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Exposure to tributyltin induces endoplasmic reticulum stress and the unfolded protein response in zebrafish

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ABSTRACT

Tributyltin (TBT) is a major marine contaminant and causes endocrine disruption, hepatotoxicity, immunotoxicity, and neurotoxicity. However, the molecular mechanisms underlying the toxicity of TBT have not been fully elucidated. We examined whether exposure to TBT induces the endoplasmic reticulum (ER) stress response in zebrafish, a model organism. Zebrafish-derived BRF41 fibroblast cells were exposed to 0.5 or 1 μM TBT for 0.5–16 h and subsequently lysed and immunoblotted to detect ER stress-related proteins. Zebrafish embryos, grown until 32 h post fertilization (hpf), were exposed to 1 μM TBT for 16 h and used in whole mount *in situ* hybridization and immunohistochemistry to visualize the expression of ER chaperones and an ER stress-related apoptosis factor. Exposure of the BRF41 cells to TBT caused phosphorylation of the zebrafish homolog of protein kinase RNA-activated-like ER kinase (PERK), eukaryotic translation initiation factor 2 alpha (eIF2α), and inositol-requiring enzyme 1 (IRE1), characteristic splicing of X-box binding protein 1 (*XBP1*) mRNA, and enhanced expression of activating transcription factor 4 (ATF4) protein. In TBT-exposed zebrafish embryos, ectopic expression of the gene encoding zebrafish homolog of the 78 kDa glucose-regulating protein (*GRP78*) and gene encoding CCAAT/enhancer-binding protein homologous protein (*CHOP*) was detected in the precursors of the neuromast, which is a sensory organ for detecting water flow and vibration. Our *in vitro* and *in vivo* studies revealed that exposure of zebrafish to TBT induces the ER stress response *via* activation of both the PERK–eIF2α and IRE1–XBP1 pathways of the unfolded protein response (UPR) in an organ-specific manner.

1. Introduction

Tributyltin (TBT), an organotin derivative, has been used mainly as an antifouling paint for vessels and fishing nets and as a wood preservative. Although the use of organotins in paints for vessels is virtually banned by the international treaty at present, TBT was once the major organotin compound used in antifouling paints worldwide and its residue in the marine environment is still an important concern (Horiguchi, 2012). One of the typical biological activities of TBT is endocrine disruption, and it has been a longstanding issue that TBT and other organotin species cause imposex, represented by the masculinization of females, in gastropod mollusks (Blaber, 1970; Sternberg, 2012). Other adverse effects of TBT (Pagliarani et al., 2012; Snoeij et al., 1987), such as hepatotoxicity (Reader et al., 1999; Ueno et al., 1994), immunotoxicity (Cima and Ballarin, 2012; Rice et al., 1995; Snoeij et al., 1988), and neurotoxicity (Boyer, 1989; Inageda and Matsuoka, 2009; Zuo et al., 2009), have also been reported. We previously determined that exposure of SH-SY5Y human neuroblastoma cells to TBT causes endoplasmic reticulum (ER) stress and suggested a possible role for the ER stress response in the neurotoxicity of TBT (Inageda and Matsuoka, 2009). However, the toxicological significance of the ER stress response in TBT-induced toxicities in aquatic organisms has not been investigated.

The ER is the intracellular organelle responsible for various aspects of the quality control of biologically active proteins, such as synthesis, folding, posttranslational modification, and delivery (Ron and Walter, 2007). The accumulation of unfolded proteins in the lumen of the ER contributes to the ER stress and induces an adaptive program called the unfolded protein response (UPR) (Schröder and Kaufman, 2005). It has been well documented that when cells are subjected to stress, three UPR branches, the protein kinase RNA-activated-like ER kinase (PERK) – eukaryotic

Abbreviations: ATF4, activating transcription factor 4; DAPI, 4,6-diamidino-2-phenylindole; DIG, digoxygenin; DMSO, dimethyl sulfoxide; eIF2α, eukaryotic translation initiation factor 2 alpha; ER, endoplasmic reticulum; FBS, fetal bovine serum; hpf, hours post fertilization; IRE1, inositol-requiring enzyme 1; MAB, maleic acid buffer; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PERK, protein kinase RNA-activated-like ER kinase; RT, reverse transcription; SERCA, sarcoplasmic–endoplasmic reticulum Ca^{2+}-ATPase; TBS, Tris buffered saline; TBT, tributyltin; Tg, thapsigargin; Tm, tunicamycin; UPR, unfolded protein response; XBP1, X-box binding protein 1.

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translation initiation factor 2 alpha (eIF2α) pathway, the inositol-requiring enzyme 1 (IRE1) – X-box binding protein 1 (XBP1) pathway, and the activating transcription factor 6 (ATF6) pathway, are activated and promote the expression of ER chaperones and factors involved in the ER-associated protein degradation (Wang and Kaufman, 2012). The primary purpose of the UPR is to facilitate adaptation of the cells to environmental changes and to reestablish normal ER functions; however, the UPR also results in cell death when ER stress is severe and prolonged (Inagi, 2010).

Zebrafish (*Danio rerio*) is currently accepted as a useful model organism in various fields of biological and medical science. Zebrafish has many advantages for animal experiments, including the external fertilization of eggs, rapid development and the optical transparency of embryo, and a small size and low-cost breeding of adult fish. These features are also useful for investigations in ecotoxicological disciplines, and numerous studies have been performed using zebrafish (Froehlicher et al., 2009; Scholz et al., 2008; Sipes et al., 2011). Although the adverse effects of TBT in zebrafish, such as masculinization (McAllister and Kime, 2003; Santos et al., 2006), sperm damage (McAllister and Kime, 2003; Thresher et al., 2011), decreased fertility (Thresher et al., 2011), and genotoxicity, have been studied, there is no mention of the potential contribution of the ER stress response in TBT toxicity. In this study, we investigated whether exposure to TBT evokes the ER stress response in a zebrafish model system and report that exposure of zebrafish to TBT induces the UPR in an organ-specific manner.

2. Material and methods

2.1. Cell culture and exposure

BRF41 fibroblast cells established from the fin scale of adult zebrafish were provided by Dr. Hiroshi Mitani through RIKEN Cell Bank (RIKEN Bioresource Center, Tukuba, Japan) and grown in Leibovitz's L-15 medium (Gibco, Carlsbad, CA, USA) supplemented with 15% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco) in a humidified atmosphere at 33 °C. For each experiment, an appropriate number of exponentially growing BRF41 cells were seeded 48 h before treatment with chemicals. Tributyltin (IV) chloride (TBT) (Wako fine chemicals, Osaka, Japan) and thapsigargin (Tg) (Calbiochem, Darmstadt, Germany) were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 1 mM and stored at −20 °C. Tunicamycin (Tm) (Sigma–Aldrich, St. Louis, MO, USA) was dissolved in DMSO at a concentration of 1 mg/ml and store at −20 °C. Cells were exposed to DMSO vehicle, 0.5 or 1 μM TBT, 2 μg/ml Tm, and 2 μM Tg for 0.5–16 h. In each exposure, the concentration of DMSO was adjusted to 0.2% because DMSO had no effect on viability and morphology of BRF41 cells and negligible effects on the assessed parameters under this condition (data not shown). Both Tg and Tm were used as positive controls for induction of the ER stress and the UPR. During treatment with chemicals, the FBS concentration was reduced to 1%. A series of these exposure experiments was independently performed three times using BRF41 cells of different passage number and a set of treated cells was collected in each exposure.

2.2. Maintenance of adult zebrafish and exposure of embryos

Adult zebrafish (*Danio rerio*) were maintained at 28.5 °C under 14-h light/10-h dark cycle conditions. Fertilized eggs from natural crosses were collected immediately after spawning and cultured at 28.5 °C in the egg culture water (0.006% NaCl; 0.00025% methylene blue in deionized water). To avoid the pigmentation of the embryos, 0.003% of phenylthiourea was added to the egg culture water at 10 hpf (bud stage). The chorion of the eggs was removed with fine forceps under microscopic observation at 24 h post fertilization (hpf), and the embryos were exposed to DMSO vehicle, 1 μM TBT, 0.5 μg/ml Tm, and 0.5 μM Tg in the egg culture water from 32 to 48 hpf. In each exposure, the concentration of DMSO was adjusted to 0.1%, because during this developmental period, 0.1% DMSO had no effect on viability, morphology, and motility of zebrafish embryos (data not shown). Thirty embryos were used in each exposure and a set of these treatments was independently done three times using the different siblings. Embryos were fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) at 48 hpf for 1 h at 4 °C. All animal experiments were designed according to the institutional ethical code for laboratory animals and approved by the institutional review committee (Tokyo Women's Medical University, Tokyo, Japan).

2.3. Gene names and parameters

The name of the ER stress response-related genes of zebrafish and those human homologs, the analyzed parameters, the samples and the methods for analyzing the parameters, and the implications of the changes of these parameters studied in this work were summarized in Table 1.

2.4. Immunoblotting

BRF41 cells were lysed in cell lysis buffer (50 mM Tris–HCl (pH 6.8), 2% sodium dodecyl sulfate, and 10% glycerol) containing complete Mini EDTA-free (Roche Applied Science, Mannheim, Germany) and Phosphatase Inhibitor Cocktail 1 & 2 (Sigma–Aldrich). Bromophenol blue and 2-β-mercaptoethanol were added to cell lysates at a final concentration of 0.006% and 6%, respectively, and boiled for 5 min. Proteins were separated by electrophoresis on the Mini-Protean TGX gel (Bio-Rad, Hercules, CA, USA) and transferred to polyvinylidene difluoride membrane Immobilon-P (Millipore, Billerica, MA, USA). Membranes were briefly rinsed with 0.1% Tween 20 in Tris-buffered saline (TBST), and non-specific binding of the antibody was blocked by incubating the membranes in 5% skim milk in TBST for 1 h at room temperature. Subsequently, the membranes were immersed in working dilutions of primary antibodies overnight at 4 °C. Blots were washed with TBST and incubated with the appropriate secondary antibodies for 1 h at room temperature. Proteins were detected with a Phototope-HRP Western blot detection kit (Cell Signaling Technology, Danvers, MA, USA) and Amersham Hyperfilm ECL (GE Healthcare, Buckinghamshire, UK), and exposed photographic films were developed in Fuji medical film processor FPM100 (Fuji photo film, Tokyo, Japan). The bands on the developed film were quantified with ImageJ 1.42 (National Institutes of Health, Bethesda, MD, USA). Working dilution and distributor of each antibody were as follows. Anti-Atf4b1/CREB-2 (C-20) (1:1000), Hspa5/GRP78 (76-E6) (1:2500), and phospho-Eif2ak3/PERK (Thr 981) (1:500) antibodies and horseradish peroxidase (HRP)-conjugated anti-rat IgG antibody (1:2000) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-phospho-Eif2s1/eIF2α (Ser 51) antibody (1:1000) and HRP-conjugated anti-rabbit IgG antibody (1:2000) were obtained from Cell Signaling Technology. Anti-β-Actin/Actb1 (AC-15) antibody (1:5000) was obtained from Sigma–Aldrich. Anti-phospho-Ern1/IRE1 (Ser 724) antibody (1:500) was obtained from Abcam (Cambridge, MA, USA). Anti-Hsp90b1/GRP94 antibody (1:5000) was obtained from Stressgen (Farmingdale, NY, USA). HRP-conjugated anti-mouse IgG antibody (1:2500) was obtained from GE Healthcare. A series of immunoblotting was replicated three times with use of cell lysates prepared from independently treated BRF41 cells (see Section 2.1).