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Glutathione (GSH) Response as a Metabolic Biomarker to Benzo(α)pyrene and Aroclor 1254 Exposure in the Pacific Oyster *Crassostrea gigas*

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We measured activities of the ubiquitous tripeptide non-protein thiol (L-γ-glutamyl-L-cysteinyl-glycine), glutathione (GSH), which is believed to play a fundamental role in detoxifying xenobiotics in biological systems, as a metabolic biomarker for benzo(α)pyrene and Aroclor 1254 exposure in the Pacific oyster *Crassostrea gigas*. Reproductive oysters were exposed to the pollutants for 50 days by the algal vectoring method in which the oysters were fed with concentrated standard algal foods grown in culture media containing Aroclor 1254 (0, 5, 50, 500 ng/g) or benzo(α)pyrene (0, 10, 100, 1,000 ng/g). Both pollutants induced maternal GSH activities in 10 days in a dosage-dependent manner ($p<0.05$), although Aroclor 1254 was stronger. The pollutant-driven GSH elevation persisted for 20 to 30 days depending on the pollutants and concentrations. Thereafter, a drastic decline in the GSH activity was observed due to metabolic failure, after which the oyster GSH remained at low levels throughout the remainder of the experiment. The pollutant exposures influenced maternal reproductive output in terms of fertilization, hatching, and morphology. These results imply that changes in activity of the GST-catalyzing molecule can be used as an oyster biomarker for Aroclor 1254 and benzo(a)pyrene exposure.

Key words: Glutathione, Biomarker, Aroclor 1254, Benzo(α)pyrene, *Crassostrea gigas*

Introduction

As anthropogenic pollutants continuously accumulate in marine waters, several biomonitoring methods capable of assessing chemical-induced damage to marine organisms have been developed. The biological effects of chemicals on marine organisms can be detected by early warning assays used for diagnostic and prognostic purposes (Depledge et al., 1994; Peakall, 1994). During the last decade, interest has been growing in the use of biochemical parameters or biomarkers for diagnostic tests to detect the effects of chemical pollutants, includeing polychlorinated biphenyls (PCBs) and polymust be specific and sensitive cyclic aromatic hydrocarbons (PAHs). Such assays for the discrimination of aquatic contaminants.

Marine organisms exposed to xenobiotics usually have the capacity to metabolize and depurate directly, minimizing any cellular damage from the chemicals. Such protective mechanisms often include alterations to enzyme activities, which can be regarded as biomarkers of exposure or effects (Cheung et al., 2001). Four lines or phases of protection have been widely studied, including multixenobiotic resistant protein (phase 0), cytochrome P450 (phase I), glutathione-S-transferases (phase II), and multixenobiotic-related protein (phase III; Kurelec and Pivcevic, 1991; Kurelec, 1992; Bard, 2000; Achard et al., 2004). Glutathione-S-transferase (GST) catalyzes the conjugation of various electrophilic chemicals (e.g., epoxides of PAHs) with a tripeptide glutathione (L-γ-

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glutamyl-L-cysteinyl-glycine, GSH), and the resulting conjugates are water-soluble and thus more easily excretable. In contrast to vertebrate species, enzyme activities associated with the P450 system remain poorly identified in bivalves and are thought to be less inducible in the digestive gland compared to vertebrate liver (Livingstone, 1991; Livingstone et al., 1995; Akcha et al., 2000). Therefore, measurement of bivalve GST-GSH activities may provide an easily assayed biological indicator in response to changes in the pollution status of marine environments (Pandey et al., 2003; Cheung et al., 2004; De Luca-Abbott et al., 2005).

Here we focused on the expression of GSH in the Pacific oyster *Crassostrea gigas* as a biomarker for two xenobiotic pollutants, the PAH benzo(α)pyrene and the PCB Aroclor 1254. The expressions of the maternal biomarker were further compared to reproductive potential and reproductive output in terms of fertilization, hatching, and morphology.

Materials and Methods

Oysters

Reproductive *C. gigas* weighing 87.4 ± 15.1 g (mean ± SD) were obtained from a local oyster farm in Tongyoung, Korea, and brought to the laboratory, where epibiota on the oyster shells were removed prior to acclimation in captivity for 10 days. Following the acclimation, oysters were distributed into ten 50-L flow-through chambers (30 oysters per tank): five for the Aroclor 1254 test and five for the benzo (α)pyrene test. The cultures were replicated three times.

Preparation and feeding of chemical-carrying algal vectors

Algal vectors of Aroclor 1254 and benzo(α)pyrene were prepared by keeping algal foods in a series of chemical solutions for 30 min. In brief, a suggested amount of algal paste ($7\text{-}40\times10^8$ cells/mL; Reed Mariculture, Campbell, CA, USA) was distributed into glass beakers (water volume, 1 L). Each was then contaminated with one of four concentrations of benzo(a)pyrene (10, 100, 500, 1,000 ng/g) or Aroclor 1254 (5, 50, 500 ng/g) by adding corresponding amounts of the chemicals in pure acetone. Chemical-free controls (0 ng/g benzo(α)pyrene or Aroclor 1254) were prepared with the acetone vehicle only. All preparations were kept under gentle aeration for 30 min prior to use. The amount of algal paste added to a tank ranged from 5 to 20 mL, depending on the number of oysters in the culture tank. The algae used were *Isochrysis galbana*, *Tetraselmis suecica*, *Thalassiosira* sp., and *Chaetoceros* sp. The feeding scheme was twice a day, alternating among the four different algal vectors to ensure nutritional balance. Feeding was allowed for 1 to 2 h. After each feeding, the tanks were maintained in a flow-through manner until the next feeding. Tank water was kept at a temperature of 10 to 15°C and specific gravity of 1.0253 to 1.0286 during the 50-day experiment.

Determination of GSH and protein

Six oysters (two from each replicate) were killed every 10 days for the determination of digestive gland GSH. Total GSH concentration was determined by the DTNB-GSSG reductase recycling assay (Anderson, 1985). Digestive glands were homogenized in 5% sulfosalicyclic acid (SSA; 1:10 wet weight: volume) and centrifuged (14,000 rpm, 4°C, 5 min). Supernatants were collected and acidified (1:1) with 5% SSA. Some of the supernatant was added to sodium phosphate buffer (143 mM, pH 7.5), which contained 200 μM β-NADPH and 1 mM 5,5'-dithiobis (2-nitrobenzoic acid), for a final volume of 1 mL. Samples were vortexed and warmed (30°C) in a water bath for 10 min. Glutathione reductase (15 mL at 50 units/mL) was then added to initiate the enzymatic reaction. The rate of 5-thio-nitrobenzoic acid formation was followed spectrophotometrically at 412 nm at 30-s intervals for 90 s. Standards were prepared from reduced glutathione, and the results were expressed as nmol/g-wet weight. Protein concentration was determined following the method of Lowry et al. (1951), using bovine serum albumin as a standard protein.

Reproductive potential, fertilization, hatching rate, and D-larval morphology

After the 50-day experiment, all oysters were conditioned to maturation by feeding and temperature manipulation for 10 days. For the measurements, 30 oysters (ten each from three replicates) for each experimental culture were individually stripped into 2-L glass beakers containing sterilized seawater (Park et al., 2002). The suspended egg solution was sampled for determination of reproductive potential expressed as a percentage of normal to total eggs. Measurements differentiating abnormal from normal eggs were based on external characters under light microscopy, such as the occurrence of necrosis, loss of roundness in shape, and albinism, which can be a criterion for fertilization potential. Gametes that could not be clearly differentiated as being either normal or abnormal were considered abnormal. Per-

cent reproductive potential (percentage of normal to total eggs) was graded from 1 to 10, where 1 = <10%, 2 = 10-20%, 3 = 20-30%, ..., and 10 = 90-100%.

After evaluating the reproductive potential, eggs that were considered viable were separately rinsed in sterilized seawater using a net (mesh size, 20 μm), fertilized by the addition of pooled sperm solution, and distributed evenly in hatching tanks. The hatching rate was calculated as the number of larvae hatched/total eggs fertilized×100. For the fertilization measurement, any embryos that had undergone cell division, even if they were dead, were counted as a fertilized. The percent abnormality was calculated 26 h after fertilization at 22°C following the method of Jo et al. (2005a,b), which was primarily based on the criteria employed by His et al. (1997). These criteria include segmented eggs, normal or malformed embryos that failed to reach the D-larval stage, and D-larvae with either convex hinges, indented shell margins, incomplete shells, or protruded mantles. D-larvae that did not match any of the abnormal criteria but were different from normal larvae were also counted as abnormal.

Results and Discussion

We determined the expression mode of the phase II defense against Aroclor 1254 and benzo(α)pyrene burdens in *C. gigas* (Figs. 1,2). Bivalves are able to survive in environments polluted by hydrophobic xenobiotics if the pollution levels are within their metabolic capabilities. The defense lines are collectively termed phases 0-III (Kurelec and Pivcevic, 1991; Livingstone et al., 1992; Dellali et al., 2001; Moreira et al., 2001). Among these four defense lines, phase II assessed in the present study is a line for a conjugation after transformation by phase I. All experimental oysters fed both of the chemical vectors showed a noticeable elevation in GSH after 10 days relative to the lowest level (control level). The elevations attained in 10 days were significant and vector dosage-dependent ($p<0.05$). By day 10, the GSH levels in the digestive glands of oysters fed algal vectors with 5, 50, and 500 ng/g Aroclor 1254 were on average 6.5, 8.9, and 11.9 nmol/mg-protein, respectively. A similar trend was found in the oysters fed benzo(α)pyrene. In a comparison of the GSH sensitivity of the two pollutant species, Aroclor 1254 induced a higher magnitude of GSH response than benzo(α)pyrene. This is particularly interesting because the vector dosages of Aroclor 1254 were lower than those of the compatible benzo(α)pyrene levels, suggesting species-specific metabolic capacities for the oyster.

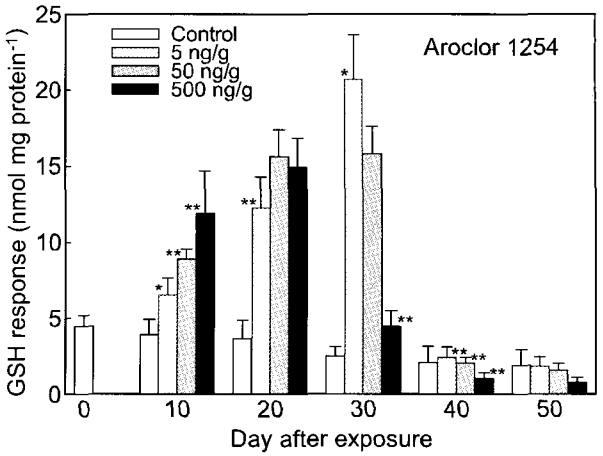

Fig. 1. GSH responses of *Crassostrea gigas* fed algal vectors of PCBs prepared in the presence of Aroclor 1254 concentrations. Control, 5, 50, and 500 ng/g represent chemical concentrations in which algal vectors were prepared. Error bars are standard deviations. Asterisks stand for statistical difference from its previous measurement; single for $p<0.05$, double for $p<0.01$.

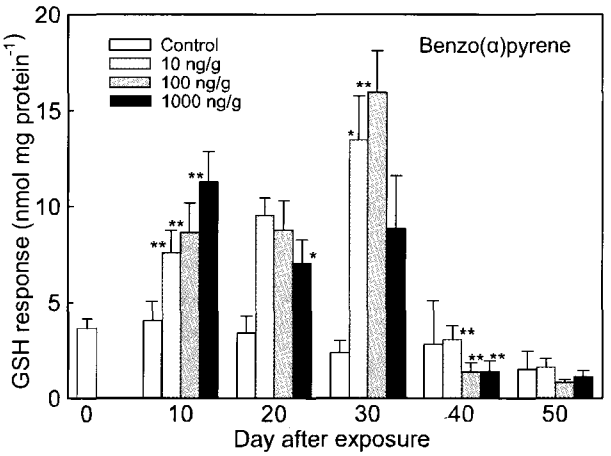

Fig. 2. GSH responses of *Crassostrea gigas* fed algal vectors of PAH prepared in the presence of benzo(α) pyrene concentrations. Control, 10, 100, and 1,000 ng/g represent chemical concentrations in which algal vectors were prepared. Error bars and asterisks are as in Fig. 1.

Although the chemical exposure caused an elevation in GSH levels, it was not always persistent. The persistence was dependent on the chemicals and burden. The GSH response of the oysters was more sensitive to Aroclor 1254 than to benzo(α)pyrene. At the same time, it was concentration-dependent only over a given time; higher burdens induced a negative

response in the long run, probably due to cellular damage caused by the large dosage (Bard, 2000). Studies have revealed that activities of transformation enzymes fluctuate with bivalve species and chemicals tested (Livingstone, 1991; Bucheli and Fent, 1995). However, conjugation enzymes are much more effective, as they catalyze the con-jugation between xenobiotics with an electrophilic site and endogenous molecules such as GSH for glutathione-*S*-transferases (GSTs), yielding xenobiotic excretable metabolites (Mannervik and Danielson, 1988). The oyster GSH responses in this study, which showed higher magnitude and stability against Aroclor 1254, suggest availability of the phase II defense line as a biomarker candidate at least for PCB pollution.

Under normal conditions, GSH levels vary depending on the species and types of physiological stress (Lange et al., 2002). The content of GSH in tissue depends on the organ, with the highest levels in the liver or the digestive gland (Valencia et al., 2001). In our study, the GSH content of the digestive gland remained between 3 and 5 nmol/mg-protein in intact control oysters. These control levels in *C. gigas* are similar to those in the mussel *Perna viridis* (Cheung et al., 2004). However, additional studies of GSH responses in different organs at higher burdens are needed to generate a stable biomarker.

The culture stress appeared to influence the response of GSH. Therefore, a parameter known as culture stress can be another consideration in the generation of a stable biomarker. In our small-scale system, the oysters appeared to be stressed by the prolonged culture. The GSH responses of control oysters were gradually lowered with the duration of the culture (Figs. 1,2). The control GSH response for the Aroclor 1254 test, 4.47 nmol/mg-protein, continuously decreased over time, amounting to 1.5 nmol/mg-protein by day 50. A similar trend was evident in the benzo(α)pyrene control.

GSH is a ubiquitous molecule occurring in eukaryotic cells. It is involved in a variety of cellular reactions, including synthesis, reduction, oxidation, conjugation, and other processes. It is also required for synthesis of proteins and nucleic acids, maintenance of enzymes in their active forms, and maintenance of cell membranes (Deneke and Fanburg, 1989; Meister, 1995; Canesi et al., 1999; Griffith, 1999; Valencia et al., 2001). Therefore, it is important to minimize culture stress, which can be an additional parameter influencing experimental data.

We measured the reproductive status of oysters stressed by pollutants. The results are summarized in Table 1 for Aroclor 1254 and Table 2 for benzo(α) pyrene. The chemical pollutants adversely affected the reproductive potential and reproductive output of the oysters. The damage was particularly significant for reproductive potential and the occurrence of abnormal D-larvae ($p<0.01$). Fertilization and hatching rates of highly burdened oysters were still high, although they were significantly lower than those of the controls. Similar results were found in our previous studies, in which the two measurements were far less influenced than morphogenesis by polluted sediments (Jo et al., 2005a,b).

Several studies have confirmed that reproduction of aquatic organisms is seriously damaged by exposure to benzo(α)pyrene (Casillas et al., 1991; Hall and Oris, 1991; Johnson et al., 1998; Hoffmann and

Table 1. Aroclor 1254-induced *Crassostrea gigas* damage expressed as events in reproduction and reproductive outputs

Vector conc.	Reproductive success	Fertilization (%)	Hatching (%)	Normal D-larva (%)
Control	9.7 (± 0.49)*	88.0 (± 1.65)	83.3 (± 2.09)	88.5 (± 1.06)
5 ng/g	9.5 (± 0.84)	87.8 (± 1.20)	87.6 (± 1.14)	91.2 (± 1.37)
50 ng/g	7.2 (± 0.75)+	88.7 (± 2.08)	81.2 (± 3.15)	22.3 (± 3.74)++
500 ng/g	2.8 (± 1.30)++	62.0 (± 4.42)++	58.3 (± 6.73)++	14.7 (± 3.77)++

*Mean (± SD).
+Different from control with $p<0.05$, ++with $p<0.01$.

Table 2. Benzo(α)pyrene-induced *Crassostrea gigas* damage expressed as events in reproduction and reproductive outputs

Vector conc.	Reproductive success	Fertilization (%)	Hatching rate (%)	Normal D-larva (%)
Control	9.2 (± 0.84)*	89.6 (± 1.32)	81.1 (± 2.17)	85.2 (± 2.11)
10 ng/g	9.3 (± 0.82)	91.3 (± 1.12)	84.0 (± 1.22)	82.9 (± 2.38)
100 ng/g	5.3 (± 0.96)++	87.1 (± 3.53)	79.2 (± 2.04)	16.1 (± 3.08)++
1,000 ng/g	4.5 (± 1.64)++	79.5 (± 3.05)+	61.7 (± 5.25)+	9.7 (± 2.45)++

*Mean (± SD).
+Different from control with $p<0.05$, ++with $p<0.01$.

Oris, 2006). Exposure to PCBs could result in regional declines in fish and invertebrate populations via some biochemical, endocrine, and physiological effects in individuals (Vasseur and Cossu-Leguille, 2006). Recently, the underlying mechanisms of chemical pollutantinduced reproductive toxicity have been explained in more detail. For example, these chemicals alter the transcription of genes important in regulating reproduction such as 20β-HSD, CYP19A2, and vitellogenin (Hoffmann and Oris, 2006). They are also proposed to block the 17β-estradiol-estrogen receptor (ER) complex from binding to DNA by the arylhydrocarbon receptor (AhR)/Ah-receptor nuclear trans-locator protein (ARNT) complex (Klinge et al., 1999), inhibit P450 enzymes (Monteiro et al., 2000; Moran et al., 2003), and alter transcription of steroidogenic enzymes critical to the production of E_2 and DHP (Dasmahapatra et al., 2000). Accumulated knowledge about the underlying mechanisms of chemical damage to reproduction provides a precise linkage between chemicals and their toxicities, which will provide baseline information for the generation of reliable biomarkers. In our study, reproductive potential/output did not completely correspond to GSH activities, although overall, a reliable relationship was observed between the two parameters (cf., Figs 1 and 2 with Tables 1 and 2).

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