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# Effects of 17β-estradiol, Bisphenol A and Genistein on the Expression of the Glutathione Peroxidase Gene of *Philasterides dicentrarchii* (Ciliophora: Scuticociliata)

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A subtracted cDNA library of a marine scuticociliate, *Philasterides dicentrarchii*, in response to 17β-estradiol exposure was constructed using suppression subtractive hybridization (SSH). As a result of SSH, 275 clones were isolated, and among them, only glutathione peroxidase (GPX) gene was isolated as an antioxidative enzyme responding to 17β-estradiol. The semi-quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) analysis revealed that the transcription of GPX gene of *P. dicentrarchii* was clearly increased by exposure to 17β-estradiol. The GPX transcription was also clearly increased by exposure to xenoestrogens such as bisphenol A (BPA) and genistein.

*Key Words*: *Philasterides dicentrarchii*, Glutathione peroxidase, 17β-estradiol, Bisphenol A , Genistein, Suppression subtractive hybridization, Semiquantitative RT-PCR

## INTRODUCTION

In recent years, there have been many concerns about endocrine disrupting chemicals (EDCs), because of their possible adverse effects to the health of humans and wildlife (Stone, 1994; Colborn, 1995; Cooper and Kavlock, 1997; McLachlan, 2001). Many known EDCs are estrogenic, and so far, about 50 different compounds were identified as possible EDCs (Matthiesen *et al*., 1996). Xenoestrogens, which include natural plant compounds (phytoestrogens) and industrial byproducts (industrial estrogens), can modulate estrogen receptor (ER) activity like the endogenous hormone estradiol (17β-estradiol; E2). Although a variety of *in vitro* biological assays such as yeast based recombinant estrogen receptor-reporter assay (YES: Jurgens *et al*., 2001), MCF-7 cell proliferation (E-screen: Oh *et al*., 2000), estrogen receptor-mediated chemical activated luciferase gene expression assay (ER-CALUX: Murk *et al*., 1996), and RTG-2 reporter gene assay (Ackermann *et al*., 2002) have been developed to characterize estrogenic activity in environmental samples, *in vitro* biological assay systems using marine organisms, especially marine protozoans, have not been developed.

Ciliated protozoans have a number of useful features for use as experimental organisms to assess the toxicological effects of environmental pollutants or various pharmaceuticals. Several ciliate species can be cultured axenically in laboratory and their generation time is as little as 2-3 hours. Moreover, ciliates have a well developed signal cascade mechanisms as in multi-cellular eukaryotes (Csaba, 1985, 1994; Köhidai *et al*., 1992; Kovács *et al*., 1996). These features have allowed the ciliates to be

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used as a biological indicators of environmental pollution and pharmacological tools in different bioassays to detect toxicants (Nilsson, 1989; Cronin *et al*., 1991; Sauvant *et al*., 1999; Chen and Leick, 2004). However, almost all these studies had used freshwater ciliates, especially *Tetrahymena pyriformis*, as experimental organisms.

To date, little information is available on the molecular or biological markers for analyzing toxicological effects of marine pollution using marine protozoans. A marine ciliate, *Philasteides dicentrarchii*, known as a culprit of scuticociliatosis in diverse marine fish species (Iglesias *et al*., 2001; Kim *et al*., 2004), is a facultative parasite. Since *P. dicentrarchii* can live not only in sea water as a free-living organism but also in fish as a parasite, this ciliate has peculiar advantages to assess toxicological effects of various pollutants at both seawater salinity and physiological salinity. The ultimate aim of this study is to find the ciliate genes up-regulated by exposure to EDCs and to use those up-regulated genes as molecular markers for environmental EDCs pollution. In the present study, up-regulation of the glutathione peroxidase (GPX) gene of *P. dicentrarchii* in response to 17$\beta$-estradiol and xenoestrogens was demonstrated by suppression subtractive hybridization (SSH) and semi-quantitative reverse-transcriptase polymerase chain reaction (RT-PCR).

## MATERIALS AND METHODS

### Ciliates

*Philasterides dicentrarchii* were isolated from the abdominal cavity of infected olive flounder (*Paralichthys olivaceus*), which were obtained from a fish farm in South Korea. Polymerase chain reaction was performed to confirm *P .dicentrarchii* using *P. dicentrarchii* 18S rRNA gene-specific primers (Kim *et al*., 2004). The isolated ciliates were cultured in chinook salmon epithelia (CHSE)-214 cell line supplemented with 10% fetal bovine serum.

### Exposure of *P. dicentrarchii* to 17$\beta$-estradiol, bisphenol A, and genistein

The ciliates were harvested at the logarithmic phase by centrifugation at 1000 $\times$ g for 5 min, and incubated in the culture medium containing the final concentration of $10^{-6}$ M water-soluble 17$\beta$-estradiol (Sigma), 1 $\mu$M bisphenol A (BPA; Sigma) dissolved with dimethyl sulphoxide (DMSO; Sigma), or 10 $\mu$M genistein (Sigma) dissolved with DMSO for 2 hours at 20℃. Ciliates in control groups of BPA and genistein treatment were also incubated in the medium containing the corresponding concentration of DMSO for 2 hours. The incubated ciliates were harvested by centrifugation at 1,000 x g for 2 min at 0℃ and 1 ㎖ of RNAlater (Ambion) was added to the pellets immediately. Ciliates in RNAlater were stored at -20℃ until used.

### RNA purification

Total RNA was isolated using Tri Reagent (Sigma). $1\times10^7$ cells of *P. dicentrarchii* were lysed in 1.5 ㎖ tube by adding 1 ㎖ Tri Reagent and incubated for 5 min at room temperature to permit complete dissociation of nucleoprotein complexes. Then, 0.2 ㎖ of chloroform was added to the lysate and the tube was vigorously shaken by hand for 15 sec. The sample was centrifuged at 12,000 $\times$ g for 15 min at 4℃. The aqueous phase was transferred to a new tube and the RNA was precipitated by adding same volume of isopropyl alcohol for 10 min at room temperature. RNA was precipitated by centrifugation at 12,000 $\times$g for 10 min and washed with 70% ethanol. The pellet was air-dried for 5

min and dissolved in diethylpyrocarbonate (DEPC) treated water. Poly $A^+$ RNA from total RNA was isolated using PolyATract mRNA isolation system IV (Promega) according to the manufacturer's instructions. After total and poly $A^+$ RNA isolation, RNA's integrity was determined by ultraviolet (UV) spectrophotometry and formaldehyde gel electrophoresis.

**Suppression Subtractive Hybridization (SSH)**

SSH was performed using the PCR-Select™ cDNA Subtraction Kit (Clontech) according to the manufacturer's instructions. Poly $A^+$ RNA (2 ㎍) was used for synthesis of tester (treated with 17β-estradiol) and driver (non-treated control) cDNA for use in SSH. The PCR product from the secondary PCR was purified with Wizard SV Gel and PCR clean-up system (Promega), and 3 ㎕ of purified PCR product was directly ligated into pGEM-T easy plasmid vector (Promega) to make a subtracted library and then transformed to *Escherichia coli* strain DH5α competent cells. The transformed *E. coli* cells were plated onto Luria-Bertani (LB) agar plate containing ampicillin (50 ㎍ per ㎖), 5-bromo-4-chloro-3- indolyl-D-galactoside (X-GAL) and Isopropyl 1-thio-β-D-galactopyranoside (IPTG) and incubated at 37℃ overnight. Individual white colonies were then picked randomly and incubated in LB medium containing ampicillin overnight at 37℃. Plasmid DNAs were isolated from these cultures using GeneALL Plasmid miniprep kit (General Biosystem) according to the manufacturer's instruction. Sequencing reaction was carried out using BigDye Terminator Ready Reaction Mix (Applied Biosystems) and the sequences were analyzed with an Automated DNA Sequencer (ABI Prism 377, Applied Biosystems). Homology searches of the translated amino acid sequences were performed using the BLAST X program of National Center for Biotechnology Information (NCBI).

**Semi-quantitative reverse-transcriptase polymerase chain reaction (RT-PCR)**

To further investigate and quantify differential expression of the isolated gene glutathione peroxidase (GPX), semi-quantitative reverse-transcriptase polymerase chain reaction (RT-PCR) was performed. RT-PCR was performed using AccuPower® RT/PCR PreMix (Bioneer). For GPX the primers were F:5'-GAAGTTCCCAATTTGCTGCCAAAACTTCC-3' and R:5'-GTGTATCCAGGGAAGTTGGCATCAGCTTC-3'. GAPDH, a housekeeping gene, was used to normalize for variations in the amount of starting material, and primers were F:5'-GTTCGGAATTGCTGAAGGATTAATG-3' and R:5'-GTACGACAACGAATGGGGATACTCCAACAG-3'. The reaction products were electrophoresed on 1% agarose gel and stained with ethidium bromide.

## RESULTS

As a result of SSH, 275 clones were isolated, and among them, only glutathione peroxidase (GPX) gene was isolated as an antioxidative enzyme responding to 17β-estradiol. The nucleotide and deduced amino acid sequences of the *P. dicentrarchii* GPX (Fig. 1) showed the highest similarity with the GPX of marine sponges, *Hymeniacidon perlevis* and *Suberites domuncula* at *E* values $1e^{-73}$ and $1e^{-70}$, respectively.

As SSH can detect either significantly increased or decreased expression of genes compared to control, it could not be differentiated whether the GPX gene expression was increased or decreased in response to 17β-estradiol exposure. Therefore, semi-quantitative RT-PCR was adopted to analyze

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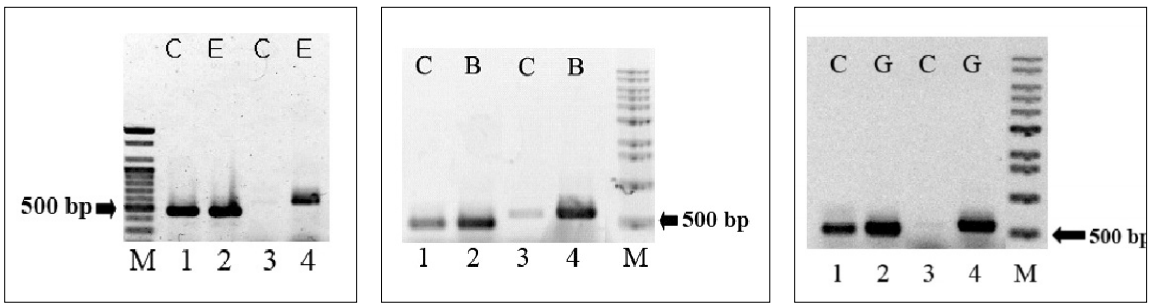
ATGCAAACCTAATAAAAAAACACTGCTTTGCTCCTCGGAGATGAAGTTCCCAATTTTGCT
 M  Q  T  Q  Q  K  N  T  A  L  L  L  G  D  E  V  P  N  F  A
GCCAAAACTTCCGCTGGAGATATTTAATTCCACAATTACATCAAAGATTCTTGGGCCATT
 A  K  T  S  A  G  D  I  Q  F  H  N  Y  I  K  D  S  W  A  I
CTTTTCTCTCACCCTGCTGACTACACTCCTGTTTGCACCACAGAATTGGGAAGAGTTGCG
 L  F  S  H  P  A  D  Y  T  P  V  C  T  T  E  L  G  R  V  A
AAATTGACTTCAGAATTCGCCAAAAGAAATGTTAAAGTGTTAGCCCTTTCTTGTGATAGT
 K  L  T  S  E  F  A  K  R  N  V  K  V  L  A  L  S  C  D  S
GTCGAAGACCACAATGGATGGATTAGTGACGTTAACTAATACAGCAGCTGTGAAGTCAAG
 V  E  D  H  N  G  W  I  S  D  V  N  Q  Y  S  S  C  E  V  K
TTTCCTATCATCGCTGATCCCACCAGAGATATCTCTAGATAGTGGGGAATGATCTAAACT
 F  P  I  I  A  D  P  T  R  D  I  S  R  Q  W  G  M  I  Q  T
GAAGGACCTACCGACTAAAAAGGAATGCCCCTTACTGTTAGAAGTGTTTTCATTATCGGA
 E  G  P  T  D  Q  K  G  M  P  L  T  V  R  S  V  F  I  I  G
CCTGATAGAAAATTGAAATTATCCATCACTTATCCCGCATCTACAGGAAGAAACTTTGAC
 P  D  R  K  L  K  L  S  I  T  Y  P  A  S  T  G  R  N  F  D
GAAATTTTGAGAGTTGTCGACTCTTTACAATTGACAGCTGGAAGAAAACTGGCTACTCCT
 E  I  L  R  V  V  D  S  L  Q  L  T  A  G  R  K  L  A  T  P
GTAGACTGGAAACATGGATAAGATTGTATCATCTTACCTTCTGTTACTAAAGAAGAAGCT
 V  D  W  K  H  G  Q  D  C  I  I  L  P  S  V  T  K  E  E  A
GATGCCAACTTCCCTGGATACACTACAGCTGATTTACCTTCTAAAAAATAATACTTAAGA
 D  A  N  F  P  G  Y  T  T  A  D  L  P  S  K  K  Q  Y  L  R
TCTTTCAACCCTGACAAAAAATGA 684
 S  F  N  P  D  K  K  *
```

Fig. 1. *Philasterides dicentrarchii* glutathione peroxidase cDNA sequence and predicted amino acid sequence, which is indicated below the nucleotide sequence in single letter code.




(a) (b) (c)

Fig. 2. Semiquantitative RT-PCR analysis of RNA from *Philasterides dicentrarchii* exposed to 17$\beta$-estradiol (a), bisphenol A (b) or genistein (c). Lane 1 and lane 2 indicate GAPDH as an internal control and Lane 3, and lane 4 indicate glutathione peroxidase. "C" indicates the nonexposed control, and "E" indicates 17$\beta$-estradiol exposed, "B" indicates bisphenol A exposed, and "G" indicates genistein exposed ciliates.

the expression pattern of GPX. As a result, increased expression of the GPX gene by exposure to 17$\beta$-estradiol was demonstrated (Fig. 2a).

To know xenoestrogens also affect on the expression of GPX gene of *P. dicentrarchii*, ciliates were exposed to bisphenolA and genistein, and analyzed the GPX gene expression by semiquantitative RT-PCR. As a result, expression of the GPX gene was

significantly increased by exposure to the xenoestrogens (Fig. 2 b,c).

## DISCUSSION

In the present results, the transcription of glutathione peroxidase (GPX) gene of *P. dicentrarchii* was clearly increased by exposure to 17β-estradiol. GPX is an antioxidant enzyme, which is involved in the metabolism of both hydrogen and lipid peroxides, and plays a role in the host defense mechanisms. Estrogens regulate the expression of specific genes through the interaction of the ligand-bound estrogen receptor dimer complex with specific DNA sequences called estrogen responsive elements (ERE). As a consequence of this action, specific mRNA and subsequently proteins are synthesized by the cells that regulate physiological function. In mammals, the increase of GPX activity in response to estrogen has been demonstrated (Ohwada *et al*., 1996), and Brigelius-Flohe *et al*. (1994) reported that pig GPX has several estrogen responsive elements in its genomic site, indicating association of GPX transcription with estrogen. However, in protozoans, little information is available on the GPX regulation in response to estrogen. Although the receptors for steroid hormones are not known in ciliates, the possession of endogenous steroids, such as dehydroepiandrosterone, testosterone and estradiol, had been reported from a fresh-water ciliate, *Tetrahymena pyriformis* (Csaba *et al*., 1985). Furthermore, it was demonstrated that pretreatment the ciliates with steroids including estradiol provoked the appropriate binding structures in the cytoplasm and affected various biological processes (Csaba and Ubornyak, 1981; Csaba and Cserhalmi, 1982; Csaba *et al*., 1985; Köhidai and Csaba, 1990). Therefore, the increase of GPX transcription in *P. dicentrarchii* by exposure to 17β-estradiol in this study might be the result of ERE-mediated specific response.

In the present results, the GPX transcription was also clearly increased by bisphenol A (BPA) and genistein. BPA, a monomer of epoxy resins and polycarbonate plastics, widely used in consumer products, are released from canned foods and dental sealants, and has been implicated as an endocrine disrupter. The isoflavone genistein is a naturally occurring phytoestrogen found in soy products. In contrast to 17β-estradiol, xenoestrogens have been reported not only to induce estrogenic responses but also to elicit depletion of antioxidant defense system and to induce oxidative stress in mammals (Obata and Kubota, 2000; Bindhumol *et al*., 2003). Therefore, the present GPX transcription increase might be the result of the defense response of the ciliates against xenoestrogens-mediated production of oxygen radicals.

In conclusion the present study reveals that exposure of ciliates to 17β-estradiol, BPA or genistein increased GPX transcription, suggesting possible use of GPX as a biological indicator for pollution including endocrine disruptors in marine environments.

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