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Small Heat Shock Protein 20 Gene (*Hsp20*) of the Intertidal Copepod *Tigriopus japonicus* as a Possible Biomarker for Exposure to Endocrine Disruptors

J. S. Seo,[1] T.-J. Park,[1] Y.-M. Lee,[1] H. G. Park,[2] Y.-D. Yoon,[3] J.-S. Lee[1]

[1] Department of Molecular and Environmental Bioscience, Graduate School, Hanyang University, Seoul 133-791, South Korea
[2] Faculty of Marine Bioscience and Technology, College of Life Sciences, Kangnung National University, Gangneung 210-702, South Korea
[3] Department of Life Science, College of Natural Sciences, Hanyang University, Seoul 133-791, South Korea

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The harpacticoid copepod *Tigriopus japonicus* is widely distributed in the intertidal zones of Korea, Japan, and China (Jung et al. 2006). *T. japonicus* is a good model species to assess the effects of endocrine-disrupting chemicals (EDCs) and toxic chemicals on gene expression, as it is easily reared in the laboratory and its genome is relatively well characterized (Ara et al. 2002; Kim et al. 2003; Lee 2003; Kim et al. 2004; Kwok and Leung 2005; Lee et al. 2006; Seo et al. 2006). To explore the use of the copepod *T. japonicus* as an ecotoxicogenomic model species, we evaluated expressed sequence tags (ESTs) from this species which were described in Lee et al. (2005). During the evaluation, we noticed that the small heat shock protein 20 (*Hsp20*) gene was consistently modulated after exposure to some EDCs (unpublished data). We, therefore, decided to further characterize *Tigriopus Hsp20* expression following exposure to model EDCs in order to evaluate this gene as a possible biomarker.

Several heat shock proteins (Hsps) have been proposed as general biomarkers of cellular aggregation and been used in environmental monitoring (Ait-Aissa et al. 2003; Kubo et al. 2004). For example, uterine hsp mRNA levels in human hepatoma cells are increased by 17β-estradiol (E2) and bisphenol A (BisA) through a direct effect on transcription and an increase in heat shock factor-1 mRNA (Kubo et al. 2004). Ait-Aissa et al. (2003) also showed that EDCs such as polychlorinated biphenyl and 17β-estradiol can induce gene expression of *Hsp70* and *Hsp90* in fish.

To date, however, only a few studies have examined the gene expression of small Hsps following EDC exposure, especially in aquatic invertebrates. Hence, this study investigated gene expression patterns and regulation of *Hsp20* in *T. japonicus* after waterborne exposure to six different model EDCs at sublethal concentrations. In light of the results, the potential use of *Hsp20* as a biomarker for monitoring and assessing EDC exposure is briefly discussed.

MATERIALS AND METHODS

T. japonicus specimens were raised in aquaculture at Kangnung National University, Gangneung, South Korea, and maintained in the Department of Molecular and Environmental Bioscience, Hanyang University, Seoul in South Korea. *T. japonicus* were identified using morphological characteristics and sequence homology in the mitochondrial genome sequence (Jung et al. 2006). *Tigriopus Hsp20* gene expression was analyzed following exposure to six model

Correspondence to: J.-S. Lee

EDCs: 17β-estradiol (E2), Di(ethyl-hexy)-phthalate (DEHP), 4-*t*-octylphenol (OP), 4-nonylphenol (NP), bisphenol A (BisA), and benzo[α]pyrene (B[α]P). An appropriate amount of each chemical tested was firstly dissolved in 100% dimethyl sulfoxide (DMSO). Then a stock solution for each chemical was made by an adequate dilution of the EDC-DMSO solution with ultra-pure distilled water. Working solutions were prepared by adding appropriate volumes of the stock solution to artificial seawater (20‰ salinity). Final testing solutions contained no more than 0.001% DMSO.

To determine the appropriate sublethal exposure concentration for the gene expression study, a standard 48-hr acute toxicity test for adult *T. japonicus* was conducted for each of the six EDCs, following the procedures described in Marcial et al. (2003). In brief, copepods were exposed to each of the six EDCs at various concentrations in filtered seawater (salinity 20‰; 18°C with a 12L/12D photoperiod) for 48 hr. Triplicates were applied for each treatment or control group; 20 individuals were placed in each replicate well with 10 ml of test solution (six-well plate; Nunc, Inc., USA). All test solutions were renewed once daily. At the end of 48 hr exposure, mortality of *T. japonicus* was examined under a stereomicroscope, following the method described in Kwok and Leung (2005). Based on the dose-response curve (Figure 1), both the no observed effect concentration (NOEC) and the lowest observed effect concentration (LOEC) of each EDC were determined and chosen for subsequent sublethal gene expression study.

The sublethal concentrations for all chemicals chosen were 0 (as control), 12.5, 25, 50, and 100 μg/L, except for DEHP, with corresponding concentrations of 0 (as control), 125, 250, 500, and 1,000 μg/L. In the sublethal gene expression study, triplicates of 50 adult copepods were placed in 50 ml centrifuge tubes (Corning, USA) and exposed to each of the selected concentrations (20 ml) for 48 hr while the other conditions were identical to the acute toxicity text. After the exposure period, viable *T. japonicus* were carefully collected from each replicate using a sieve (max 90 μm; Cheonggye Inc., Korea). To extract total RNAs, the copepods were fixed with TRI reagent (Molecular Research Center, Inc., USA) at a 3:1 ratio (volume:wet wt of the animals). Total RNAs were extracted according to manufacturer's instructions (Molecular Research Center, Inc., USA). Briefly, fixed *T. japonicus* were quickly collected using 90 μm sieve and transferred to a 1.5 ml of Eppendorf tube, then homogenized in 3 volumes of the TRI reagent with a 1.5 ml double-ended pestle (Sigma, USA), and stored at -80°C prior to use.

To make the first-strand of cDNA from each exposed group, a 2 μg sample of total RNA was reverse transcribed to cDNA using the reverse transcriptase-polymerase chain reaction (RT-PCR) in 20 μl of reaction volume containing 1 μl of SuperScript™ III reverse transcriptase (Invitrogen), 10 p*M* of oligo d(T), 1 μl of 10 mM dNTPs, 4 μl of 5 × PCR reaction buffer, 1 μl of 0.1 M dithiothreitol and 1 μl of RNase OUT (Invitrogen). After priming for 5 min at 65°C, synthesis of the first-strand cDNA was sequentially carried out for 60 min at 50°C, 15 min at 70°C, and finished with 20 min at 37°C using an iCycler (Bio-Rad).

The *Tigriopus Hsp20* gene was amplified with RT-PCR using primers, TJ-*Hsp20*-F: 5'-AAT CGG AAT ACA AAG ATG GAA CA-3' and TJ-*Hsp20*-R: 5'-CTT CAC ATC CTT CAT TTG ACA ATT-3' (expected product of 350 bp). This was done based on the first-strand cDNA obtained from *T. japonicus* as described above. The *Tigriopus* GAPDH gene (primers TJ-GAPDH-F, 5'-GAT CTG GAC AGA ACA TCA TC-3' and TJ-GAPDH-R, 5'-GAA TAC CCC AAG TAT CCC

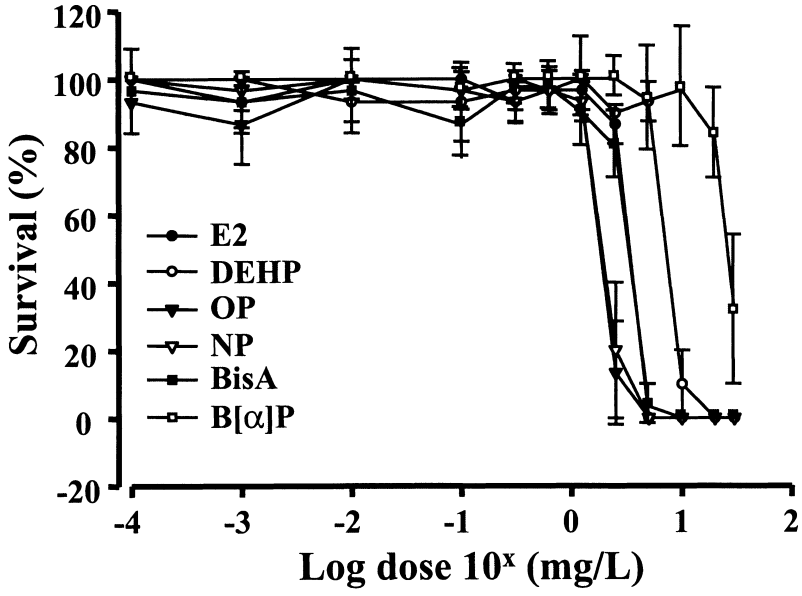

Figure 1. Semi-log curve of acute toxicity tests in adult *Tigriopus japonicus* after 48 hr of exposure to various EDCs at 18°C and 20‰. Charts show mean±SD of three replicate experiments. E2, 17β-estradiol; DEHP, 2-ethylhexyl phthalate; OP, 4-*t*-octylphenol; 4-NP, 4-nonylphenol; BisA, bisphenol A; B[α]P, benzo[α]pyrene.

TTC-3'; expected product of 250 bp) served as an internal control to normalize the input amount of template first-strand cDNAs. Each RT-PCR reaction mixture contained 1 μl of first-strand cDNA, 5 μl of 10 × PCR reaction buffer, 1 μl of 10 mM dNTPs, 10 p*M* of each primer, and 0.5 μl of NeoTherm *Taq* polymerase (GeneCraft Co., Germany). Cycling conditions consisted of 5 min at 95°C, followed by 30 cycles of 25 sec at 95°C, 30 sec at 55°C, and 30 sec at 72°C, and finished with 10 min at 72°C using an iCycler (Bio-Rad). Amplification products were quantified following electrophoresis by evaluating the intensity of ethidium bromide-stained bands and visualized on a Fluor-STM Multimager system (Bio-Rad), and the densitometric signals were measured using the Bio1D® image analysis system software (Bio-Rad). The levels of gene expression between the control group and each treatment group were compared using 1-way ANOVA, followed by post hoc Dunnett's multiple comparison test. The *Tigriopus Hsp20* (GenBank Accession no. AY522572) and *Tigriopus* GAPDH (GenBank Accession no. DQ088367) sequences were deposited into GenBank.

RESULTS AND DISCUSSION

The survivorship of *T. japonicus* varied amongst the tested chemicals; their mortality increased significantly at above 1 to 10 mg/L, depending on the chemical (Figure 1). The survival rate of *T. japonicus* was dramatically decreased by exposure to between 1 and 5 mg/L of most EDCs, except B[α]P (10 to 50 mg/L) and DEHP (7 to 10 mg/L). Based on these dose-response relationships, sublethal concentrations (0 μg/L to LOEC) were chosen for the gene expression study.

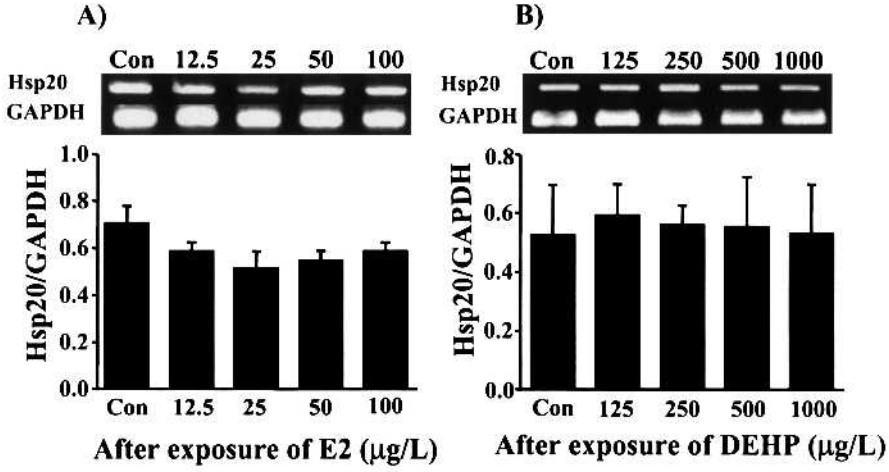

Figure 2. Expression of *Tigriopus japonicus Hsp20* gene after exposure to E2 (A) or DEHP (B). The relative expression of *T. japonicus Hsp20* was normalized with GAPDH expression. These experiments were performed in triplicate (n =3, mean ± SD). Con, control group.

Table 1. Annual survey data of average concentrations of exposed chemicals at several reference sites in Korea*.

Chemicals	Freshwater (μg/L) (41 sites)	Freshwater sediment (μg/kg) (15 sites)	Soil (μg/kg) (38 sites)
DEHP	0.15	655.2	170.3
B[α]P	-**	2.95	9.22
4- NP	0.16	7.39	2.78
OP	0.005	ND***	ND
BisA	0.01	1.50	1.01

*Annual survey data for the years 2004 to 2005 (NIER 2005).
**-, not tested
***ND, not detected

Indeed, the six selected model EDCs in this study are commonly found in urban and industrialized environments. In Korea, there were annual survey data of 67 pollutants at 129 reference sites including waters (41 sites), sediments (15 sites), soils (38 sites), and atmosphere (35 sites) nationwide (NIER 2005). Of the 6 tested EDCs, 5 chemicals (DEHP, B[α]P, OP, 4-NP and BisA) were included in the list of the annual survey. The average concentrations of these chemicals in different environmental compartments in Korea are shown in Table 1. The average concentrations of these chemicals in the aquatic environment ranged from 0.005 to 0.16 μg/L while even higher concentrations could be found in marine sediments. In the present study, the sublethal concentrations of these chemicals were therefore considered to be environmentally realistic.

The results from the sublethal gene expression study are shown in Figures 2 and 3.

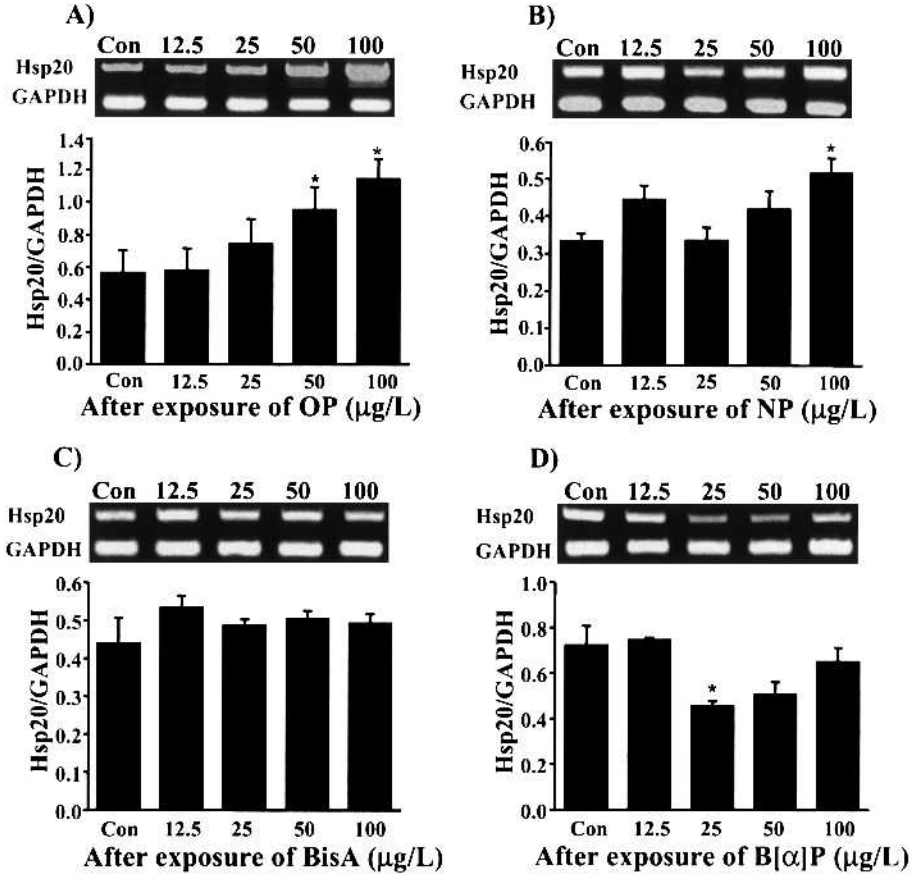

Figure 3. Expression of *Tigriopus japonicus Hsp20* gene after exposure to OP (A), NP (B), BisA (C) or B[α]P (D). The relative expression of *T. japonicus Hsp20* was normalized with GAPDH expression. These experiments were performed in triplicate (n = 3, mean ± SD), and 1-way ANOVA, followed by post hoc Dunnett's multiple comparison test, was applied for statistical analysis. Con, control group. * Significantly different from the control ($p<0.05$).

No down-regulation of the *T. japonicus Hsp20* gene was observed following exposure to E2 or DEHP. There was a trend towards down-regulation in the E2-exposed group (Figure 2), but it was not statistically significant. In contrast, some alkylphenol compounds did alter gene expression of *Tigriopus Hsp20*. The *Hsp20* gene expression in OP or NP exposed copepods increased in a dose-dependent manner, however, this was not observed in those exposed to BisA (Figures 3A to 3C). These differences in sensitivity may be explained by either structural differences between NP and OP (two alkylphenol rings) and BisA (one alkylphenol ring), or by differences in chemical action mechanisms. Interestingly, the *Hsp20* gene expression in B[α]P exposed *Tigriopus* was significantly decreased at a concentration of 25 μg/L, but up-regulated at higher doses (Figure 3D). These findings may be explained by the fact that small Hsps (e.g. *Hsp20*) play an important role in repairing altered conformations in malfunctioning

proteins in mammals, bacteria or plants (Bukach et al. 2004; Sreelakshmi et al. 2004), although such a function of *Hsp20* has not been reported in aquatic invertebrates such as the copepod.

In summary, *Tigriopus Hsp20* gene expression varied amongst the six model EDCs following water exposure for 48 hr. Based on our results, up- or down-regulation of the *Tigriopus Hsp20* gene by RT-PCR may be potentially applied as a biomarker for monitoring and assessing the exposure effect of alkylphenol compounds on the copepod *T. japonicus* in coastal environments through out Korea, Japan and China.

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