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Assaying uptake of endocrine disruptor compounds in zebrafish embryos and larvae

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ABSTRACT

To study the effects of environmental endocrine disruptor compounds (EDCs) on aquatic animals, embryos and larvae are typically incubated in water containing defined concentrations of EDCs. However, the amount of EDC uptake into the animal is often difficult to determine. Using radiolabeled estradiol ([^{3}H]E2), we previously developed a rapid, straightforward assay to measure estradiol uptake from water into zebrafish embryos and larvae. Here, we extend this approach to measure the uptake of two additional EDCs, bisphenol A (BPA) and ethinyl estradiol (EE2). As with E2, the uptake of each compound by individual larvae was low (< 6%), and increased with increasing concentration, duration, and developmental stage. We found that E2 and EE2 had similar uptake under equivalent exposure conditions, while BPA had comparatively lower uptake. One application of this assay is to test factors that influence EDC uptake or efflux. It has been suggested that persistent organic pollutants (POPs) inhibit ABC transporters that may normally efflux EDCs and their metabolites, inducing toxicity in aquatic organisms. We measured [^{3}H]E2 levels in zebrafish in the presence or absence of the POP PDBE-100, and cyclosporine A, a known inhibitor of ABC transporters. Neither chemical significantly affected [^{3}H]E2 levels in zebrafish, suggesting that zebrafish maintain estradiol efflux in the presence of PDBE-100, independently of cyclosporine A-responsive transporters. These uptake results will be a valuable reference for EDC exposure studies in developing zebrafish, and provide a rapid assay to screen for chemicals that influence estrogen-like EDC levels in vivo.

1. Introduction

Environmental endocrine disruptor compounds (EDCs) are small molecules that mimic endogenous hormones. EDCs can negatively impact the health of humans and wildlife by disrupting endogenous hormone signaling (Diamanti-Kandarakis et al., 2009). Estrogen-like EDCs are a broad class of EDCs including endogenous compounds like 17-β-estradiol (E2) and synthetic compounds like bisphenol A (BPA), commonly found in manufactured plastics.

Aquatic animal models, such as zebrafish, are used to study the environmental impact of EDCs. One common approach is to expose zebrafish embryos to known and suspected EDCs and assay their toxicity (Bouwmeester et al., 2016; Carroll et al., 2014; Gorelick et al., 2014; Padilla et al., 2012; Tal et al., 2016). However, for the majority of EDCs, information regarding the precise uptake and excretion is lacking. We previously developed an assay to measure [^{3}H]E2 uptake in zebrafish embryos, and found that supraphysiologic concentrations of E2 in fish water are required to achieve physiologically-relevant doses in embryos (Souder and Gorelick, 2017). We also found that E2 uptake is dependent on exposure concentration, duration and developmental stage (Souder and Gorelick, 2017). We sought to determine if this is also true for other estrogen-like EDCs, by testing the uptake of the pharmaceutical estrogen analog, ethinyl estradiol (EE2) and the non-steroidal synthetic estrogen, bisphenol A (BPA).

In addition to quantifying EDC uptake, it would also be useful to identify chemicals or chemical mixtures that influence EDC uptake or efflux, to discover novel mechanisms of toxicity and to potentially inhibit the uptake of toxic EDCs. Major drug transporters like P-glycoprotein (P-gp) are known to regulate uptake and efflux of an array of structurally diverse substrates, including xenobiotics and pharmaceuticals (Aller et al., 2009; Ambudkar et al., 1999). Though previous efforts have investigated P-gp transport of steroids and xenobiotics in vitro (Kim and Benet, 2004), the degree to which P-gp influences EDC efflux in vivo is less well understood.

Using our assay for measuring radiolabeled estradiol uptake, we quantified the uptake of [^{3}H]EE2 and [^{3}H]BPA in zebrafish embryos at multiple concentrations, exposure durations, and developmental stages. We found that < 5% of EE2 and BPA are taken up following 24 h

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exposure, and that EE2 and BPA uptake are dependent on concentration, duration, and developmental stage. When comparing E2 uptake to EE2 and BPA, we found that EE2 uptake is similar to E2, whereas BPA uptake is substantially lower. Additionally, we found that inhibition of *abcb4*, a zebrafish P-gp orthologue (Fischer et al., 2013), did not affect E2 uptake. Our results support the hypotheses that supraphysiologic concentrations of EDCs in water are required to achieve physiologic concentrations in vivo. Our results also suggest that environmentally-relevant concentrations of E2 are not influenced by the drug transporter *abcb4*.

2. Methods

2.1. Zebrafish

Adult zebrafish were raised at 28.5 °C on a 14-h light, 10-h dark cycle in the UAB Zebrafish Research Facility in a recirculating water system (Aquaneering, Inc., San Diego, CA). All zebrafish used for experiments were wildtype, AB strain (Westerfield, 2000). All procedures were approved by the UAB Institutional Animal Care and Use Committee.

2.2. Embryo collection

Adult zebrafish were allowed to spawn naturally in groups of 2–3 females with 2 males per breeding tank. Embryos were collected in intervals of 10 min to ensure precise developmental timing, placed in 100 mm × 15 mm Petri dishes at a density of no > 100 per dish in E3B media (60 × E3B: 17.2 g NaCl, 0.76 g KCl, 2.9 g $CaCl_2$-$2H_2O$, 2.39 g $MgSO_4$ dissolved in 1 L Milli-Q water; diluted to 1 × in 9 L Milli-Q water plus 100 μL 0.02% methylene blue), and then stored in an incubator at 28.5 °C on a 14-h light, 10-h dark cycle until treatment.

2.3. Embryo treatments

For uptake experiments, embryos were treated in tritiated ethinyl estradiol ([6,7-^{3}H(N)]-17α-ethynylestradiol, 1 mCi/mL, 60 Ci/mmol, American Radiolabeled Chemicals Inc. #ART1321, Lot #170203), tritiated bisphenol A ([ring-3H]-bisphenol A, 1 mCi/mL, 25 Ci/mmol, American Radiolabeled Chemicals Inc. #ART1676, Lot #170320), tritiated estradiol ([6,7-^{3}H(N)]-17β-estradiol, 1 mCi/mL, Perkin Elmer NET013250UC), or vehicle (ethanol) and diluted to final concentration (1–20 nM) in E3B at the time of treatment. Vehicle concentration did not exceed 0.1% in E3B. ABC transporter inhibitor experiments were conducted using cyclosporin A (Enzo Life Sciences, #380-002-M100) or 2,2′,4,4′,6-pentabromodiphenyl ether (PDBE-100) (AccuStandard, #FF-BDE-100 N, Lot #26813) dissolved in dimethylsulfoxide (DMSO), or vehicle control (DMSO), diluted to final concentration (20 nM–5 μM) in E3B at the time of treatment. Rhodamine B (Acros Organics, ≥ 98% pure, #AC296570100) was dissolved in methanol (MeOH) and diluted to final concentration (0.5 μM) in E3B. DMSO or methanol at the concentrations used (0.1%) did not affect [^{3}H]E2 or [^{3}H]BPA uptake (data not shown).

2.4. [^{3}H] uptake assay

Isotopic uptake assays were performed as described previously (Souder and Gorelick, 2017). Briefly, embryos between 6 and 96 hpf were exposed in 24-well plates to 2 mL of treatment solution per well, ten embryos per well, for 1 h or 24 h. All embryos were manually dechorionated prior to exposure and incubated at 28.5 °C on 14-h light, 10-h dark cycle unless noted. Radioactivity of individual homogenized embryos was measured using liquid scintillation counting. To ensure compounds are not adhered to the outside of the embryo, embryos are individually washed 2 × in fresh embryo medium prior to measurement. The final wash step was measured to ensure the absence of radioactivity. Background radioactivity of vehicle-control groups was negligible and is therefore excluded from graphs. [^{3}H] radioactivity was converted to pmol using a standard curve generated for each chemical (Fig. S1) with a limit of detection of 0.01 pmol for each chemical. For experiments requiring addition of non-tritiated compounds, drugs were added to the well at the same time as the tritiated compound and DMSO was used as a vehicle control. No toxicity was observed at the concentrations used for treatment (not shown).

2.5. Rhodamine B uptake assay

For rhodamine B (RhB) uptake experiments, embryos were co-exposed to RhB, cyclosporin A, and/or E2 at 96 hpf for 24 h, except that embryos were exposed in the dark to prevent the loss of fluorescence during treatment. At the end of the exposure period, embryos were washed three times in fresh E3B and 0.01 mg/mL tricaine was added to immobilize embryos for imaging. Anesthetized embryos were embedded in 3% methyl cellulose in E3B and imaged on a Nikon AZ100 microscope with Andor Clara digital camera. Fluorescence was quantified from whole embryos using ImageJ software (Schneider et al., 2012) by tracing the outline of the entire embryo and averaging the mean gray value of 10 embryos per experiment.

2.6. Experimental design and data analysis

Experiments were performed on 10 embryos from a single clutch per treatment group or vehicle control group. Experiments were performed at least 3 times (n ≥ 3) using embryos from different clutches. Mean pmol uptake and mean percent uptake from each group were used for comparing treatment groups between experiments. Mean integrated density was used to compare fluorescence between RhB-treated groups. A two-tailed, unpaired Student's *t*-test was used when testing for statistical significance between two groups, one-way ANOVA with Tukey's test for multiple comparisons was used when comparing uptake between > 2 groups, and one-way ANOVA with Dunnett's test for multiple comparisons was used when comparing fold-change in uptake of [^{3}H]EE2 and [^{3}H]BPA versus [^{3}H]E2. Statistical significance was set at $p < 0.05$. GraphPad Prism 7.0a software was used for all statistical analyses and for producing graphs.

3. Results

3.1. EDC uptake is concentration- and duration-dependent

To test the effect of increasing concentration on chemical uptake, we exposed 48 h post fertilization (hpf) dechorionated zebrafish embryos to three different concentrations of [^{3}H]EE2 (1 nM, 5 nM, 10 nM) or [^{3}H]BPA (5 nM, 10 nM, 20 nM) for one hour. Consistent with previous results measuring [^{3}H]E2 uptake(Souder and Gorelick, 2017), the absolute amount absorbed in pmol of both EE2 and BPA increased with increasing exposure concentration. [^{3}H]EE2 absorption increased by 11-fold between 1 nM and 10 nM exposure (Figs. 1A, S2, Tables 1, S1). [^{3}H]BPA absorption was less robust, increasing by 3.6-fold between 5 nM and 20 nM exposure (Figs. 1D, S2, Tables 1, S1).

We next tested the hypothesis that increasing exposure duration would increase EDC uptake. We exposed 48 hpf dechorionated embryos to identical concentrations of [^{3}H]EE2 or [^{3}H]BPA for 24 h. We found that pmol absorption increased as concentration increased. [^{3}H]EE2 absorption increased by 9-fold between 1 nM and 10 nM exposure (Fig. 1B, Tables 1, S1). [^{3}H] BPA absorption increased less robustly, with a 3.3-fold increase between 5 nM and 20 nM (Fig. 1E, Tables 1, S1). For each concentration, absorption increased when increasing exposure duration from 1 h to 24 h by an average of 5.5 ± 0.69 fold for [^{3}H]EE2 (Fig. 1C), and an average of 12.4 ± 0.45 fold for [^{3}H]BPA (Fig. 1F). Together, these results demonstrate that the uptake of [^{3}H]EE2 and [^{3}H]BPA are concentration- and duration-dependent.

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