Fish & Shellfish Immunology 44 (2015) 302–306

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Full length article

Immunotoxic effects of perfluorooctane sulfonate and di(2-ethylhexyl) phthalate on the marine fish *Oryzias melastigma*

Qiansheng Huang [a, b, 1, **], Yajie Chen [a, 1], Yulang Chi [a], Yi Lin [a], Huanteng Zhang [a], Chao Fang [a], Sijun Dong [a, *]

[a] Key Laboratory of Urban Environment and Health, Institute of Urban Environment, Chinese Academy of Sciences, Xiamen 361021, PR China
[b] Universidad Tecnica de Manabi, Portoviejo, Ecuador

ARTICLE INFO

Article history:
Received 16 November 2014
Received in revised form
28 January 2015
Accepted 5 February 2015
Available online 14 February 2015

Keywords:
Immunotoxicity
Perfluorooctane sulfonate
di(2-ethylhexyl) phthalate
Oryzias melastigma
Combined exposure

ABSTRACT

Perfluorooctane sulfonate (PFOS) and di(2-ethylhexyl) phthalate (DEHP) have both been reported to induce adverse effects including immunotoxicity. Despite the widespread presence of these two chemicals in estuaries and seawater, their health effects on marine fish have received little attention. *Oryzias melastigma* is a potential marine fish model for immunological studies. In the present study, immune-related genes in *O. melastigma* were enriched at the transcriptome level. Three-month-old fish were exposed to PFOS and DEHP (single or combined) for one week. The liver index-hepatosomatic index (HSI) of the fish was higher in the PFOS-exposed group and combined group than in the control group. This result indicates that PFOS might lead to liver toxicity. The mRNA level of interleukin-1 beta (*IL1β*) was upregulated after exposure. For catalase (*CAT*), glutathione peroxidase (*GPx*) and cluster of differentiation 3 (*CD3*), single exposure did not affect mRNA levels, but the combined exposure did significantly alter the expression of these genes. In all, our study provides a useful reference for immunotoxicological studies with *O. melastigma*; it also highlights the importance of assessing the combined effects of pollutant mixtures when determining the risk to aquatic organisms.

1. Introduction

The immune system of fish consists of both innate immunity and acquired immunity. They work together to protect against infection. Pro-inflammatory cytokines are released as part of the non-specific innate immune response. Both interleukin-1beta (*IL1β*) and tumor necrosis factor-α (*TNFα*) are essential proteins in the induction of immunity. The complement system plays an important role in the adaptive immune response and is involved in chemotaxis, opsonization, phagocytosis and degradation of pathogens in teleosts [1,2]. *Oryzias melastigma* is becoming a potential marine fish model for immunological studies [3]. The fish has wide salinity adaptability and is very suitable for marine and estuarine ecotoxicological studies [4,5]. However, genomic information related to immunity for *O. melastigma* is currently very limited.

Health concerns regarding the effects of organic pollutants on the immune system of wildlife have increased in recent years. T-lymphocyte proliferation and indices of innate immunity decreased with polychlorinated biphenyl (PCB) levels in the blubber of bottlenose dolphins [6]. The expression of genes involved in the innate immune system such as *TNFα*, *IL1β* and CC-chemokines were altered in newly hatched zebrafish after embryonic exposure to nonylphenol [7]. Similar effects were also observed in the liver of male Japanese medaka (*Oryzias latipes*) when exposed to high concentrations of PFOA (10–100 mg/L) [8].

Perfluorinated chemicals (PFCs) and phthalates are two widely detected groups of organic compounds that generate great health concerns. Perfluorooctane sulfonate (PFOS) and di(2-ethylhexyl) phthalate (DEHP) are the most common monomers of PFCs and phthalates, respectively. They are widely detected in estuarine and

Abbreviations: C8, complement component C8; *CAT*, catalase; *CCL20*, chemokine (C–C motif) ligand 20; *CD3*, cluster of differentiation 3; DEHP, di(2-ethylhexyl) phthalate; *GPx*, glutathione peroxidase; *IL1β*, interleukin-1 beta; PFCs, perfluorinated chemicals; PFOS, perfluorooctane sulfonate; qRT-PCR, quantitative RT-PCR; *RPL-7*, ribosomal protein L7.

* Corresponding author. Tel./fax: +86 592 6190779.
** Corresponding author. Key Laboratory of Urban Environment and Health, Institute of Urban Environment, Chinese Academy of Sciences, Xiamen 361021, PR China.
E-mail addresses: qshuang@iue.ac.cn (Q. Huang), sjdong@iue.ac.cn (S. Dong).
[1] Qiansheng Huang and Yajie Chen contributed equally to this paper.

http://dx.doi.org/10.1016/j.fsi.2015.02.005
1050-4648/

marine water as well as in aquatic organisms. The concentrations of PFOS reach up to 703 ng/L in the Yangtze river estuary in China [9]. PFOS bioaccumulates through the food chain. The concentration was found to be 7 μg/g (wet weight) in the liver of the seawater organism plaice (*Pleuronectes platessa*) [10]. DEHP is one of the dominant phthalates in the Seine river estuary with concentrations ranging from 160 to 314 ng/L [11]. In the Gulf of Mexico, an average DEHP level of 5 ng/g has been detected in marine biota (mainly fish) [12]. As pollutants exist as mixtures in the environment, their combined effects have received significant attention recently. Nonylphenol and di-n-butyl phthalate (DBP) show additive effects on rat Sertoli cell toxicity [13]. Hayes et al. concluded that interactions of pesticides that are concentration additive or response additive should be addressed to clearly assess the adverse impacts of pesticides on amphibian [14]. PFOS and DEHP are two potentially damaging pollutants that are widely distributed in the environment. However, to our knowledge, their combined effects are not well understood.

The aim of this study was to investigate the immunotoxic effects of PFOS and DEHP on marine fish. We first obtained candidate genes in *O. melastigma* immunity at the transcriptome level. Then, the immunotoxic effects of PFOS and DEHP (single or combined) were analyzed at the mRNA level.

2. Materials and methods

2.1. Fish, doses and experimental procedures

O. melastigma was cultured in artificial seawater at a salinity of 30‰ and temperature of 28 ± 2 °C with a constant 14 h-light/10 h-dark photoperiod. They were fed with live brine shrimp nauplii (Artemia sp.) twice a day. Three-month old fish were used in the current study. PFOS and DEHP were both dissolved in dimethyl sulfoxide (DMSO). The nominal exposure concentrations were 0.25, 1 mg/L for PFOS and 0.1, 1 mg/L for DEHP. The combined exposure consisted of 1 mg/L PFOS and 1 mg/L DEHP. These doses were commonly used in previous studies with PFOS ranging from 0.1 to 25 mg/L [15–17] and DEHP ranging from 0.4 to 4 mg/L [18]. Three replicates were set for each exposed group. Each replicate contained four fish (two males and two females) in one tank. Fish treated with 0.1% DMSO were used as the control group. The media were renewed every day and the exposure period was one week.

2.2. Hepatosomatic index (HSI)

After one week's exposure, the fish were euthanized with ice water. After measurement of body weight, the liver was dissected out and weighed. HSI was calculated as follows.

$$\mathrm{HSI} = \frac{\text{Liver weight}}{\text{Total body weight}} \times 100$$

The HSI was calculated (n = 12) and the data are presented as the mean value ± standard deviation (SD).

2.3. Enrichment of gene information on immune-related genes

The transcriptome sequencing of *O. melastigma* was reported in our previous study [19]. In the current study, we further enriched immune-related genes and pathways using bioinformatics approaches including Swiss-Prot, Gene Ontology database (GO), Clusters of Orthologous Groups (COG) and Kyoto Encyclopedia of Genes and Genomes (KEGG).

2.4. RNA extraction and quantitative RT-PCR

Four fish from each tank were randomly collected for RNA extraction. RNA was extracted from the liver of fish using a commercial kit (Omega Bio-Tek, Inc. Norcross, USA) according to the manufacturer's protocol. Equal amounts (1 μg) of RNA were used for cDNA synthesis by SYBR Premix Ex Taq™ kit (TaKaRa, China). The second step PCR was performed on a Roche LightCycler® 480 with the thermal cycling conditions as follows: an initial step at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 60 °C for 20 s. Four repeats of the PCR reaction were performed for each individual gene. The primer sequences are provided in Table 1. Two genes, 18s ribosomal RNA (*18S*) and ribosomal protein L7 (*RPL-7*), were selected for normalization of gene expression levels [20]. As the results from both reference genes were very similar, the data normalized to *RPL-7* are presented in the study. The $2^{-\Delta\Delta Ct}$ method was used to calculate the relative expression levels of the target genes within the various exposed groups [21]. The results are presented as the mean ± standard deviation (SD).

2.5. Data analysis

The data are shown as the mean ± SD. SPSS (Statistical Package for the Social Sciences) 16.0 software was used for statistical analysis. Significant differences were examined by one-way ANOVA combined with the Tukey test (post hoc test); $p < 0.05$ was regarded as significant.

3. Results

3.1. Information on immune-related genes and pathways

The transcriptome of *O. melastigma* was obtained as reported in our previous study [19]. Candidate immune-related genes were enriched in the current study. The annotation results showed that a total of 6,415 unigenes were regarded as homologous to known immune-related genes. Members of the interleukin and chemokine families were included in these genes. A total of 854 and 5,561 unigenes were enriched in the GO terms of immune system process and response to stimulus, respectively. A total of 197 unigenes were included in defense mechanisms by COG annotation. KEGG analysis showed that ten pathways were related to immunity (Table 2). In total, 992 unigenes were enriched in the chemokine signaling pathway (ko04062). Further annotation of these unigenes extended to almost all of the genes involved in this pathway (Fig. 1).

Table 1
Primer sequences used in SYBR qRT-PCR analysis.

Gene name	GenBank accession no.	Primer sequences used for qRT-PCR (5′ to 3′)
CAT	KC138555	F: GCCAACTACCTGCAGATCCCCGTCA
		R: AGTTTGGAGCGCCGCCTTGGTTGT
GPx	KC138557	F: GTGTGCAGAAACGACGTGGCCTGGA
		R: TCGCCTTCGATGTCGCTGGTGAGGA
IL1β	KC138556	F: AGGCAGCGACAGCCGCAAAGTTCA
		R: TGGTGTCCTTGATGCCCAGAGCCA
CD3	KC138558	F: CGGACCTGCGACAACTGTGTGGAGTT
		R: ATCCCAGCGGTCGCCACCAAGTTT
C8	KC138554	F: ACCCTCTCAGAGCCCATGCTCACCA
		R: TGGTCCTGGTCCACTTCACCACAGT
CCL20	KC138559	F: CGGTGCTGCCGAAAATACATGAAAGGC
		R: GCTCAGAGCAGGATTGGTGCAAGTGT
RPL-7	KC138553	F: TGGCACACAAGTACCGGCCAGAGA
		R: TTGGTGGGGGTGTCTCCTTTCCCA
18S	DQ105650	F: GCAGCGTCCGGGAAACCAAAGTCTT
		R: TGGTGGTGCCCTTCCGTCAATTCCT