Fish & Shellfish Immunology 34 (2013) 142–146

Contents lists available at SciVerse ScienceDirect

Fish & Shellfish Immunology

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

Characterisation of immune-related gene expression in clam (*Venerupis philippinarum*) under exposure to di(2-ethylhexyl) phthalate

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ARTICLE INFO

Article history:
Received 27 June 2012
Received in revised form
7 October 2012
Accepted 11 October 2012
Available online 23 October 2012

Keywords:
Venerupis philippinarum
Di(2-ethylhexyl) phthalate
Gene expression

ABSTRACT

Di(2-ethylhexyl) phthalate (DEHP) mediates the immune system mainly by triggering the production of reactive oxygen species (ROS) and nitric oxide (NO) in higher animals. In the present study, spatial variation in the expression of immune-related genes in clam (*Venerupis philippinarum*) under acute short-term DEHP treatment was assessed by qPCR. The expression of six genes including glutamine synthetase (GS), IkB (IK), transcription factor activator protein-1 (AP-1), cyclophilin A-1 (CypA-1), heat shock protein 90 (HSP90) and superoxide dismutase (SOD) was dose-dependent. A negative correlation between expression and DEHP treatment was observed for big defensin (BD), glutathione S-transferase (GST), and thioredoxin peroxidase (TP). Surprisingly, lysozyme (LYZ) exhibited two distinct expression patterns at two DEHP doses. Significant differences between the experimental and control groups were observed for all tested genes at the various time points. Overall, our results revealed that DEHP mediates immune responses in clams by various means, and certain genes are promising candidate for biomarkers in DEHP monitoring.

1. Introduction

Environmental pollution is already at a critical level and they are worsening over time, especially for the global ocean. The impact of pollutants on marine ecosystems is evident at various levels of biological complexity from molecules to communities [1]. Accumulating evidence indicates that environmental contaminants adversely affect the immune systems of marine organisms and may be partially responsible for disease outbreaks in these organisms [2]. Therefore, immune-related genes were considered to be promising biomarkers for early monitoring of environmental conditions due to their expeditious response to environmental perturbations and contamination [3].

Recently, environmental hormones such as di(2-ethylhexyl) phthalate (DEHP) have attracted much attention from the development industry as these compounds are increasingly being used as plasticisers, wire insulators and pesticide carriers. It was estimated that the concentrations of phthalate esters (PAEs) in Quanzhou Bay seawater and sediments typically range from 18.77 to 191.51 ng L^{-1} and 171.50–1435.61 μg kg^{-1}, respectively, and DEHP is a major component of PAEs [4]. Experimental evidence revealed that DEHP in seawater could be absorbed, metabolised and accumulated at high levels in the tissue of marine animals such as penaeid shrimp, and DEHP had adverse affects on the immune systems of these animals [5]. It had also been well documented that exposure to DEHP altered the activity of several proteins involved in apoptosis, signalling and metabolism in addition to cell cytoskeletal proteins [6,7]. Recent research in higher animals also revealed that DEHP had the potential to interact with the immune system through its enhancement of IL-4 production [8], which caused an inflammatory response [9]. In the lower aquatic animals *Chironomus riparius* and *Tigriopus japonicus*, DEHP was able to induce the expression of many genes including cytoskeletal proteins, alcohol dehydrogenases and heat shock proteins [6,10,11]. However, to our knowledge, very little information is available on the interaction between aquatic immune responses and DEHP exposure. Because DEHP interacts with the immune systems of higher animals by regulating ROS and NO production, the goal of the present study was to evaluate the *in vivo* effects of DEHP on genes known to be involved in the immune system of the clam *Venerupis philippinarum* under conditions simulating the natural environment.

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http://dx.doi.org/10.1016/j.fsi.2012.10.015

2. Materials and methods

2.1. Clams

Clams (*V. philippinarum*; 10 ± 1.65 g in weight) were purchased from Ningbo, Zhejiang Province, China. The clams were acclimated for a week before commencement of the experiment. The temperature was maintained at 20–22 °C throughout the experiment, and the salinity of the supplied seawater was maintained at 3%.

2.2. DEHP exposure

DEHP (analytical purity >99.0%) was first dissolved in absolute ethanol and subsequently diluted with an equal volume of seawater to obtain a stock solution of 40 mg L^{-1}. The clams were distributed randomly into three groups containing 50 individuals each. The DEHP stress experiment was performed by exposing the clams to two doses of DEHP with final concentrations of 0.4 and 4.0 mg L^{-1}. The control group was exposed to the same volume of ethanol and seawater. Samples from both the experimental and control groups were collected at 0, 12, 36, 60 and 96 h. Haemolymphs from the control and the experimental groups were collected individually using a syringe and centrifuged at 2000 × g for 10 min at 4 °C to harvest the haemocytes. We performed five replicates for each of the experimental groups as well as the control group.

2.3. Temporal expression profiles of candidate genes under DEHP exposure

Total RNA was isolated from clam haemocytes using TRIzol reagent (Invitrogen). First-strand cDNA synthesis was performed using the MMLV First Strand cDNA Synthesis Kit according to the manufacturer's instructions (Bio Basic Inc.). The expression levels of candidate genes after exposure of haemocytes to DEHP were measured by qPCR. Two clam β-actin primers, P1 and P2 (Table 1), were used to amplify a 121 bp fragment as an internal control to verify successful reverse transcription and to calibrate the cDNA template. Two specific primers for each gene (Table 1) were designed to amplify desired product. Real-time PCR amplification was performed using a Rotor-Gene 6000 real-time PCR detection system. We utilised the reaction components and thermal profiles suggested by the manufacturer. The $2^{-\Delta\Delta CT}$ method was used to analyse the expression level of the candidate genes, and the value obtained denoted the *n*-fold difference relative to the calibrator (untreated samples). The data are presented as relative mRNA expression levels (means ± S.D, $n = 5$). The data were subjected to a one-way analysis of variance (ANOVA). Significant differences between the DEHP exposure group and the corresponding control group at each time point are indicated with one asterisk for $P < 0.05$ and two asterisks for $P < 0.01$.

Table 1
Primers used in the present study.

Primer	Sequence (5′–3′)	Product size	Gene ID
P1(forward)	CTCCCTTGAGAAGAGCTACGA	121 bp	EF520696.1
P2(reverse)	GATACCAGCAGATTCCATACCC		
P3(forward)	CATTGCCCGTGCTTACTATTGAC	207 bp	GQ384392
P4(reverse)	TGTTCCTTTCTTCCTCGTTATCC		
P5(forward)	ATTCTTTCGCAGCACTTTCGTTG	253 bp	GQ384413
P6(reverse)	ACCCGGTTTACCGCAGTCTATC		
P7(forward)	GTGCAGGTCCTCACTATAACCCA	226 bp	GQ384412
P8(reverse)	GACAACTCGTGACCACCTTTACC		
P9(forward)	CTGGTCGCCATGACGACTCTATC	101 bp	HM562672
P10(reverse)	CGTTGTCGGGATGGTTCAAGTGC		
P11(forward)	CACGGGACTTCACGAGACTTCAG	145 bp	AM877049.1
P12(reverse)	TGGGTCACAGTTAGAGGACGGGC		
P13(forward)	AGCCACTTCTCCCATCTTGCCTG	235 bp	AM877524.1
P14(reverse)	ACGGAGCACCTCGTCAACATTTC		
P15(forward)	TGGTGACGATGTATCTGGACGAA	307 bp	JF683414.1
P16(reverse)	CACAATGTCTTCAGCGTTTCTCC		
P17(forward)	ATGACATCGCAGAGGACAAGGAG	225 bp	HM581640.1
P18(reverse)	CTCTGCTCTCGCCTGTGATGTAG		
P19(forward)	AGGAGTCGGTTCCCAAAGTG	248 bp	GQ384405.1
P20(reverse)	TATTGCATCCGGGGGTAGGT		
P21(forward)	TTGTGACGGATGAGCAGGAGGCA	280 bp	HQ918289.1
P22(reverse)	TCTATTGGAGATGGCGGAGGTGA		

3. Results and discussion

Di(2-ethylhexyl) phthalate (DEHP) is frequently observed in marine ecosystems due to its widespread use in plastics; thus, many marine species are constantly exposed to various levels of DEHP [6]. In higher animals, it was well established that DEHP disrupted oxidative balance by increasing the production of reactive oxygen species (ROS) and nitric oxide (NO), further leading to lipid oxidate, DNA damage and ERK/NFκB signalling pathway activation [12,13]. In cultured prawn *Macrobrachium rosenbergii*, DEHP exposure were able to inhibit and decrease the immune responses of prawn haemocytes through mediating O_2^- generation [14]. To better understand its toxic effects on the immune systems of marine clams, the expression profiles of 10 clam genes potentially involved into these physiological process were investigated by qPCR. The transcript levels in DEHP-exposed groups were compared to that in control groups at each sampling time point, which allowed us to classify the 10 genes into three response groups (Figs. 1–4). Six genes including glutamine synthetase (GS), IkB (IK), transcription factor activator protein-1 (AP-1), cyclophilin A-1 (CypA-1), heat shock protein 90 (HSP90) and superoxide dismutase (SOD) were expressed in a DEHP dose-dependent manner. The expression levels of big defensin (BD), glutathione S-transferase (GST), and thioredoxin peroxidase (TP) were negatively correlated with DEHP dose. Surprisingly, lysozyme (LYZ) exhibited two distinct expression patterns at two DEHP doses. Significant differences between the DEHP-treated and control groups were observed for all tested genes at the various time points. Either no

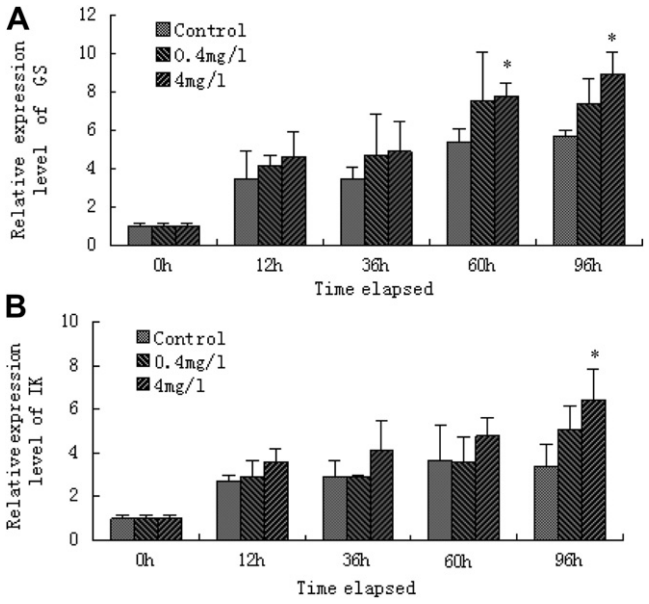

Fig. 1. Time course expression of GS (A) and IK (B) in haemocytes after exposure to different doses of DEHP measured by qPCR at 0, 12, 36, 60, and 96 h. Each symbol and vertical bar represent the mean ± SD ($n = 5$).