Contribution of G protein-coupled estrogen receptor 1 (GPER) to 17 β -estradiol-induced developmental toxicity in zebrafish

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ABSTRACT

Exposure to 17 β -estradiol (E2) influences the regulation of multiple signaling pathways, and E2-mediated disruption of signaling events during early development can lead to malformations such as cardiac defects. In this study, we investigated the potential role of the G-protein estrogen receptor 1 (GPER) in E2-induced developmental toxicity. Zebrafish embryos were exposed to E2 from 2 h post fertilization (hpf) to 76 hpf with subsequent transcriptional measurements of heart and neural crest derivatives expressed 2 (*hand2*), leucine rich repeat containing 10 (*lrrc10*), and *gper* at 12, 28 and 76 hpf. Alteration in the expression of *lrrc10*, *hand2* and *gper* was observed at 12 hpf and 76 hpf, but not at 28 hpf. Expression of these genes was also altered after exposure to G1 (a GPER agonist) at 76 hpf. Expression of *lrrc10*, *hand2* and *gper* all coincided with the formation of cardiac edema at 76 hpf as well as other developmental abnormalities. While co-exposure of G1 with G36 (a GPER antagonist) rescued G1-induced abnormalities and altered gene expression, co-exposure of E2 with G36, or ICI 182,780 (an estrogen receptor antagonist) did not rescue E2-induced cardiac deformities or gene expression. In addition, no effects on the concentrations of downstream ER and GPER signaling molecules (cAMP or calcium) were observed in embryo homogenates after E2 treatment. These data suggest that the impacts of E2 on embryonic development at this stage are complex and may involve multiple receptor and/or signaling pathways.

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1. Introduction

Xenobiotics in the environment can alter the normal functions of the endocrine system by acting as antagonists or agonists to hormonal receptors, and/or alter the synthesis/metabolism of endogenous hormones (Guillette et al., 1995; Sonnenschein and Soto, 1998). For example, phenolic xenobiotic compounds structurally similar to the endogenous hormone, 17 β -estradiol (E2), may alter the regulation of a variety of signal transduction events during development. Studies in zebrafish have been utilized extensively as a model to evaluate the effects of xenobiotics on development, and have shown that exposure to agents such as Bisphenol-A (BPA) and the natural hormone E2 during development resulted in a range of gross malformations, including curved body axis, yolk-sac edema, and pericardial edema (Brion et al., 2004; Duan et al., 2008; Saili et al., 2012). Similar cardiac phenotypes have been observed following polycyclic aromatic hydrocarbon (PAH) expo-

sure in zebrafish embryos (Incardona et al., 2004). Although many studies have evaluated the impacts of xenobiotics such as PAHs on heart development, the molecular mechanisms by which estrogenic compounds cause cardiotoxicity is poorly understood.

Embryonic development depends on the precise spatio-temporal expression of signaling genes, and alterations can result in abnormal phenotypes. While nuclear E2 receptors have been primarily studied in development, limited work has been reported on the G-protein-coupled estrogen receptor 1 (GPER). Activation of GPER by G1 (a GPER agonist), E2 and other estrogenic compounds increases levels of secondary messengers such as Ca²⁺ (Brailoiu et al., 2013; Szego and Davis, 1967; Thomas et al., 2005), which can affect the expression of genes vital to cardiovascular development, such as *lrrc10* and *hand2* (Brody et al., 2013; Dirkx et al., 2013).

To better understand the effects of E2 and other potential phenolic contaminants that have estrogenic activities, the purpose of this study was to evaluate the role of GPER in the toxicity of E2 on zebrafish embryo development; G1 was used as a positive control for all exposures. To analyze the potential role of GPER in E2 and G1 toxicity, signal transduction and the expression of *gper* and cardiac genes, *lrrc10* and *hand2* were evaluated.

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2. Materials and methods

2.1. Chemicals

G1 (*rel*-1-[4-(6-Bromo-1,3-benzodioxol-5-yl)-3aR,4S,5,9bS-tetrahydro-3H-cyclopenta[c]quinolin-8-yl]-ethanone; $\geq 98\%$ purity, Cayman Chemical, Ann Harbor, MI), G36 ((4S)-*rel*-4-(6-bromo-1,3-benzodioxol-5-yl)-3aR,4,5,9bS-tetrahydro-8-(1-methylethyl)-3H-cyclopenta[c]quinoline; $\geq 98\%$ purity, Cayman Chemical); ICI 182,780 182, 780 (7 α ,17 β -[9-[(4,4,5,5,5-pentafluoropentyl)sulfinyl]nonyl]estra-1,3,5(10)-triene-3,17-diol; $\geq 99\%$ purity, Tocris, Minneapolis, MN) and 17 β -estradiol (E2; $\geq 98\%$ purity, Sigma-Aldrich, St. Louis, MO) were dissolved in ethanol. Stock solutions were kept at 4 °C in dark conditions. Exposure solutions were prepared by diluting working stocks in dechlorinated water at a final vehicle concentration of 0.1–0.2% ethanol within all vehicle control and treatment groups.

2.2. Maintenance of zebrafish culture

Zebrafish (*Danio Rerio*) 5D adults were purchased from Oregon State University. Adults were maintained and embryos were treated according to an animal use protocol (AUP #20130005) that was reviewed and approved by the University of California, Riverside (UCR) Institutional Animal Care and Use Committee (IACUC). Adults were bred in tanks containing a mesh-spawning basket. The morning of treatment, embryos were collected 30 min after the light turned on. Embryos were cleaned and microscopically evaluated for viability. The stage of the embryos was visualized before treatment to ensure that exposure was conducted at a consistent developmental stage throughout all experiments. The different stages were determined using previously published standards (Kimmel et al., 1995). The embryos were maintained at 28 °C in dechlorinated water with a light:dark cycle of 14 h:10 h.

2.3. Exposure regime

After microscopic evaluation, 30–35 randomly selected embryos were placed in petri dishes (100 mm $\times$ 15 mm) for exposure. Treatment was initiated at 2 h post fertilization (hpf) and embryos were statically exposed until 76 hpf. At this time point, there is no cardiac progenitor cell formation which allows for sufficient time to determine the embryo viability without confounding cardiac effects. The embryos were exposed to 0.1% ethanol as the solvent control and to various nominal concentrations of E2 (0.1, 2, 5, 7 and 8 μ M). The concentrations were selected based on range finding studies and our own dose response experiment (data not shown). Each dish of treatment contained 20 ml of the appropriate treatment solution. There were 4–9 replicates for each treatment depending on the specific experiment.

After exposure, the plates were incubated at 28 °C in a light:dark cycle of 14 h:10 h. Embryos were statically exposed for 74 h and were checked daily for viability based on a transparency, yellowish appearance and presence of a heartbeat. Embryos that were determined to be dead were removed immediately at each observation time. At 76 hpf, treatment was terminated and surviving embryos were used for molecular endpoints (see below) and analyzed for abnormalities. Prevalence of the following gross malformations were assessed microscopically and quantified: curved body axis, bent tail, yolk-sac edema, looping defects and pericardial edema.

To investigate the role of GPER in E2-induced toxicity expression of GPER, Hand2, and *Lrrc10* mRNA was analyzed at several time points during development including the segmentation (12 hpf) and pharyngula stages (28 hpf). We also analyzed expression of these genes and Vtg mRNA at the same time point morphological

responses occurred (76 hpf). Vtg was included as a positive control for ER activation.

To determine and compare whether GPER activation resulted in similar effects as E2 during development, embryos were exposed to G1, a GPER agonist. Embryos were exposed at various concentrations (0.5, 2 and 5 μ M) of G1 at the same developmental time point as the E2 treatment. Doses were determined using range finding dose response experiments based on previous studies (Chandrasekar et al., 2010). To determine the contribution of GPER, co-exposure experiments were conducted with the GPER antagonist, G36 (5 μ M) and ICI 172, 780 (14 μ M). Concentrations of the antagonists were selected based on the solubility of these compounds in ethanol and the highest concentration that did not cause adverse effects on embryonic development, based on a range finding dose response experiment (data not shown).

2.4. RNA isolation and RT-PCR

Post-treatment embryos at 12, 28, and 76 hpf were pooled for mRNA expression analysis. Approximately 35 embryos were pooled per treatment replicate ($n=4-9$) and used for total RNA extraction using RNeasy Lipid Tissue Mini Kit purchased from Qiagen (Valencia, CA). After RNA extraction, the purity and integrity of the samples were analyzed using the OD₂₆₀/OD₂₈₀ ratio. Using the Promega Reverse Transcription System kit (Madison, WI), 1000 ng of RNA was used for cDNA synthesis following the manufacturer's instructions. Upon completion, cDNA was stored at -20°C until qPCR was performed. qPCR was done using the iTAQ Universal SYBR green kit from BioRad (Hercules, CA). Each SYBR reaction mix had 100 ng of cDNA and a specific primer set for the genes of interest. The primer sequence is as follows:

gper forward-5' TGG CTG TGG CAG ATC TTA TTC 3'
gper reverse-5' CAA TGG ACT GCT GCT CAT AGA 3'
hand2 forward-5' AGA GAT GTC TCC TCC TGA CTA TAC 3'
hand2 reverse-5' TTC CCT GAG TTC TGC AAA GG 3'
lrrc10 forward-5' AGG AGC TTC CTC TGG TCA TA 3'
lrrc10 reverse-5' AGC CTA AAT GGA GCG TCT TG 3'
vtg forward-5' CTG CAA GAG TGC AAC TGA TAG TTT C 3'
vtg reverse-5' ACT TGC CAG TGA CTT TGT GCT T3'
ef- α forward-5' CTA CAT CAA GAA GAT CGG CTA CAA 3'
ef- α reverse-5' CGA CAG GGA CAG TTC CAA TAC 3'

The thermal cycling conditions used for all three genes analyzed via qPCR were as follows: The denaturation step was done at 95 °C for 5 min, followed by annealing and extension at 95 °C for 10 s and at 55 °C for 30 s. This was repeated for 40 cycles. Afterward, a melt curve analysis was done from 54 to 95 °C in increments of 0.5 °C. All primer sets displayed one peak demonstrating the specificity. Data was normalized using *ef- α* and the efficiency for all genes were optimized and monitored using PCRminer (Zhao and Fernald, 2005).

2.5. Calcium measurements

GPER signaling occurs through second messengers such as Ca²⁺ and cAMP (Brailoiu et al., 2013; Szego and Davis, 1967; Thomas et al., 2005). To measure intracellular Ca²⁺, embryos after E2 and/or agonist/antagonist treatment were collected (~30–35 embryos per treatment replicate; $n=6$) and homogenized on ice using a lysis buffer (150 mmol L⁻¹ NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 50 mmol L⁻¹ Tris-HCl, 1 mmol L⁻¹ EDTA, 1 mmol L⁻¹ phenylmethanesulfonyl fluoride, 0.1% SDS). After incubation for 30 min on ice, lysed samples were centrifuged at 15,000 $\times$ g for 20 min at 4 °C. The resulting supernatant was aspirated and used for the assay. Supernatant was mixed with 150 μ l of the cal-

Fig. 1. Effects of 17 β -estradiol (E2) on cardiac development and mRNA levels of GPER in zebrafish embryos at different times during development. Cumulative fold change of GPER mRNA of zebrafish embryos exposed to 0.1% ethanol (ETOH) and 0.1–8 μ M of E2 at 2 hpf. Each graph represents different time points (A) 12 hpf, (B) 28 hpf and (C) 76 hpf. Data presented as mean $\pm$ standard error (SE) of four to nine independent replicates. (D) Average percent cardiac deformities at 76 hpf after treatment with 0.1% ethanol (ETOH) and 0.1–8 μ M of E2 at 2 hpf. Data presented as mean $\pm$ standard error (SE) of four to six independent replicates. Deformities data were analyzed using a logistic regression model. Normal data was analyzed by ANOVA followed by Tukey's HSD multiple comparisons test. Data that was not normal a Wallis test was used followed by a Dunn's Multiple Comparison Test. Asterisks (*) represent the significant difference ($p < 0.05$).

cium reagent Arsenazo III (Sigma Aldrich, St. Louis, MO) and the absorbance determined at 650-nm with a spectrophotometer. The readings were then normalized to protein content, which was measured using a PierceTM BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA) per manufacturer's instructions.

To measure cAMP, treated zebrafish embryos at 76 hpf (30–35 embryos per replicate, $n = 4$ –6) were snap-frozen in liquid nitrogen. cAMP concentrations was determined using Direct cAMP ELISA kit (Enzo Life Sciences, Farmingdale, NY) following the manufacturers' instructions. Proteins levels were determined using a Bradford assay (Thermo Fisher Scientific, Waltham, MA) for normalization.

2.6. Statistical analysis

All statistical analyses were done using the statistical program R (R version 3.1.2). For parametric analyses, one-way ANOVA was used followed by a Tukey's HSD test ($p < 0.05$). Data sets that were not normally distributed were analyzed using a nonparametric method, the Kruskal–Wallis one-way analysis of variance. If the Kruskal–Wallis test showed significance ($p < 0.05$), then Dunn's post hoc test was used. Co-exposures were analyzed by a two-way ANOVA. Survival and deformity datasets were analyzed via logistic regression model.

3. Results

Sublethal malformations were observed after treatment with E2, including curved body axis, yolk-sac edema and pericardial edema (Supplemental Fig. 1). At 76 hpf, 35.5 $\pm$ 15.9% and 61.1 $\pm$ 28.9% of the surviving embryos exhibited pericardial edema and cardiac impairment (such as looping defects) after exposure to 7 μ M and 8 μ M of E2, respectively, which were both statistically significant compared to all groups (Fig. 1A). To assess ER activation

in embryos, *vtg* expression was also analyzed at 76 hpf after treatment and an upward trend was observed following E2 treatment (Supplemental Fig. 2).

To investigate the role of GPER in E2-induced toxicity, expression of *gper* was analyzed at several time points during development including segmentation (12 hpf) and pharyngula stages (28 hpf). We also analyzed *gper* expression at the same time point morphological responses occurred (76 hpf). At 12 hpf, a 9-fold increase in *gper* expression was observed in the 8 μ M E2 treatment group (Fig. 1B). No significant changes were observed in *gper* mRNA levels at 28 hpf (Fig. 1C). At 76 hpf, when abnormalities were observed, a significant increase in *gper* expression occurred after exposure to 2 μ M (6.9-fold increase), 5 μ M (8.5-fold increase) and 7 μ M of E2 (13.4-fold increase), respectively (Fig. 1A and D).

The expression of two cardiac genes *lrrc10* and *hand2* regulated by GPER through Ca²⁺ and cAMP signaling also increased after exposure to E2. The patterns of *lrrc10* and *hand2* expression were correlated with an r^2 value of 0.88. Expression of *lrrc10* transcripts at 12 hpf showed an increasing trend after exposure to 5 μ M and 7 μ M of E2, with a significant 9.6-fold increase observed at 8 μ M E2 (Fig. 2A). At 28 hpf, *lrrc10* expression did not significantly change (Fig. 2B). At 76 hpf, an upward trend in *lrrc10* expression was seen after exposure to 2, 7 and 8 μ M of E2, with a significant change at 5 μ M exposure (Fig. 2C). At 12 hpf, the expression of *hand2* after exposure to 8 μ M of E2 resulted in a significant 5.7-fold increase (Fig. 3A). At 28 hpf, *hand2* expression did not significantly change (Fig. 3B). At 76 hpf, *hand2* expression showed an upward trend after exposure to 2, 7 and 8 μ M of E2, but a significant change only occurred at 5 μ M exposure (Fig. 3C).

After exposure to 2 μ M and 5 μ M of G1 for 74 h, there was a significant increase in cardiac deformities (e.g., pericardial edema and looping defects). Treatment with 2 μ M and 5 μ M of G1 resulted in 87.7 $\pm$ 19.4% and 100 $\pm$ 0% of embryos with cardiac deformi-

Fig. 2. Effects of E2 on the expression of *lrrc10* in zebrafish embryos at different times during development. Cumulative fold change of *lrrc10* mRNA of zebrafish embryos exposed to 0.1% ethanol (ETOH) and 0.1–8 μM of 17β-estradiol (E2) at 2 hpf. Each graph represents different time points (A) 12 hpf, (B) 28 hpf and (C) 76 hpf. Data presented as mean ± standard error (SE) of four to nine independent replicates. Normal data was analyzed by ANOVA followed by Tukey's HSD multiple comparisons test. Data that was not normal a Kruskal–Wallis test was used followed by a Dunn's Multiple Comparison Test. Asterisks (*) represent the significant difference compared to control ($p < 0.05$).

Fig. 3. Effects of E2 on the expression of *hand2* in zebrafish embryos at different times during development. Cumulative fold change of *hand2* mRNA of zebrafish embryos exposed to 0.1% ethanol (ETOH) and 0.1–8 μM of 17β-estradiol (E2) at 2 hpf. Each graph represents different time points (A) 12 hpf, (B) 28 hpf and (C) 76 hpf. Data presented as mean ± standard error (SE) of four to nine independent replicates. Normal data was analyzed by ANOVA followed by Tukey's HSD multiple comparisons test. Data that was not normal a Kruskal–Wallis test was used followed by a Dunn's Multiple Comparison Test. Asterisks (*) represent the significant difference compared to control ($p < 0.05$).

ties (Fig. 4A). After treatment with 2 μM of G1, expression of *lrrc10*, *hand2* and *gper* were significantly induced. Expression of *gper* resulted in a 9-fold increase compared to the vehicle control (Fig. 4B). G1 exposure also resulted in a 8- and 9-fold increase in the expression of *lrrc10* and *hand2*, respectively (Fig. 4C and D).

To assess whether the impacts on cardiac development were mediated through activation of GPER, E2- and G1-exposed embryos were co-exposed with the GPER antagonist G36. Exposure up to 5 μM of G36 did not cause cardiac deformities. Embryos exposed to 7 μM of E2 and 1.75 μM of G1 both caused a significant increase in the percentage of embryos that had cardiac deformities ($45.4 \pm 5.3\%$ and $52.8 \pm 6.1\%$ respectively). Co-exposure of embryos to G1 and G36 decreased the abnormalities observed with G1 to $13.3 \pm 2.5\%$ (Fig. 5A). In addition, the alterations observed in *gper*, *lrrc10* and *hand2* transcripts after G1 exposure were also abolished (Fig. 5B and C). This suggests that expression of these genes may be associated with GPER-induced cardiotoxicity.

The increase in the expression of *gper*, *lrrc10* and *hand2* observed in the E2 exposed embryos were reduced when co-exposed with G36 (Fig. 6B and C). However, the deformities were not rescued in the embryos that were co-exposed with E2 and G36 (Fig. 6A). Since E2 deformities were not rescued by G36, we investigated whether the nuclear estrogen receptors were involved in E2 developmental toxicity. Embryos were co-exposed to 14 μM of ICI, 182,780 (ICI). The co-exposed samples did not significantly decrease the num-

ber of observed deformities, although levels of *vtg* expression was diminished confirming the concentration of ICI necessary for ER antagonism (Fig. 7 and Supplemental Fig. 2).

Activation of GPER enhances levels of intracellular secondary messengers such as cAMP and Ca^{2+} , which can moderate the expression of *lrrc10* and *hand2* (Brody et al., 2013; Dirks et al., 2013). Levels of both secondary messengers were analyzed after exposure to 2, 5 and 7 μM of E2, and the GPER agonist G1. There was an increase in the levels of Ca^{2+} following treatment with 5 μM of G1. However, significant changes were not seen after exposure to E2 for 74 h at any of the concentrations analyzed (Supplemental Fig. 3). There was also no increase in cAMP after treatment with E2 and a trend towards a decrease after G1 exposure (Supplemental Fig. 4).

4. Discussion

Activation of GPER by the GPER agonist G1 induces similar malformations as those of embryos exposed to other estrogenic compounds (Jayasinghe and Volz, 2012). Binding of E2 or G1 to GPER can alter transcriptional regulation of genes required for development. In the present study, the role of GPER on cardiac impacts by E2 during development was evaluated. We showed that exposing zebrafish embryos to E2 and G1 during embryogenesis altered the transcription of *gper* and genes involved in cardiac

Fig. 4. Effects of G1 exposure on cardiac development and expression of *lrrc10*, *hand2* and *gper* in zebrafish embryos. (B–D) Cumulative fold change of *gper* (B), *lrrc10* (C) and *hand2* (D) mRNA of zebrafish embryos exposed to 0.1% ethanol (ETOH) and 0.5–5 μ M of G1 for 74 h starting at 2 hpf. Data presented as mean $\pm$ standard error (SE) of four to nine independent replicates. (A) Data represents average percent cardiac deformities at 76 hpf after treatment with 0.1% ethanol (ETOH) and 0.5 μ M of E2 at 2 hpf. Average percent cardiac deformities is presented and was analyzed via a logistic regression model. Normal data was analyzed by ANOVA followed by Tukey's HSD multiple comparisons test. Data that was not normal a Kruskal–Wallis test was used followed by a Dunn's Multiple Comparison Test. Different letters represent the significant differences ($p < 0.05$).

Fig. 5. Effects of GPER agonist G1 and GPER antagonist G36 co-exposure on deformities and the expression of *lrrc10*, *hand2* and *gper* in zebrafish embryos. (A) Data represents average percent cardiac deformities at 76 hpf after treatment with 0.2% ethanol (ETOH), 5 μ M of G36 and 1.75 μ M of G1 for 74hr starting at 2hpf. (B–D) Cumulative fold change of *gper* (B), *lrrc10* (C) and *hand2* (D) mRNA of zebrafish embryos after exposure at 2 hpf. Data presented as mean $\pm$ standard error (SE) of four to nine independent replicates. Normal data was analyzed by two-way ANOVA followed by Tukey's HSD multiple comparisons test. Deformities data was analyzed using a logistic regression model. Different letters represent the significant difference ($p < 0.05$).

Fig. 6. Effects of co-exposure to E2 and GPER antagonist G36 on deformities and the expression of *lrrc10*, *hand2* and *gper* in zebrafish embryos. (A) Data represents average percent cardiac deformities at 76 hpf after treatment with 0.2% ethanol (ETOH), 5 μ M of G36 and 7 μ M E2 for 74 h starting at 2 hpf. (B–D) Cumulative fold change of *gper* (B), *lrrc10* (C) and *hand2* (D) mRNA of zebrafish embryos after exposure at 2 hpf. Data presented as mean $\pm$ standard error (SE) of four to nine independent replicates. Normal data was analyzed by two-way ANOVA followed by Tukey's HSD multiple comparisons test. Deformities data was analyzed using a logistic regression model. Different letters represent the significant difference ($p < 0.05$).

Fig. 7. Percent of cardiac deformities after co-exposure of ER antagonist ICI 182,780 with 17 β -estradiol (E2). Data represents average percent cardiac deformities at 76 hpf after treatment with 0.2% ethanol (ETOH), 7 μ M of E2, 14 μ M of ICI 182,780 and 2 μ M of Ral at 2 hpf. Data presented as mean $\pm$ standard error (SE) of four to six independent replicates. Data was analyzed using a logistic regression model. Asterisks (*) represent the significant difference ($p < 0.05$).

development (*lrrc10* and *hand2*). Embryo lethality and developmental abnormalities such as cardiac deformities (e.g., pericardial edema) were temporally related to the altered expression patterns.

A significant increase in cardiac deformities (primarily pericardial edema) was observed after exposure to 7–8 μ M of E2 at 76 hpf. Exposure to E2 (7–96 hpf) was reported to cause cardiac abnormalities in zebrafish embryos, but was observed at lower concentrations (2 μ M) (Chandrasekar et al., 2010). The inconsistency in effect thresholds are likely due to experimental design differences such as different treatment vessels, duration and developmental stage. In our study, exposure was only for 74 h with exposure occurring at 2 hpf. In contrast, Chandrasekar et al. (2010) exposed 7-hpf embryos to E2 for 96 h. Cardiac deformities have also been observed with other estrogen receptor ligands including EE2, BPA, bifenthrin,

and genistein (Duan et al., 2008; Jin et al., 2009; Tse et al., 2013; Santos et al., 2014). EE2 was shown to alter heart rate and cause pericardial edema after exposure for 142 h (2–144 hpf) (Santos et al., 2014). Exposure to BPA in embryos from 8 to 120 hpf resulted in cardiac abnormalities at concentrations between 30 and 70 μ M (Saili et al., 2012). Similarly, exposing 24-hpf zebrafish embryos to genistein (25 and 50 μ M) for 60 h resulted in a reduction of heart rate and increased pericardial edema (Kim et al., 2009).

Consistent with earlier studies, G1 also caused similar developmental cardiac toxicity in the current study (Jayasinghe and Volz, 2012). In addition, expression of *gper* mRNA was significantly increased following E2 treatment. Previous studies have shown that E2 induced the levels of both GPER mRNA and protein in breast cancer cells via the epidermal growth factor receptor (EGFR) pathway (Vivacqua et al., 2009). Overexpression of GPER has been observed in breast and lung cancer cells, indicating its role in different chronic diseases (Filardo et al., 2006; Jala et al., 2012). The role of EGFR in the developmental toxicity of estrogenic compounds in zebrafish is unclear, but warrants further study.

GPER signaling is important for reproductive physiology and cardiovascular function (e.g. vasodilation) (Bopassa et al., 2010; Deschamps and Murphy, 2009; Meyer et al., 2011). In this study, E2 altered *hand2* transcripts at 12 hpf and 76 hpf and treatment with G1 also caused altered expression of *hand2*. The transcription factor, *hand2* belongs to the beta helix-loop-helix (bHLH) family of proteins and regulates cardiac differentiation, especially during early somitogenesis, which occurs ~10–24 hpf in zebrafish (Kimmel et al., 1995; Stainier, 2001; Yelon et al., 2000). In addition, *hand2* plays a role in Notch and NFAT signaling in the endocardium (VanDusen et al., 2014). Overexpression of *hand2* in zebrafish embryos increases the number of cardiomyocyte cells and causes heart enlargement (Schindler et al., 2014). For example, mice that overexpressed *hand2* developed cardiac hypertrophy (Dirkx et al., 2013). Thus, the increase of *hand2* observed in our study may neg-

actively affect cardiac development, but further study is needed to test this hypothesis.

Changes in the expression of *lrrc10* during development were also observed in the study after exposure to E2 at 12 hpf and 76 hpf. Transcripts of *lrrc10* were also altered by G1 exposure, showing that activation of GPER is a potential pathway for E2-induced expression of *lrrc10*. LRRC10 is a cardiac specific protein that contains leucine-rich repeat (LRR) motifs and is found in mice, humans and zebrafish. LRRC10 also has a significant role in heart function and development in mice as well as zebrafish (Brody et al., 2013; Kim et al., 2007; Nakane et al., 2004). A zebrafish study using *lrrc10* morpholinos showed that knocking down this gene caused cardiac morphogenic defects, such as cardiac looping errors, in addition to pericardial edema (Kim et al., 2007). This, along with the results from our study indicates that any disturbance in the levels of *lrrc10* may also result in abnormal development.

To verify that the altered gene expression of *gper*, *lrrc10* and *hand2* were mediated through the activation of GPER, embryos were co-exposed to the GPER antagonist, G36. Co-exposure of G1 with G36 rescued the altered expression of all three genes back to control levels. In addition, the rescue of *gper*, *lrrc10* and *hand2* expression was associated with a reduced percentage of cardiac deformities in the G1–G36 co-exposure. This shows that *gper*, *lrrc10* and *hand2* may be involved in the cardiac effects of G1.

In contrast to G1, G36 was unable to rescue the cardiac deformities induced by E2 exposure, although the expression of *gper*, *hand2* and *lrrc10* were reduced in the E2–G36 co-exposure. To investigate the involvement of the estrogen receptor pathway in E2 toxicity, a co-exposure study was conducted using the ER antagonist, ICI 182, 780 (ICI). Interestingly, neither ICI 182, 780 nor G36 rescued the observed cardiac effects caused by E2. While these results were unexpected, it is important to note the limitations of chemical co-exposure experiments in evaluating the mechanism of toxicity of compounds. The inability of G36 to rescue E2 cardiac deformities does not eliminate the contribution of other rapid estrogen signaling pathways involvement such as ERs variants (i.e. mER36) that can localize in the membrane (Razandi et al., 1999; Levin, 2002; Chaudhri et al., 2012). In addition, E2 may induce expression of additional ERs that may not be inhibited by ICI. Although these compounds are known bind to specific receptors and initial downstream effects, it is currently not possible to eliminate all other pathways that might be affected by E2 at this concentration, time-point and developmental stage. Thus, the inability of ICI 182, 780 or G36 to rescue the E2 induced morphological effects may be due to the possibility that concentrations used for the study were insufficient to reverse E2 effects at the specific stage of development examined. Clearly, concentrations of G36 were sufficient to reduce G1-mediated effects and expression of GPER regulated cardiac genes, but morphological effects of E2 were unchanged. Concentrations of agonists and antagonists for all exposures were optimized based on solubility and overt toxicity. Antagonist concentrations above the solubility limit or that caused overt toxicity were not used. Although other studies in zebrafish adults have shown that 1 μ M ICI can rescue effects of 10 nM E2, this was seen in adult male fish (Lam et al., 2011). Given that the concentrations of ICI in our study (14 μ M) were more than 10-fold greater than those used in the adult male exposure, and below limits of solubility and overt toxicity, it seems likely that concentrations of ICI should have been high enough to elicit anti-estrogenic activity. In fact, the ICI-mediated inhibition of *vtg* induction by E2 is also consistent with ICI concentrations being relevant. However, the kinetic disposition of E2 and antagonists to specific molecular targets in adults relative to embryos is unknown, and suggests morphological effects may be dependent upon the time of exposure.

Alteration in the transcriptional regulation of *gper*, *hand2* and *lrrc10* was observed at 12 hpf and 76 hpf but not at 28 hpf. Enhanced

expression at unique temporal periods may indicate sensitive windows for E2 toxicity during zebrafish development. At 12 hpf, the embryo is in the segmentation period of development. At this stage, cardiac precursor cells begin to move towards the embryonic axis and segregate (Stainier, 2001; Bakkers, 2011). At 28 hpf, ventricular and atrial chambers of the heart tube are becoming more distinct (Stainier, 2001). At 76 hpf, rapid growth of all tissues including the heart is occurring (Staudt and Stainier, 2012). This is the time when deformities were observed in the current study. Proliferation of cardiac myocytes, and development of the primitive leaflet valves occur during 76 hpf and are critical for regulating blood flow (Scherz et al., 2008). The degree of reduced blood flow needed to cause embryo or larval mortality is unclear but would be a fruitful area of further study.

Due to the simultaneous increases in *lrrc10*, *hand2* and *gper* expression after E2 treatment at specific time and developmental stages, the levels of secondary messengers, which theoretically link the downstream genes with GPER were analyzed. Ca^{2+} and cAMP concentrations did not significantly change after E2 exposure at 76 hpf. These findings suggest that *hand2* and *lrrc10* expression may not be dependent on significant increases in the concentrations of secondary messengers, suggesting the role of other pathways. It should be noted, however, that the use of whole embryo homogenates also could decrease the signal-to-noise ratio potentially masking site-specific alterations in Ca^{2+} concentration such as the heart. Many studies have shown that E2 can alter cellular concentrations of Ca^{2+} but most studies have been conducted in isolated cells, which allow more sensitive detection (Filardo et al., 2002; Revankar et al., 2005; Thomas et al., 2005). Embryonic exposure to 5 μ M of G1 resulted in a significant increase of Ca^{2+} concentrations and a significant decrease in cAMP in embryos even though alterations in downstream gene expression (*hand2* and *lrrc10*) were observed at 2 μ M and not at 5 μ M of G1. These findings suggest that activation of these genes via GPER may occur independently or have cell-specific second messenger pathways, which may require microscopic imaging to identify in whole embryos.

In summary, our data suggest that E2-induced cardiotoxicity in developing zebrafish embryos may not be attributed to a specific estrogen pathway. This further raises the question of the role of GPER and ERs during development. Given the widespread occurrence of structurally similar environmental pollutants, such as hydroxy-PAHs or phenolic compounds, that affect estrogen pathways, it is necessary to better characterize this pathway in vertebrate development and environmental toxicology.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.aquatox.2017.02.024>.

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