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A brominated flame retardant 2,2′,4,4′ tetrabrominated diphenyl ether (BDE-47) leads to lipogenesis in the copepod *Tigriopus japonicus*

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ABSTRACT

De novo lipogenesis (DNL) is a fatty acid synthesis process that requires several genes, including sterol regulatory element binding protein (SREBP), ATP-citrate lyase (ACLY), and acetyl-CoA carboxylase (ACC). DNL up-regulation is able to induce fat accumulation through an increase in fatty acids. To investigate the relationship between DNL up-regulation and the accumulation of fatty acids and lipid droplets in response to 2,2′,4,4′ tetrabrominated diphenyl ether (BDE-47), we examined DNL in the copepod *Tigriopus japonicus*. Transcription levels of DNL-related genes were increased after exposure to 2.5 μg/L BDE-47 for 24 h. After exposure to 2.5 μg/L BDE-47, palmitic acid was significantly increased ($P < 0.05$) at days 1 and 4, along with upregulation of fatty acid synthesis-related genes (*e.g.*, desaturases and elongases). However, docosahexaenoic acid and arachidonic acid were down-regulated at days 1 and 4, showing an antagonistic effect. Lipid droplet area significantly increased in Nile red staining analysis after 24 h of exposure to 2.5 μg/L BDE-47 in *T. japonicus*, while DNL was down-regulated in response to 500 μM salicylate (a lipogenesis inhibitor), indicating that BDE-47 exposure is closely associated with an increase in fatty acids in this copepod. This study provides a better understanding of the effects of BDE-47 on DNL in copepods.

1. Introduction

An imbalance between energy production and consumption leads to obesity and malnutrition. While several factors (*e.g.*, eating habits, family history, and environmental factors) contribute to obesity, the causes are not completely understood. Endocrine disrupting chemicals (EDCs) have emerged as important environmental obesogens, foreign chemicals that could lead to obesity (McAllister et al., 2009). EDCs have been steadily increasing in the environment and are found in various human organs (Guvenius et al., 2003; McAllister et al., 2009). In humans, *Xenopus laevis*, rats, and mice, exposure to EDCs (*e.g.*, tributyltin, bisphenol A, diethylstilbestrol) led to increased body weight with expression of some obesity biomarkers (Grun et al., 2006; Nikaido et al., 2004).

To date, many studies have detected EDCs in the ocean (Dorneles et al., 2015; Munschy et al., 2015; Peltonen et al., 2014; Wild et al., 2015). However, several studies on the mechanism of lipid synthesis caused by EDCs have been mainly performed in a mouse adipocyte cell line (3T3-L1 preadipocyte), humans, and mice (Kamstra et al., 2014; Stel and Legler, 2015). The understanding of DNL in crustaceans is poor. 2,2′,4,4′-tetrabrominated diphenyl ether (BDE-47) is a congener of polybrominated diphenyl ethers (PBDEs) and is a type of EDC. BDE-47 is a lipophilic substance that accumulates in fat tissue and belongs to a commercial flame retardant used in the manufacturing of electronics and furniture (Birnbaum and Staskal, 2004; Harrad et al., 2004). Many EDCs can be stored in fat tissue, as they have lipophilic characteristics (Jandacek and Tso, 2001). Also, BDE-47 is known to be the strongest toxicant among PBDEs and is easily found in the environment (Usenko et al., 2011). Due to the strongest toxicant and lipophilic characters which

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BDE-47 possess, studies have investigated its potential. For example, in several cell lines (*e.g.*, murine N2A, murine preadipocyte fibroblasts [3T3-L1], human SK-N-AS neuroblastoma cells) and mice with *in vitro* adipocyte differentiation, BDE-47 likely affects lipid accumulation through an increase in fatty acid-related mRNA expression in vertebrates *in vivo* and *in vitro* (Bastos Sales et al., 2013; Gee and Moser, 2008; Kamstra et al., 2014). Recently, in marine species, studies about the toxicity of BDE-47 have been conducted. BDE-47 has shown to affect gender-specific transcriptional profiling in the marine medaka *Oryzias melastigma*, (Yu et al., 2013). BDE-47 has also caused developmental retardation, reduced fecundity, and modulated expression of the defensome, which specifically participates in defense mechanisms to environmental stressors, in the copepod *Tigriopus japonicus* (Han et al., 2015). However, the mechanistic effects of BDE-47 on lipid synthesis have not been reported in copepods, despite the lipophilic characteristics of BDE-47.

The intertidal copepod *T. japonicus* has several ideal traits for an experimental model, including ease of maintenance in the laboratory, small body size (less than 1 mm), high fecundity, and a short life cycle (~2 weeks). Therefore, *T. japonicus* has been widely used in ecotoxicology (Raisuddin et al., 2007; Jeong et al., 2014; Lee et al., 2013). The genus *Tigriopus* has a worldwide distribution with its congeneric species (*e.g.*, *T. californicus, T. fulvus, T. brevicornis*) and is important in transferring energy via the food chain (Raisuddin et al., 2007). Therefore, examining lipid metabolism of the copepod *T. japonicus* may be important for understanding energy transfer in the food chain as primary consumer. So, this study will provide a better understanding of the mechanistic pathway of *de novo* lipogenesis (DNL) in aquatic invertebrates.

In this paper, we measured the mRNA expression of DNL genes with markers (fatty acid composition, distribution of triglycerides) in *T. japonicus* in response to different doses of BDE-47 and examined the effects of BDE-47 on the accumulation of fatty acids in a time-dependent manner by Nile red staining analysis. This study sought to identify the DNL pathway and the potential role of BDE-47 in *T. japonicus*. This paper will provide a better understanding of how BDE-47 affects lipogenesis in the copepod *T. japonicus*.

2. Materials and methods

2.1. Chemicals

BDE-47 (CAS No. 5436-43-1; purity >99.0%) and salicylate (purity >99.5%) were purchased from Sigma (Sigma-Aldrich, Inc., St. Louis, MO, USA). Nile red (9-diethylamino-5-benzo[α]phenoxazinone) (purity>98.0%) was purchased for lipid staining (Sigma-Aldrich, Inc.).

2.2. Culture and maintenance

The copepod *T. japonicus* was maintained and reared in artificial sea water (TetraMarine Salt Pro, Tetra™, Cincinnati, OH, USA) adjusted to 25 °C with a 12:12 h light:dark photoperiod and a salinity of 30 practical salinity units (psu). The copepods were fed the green algae *Chlorella vulgaris* daily (approximately 1×10^6 cells/mL). Species identification was performed by morphological characteristics and the mitochondrial DNA *COI* sequence was used as a universal barcode marker (Jung et al., 2006).

2.3. Total RNA extraction and single-strand cDNA synthesis

Approximately 300 copepods were homogenized in five volumes of TRIZOL® reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) with a tissue grinder and stored at −80 °C. Total RNA was isolated according to the manufacturer's instructions. Genomic DNA was removed using DNase I (Sigma-Aldrich, Inc.). Total RNAs were measured at 230, 260, and 280 nm (A230/260, A260/280) using a spectrophotometer (Ultrospec 2100 pro, Amersham Bioscience, Freiburg, Germany). Genomic DNA contamination of RNA was checked with 1% agarose gels with ethidium bromide and a UV transilluminator (Wealtec Corp., Sparks, NV, USA). RNA quality was determined with 1% formaldehyde/agarose gels with ethidium bromide to determine the *18/28S rRNA* integrity and band ratio. Single-stranded cDNA was synthesized from 1 μg total RNA using an oligo $(dT)_{20}$ primer for reverse transcription in 20 μL reactions (SuperScript™ II RT kit, Invitrogen, Carlsbad, CA, USA).

2.4. *In silico analysis of* de novo *lipogenesis genes*

To obtain the cDNA sequences of DNL genes, we searched *T. japonicus* RNA-seq information (contig number 54,761; average read length 1515 bp) (Kim et al., 2015). DNL genes were subjected to BLAST analysis in the GenBank non-redundant (NR; including all GenBank, EMBL, DDBJ, and PDB sequence except for EST, STS, GSS, or HTGS) amino acid sequence database (http://blast.ncbi.nlm.nih.gov/) to confirm identity.

2.5. Sequence alignment and phylogenetic analysis

To place the identified lipogenesis genes (ecdysone receptor, sterol regulatory element binding protein, carbohydrate regulatory element binding protein, ATP-citrate lyase, acetyl-coA carboxylase, β-ketoacyl-acyl-carrier-protein synthase, desaturases, and elongases) in the phylogenetic tree, we aligned the DNL genes of other species and deduced the amino acid sequences using Clustal W (Thompson et al., 1994). Gaps and the missing data matrix were excluded from the analysis. The generated data matrix was converted to NEXUS format, and was analyzed using maximum likelihood with optimum substitution models that determined each alignment based on the Bayesian information criterion (BIC) and the corrected Akaike information criterion (AIC) using MEGA 6.0 (Tamura et al., 2013). The LG + I model was identified as the best-fit model and was used in subsequent phylogenetic analyses. Maximum likelihood phylogenetic analysis was conducted using MEGA 6.0 with 10,000× bootstrap resampling. After analysis, a consensus tree was constructed and then visualized with MEGA 6.

2.6. Real-time reverse transcriptase-polymerase chain reaction

Transcripts of DNL genes were measured with real-time reverse transcriptase-polymerase chain reaction (real-time RT-PCR). Optimized conditions were used according to the CFX96™ real-time PCR protocol (Bio-Rad, Hercules, CA, USA). All real-time RT-PCR reactions were performed in unskirted low 96-well clear plates (Bio-Rad, Hercules, CA, USA). One μg of total RNA was used to synthesize cDNA. 0.2 mM RT-F/R primer for DNL genes was used in each reaction (Table 1). Reaction conditions were: 94 °C/4 min; 40 cycles of 94 °C/30 s, 58 °C/30 s, 72 °C/30 s; and 72 °C/10 min. SYBR® Green (Molecular Probe Inc., Invitrogen, Carlsbad, CA, USA) was used to detect specific PCR products. To confirm amplification of specific products, melting curves were obtained under the following conditions: 95 °C/min, 55 °C/min, and 80 cycles of 55 °C/10 s with 0.5 °C increase per cycle. All PCR products were sequenced at Bionics Co. (Seoul, South Korea). Amplification and detection of SYBR Green-labeled products were performed using CFX96 real-time PCR system (Bio-Rad). Data from triplicate experiments were normalized to *18 S rRNA* to account for differences in reverse transcriptase efficiency. Fold-change for relative gene expression