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17α-Ethinylestradiol decreases expression of multiple hepatic nucleotide excision repair genes in zebrafish (*Danio rerio*)

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Abstract

Waterborne 17α-ethinylestradiol (EE_2) alters hormone-mediated biological indicators in fish. These alterations include increased plasma vitellogenin, increased intersex individuals, decreased egg and sperm production, reduced gamete quality, and complete feminization of male fish. Together, these observations implicate aquatic estrogens in a broad range of detrimental effects on fish reproduction and fitness. In addition to impairing reproductive processes, EE_2 is also a strong promoter of hepatic tumor formation. Since many ubiquitous, aquatic hepatocarcinogens form DNA adducts that are preferentially repaired by nucleotide excision repair (NER) processes, we hypothesized that EE_2 may exert co-carcinogenic effects by reducing an organisms ability to repair DNA adducts via this mechanism. The present study used fluorescence-based quantitative RT-PCR to examine effects of environmentally relevant concentrations of the semisynthetic estrogen, EE_2, on hepatic nucleotide excision repair (NER) gene expression. Adult male and female zebrafish (*Danio rerio*) were exposed to 1 ng/L, 10 ng/L or 100 ng/L concentrations of EE_2, or to a solvent control (0.05%, v/v ethanol), for 7 days with static water renewal every 24 h. Effectiveness of EE_2 exposure in the liver was confirmed by examining hepatic expression of two estrogen-responsive biomarkers, vitellogenin-1 and cytochrome P450-1A1 (CYP1A1). Quantitative analysis confirmed that exposure to 100 ng/L EE_2 caused significant decreases in transcript abundance of several hepatic NER genes in male zebrafish, including XPC (>17-fold), XPA (>7-fold), XPD (>8-fold), and XPF (>8-fold). Adult female zebrafish exhibited a four-fold decreased in XPC mRNA abundance at all exposure concentrations. Decreased mRNA abundance of NER genes was also seen to a lesser degree at lower concentrations of EE_2. Adult male zebrafish showed greater reduction of hepatic NER transcript levels than their female counterparts, which is consistent with the sexually dimorphic incidence of hepatocellular carcinoma in many species. Decreased transcript levels of NER genes have been shown to be an important epidemiological marker for increased cancer risk and decreased repair capacity in humans.

Keywords: 17α-Ethinylestradiol; EE_2; Nucleotide excision repair; NER; Zebrafish; *Danio rerio*

1. Introduction

Documentation of pharmaceuticals and personal care products in surface waters has stimulated diverse research examining their environmental impacts (Daughton and Ternes, 1999). As a result of numerous studies on a wide variety of pharmaceuticals, organizations nationwide are beginning to implement drug recovery and disposal plans to help alleviate impacts of improperly disposed prescription drugs. Such proactive efforts are a reasonable step toward reduction of environmental inputs of pharmaceuticals and personal care products via public sewers and landfills. However, pharmaceuticals and their metabolic products will remain a constant source of wastewater contamination as long as pharmaceuticals are consumed and excreted (Desbrow et al., 1998; Aguayo et al., 2004; Servos et al., 2005).

One group of environmentally relevant pharmaceuticals found in wastewater is comprised of endocrine active compounds such as sex steroids and chemical hormone mimics. The most potent of these xenoestrogens in the aquatic environment is 17α-ethinylestradiol (EE_2), the semisynthetic hormone found in numerous oral contraceptives and hormone replacement therapies (Gutendorf and Westendorf, 2001). A recent survey of more than 100 streams in the U.S. revealed a median EE_2 concentration of 73 ng/L (Kolpin et al., 2002). Given that estrogens elicit response in aquatic organisms at concentrations in the low ng/L range, the survey suggests that EE_2 is present in suffi-

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cient amounts in the aquatic environment to induce biological effects (Kolpin et al., 2002). Additionally, EE_2 is more resistant to degradation than natural steroids such as 17β-estradiol (E_2) (Jurgens et al., 2002). Due to its greater stability and higher potency *in vivo*, EE_2 may be of disproportional toxicological importance despite being found at much lower concentrations in surface waters than natural steroids such as estrone (E_1) (Jurgens et al., 2002).

The effects of estrogens on a variety of reproductive processes in teleosts have been well delineated. These include increased plasma vitellogenin in male and female fish, increased proportions of intersex fish, decreased egg and sperm production, reduced gamete quality, and complete feminization of male fish (Panter et al., 1998; Rodgers-Gray et al., 2001; Sohoni et al., 2001; Jobling et al., 2002; Van den Belt et al., 2002; Sole et al., 2003). A wide range of estrogens and estrogen mimics are known to increase plasma vitellogenin in male fish (Jones et al., 2000; Rose et al., 2002; Van den Belt et al., 2003; Versonnen and Janssen, 2004; Fenske et al., 2005). Increased plasma vitellogenin, altered estrogen levels in bile, and increased intersex proportions have been found in fish downstream of sewage treatment plant outflows with known estrogenicity (Jobling et al., 1998; Carballo et al., 2005; Gibson et al., 2005). This suggests that estrogens affect reproductive processes in the environment in addition to laboratory investigations.

In contrast to the wealth of information about effects of exogenous estrogen exposure on fish reproduction, very little is known about effects on non-reproductive processes. Estrogens, including EE_2, are known to promote mutagen-induced hepatic neoplasia in medaka (*Oryzias latipes*) and rainbow trout (*Oncorhynchus mykiss*) (Cooke and Hinton, 1999; Tilton et al., 2006). However, the mechanism of action of estrogens in increased rates of mutagen-induced neoplastic transformation is not known (Cooke and Hinton, 1999; Tilton et al., 2006). Increased somatic mutations, regardless of resultant neoplastic transformation, can lead to genomic instability of individuals and decreased fitness of populations (Wirgin and Waldman, 1998). One biological process that circumvents mutations caused by DNA lesions is DNA repair. Previous research has not examined the effects of xenoestrogens on DNA repair processes in fish, despite known carcinogenic effects of estrogen including attenuated nucleotide excision repair in human cells (Klaunig et al., 2000; Evans et al., 2003).

Nucleotide excision repair (NER) is the primary DNA repair pathway responsible for removing a variety of lesions caused by bulky adduct forming mutagens (de Laat et al., 1999; Sancar et al., 2004). Bulky adduct forming mutagens, such as benzo(*a*)pyrene, are ubiquitous in the environment and are concentrated in areas impacted by anthropogenic pollution (Wirgin and Waldman, 1998; Kolpin et al., 2002; Ohe et al., 2004). NER removes small sections of adducted DNA via a multiple step process involving the assembly of numerous proteins at the site of DNA damage (Evans et al., 1997; de Laat et al., 1999; Sancar et al., 2004). This heterologous assembly of repair factors consists of proteins that carry out initial damage recognition, damage verification and open complex formation, incision on either side of the lesion, DNA synthesis, and DNA ligation.

Two sub-pathways exist in NER: global genome repair and transcription coupled repair. Many of the core NER proteins are functional in both sub-pathways with the primary difference occurring in initial damage recognition. The rate-limiting step in either pathway is the initial detection of DNA damage (Thoma and Vasquez, 2003). In global genome repair, XPC and XPA work in conjunction to recognize and verify DNA damage and initiate open complex formation prior to excision of damaged DNA (de Laat et al., 1999). Helical distortion attracts one XPC-HR23B heterodimer to the site of DNA damage but dual incision of the adducted oligomer only occurs if a lesion is present (Sugasawa et al., 2001). Thus, a multi-step process involving damage verification is necessary before damage excision. Once the XPC-HR23B complex has recognized damaged DNA, sequential repair factors are recruited to form an open complex around the damaged site (de Laat et al., 1999; Hanawalt, 2002; Sancar et al., 2004). Formation of the open complex begins with association of the damage verification heterodimer XPA-RPA, and recruitment of the TFIIH complex which contains helicases XPB and XPD that unwind DNA in 3′–5′ and 5′–3′ directions, respectively (de Laat et al., 1999; Volker et al., 2001). Two nucleases, XPG and XPF, then cleave 3′ and 5′ ends of the open complex (Sancar et al., 2004). After the adducted segment of DNA is removed, DNA synthesis and ligation complete the process to replace the excised DNA oligomer.

The present study examined effects of EE_2 exposure on hepatic gene expression of NER damage recognition, damage verification, helicase, and endonuclease proteins in sexually mature zebrafish (*Danio rerio*). Concentrations of nucleotide excision repair mRNAs that code for proteins involved in progressive steps of the NER pathway were quantified in zebrafish livers after 7-day exposure to environmentally relevant concentrations of EE_2. Results from this investigation showed a significant, sexually dimorphic alteration of NER gene expression after EE_2 exposure and indicate a novel synergistic mechanism for estrogens in environmental carcinogenesis.

2. Materials and methods

2.1. Adult zebrafish exposures

One-year-old zebrafish were maintained at the University of Maine zebrafish facility with a light:dark cycle of 14:10 h. Prior to EE_2 exposure, 20 male and 20 female fish were placed in separate 3.5 L tanks for each exposure regime with water from the University of Maine zebrafish facility (carbon filtered and UV-treated Orono, ME city water, with 7.5 mg/L dissolved oxygen and 42 mg/L hardness) and maintained at 27.6 °C. Aqueous 17α-ethinylestradiol (CAS 57-63-6, Sigma E4876) was diluted in ethanol to produce a stock concentration of 2 mg/L and added to tanks to yield final EE_2 concentrations of 1 ng/L, 10 ng/L or 100 ng/L. Maximum ethanol levels were 0.05%, two orders of magnitude below the lowest observed effect concentration of ethanol for zebrafish (Dlugos and Rabin, 2003). Although no discernable difference in transcript abundance of NER genes could be detected between 0.05% ethanol exposed and unexposed zebrafish (data not shown), control fish