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Triphenyl phosphate-induced developmental toxicity in zebrafish: Potential role of the retinoic acid receptor

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ABSTRACT

Using zebrafish as a model, we previously reported that developmental exposure to triphenyl phosphate (TPP) – a high-production volume organophosphate-based flame retardant – results in dioxin-like cardiac looping impairments that are independent of the aryl hydrocarbon receptor. Using a pharmacologic approach, the objective of this study was to investigate the potential role of retinoic acid receptor (RAR) – a nuclear receptor that regulates vertebrate heart morphogenesis – in mediating TPP-induced developmental toxicity in zebrafish. We first revealed that static exposure of zebrafish from 5–72 h post-fertilization (hpf) to TPP in the presence of non-toxic concentrations of an RAR antagonist (BMS493) significantly enhanced TPP-induced toxicity (relative to TPP alone), even though identical non-toxic BMS493 concentrations mitigated retinoic acid (RA)-induced toxicity. BMS493-mediated enhancement of TPP toxicity was not a result of differential TPP uptake or metabolism, as internal embryonic doses of TPP and diphenyl phosphate (DPP) – a primary TPP metabolite – were not different in the presence or absence of BMS493. Using real-time PCR, we then quantified the relative change in expression of cytochrome P450 26a1 (*cyp26a1*) – a major target gene for RA-induced RAR activation in zebrafish – and found that RA and TPP exposure resulted in a ~5-fold increase and decrease in *cyp26a1* expression, respectively, relative to vehicle-exposed embryos. To address whether TPP may interact with human RARs, we then exposed Chinese hamster ovary cells stably transfected with chimeric human RARα-, RARβ-, or RARγ to TPP in the presence of RA, and found that TPP significantly inhibited RA-induced luciferase activity in a concentration-dependent manner. Overall, our findings suggest that zebrafish RARs may be involved in mediating TPP-induced developmental toxicity, a mechanism of action that may have relevance to humans.

1. Introduction

Triphenyl phosphate (TPP) is an unsubstituted aryl phosphate ester historically used as a high-production volume flame retardant within polyvinyl chloride, polymers, printed circuit boards, photographic films, and hydraulic fluids (Brooke et al., 2009). Based on estimates from 10 to15 years ago, the combined production and use of TPP within Western Europe was 20,000–30,000 tons (or 44–66 million pounds) in 2000, while the combined production and use of TPP within the United States alone was 4500–22,700 tons (or 10–50 million pounds) (van der Veen and de Boer, 2012). Since 2005, the use of TPP as a flame retardant within polyurethane foam likely increased within the United States following the phase-out of pentabrominated diphenyl ether and subsequent replacement with alternative TPP-containing flame retardant formulations (USEPA, 2005). Similar to pentabrominated diphenyl ether, TPP is an additive flame retardant that can migrate from end-use products into indoor and outdoor environmental media (van der Veen and de Boer, 2012). As such, environmental exposure to TPP may pose a health risk to humans and ecological species, particularly during sensitive windows of development.

Using zebrafish embryos, we recently evaluated the potential developmental toxicity of brominated and aryl phosphate components (including TPP) present within Firemaster 550 (McGee

Abbreviations: AHR, aryl hydrocarbon receptor; BMS4934, -[(1E)-2-[5,6-dihydro-5,5-dimethyl8-(2-phenylethynyl)-2-naphthalenyl]ethenyl]benzoic acid; *cyp26a*1, cytochrome P450 26a1; DMSO, dimethyl sulfoxide; DPP, diphenyl phosphate; hpf, hours post-fertilization; MS-222, tricaine methanesulfonate; RA, retinoic acid; RAR, retinoic acid receptor; TPP, triphenyl phosphate.

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et al., 2013), an increasingly used alternative formulation for polyurethane foam. Within this study, exposure to TPP and mono-isopropylated triaryl phosphate – but not the remaining brominated and aryl phosphate components of Firemaster 550 – blocked normal looping of the atrium and ventricle, resulting in a "tube heart" phenotype. In addition, heart malformations resulting from mono-isopropylated triaryl phosphate exposure – but not TPP exposure– were blocked in the presence of an aryl hydrocarbon receptor (AHR) antagonist (1-methyl-*N*-[2-methyl-4-[2-(2-methylphenyl) diazenyl]phenyl-1*H*-pyrazole-5-carboxamide, or CH223191). Therefore, since TPP-induced malformations appear to be AHR-independent, it is currently unclear how TPP induces developmental toxicity in zebrafish and whether this mechanism of action is conserved within other vertebrates.

Although TPP is known to activate human pregnane X receptor (Honkakoski et al., 2004; Kojima et al., 2013) and peroxisome proliferator-activated receptor γ (Belcher et al., 2014; Pillai et al., 2014), these two receptors are not known to play a major role in regulating cardiac development within vertebrates. However, retinoic acid receptor (RAR) is a ligand-activated nuclear receptor that; (1) directly controls heart morphogenesis in zebrafish (Stainier and Fishman, 1992) and mice (Niederreither et al., 2001); (2) when over-activated by excess retinoic acid (RA), blocks normal cardiac looping within zebrafish (Chen et al., 2008); and (3) is activated by structurally diverse xenobiotics (Kamata et al., 2008). While mammals have three RAR orthologs (RARα-, RARβ-, and RARγ) (Bastien and Rochette-Egly, 2004), zebrafish lack RARβ and possess two different paralogs of RARα (*raraa* and *rarab*) and RARγ (*rarga* and *rargb*) (Waxman and Yelon, 2007). Despite these differences, cytochrome P450 26a1 (*cyp26a1*) is a major target gene for RA-induced RAR activation in zebrafish (White et al., 1996), mice (Ray et al., 1997), and humans (White et al., 1997), representing a biomarker for assessing potential RAR activation *in vivo*.

Based on our previous findings relative to data available within the literature, the overall objective of this study was to begin investigating the potential role of RAR in mediating TPP-induced developmental toxicity in zebrafish. To accomplish this objective, we relied on a combination of high-content screening assays, real-time PCR, and human RAR reporter assays to test the hypothesis that TPP-mediated RAR activation results in developmental toxicity, a mechanism that, similar to our findings with mono-isopropylated triaryl phosphate (McGee et al., 2013), may have relevance to humans. We also quantified internal embryonic doses of TPP and diphenyl phosphate (DPP) – a primary TPP metabolite – to confirm uptake of TPP and determine whether TPP and DPP doses were affected by the presence of an RAR antagonist.

2. Materials and methods

2.1. Animals

For all assays described below, we relied on a robust line of transgenic zebrafish (*fli1:egfp*) that stably express enhanced green fluorescent protein within vascular endothelial cells (Lawson and Weinstein, 2002). Although we did not assess the potential impacts of chemical exposure on angiogenesis within this study, we relied on fluorescent *fli1:egfp* zebrafish to analyze heart rate and body length using previously optimized protocols (Yozzo et al., 2013). Adult *fli1:egfp* zebrafish were maintained on a 14-h:10-h light:dark cycle within a five-shelf stand-alone system (Aquatic Habitats, Inc., Apopka, FL, USA) containing photoperiod light-cycle enclosures and recirculating conditioned reverse osmosis water. Dissolved oxygen, pH, conductivity, salinity, alkalinity, and temperature within recirculating water were maintained at 4-6 mg/L, 6.5–7.5, 425–475 μS, <1 ppt, 50–100 mg/L, and 27–28 °C, respectively; in addition, levels of ammonia, nitrite, and nitrate within recirculating water were consistently below 0.1 mg/L, 0.05 mg/L, and 2 mg/L, respectively. Adult females and males were bred directly on-system using in-tank breeding traps suspended within 3-L tanks, or bred off-system within a light- and temperature-controlled incubator using breeding traps suspended within 1-L tanks. For all experiments described below, newly fertilized eggs were staged according to previously described methods (Kimmel et al., 1995). All fish were handled and treated in accordance with approved Institutional Animal Care and Use Committee protocols at the University of South Carolina – Columbia.

2.2. Chemicals

TPP (99.5% purity) was purchased from ChemService, Inc. (West Chester, PA, USA), whereas all-*trans*-RA (99.7% purity) and 4-[(1*E*)-2-[5,6-dihydro-5,5-dimethyl-8-(2-phenylethynyl)-2-naphthalenyl]ethenyl]benzoic acid (BMS493, 98.2% purity) were purchased from R&D Systems (Minneapolis, MN, USA). Stock solutions of each chemical were prepared by dissolving chemicals in high performance liquid chromatography-grade dimethyl sulfoxide (DMSO) (50 mM), and then performing two-fold serial dilutions into DMSO to create stock solutions for each working solution. All stock solutions were stored at room temperature within 2-mL amber glass vials containing polytetrafluoroethylene-lined caps. Working solutions of tricaine methanesulfonate (MS-222) (Western Chemical, Inc., Ferndale, WA, USA) were freshly prepared by dissolving MS-222 into embryo media (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$), and working solutions of all treatments were freshly prepared by spiking stock solutions into embryo media, resulting in 0.1% DMSO within all vehicle control and treatment groups.

2.3. High-content screening assays with RA, TPP, and BMS493

2.3.1. Exposure setup

Black 384-well microplates containing 0.17-mm glass-bottom wells (Matrical Bioscience, Spokane, WA, USA) were used for all high-content screening assays. Immediately following spawning, newly fertilized eggs were collected and placed in groups of approximately 50 per plastic petri dish within a light- and temperature-controlled incubator until 5 h post-fertilization (hpf). For each assay, 384 viable *fli1:egfp* embryos were arrayed at 5 hpf into a 384-well plate (one embryo per well; 32 embryos per treatment) containing 50 μL per well of vehicle (0.1% DMSO) or treatment solution (0.1–100 nM RA; 0.05–50 μM TPP; or 0.05–50 μM BMS493), and then incubated at 28 °C under a 14-h:10-h light:dark cycle and static conditions until 72 hpf.

2.3.2. Image acquisition

At 72 hpf, the plate was removed from the incubator, and zebrafish embryos were anesthetized with 100 mg/L MS-222 by adding 25 μL of 300 mg/L MS-222 to 50 μL of vehicle or treatment solution. The plate was then centrifuged at 200 rpm for 2 min to help orient hatched embryos into right or left lateral recumbency. Using automated image acquisition protocols and parameters previously optimized (Yozzo et al., 2013) for our ImageXpress Micro Widefield High-Content Screening System (Molecular Devices, Sunnyvale, CA, USA), each embryo was imaged to analyze the following endpoints: heart rate, pericardial area, and body length. During the entire image acquisition period, internal temperature within the ImageXpress Micro system was maintained between 25 and 27 °C by removing panels on both sides of the ImageXpress Micro system and blowing air from left to right through the ImageXpress Micro with a portable fan; internal temperature was monitored and recorded at initiation and termination of each