.* 140, 615–628.
- Wang, Y., Cheng, C.H.K. , 2004. ER α and STAT5a cross-talk: interaction through C-terminal portions of the proteins decreases STAT5a phosphorylation, nuclear translocation and DNA-binding. *FEBS Lett.* 572, 238-244.
- Watson, C.S., Bulayeva, N., Wozniak, A.L., Alyea, R.A., 2007. Xenoestrogens are potent activators of nongenomic estrogenic responses. *Steroids* 72, 124-134.

Wood, A.W., Duan, C., Bern, H.A., 2005. Insulin-like growth factor signaling in fish. *Int. Rev. Cytol.* 243, 215-285.

Figure Legends

Figure 1. Effects of environmental estrogens (EE) on the abundance of growth hormone receptor (GHR) mRNAs in liver pieces incubated *in vitro*. Liver pieces were incubated for (A) various times with 10 µg/L 17β-estradiol (E2), 75 µg/L β-sitosterol (βS), or 75 µg/L 4-nonylphenol (NP) or (B) various concentrations of E2, βS, or NP for 6 h. Data are presented as mean ± SEM (n=6). For a given GHR subtype within a treatment, groups with different letters are significantly different ($p < 0.05$) from each other; * indicates a significant difference ($p < 0.05$) between two GHR subtypes within a treatment.

Figure 2. Effects of environmental estrogens (EE) on the abundance of growth hormone receptor (GHR) mRNAs in gill filaments incubated *in vitro*. Gill filaments were incubated for (A) various times with 10 µg/L 17β-estradiol (E2), 75 µg/L β-sitosterol (βS), or 75 µg/L 4-nonylphenol (NP) or (B) various concentrations of E2, βS, or NP for 6 h. Data are presented as mean ± SEM (n=6). For a given GHR subtype within a treatment, groups with different letters are significantly different ($p < 0.05$) from each other; * indicates a significant difference ($p < 0.05$) between two GHR subtypes within a treatment.

Figure 3. Effects of environmental estrogens (EE) on the abundance of growth hormone receptor (GHR) mRNAs in muscle incubated *in vitro*. Muscle pieces were incubated for various concentrations of 17β-estradiol (E2), β-sitosterol (βS), or 4-nonylphenol (NP) for 6 h. Data are presented as mean ± SEM (n=6). For a given GHR subtype within a treatment, groups with different letters are significantly different ($p < 0.05$) from each other; * indicates a significant difference ($p < 0.05$) between two GHR subtypes within a treatment.

Figure 4. Effects of 17 β -estradiol (E2), β -sitosterol (β S), or 4-nonylphenol (NP) on binding of ¹²⁵I-salmonid growth hormone (GH) to microsomes prepared from liver, gill filaments, and muscle tissue. Tissue pieces were incubated *in vitro* with 10 μ g/L E2, 75 μ g/L β S, or 75 μ g/L NP for 6 h. Illustrated is the binding capacity (Bmax) presented as mean $\pm$ SEM (n=6). For a given tissue, treatment groups with different letters are significantly ($p < 0.05$) different from each other.

Figure 5. The effects of environmental estrogens (EE) on the abundance of hepatic growth hormone receptor (GHR) mRNAs depend on estrogen receptors. Liver pieces were incubated *in vitro* for 2 h in the presence or absence of an estrogen receptor antagonist, ICI 182,780, followed by 6 h with 10 μ g/L 17 β -estradiol (E2), 75 μ g/L β -sitosterol (β S), or 75 μ g/L nonylphenol (NP). Data are presented as mean $\pm$ SEM (n=6). For a given gene GHR subtype, groups with different letters are significantly different ($p < 0.05$) different from each other.

Figure 6. Effects of environmental estrogens (EE) on the abundance of phosphorylated JAK2, STAT5, Akt, and ERK in liver pieces incubated *in vitro*. Liver pieces were incubated for various times (A, C, E, G) with 10 μ g/L 17 β -estradiol (E2), 75 μ g/L β -sitosterol (β S), or 75 μ g/L 4-nonylphenol (NP) or various concentrations (B, D, F, H) of E2, β S, or NP for 6 h. Tissue homogenates were separated by SDS-PAGE followed by western immunoblotting and quantified with a FluorChem imager. The abundance of phosphorylated JAK2 (P-JAK), STAT5 (P-STAT), Akt (p-Akt), and ERK1/2 (p-ERK) was normalized to total JAK2, STAT5, Akt, or ERK1/2, respectively. Data are presented as means $\pm$ SEM (n=4). For a given compound, groups with

different letters are significantly different ($p < 0.05$); * significantly different ($p < 0.05$) from β S-treated group at corresponding incubation time or EE concentration.

A. Time course

B. Concentration response

A. Time course

B. Concentration response

GHR mRNA abundance
(% of control)

17 β -estradiol
 β -sitosterol
 4-*n*-nonylphenol

A. JAK-time course

B. JAK-concentration

C. STAT-time course

D. STAT-concentration

E. Akt-time course

F. Akt-concentration

G. ERK-time course

H. ERK-concentration