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Properties of Two Cellular Biomarker Parameters in the Blood of Farmed Pacific Oyster, *Crassostrea gigas*, Exposed to Polychlorinated Biphenyls

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Two cellular biomarker parameters of the farmed Pacific oyster *Crassostrea gigas* were studied *in vivo* and *in vitro* after exposure to concentrations of polychlorinated biphenyls in terms of neural red uptake (NRU) and lysozyme activity. The oysters exposed *in vivo* to the xenobiotic concentrations, 0, 30, 90, and 180 ng/g for 14 days, enhanced hemocyte NRU with occasional significant differences ($P<0.05$), depending on the chemical concentration and duration. An adverse tendency was manifest in the lysozyme activities both in the hemocyte and serum of the oyster treated with the chemical in a same manner, rendering these two cellular parameters as biomarker candidates against the chemical. The oysters exposed *in vitro* to the chemical concentrations, 0, 1, 5, 10, 100, 1,000, and 10,000 ng/g for 24 hrs at 10℃ showed a similar tendancy as those exposed *in vivo* to the chemical. Unlike *in vivo* response, however, the *in vitro* NRU was first influenced by very low concentration of the chemical. In *in vitro* results, marked but not significant increase of hemocyte NRU was noticed at the chemical concentration of 5 ng/g, where the value was almost as high as those exposed to higher chemical concentrations, up to 10,000 ng/g. An unusual result was observed in the *in vitro* lysozyme activity of hemocyte in which significant decrease was first noticed at the chemical concentration of 100 ng/g.

Key words: Polychlorinated biphenyls, Neutral red uptake, Lysozyme activity, Hemocytes, *Crassostrea gigas*

Introduction

Polychlorinated biphenyls (PCBs) are ubiquitous contaminants due to their extensive use and periodic releases to the environment from production and handling processes. Aroclor 1254 is a mixture of 77 PCB congeners with 54% total Cl content and has very low solubility in water but high solubility in oils and organic solvents of low polarity. Due to their widespread use, stability and toxicity of coplanar congeners, PCBs are of persistent environmental problem (Livingstone et al., 1992).

Bivalves such as mussels and oysters have been postulated as ideal indicator organisms as a sentinel of marine environmental pollution, as they are ubiquitous sedentary filter-feeders inhabiting coastal and estuarine areas (Widdows and Donkin, 1992). However, most of the biomarker measurements were from mussels rather than oysters. This is particularly true for Pacific oyster, *Crassostrea gigas*, one of the most economic oysters cultured globally. The oyster has contributed considerable parts of total shellfish production in Korea. In spite of recent problems in the oyster production, likely due to appearance of unhealthy broodstocks and seeds (NFRDI Report, 1997; Kang et al., 2000), biomarker research in the Korean population of the oyster is

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limited (Jo et al., 2002).

Cellular and humoral parameters have well been employed by a number of biomarker studies of bivalves (Lowe and Pipe, 1994; Cajaraville et al., 1996; Anderson et al., 1997; Livingstone et al., 2000; Cronin et al., 2001; Jo et al., 2002; Matozzo et al., 2002). Oysters have an open circulatory system which is continually exposed to fluctuating environmental factors including PCBs. The immune defense is comprised of cell-mediated and humoral mechanisms, in which the hemocytes play a key role (Cheng, 1981).

In addition to cellular molecules and pathogens, lysosomes accumulate a wide range of toxic compounds, including PCBs, PAHs, and metals, which damage cells. However, this can wreak havoc with lysosomal structure, leading to the breakdown of their limiting membranes and leakage of the hydrolase contents, as well as any accumulated toxins, into the cytosol This loss of lysosomal content into the cytosol can result in cellular dysfunction or death (Livingstone et al., 2000). Much of the damage to lysosomes is a consequence of their remarkable ability to concentrate a wide range of contaminants within the environment, including lipophilic xenobiotics and metals, resulting in increased permeability of their membranes and loss of the acid hydrolase content into the cytosol, causing cellular damage (Moore, 1990). From these reasons, the organelle has been routinely used as a biomarker in bivalves for the monitoring of environmental contamination.

The supravital dye, neutral red (3-amino-7-dimethylamino-2-methylphenazine hydrochloride), is a cytotoxic weak base (Lowe and Pipe, 1994) and has been used in the neutral red retention assay (Lowe et al., 1992) for qualifying the structural integrity of the lysosomal membranes. Lysozyme activity in oysters was first described in the hemolymph and mantle muscus of the American oyster *C. virginica* by McDade and Tripp (1967a,b). It has been suggested that the lysozyme activity could be an indication of the physiological condition and vitality of the animal defense system (Chu and La Peyre, 1989). However, despite general acceptance of the assay, the biomarker parameters are still resulting in different measurements, probably attributed to a complexity influencing the measurements.

The aim of this study is to investigate the effects of PCBs on *in vivo* and *in vitro* neutral red uptake and lysozyme activity of Korean population of *C. gigas*. The investigation might have some implication in the robust biomarker development in the cultured *C. gigas* to the PCBs.

Materials and Methods

Oysters and *in vivo* exposure to xenobiotics

A total of 400 healthy spawners of Pacific oyster, *C. gigas*, weighing 77.3 ± 13.6 g mean ± SD in total weight, were collected from a local oyster farm, Tongyoung, Korea. Upon arrival at the laboratory, the oysters were acclimated in aquaria for 1 week prior to the start of the experiment. Randomly selected 160 oysters were evenly distributed to 4 rectangular bottles (water volume, 30 L) in a static culture system for *in vivo* exposure to PCBs. Oysters were then exposed to 4 concentrations of Aroclor 1254 (0, 30, 90, 180 ng/g) with a daily based water exchange without feeding for 14 days. Ten oysters from each treatment group were sacrificed on days 0, 3, 7, and 14 for the analysis of neutral red uptake and lysozyme activity. The experiment was duplicated.

Bleeding

Hemolymph was obtained from the posterior adductor muscle sinus, by gently opening the valves near the anterior end of the oyster, inserting a needle, and withdrawing the fluid into a sterile 1 mL syringe. The hemolymph pooled from about 10 oysters was then diluted 1:1 (v:v) immediately with Tris-buffered saline pH 8.4 (TBS), and then expelled into clean Eppendorf tubes.

In vitro experiments

Hemolymph was collected in a way explained above and pooled from 100 animals. To each well of a sterile 24-well plate, 1 mL of the hemocyte suspension was added (8.8×10^5 cells). The cells were left to attach for 30 min at 10℃, the supernatants were then removed and replaced in each well by 1 mL of Aroclor 1254 concentrations (0, 1, 5, 10, 100, 1000, 10000 ng/g) with three replicates for each treatment. Control solutions contained an equivalent amount of acetone vehicle without the xenobiotics.

Neutral red uptake assay

The uptake of the cationic neutral red in the cell lysosomes was used to assess the volume of the hemocyte lysosomal system (Pipe et al., 1995). Hemolymph was withdrawn into an equal volume of anticoagulant buffer and 100 μL aliquot of each sample was pipetted into three replicates microplate

wells. The plates were centrifuged for 5 min at 60 ×g at 4℃ to facilitate hemocyte attachment. The supernatants were discarded and NR (Sigma) solution was added to the wells. The plates were then incubated for 1 hr at 10℃, centrifuged to remove supernatants, washed with fixative solution (3.7% formaldehyde and 1% $CaCl_2$ in distilled water) for 1 min, and then removed the solution. The washing procedure was practiced in three times. NR was extracted from lysosomes using 1% glacial acetic acid in 50% ethanol. After 20 min, the plates were transferred to an ELISA reader (TECAN SPECTRA, Hybaid Ltd., UK), and absorbancy at 540 nm was recorded. Data were normalized (OD/10^6 hemocytes) using hemocyte counts of hemolymph.

Lysozyme activity

Assessment of lysozyme activity was based on a microtiter plate version of the *Micrococcus lysodeikticus* turbidometric assay (Hutchinson and Manning, 1996). In brief, the hemolymph samples were centrifuged at 3000×g for 10 min at 4℃ to separate cells from serum. The cells were subsequently resuspended in 1 mL of 0.1 M phosphate buffer (pH 6.4) and then sonicated. Serum in buffer was added to 100 μL of the *M. lysodeikticus* suspension, and the decrease in absorbance was recorded at 450 nm ($\triangle A_{450}$ min^{-1}) every min using the ELISA reader.

Statistics

The data were statistically analyzed using student *t*-test in the Sigma Plot Software.

Results

***In vivo* test**

C. gigas was exposed to different concentrations of Aroclor 1254, and hemocytes were withdrawn. The NRU and lysozyme activity of the oyster were determined as a cellular biomarker to the xenobiotic compound. Fig. 1 shows the *in vivo* uptake of neutral red in the hemocytes exposed to Aroclor 1254 concentrations, 0, 30, 90, and 180 ng/g for 14 days. The initial control of NRU, expressed as an OD, 1.43 ± 0.6, remained almost constant over the experimental period. In the treatment groups, the NRU increased with duration and concentration of xenobiotic treatment even though the first significant increase was found on day 14 both in 90 and 180 ng/g treatments.

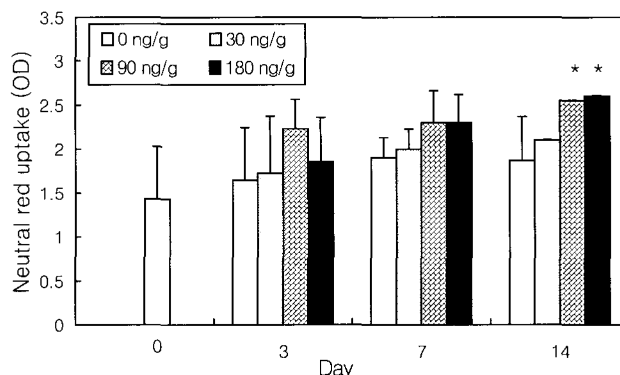

Fig. 1. The *in vivo* neutral red uptake in the hemocyte of *Crassostrea gigas* exposed to different concentrations of Aroclor 1254. The neutral red uptake was expressed as a normalized OD/10^6 hemocytes using hemocyte counts of hemolymph after ELISA reader with absorbancy at 540 nm. Vertical bars represent mean ± SD. Asterisks stand for significant difference of P<0.05 from each control of the day.

Lysozyme activities in the serum and hemocyte of the oyster were determined to compare with NRU changes. Fig. 2 (A) shows the lysozyme activities in the serum of oysters exposed to Aroclor 1254. In the 14-day exposure, the lysozyme activity of the control oysters decreased with the progress of culture, even though the decrease was not statistically significant from initial 22.7 ± 5.1 to 16.0 ± 1.5 by day 14, via 21.8 ± 5.6 on day 3 and 18.0 ± 1.2 unit/mL on day 7. The lysozyme activities of the oysters exposed to concentrations of Aroclor 1254 were significantly affected by prolonged duration and increased concentrations except for the oysters exposed to 30 ng/g Aroclor 1254, where no significant change was noticed. The first significant decrease of P<0.05 from the control was found on day 7, followed by significant decrease of P<0.01 on day 14 in the oysters exposed to 90 and 180 ng/g Aroclor 1254.

Lysozyme activity of the hemocytes exposed to the xenobiotic chemical is exhibited in Fig. 2 (B). The lysozyme activities of the hemocyte were similar to those of serum in overall sense. There was a culture stress without any significant difference, marking 27.7 ± 4.6 on day 0 to 24.0 ± 1.5 unit/mL on day 14 for the control oysters. However, the significant differences were found only in the oysters exposed to 180 ng/g Aroclor 1254 from day 7 (P<0.01), where the enzyme activities were 16.7 ± 2.3 on day 7 and 14.0 ± 4.4 unit/mL on day 14. Although significant

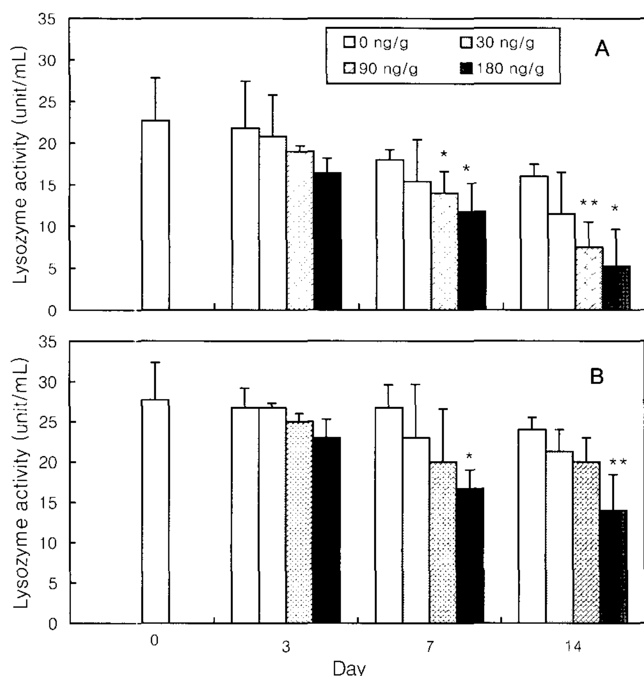

Fig. 2. The *in vivo* lysozyme activity in the serum (A) and hemocytes (B) of *Crassostrea gigas* exposed to different concentrations of Aroclor 1254. The lysozyme activity was determined by *Micrococcus lysodeikticus* turbidometric assay by recording $\triangle A_{450}$ min^{-1} in ELISA reader. Vertical bars represent mean ± SD. Asterisks stand for significant difference from control of the day, *for P<0.05 and ** for P<0.01.

changes were found only in the 180 ng/g experiment, the xenobiotic chemical affected hemocyte enzyme activities in duration and concentration-dependent ways throughout the rest of the experiments without statistical significance. In conclusion, the oysters exposed to Aroclor 1254 induced to increase NRU of hemocyte. The increased NRU, in turn, caused to decrease lysozyme activities both in serum and hemocyte.

The *in vitro* test

In the *in vitro* experiment, hemocyte of the oyster was exposed to concentrations of the xenobiotic compound. 0, 1, 5, 10, 100, 1000, and 10000 ng/g for 24 hrs at 10℃. Fig. 3 shows *in vitro* NRU of the hemocyte exposed to the concentrations of Aroclor 1254. The *in vitro* NRU was first influenced by very low concentration of the chemical. The control NRU was 1.97 ± 0.77 in OD value. The value increased to 2.65 ± 0.30 at the concentration of 1 ng/g and then remained almost constant, ranging 2.63 to 2.91 at concentrations over 5 ng/g. However, none

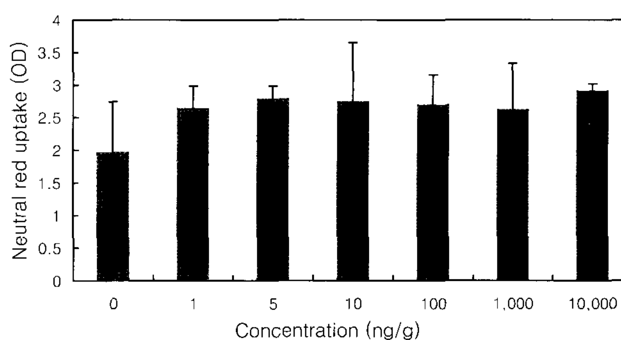

Fig. 3. The *in vitro* neutral red uptake in the hemocytes of *Crassostrea gigas* exposed to different concentrations of Aroclor 1254. The OD value for neutral red uptake is as in Fig. 1. Vertical bars represent mean ± SD.

of the changes were statistically significant.

Unlike the change of NRU value, lysozyme activity in the hemocyte was clearly affected by the chemical concentrations (Fig. 4). The activity of the control with 34.5 ± 6.9 unit/mL, first decreased at the chemical concentration of 100 ng/g with a significant manner (P<0.05). Even though significant decreases from the control were only at concentrations of 100 and 10,000, the activity showed a decreasing trend at concentrations over 5 ng/g.

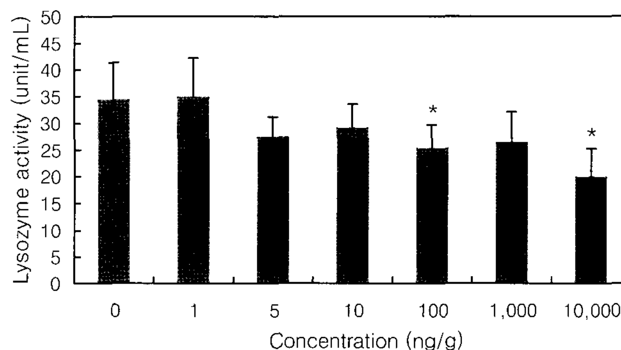

Fig. 4. The *in vitro* lysozyme activity in the hemocytes of *Crassostrea gigas* exposed to different concentrations of Aroclor 1254. The lysozyme activity determination is as in Fig. 2. Vertical bars represent mean ± SD. Asterisks stand for significant difference of P<0.05 from control.

Discussion

Bivalves have widely been used as a sentinel to monitor the quality of marine environments (Goldberg, 1986). In reality, a variety of biomarkers have been employed for monitoring toxic metals and

xenobiotics in marine environment (Livingstone et al., 2000). Some functional responses of hemocyte have been proposed to develop *in vitro* toxicity assays, indicative of the condition of the sentinel animals. However, for the Korean bivalves, the field of biomarker development is still in infancy even though some of ecotoxicological works progressed considerably.

In our experiment, the Aroclor 1254 enhanced NRU in duration and concentration dependent ways. Even though we could not explains the way and the degree the enhanced NRU affected the lysozyme activities both in the serum and hemocyte, significant decreases of lysozyme activity were found from both sites. As were in our results, decreased lysozyme activities were observed in the mussels exposed to kinds of xenobiotic and metal contaminants. Chu et al. (2002) also found the hemocyte of *C. virginica* enhanced NRU when exposed to benzo(a)pyrene, suggesting a shift in hemocyte function from normal range. The adverse effects of contaminants on the hemocytes were also reported by the previous studies (Etxeberria et al., 1994; Coles et al., 1995; Fagotti et al., 1996; Matozzo et al., 2002).

Bivalves lack a specific immune response, but possess humoral and cellular factors important in defense against pathogenic and nonpathogenic organisms. In their immune responses, lysozyme is one of the most important bacteriolytic agents against several species of bacteria (Cheng and Rodrick, 1974; Allam and Paillard, 1998), since it can hydrolyze components of bacterial walls and also participate in digestion (Cheng, 1983). However, the modes of lysozyme activity vary with species, environmental factors, and even individuals in a given species. This variation of enzymatic activity remains as a less acceptable aspect of enzymatic biomarker.

Regarding the site-specific activation of lysozyme in bivalve, our results revealed that lysozyme activity was higher in hemocytes than in serum of *C. gigas*. Cheng et al. (1975) found similar results in *Biomphalaria glabrata* and *Mercenaria mercenaria*. However, the levels of lysozyme activity were higher in serum than in hemocytes in both *C. virginica* and *Mya arenaria* (Rodrick and Cheng, 1974; Carballal et al., 1997). These differences are probably from different physiological or pathological status of the bivalves studied.

Unlike changes of lysozyme activity, the NRU of hemocyte in our study was clearly enhanced by initial slight increase of the xenobiotic concentration. Interestingly, once enhanced at lower concentrations, the NRU values remained constant even over concentrations of 3 orders of magnitude. This was particularly true when *in vitro* NRU is concerned. Neutral red is commonly used in *in vivo* and *in vitro* bioassays based on the accumulation of this cationic dye into the lysosomes of viable cells. In *in vivo* test, its uptake by hemocytes may occur by pinocytosis or passive diffusion across cell membranes. Alteration in its uptake may reflect damage to the cell membrane or, alternatively, changes in the volume of the lysosomal compartments (Coles et al., 1995). Lowe et al. (1992) suggested that free passage of lysosomal contents, including neutral red, into the cytosol might be due to impairment of the lysosomal membrane proton pump. However, our finding that the *in vitro* NRU of hemocyte was clearly enhanced by initial slight increase of the xenobiotic concentration remains unsolved. This is particularly controversial, considering that lysozyme activity reduced at PCB concentrations far over the concentration at which significant *in vitro* NRU value was first enhanced.

Normally the xenobiotic chemicals enhance NRU and lower lysozyme activities. This might be because the increase in NRU indicates lysosomal swelling as was previously observed in the hemocyte following metal exposure in the laboratory (Etxeberria et al., 1994; Coles et al., 1995). On this point controversy has been still existed in the previous studies. For example, no significant effect of TBT on release of lysozyme into the hemolymph of either oysters or mussels was observed in some previous studies (Anderson et al., 1996), whereas Pickwell and Steinert (1984) found an increase in the release of degradative enzymes into the hemolymph of *Mytilus edulis* following exposure to copper. Cytotoxicity of organic pollutants on certain cellular activities has been documented in various organisms including oysters. Exposure of *C. virginica* and *M. edulis* to PAHs reduced uridine uptake, an indication of RNA synthesis, disrupted hemocytes lysosomal integrity in terms of NRU and reduction, and hemocyte phagocytic and chemotactic ability (Chu et al., 2002; Faisal and Sami, 1994; Grundy et al., 1996a,b). While *in vitro* exposure to high concentrations of dieldrin and naphthalene depressed chemoluminescence of the oyster hemocytes, *in vivo* exposure to naphthalene and dieldrin stimulated hemocyte CL response (Larson et al., 1989).

Livingstone et al. (2000) summarized biochemical biomarkers of shellfish to detect the effects of pollutants where they explained that lysosomal alterations to cell injury can be conceptualized as increases or decreases in quantity of specific lysosomal contents, rate of specific membrane fusion events, or permeability lysosomal membranes to specific substances. According to their review, the events of lysosomes affected by xenobiotic chemicals are explained in three ways: 1) the lysosomal membrane is damaged resulting in reduced lysosomal hydrolase latency, 2) in *in vitro* studies, once the integrity of the limiting membrane is compromised, lysosomal contents leak into the cytosol, and 3) lysosomes become enlarged with an associated increase in fusion events. These explanations might support our results scientifically. However, our *in vitro* results, where the cells exposed to very low concentration of Aroclor 1254 responded as significantly as those exposed to higher concentrations, need further studies before they are used as a biomarker.

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