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Physiological responses and expression of metallothionein (MT) and superoxide dismutase (SOD) mRNAs in olive flounder, *Paralichthys olivaceus* exposed to benzo[a]pyrene

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Abstract

We cloned complementary DNA (cDNA) encoding metallothionein (MT) and superoxide dismutase (SOD) from the liver of olive flounder, *Paralichthys olivaceus*. The full-length MT cDNA consists of 183 base pairs (bp) and encodes a protein of 60 amino acids; partial SOD cDNA consists of 326 bp and encodes a protein of 109 amino acids. We investigated the dose- and time-related effects of the polycyclic aromatic hydrocarbon benzo[a]pyrene (BaP) on MT and SOD mRNA using quantitative polymerase chain reaction (QPCR). The expression levels of MT mRNA were highest at 24 h (about five times) in 10 μg/L BaP, and at 6 h (about twelve times) in 30 μg/L BaP. The expression levels of SOD mRNA were highest at 12 h (about three times) in 10 μg/L BaP, and at 6 h (about six times) in 30 μg/L BaP, and then decreased toward the end of the experiment. We also measured plasma glucose and cortisol, all of which increased with BaP exposure. These results suggest that MT and SOD play an important role in the detoxification of reactive oxygen species (ROS) caused by BaP exposure, and thus may be indicators of oxidative stress responses.

Keywords: Benzo[a]pyrene; Olive flounder; Oxidative stress; Metallothionein; Superoxide dismutase

1. Introduction

Benzo[a]pyrene (BaP), which is the representative compound of polycyclic aromatic hydrocarbons (PAHs), is generated by incomplete combustion of organic substances such as oil and coal (Mcgroddy and Farrington, 1995; Carlson et al., 2002). BaP is an organic environmental contaminant that is widespread in both terrestrial and aquatic ecosystems. When absorbed by marine organisms, it accumulates in their tissues (Boleas et al., 1998) and passes through cell plasma membranes. This may cause lipoperoxidation, resulting in carcinogenic, mutagenic, cytotoxic, teratogenic, and other adverse effects (Livingstone, 1993; Borhoumi et al., 2002). Within organisms, the BaP is broken down into BaP quinine metabolites by prostaglandin H synthase (PHS) and cytochrome P450, and then redox processes create quinines, phenol, and reactive oxygen species (ROS) (Canova et al., 1998; Kiruthiga et al., 2007).

ROS are produced naturally during oxygen metabolism (Ahmad et al., 2000), and include superoxide (O^{-}_{2}), hydrogen peroxide (H_2O_2), hydroxyl radical (HO^{-}), and singlet oxygen ($^1O_2$). Oxidative stress by ROS can increase lipid peroxidation, protein oxidation, and damage to DNA. It can also affect cell viability by damaging membranes and suppressing antioxidant enzyme activity, thereby accelerating cell senescence and apoptosis (Kim and Phyllis, 1998).

To protect themselves against xenobiotic and other toxic materials that generate oxidative stress, aerobic organisms have evolved complex antioxidant defense systems (McFarland et al., 1999). Antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione-*S*-transferase (GST), and glutathione reductase (GR) as well as antioxidant materials with low molecular weights such as glutathione (GSH), ascorbic acid, metallothionein (MT), and α-tocopherol (vitamin E) have been found in the livers and kidneys of marine organisms

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(McFarland et al., 1999; Basha Siraj and Rani Usha, 2003). Antioxidant systems are beginning to receive attention as a biological means to reduce damage to aquatic organisms.

MT is a well-known antioxidant that protects against metal toxicity (McFarland et al., 1999; Cho et al., 2006). It is a small protein (~7 kDa) that binds with and is induced by free cytosolic metal ions, especially Cd, Cu, Zn, and Hg (Klaassen and Liu, 1998; Cho et al., 2006; Choi et al., 2007a). It may also be induced by xenobiotics such as PAHs, polychlorinated biphenyl (PCB), and arecoline (Kägi, 1993; Andrews, 2000; Lee et al., in press).

SOD is an antioxidant enzyme that serves as the primary defense against lipoperoxidation caused by oxidative stress. In general, it converts O^{-}_{2} into H_2O_2 and ultimately H_2O by CAT to reduce toxicity in the living body (Marklund and Marklund, 1974). It exists in a variety of organisms, including marine organisms, and like MT, induces activation and expression when exposed to heavy metals or xenobiotic substances such as BaP, sodium dodecylbenzene sulfonate (SDBS), and parquet (Zelko et al., 2002; Jifa et al., 2006).

The olive flounder, *Paralichthys olivaceus*, is one of the most important farmed marine species in Korea. Although these fish are bottom dwellers, and thus are regularly exposed to accumulated contaminants, few studies have considered oxidative stress in olive flounder (Winzer et al., 2002). To examine oxidative stress due to BaP exposure in this species, we investigated the expression patterns of MT and SOD mRNA in the liver and measured the changes in plasma glucose and cortisol levels for physiological analysis.

2. Materials and methods

2.1. Experimental fish

Olive flounder ($n=50$, length 14.2±0.8 cm, weight 25.6±5.6 g) were collected from a commercial fish farm. The fish were reared for 4 weeks in 300-L circulation filter tanks in the laboratory. During experiments, the water temperature and photoperiod were maintained at 20±1 °C and a 12L/12D cycle, respectively. The fish were fed a commercial feed twice a day.

2.2. BaP exposure

The experimental fish were exposed to BaP (SUPELCO, USA) dissolved in acetone by waterborne in 50-L four tanks (control, acetone control, 10 μg/L and 30 μg/L BaP group) for 0, 6, 12 and 24 h. No food was supplied during experiments.

2.3. Sampling

Four fish from each treatment (control, acetone control, 10 and 30 μg/L BaP group) were randomly selected for blood and tissue sampling. The fish were anesthetized with 200 mg/L tricaine methanesulfonate (MS-222, Sigma, USA) prior to blood collection. Blood was collected from the caudal vasculature using a 3-mL syringe coated with heparin. Plasma samples were separated by centrifugation (4 °C, 13,200×*g*, 5 min), and stored at −80 °C. To collect liver tissue samples, fish were euthanized by spinal transection. Immediately after collection, the samples were frozen in liquid nitrogen and stored at −80 °C until total RNA was extracted for analysis.

2.4. Identification of MT and SOD cDNA

Primers for MT were designed using highly conserved regions of European plaice (*Pleuronectes platessa*) MT (GenBank accession no. CAA40067) and winter flounder (*Pleuronectes americanus*) MT (GenBank accession no. CAA31930): MT forward primer (5′-AAG AGT GGA ACC TGC AAC TG-3′) and MT reverse primer (5′-CAT GTC TTC CCT TTG CAC AC-3′). Primers for SOD were designed using highly conserved regions of red seabream (*Pagrus major*) SOD (GenBank accession no. AF329278) and rock bream (*Oplegnathus fasciatus*) SOD (GenBank accession no. AY613390): SOD forward primer (5′-CAT GGY TTC CAY GTC CAT TG-3′) and SOD reverse primer (5′-CCA ATN ACT CCA CAG GCC-3′). Total RNA was extracted from the liver using a TRIzol kit (Gibco/BRL, USA). Reverse transcription (RT) was performed using M-MLV reverse transcriptase (Bioneer, Korea), according to the manufacturer's instructions. Polymerase chain reaction (PCR) amplification was performed using a 2× Taq Premix I (Solgent, Korea). PCR was carried out as follows: initial denaturation at 95 °C for 2 min; 40 cycles of denaturation at 95 °C for 20 s, annealing at 54 °C (MT), 55 °C (SOD) for 40 s, and extension at 72 °C for 1 min; followed by 7 min at 72 °C for the final extension. The amplified PCR product was processed by electrophoresis in 1% agarose gels. The PCR product was purified and ligated into pGEM-T Easy Vector (Promega, USA). The colony formed by transformation was cultivated in DH5α (RBC Life Sciences, Korea), and plasmid DNA was extracted using a LaboPass Plasmid DNA Purification Kit (Cosmo, Korea) and EcoRI (Fermentas, USA). Based on the plasmid DNA, MT and SOD cDNA sequence data were analyzed using an ABI DNA Sequencer (Applied Biosystems, USA).

2.5. MT rapid amplification of cDNA 3′ and 5′ ends (3′ and 5′ RACE)

For the MT RACE reaction, total RNA was extracted from the liver using a TRIzol kit (Gibco/BRL, USA). Using 3 μg total RNA as a template, 3′ RACE cDNA and 5′ RACE cDNA were synthesized using a CapFishing™ full-length cDNA Premix Kit (Seegene, Korea). First-strand cDNA synthesis was conducted using an oligo (dT) anchor primer (5′-CTG TGA ATG CTG CGA CTA CGA $T(T)_{18}$-3′) and a CapFishing™ adaptor (Seegene, Korea).

MT-specific primers were selected from the PCR product. For 3′ RACE, the 50-μL PCR reaction mixture contained 5 μL 3′ RACE cDNA, 1 μL 10 mM 3′ RACE target primer (5′-CTG TGA ATG CTG CGA CTA CGA T-3′), 1 μL 10 mM 3′ RACE gene-specific primer (5′-GAT CTT GCA CCT GCA AGA ACT GCA CCT G-3′), and 25 μL SeeAmp Taq Plus Master Mix. PCR was performed as follows: denaturation at 94 °C for 5 min; 40 cycles of denaturation at 94 °C for 40 s, annealing at 62 °C for 40 s, and extension at 72 °C for 1 min; followed by 5 min at 72 °C for the final extension.

For 5′ RACE, the 50-μL PCR reaction mixture contained 5 μL 5′ RACE cDNA, 1 μL 10 mM 5′ RACE target primer (5′-GTC TAC CAG GCA TTC GCT TCA T-3′), 1 μL 10 mM 5′ RACE gene-specific primer (5′-CAG GTG CAG TTC TTG CAG GTG CAA GAT C-3′), and 25 μL SeeAmp Taq Plus Master Mix. PCR was carried out as follows: denaturation at 94 °C for 5 min; 35 cycles of denaturation at 94 °C for 40 s, annealing at 62 °C for 40 s, and extension at 72 °C for 1 min; followed by 5 min at 72 °C for the final extension. The amplified PCR product was processed by electrophoresis in 1% agarose gels. Transformation and sequencing were performed as described above.

2.6. Quantitative PCR (QPCR)

QPCR was conducted to determine the relative expression of MT and SOD mRNA in total RNA extracted from the BaP-exposed liver. Primers for QPCR were designed with reference to the known sequences of olive flounder as follows: MT forward primer (5′-AGT GGA ACC TGC AAC TGC-3′), MT reverse primer (5′-ATG TCT TCC CTT TGC ACA CG-3′), SOD forward primer (5′-CGT TGG AGA CCT GGG GAA TGT G-3′), SOD reverse primer (5′-ATC GTC AGC CTT CTC GTG GAT C-3′), β-actin forward primer (5′-GGA CCT GTA TGC CAA CAC TG-3′), and β-actin reverse primer (5′-TGA TCT CCT TCT GCA TCC TG-3′). PCR amplification was conducted using a BIO-RAD iCycler iQ Multicolor Real-Time PCR Detection System (Bio-Rad, USA) and iQ™ SYBR Green Supermix (Bio-Rad), according to the manufacturer's instructions. QPCR was performed as follows: denaturation at 95 °C for 5 min; 40 cycles of denaturation at 95 °C for 20 s, annealing at 55 °C for 20 s. As an internal control, experiments were duplicated with β-actin, and all data were expressed as change with respect to corresponding β-actin calculated threshold cycle (CT) levels.

2.7. Plasma parameters analysis

Plasma glucose was examined using a biochemistry auto-analyzer (model 7180; Hitachi, Tokyo, Japan). Plasma cortisol was analyzed by radioimmunoassay (RIA) using an RIA kit (Diagnostic System Laboratories, Webster, TX, USA).

2.8. Statistical analysis

All data were analyzed using the SPSS statistical package (version 10.0; SPSS Inc., USA). One way ANOVA followed by Dunnett's post hoc test was used to compare the differences to control in the data ($P<0.05$).

3. Results

3.1. Identification of full-length MT cDNA

RT-PCR was used to clone a fragment of MT cDNA using total RNA. A single PCR product of the expected size (140 base pairs [bp]) was obtained. A PCR-based cloning strategy (PCR followed by 3′ and 5′ RACE) was used to clone full-length cDNA encoding MT. The full-length MT cDNA contained 183 nucleotides, including an open reading frame (ORF) that was predicted to encode a protein of 60 amino acids (GenBank accession no. EF406132) (Fig. 1). The amino acid sequence of the olive flounder MT was about 33% cystein (20 cysteins of a protein of 60 amino acids), which is characteristic of MTs (Kojima et al., 1976). The amino acid sequence of olive flounder MT was compared to those deduced from the cDNA of other teleost species (Fig. 1). The amino acid similarities were as follows: 93% with European plaice MT (GenBank accession no. CAA40067), 90% with winter flounder MT (GenBank accession no. CAA31930), 86% with Medaka (*Oryzias latipes*) MT (GenBank accession no. AAR30249), and 85% with Atlantic salmon (*Salmo salar*) MT (GenBank accession no. ABA42827) (Fig. 1).

3.2. Identification of partial SOD cDNA

RT-PCR was used to clone a fragment of SOD cDNA using total RNA. A single PCR product of the expected size (326 bp) was obtained, and it encoded a protein of 109 amino acids (GenBank accession no. EF681883). The amino acid sequence of olive flounder SOD was compared to those deduced from the cDNA of other species. The amino acid similarities were as follows: 89% with red seabream SOD (GenBank accession no. AAO15363), 89% with rock bream SOD (GenBank accession no. AAT36615), 86% with Mozambique tilapia (*Oreochromis mossambicus*) SOD (GenBank accession no. AAR82969), and 84% with rainbow trout (*Oncorhynchus mykiss*) SOD (GenBank accession no. AAL79162) (Fig. 2).

3.3. QPCR for MT and SOD mRNA expression

Using QPCR, we measured the expression levels of the MT and SOD mRNA. At 10 μg/L BaP, the expression of MT mRNA

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ofMT  1:MDPCDCSKSGTCNCGGSCTCKNCTCTTCNKSCCPCCPSGCTKCASGCVCKGKTCDTSCCQ  60
epMT  1:MDPCECSKTGTCNCGGSCTCKNCSCTTCNKSCCPCCPSGCPKCASGCVCKGKTCDTSCCQ  60
wfMT  1:MDPCECSKTGTCNCGGSCTCKNCSCTTCTKSCCPCCPSGCPKCASGCVCKGKTCDTTCCQ  60
asMT  1:MDPCECSKTGSCNCGGSCKCANCACTSCKKSCCPCCPSGCSKCASGCVCKGKTCDTSCCQ  60
jmMT  1:MDPCDCSKTGKCNCGGSCTCTNCSCTSCKKSCCACCPSGCTKCASGCVCKGKTCDTTCCQ  60
          * *     * *   * *  * * *   ** **   * *   * *    *   **
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Fig. 1. Comparison of MT amino acid sequence of olive flounder (*Paralichthys olivaceus*) MT, European plaice (*Pleuronectes platessa*), winter flounder (*Pleuronectes americanus*), Japanese medaka (*Oryzias latipes*), and Atlantic salmon (*Salmo salar*) optimally aligned to match identical residues, indicated by a shaded box. The sequences were taken from the GenBank/EMBL/DDBJ sequence database. The GenBank accession numbers for the MT sequences used for alignment are as follows: olive flounder (ofMT, ABN50351), European plaice (epMT, CAA40067), winter flounder (wfMT, CAA31930), Medaka (mdMT, AAR30249) and Atlantic salmon (asMT, ABA42827). An asterisk indicates cystein residues.

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OfSOD   1:EHGFHVHAFGDNTNGCISAGPHFNPHGKNHAGPTDAERHVGDLGNVTAGKDNVAEINISD   60
rsSOD  43:EHGFHVHAFGDNTNGCISAGPHFNPHNKNHAGPTDAERHVGDLGNVTAGADNVAKIDITD  102
rbSOD  43:EHGFHVHAFGDNTNGCISAGPHFNPHNKNHAGPNDAERHVGDLGNVTAGADNVAKIDIKD  102
mtSOD  43:EHGFHVHAFGDNTNGCISAGPHFNPYNKNHGGPKDAERHVGDLGNVTAGADNVAKIEITD  102
rtSOD  43:EHGFHVHAYGDNTNGCMSAGPHFNPHNQTHGGPTDAVRHVGDLGNVTAGADNVAKINIQD  102

ofSOD  61:KIITLFGAHSIIGRTMVIHEKADDLGKGGNEESLKTGNAGARLACGVIG            109
rsSOD 103:KMLTLNGPFSIIGRTMVIHEKADDLGKGGNEESLKTGNAGGRLACGVIG            151
rbSOD 103:HIITLTGPDSIIGRTMVIHEKADDLGKGGNEESLKTGNAGGRLACGVIG            151
mtSOD 103:KVITLTGRDSIIGRTMVIHEKVDDLXKGGNEESLKTGNAGGRLACGVIG            151
rtSOD 103:KMLTLTGPDSIIGRTMVIHEKADDLGKGGNEESLKTGNAGGRQACGVIG            151
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Fig. 2. Comparison of SOD amino acid sequences of olive flounder, red seabream (*Pagrus major*), rock bream (*Oplegnathus fasciatus*), Mozambique tilapia (*Oreochromis mossambicus*), rainbow trout (*Oncorhynchus mykiss*), optimally aligned to match identical residues, indicated by a shaded box. The sequences were taken from the GenBank/EMBL/DDBJ sequence database. The GenBank accession numbers of the SOD sequences used for alignment are as follows: olive flounder (ofSOD, ABS12626), red seabream (rsSOD, AAO15363), rock bream (rbSOD, AAT36615), Mozambique tilapia (mtSOD, AAR82969) and rainbow trout (rtSOD, AAL79162).

in the liver was not significantly different at 6 h than that of the control group. However, it was significantly higher at 12 h and about five times higher at 24 h than that of the control group. At 30 μg/L BaP, the expression level was about twelve times higher at 6 h than that of the control group, and then decreased until the end of the experiment (Fig. 3A).

At 10 μg/L BaP, the expression of SOD mRNA in the liver was not significantly different at 6 h than that of the control group, and was about three times higher at 12 h. Thereafter, and for the remainder of the experiment, there was no significant difference. On the other hand, at 30 μg/L BaP, the level was about six times higher at 6 h than that of the control group, and then decreased until the end of the experiment (Fig. 3B).

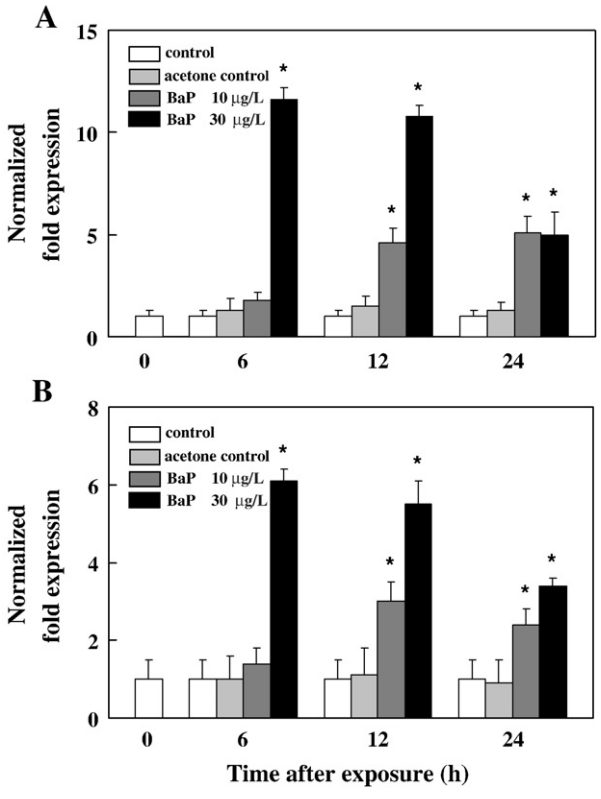

Fig. 3. Dose- and time-related effects of BaP on MT (A) and SOD (B) transcription levels in the olive flounder liver. One microgram of total RNA prepared from the liver was reverse-transcribed and amplified using gene-specific primers by quantitative PCR. Results are expressed as normalized fold expressions with respect to β-actin levels for the same sample. Values with an asterisk indicate significantly different from control in the same time of sampling ($P<0.05$). Values are means±SD ($n=4$).

3.4. Plasma parameters

The plasma glucose and cortisol levels in BaP-exposed samples are shown in Fig. 4. Plasma glucose peaked at 0.012±

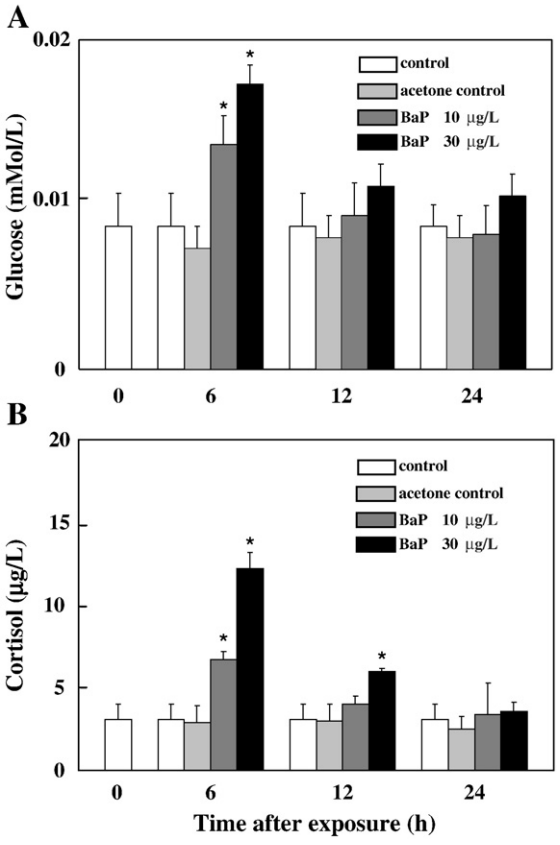

Fig. 4. Levels of plasma glucose (A) and cortisol (B) in olive flounder after exposure to BaP. Values with an asterisk indicate significantly different from control in the same time of sampling ($P<0.05$). Values are means±SD ($n=4$).

0.002 mMol/L (in 10 μg/L) and 0.015±0.002 mMol/L (in 30 μg/L) at 6 h, and then decreased. Cortisol peaked at 6.7± 0.5 μg/L (in 10 μg/L) and 12.2±1 μg/L (in 30 μg/L) at 6 h, and then decreased.

4. Discussion

We isolated MT and SOD cDNA from the liver of olive flounder and investigated the expression patterns of MT and SOD mRNA after exposure to BaP, a xenobiotic. We also analyzed the changes in plasma glucose and cortisol levels. The MT cDNA of olive flounder encodes 60 amino acids in an ORF and has a total of 183 nucleotides. Using the blast algorithm (Blastp) of the National Center for Biotechnology Information, we found that MT amino acids display high identity with those of other species: 93% with European plaice, 90% with winter flounder, 86% with Medaka, and 85% with Atlantic salmon. MT typically has high cystein content and alignment (Olafson et al., 1988); it accounts for 30% of the total amino acids and shows three major Cys-Cys motifs, namely, Cys-Cys, Cys-X-Cys, and Cys-X-Y-Cys, where X and Y are amino acids other than cystein (Kojima et al., 1976). Our results for olive flounder were in agreement. Based on the amino acid sequences deduced from the cDNA sequences, 20 of the 60 MT amino acids (approximately 33%) were cysteins, and there were six Cys-X-Cys, three Cys-X-Y-Cys, and three Cys-Cys alignments (Fig. 1).

The results of QPCR indicated that MT expression was about five times higher in tissue exposed to 10 μg/L BaP at 24 h and about twelve times higher in tissue exposed to 30 μg/L BaP at 6 h (Fig. 3A). At 30 μg/L BaP, ROS were rapidly generated, and MT was likely induced and expressed to scavenge ROS. This result is in agreement with previous studies in which MT increased in response to BaP exposure (Romèo et al., 1997; Lee et al., in press). Canova et al. (1998) and Kiruthiga et al. (2007) reported that ROS can be generated by BaP. The increase in MT mRNA expression observed in present study likely reflects a defense mechanism in response to BaP-induced ROS. Also, MT itself is involved in protecting against oxidative stress by virtue of its remarkably high content of cysteinyl residues (Andrews, 2000). Accordingly, the synthesis of MT is induced by transition metal ions and by oxidative stress (Cavaletto et al., 2002). Therefore, it can be concluded that flounder protect themselves against BaP-induced oxidative stress by increasing MT, which binds to and reduces the damaging effects of ROS (Sato and Bremner, 1993). MT genes have frequently been studied as biomarkers of heavy metals such as cadmium (Baudrimont et al., 2003; Choi et al., 2007a). However, few reports have addressed MT expression in response to BaP exposure.

The SOD cDNA of olive flounder encodes 109 amino acids in an ORF and has a total of 326 nucleotides. SOD amino acids showed high identity with those of other species: 89% with red seabream, 89% with rock bream, 86% with Mozambique tilapia, and 84% with rainbow trout (Fig. 2). QPCR of olive flounder indicated that SOD expression was approximately three times higher in tissue exposed to 10 μg/L BaP at 12 h, and about six times in tissue exposed to 30 μg/L BaP at 6 h (Fig. 3B). Our results are in agreement with Jifa et al. (2006), who reported increased SOD activity in the liver of sea bass (*Lateolabrax japonicus*) exposed to 20 μg/L BaP, and Palace et al. (1996), who reported increased SOD activity in lake sturgeon (*Acipenser fulvescens*) exposed to 2, 3, 7, 8-tetrachlorodibenzofuran (TCDF). Therefore, we conclude that the high level of ROS generated by xenobiotics induces SOD, which then scavenges the ROS (Lehtinen, 1990). This is further suggested by the fact that SOD mRNA expression peaked after 6 and 12 h of exposure to 10 and 30 μg/L BaP, respectively, and then decreased. That is, the SOD inactivated and therefore decreased the amount of ROS, leading to a subsequent decrease in SOD (Zhang et al., 2004; Jifa et al., 2006). SOD is the first enzyme to scavenge ROS, which it does by converting O^{-}_{2} and H^{+} into H_2O_2 (Marklund and Marklund, 1974). We found that SOD scavenged BaP-induced ROS via a defense mechanism against stress, in agreement with previous studies (Wells et al., 1997). Hansen et al. (2006) showed the same increases between SOD mRNA expression and enzyme activity during heavy metal exposure, support that increases of SOD mRNA increased SOD enzyme activity which means SOD scavenged BaP-induced ROS. Therefore we suggested that increase of SOD mRNA increased SOD enzyme activity which means SOD scavenged BaP-induced ROS. Like MT and SOD expression is often used as a biomarker for BaP toxicology. In the case of fish, it is used to measure the degree of oxidative stress caused by heavy metals and xenobiotics. Some studies have used SOD activity and gene expression to measure environmental pollution (Basha Siraj and Rani Usha, 2003; Jifa et al., 2006).

We also analyzed changes in plasma glucose and cortisol to better understand the stress caused by exposure to BaP. Xenobiotics such as BaP cause stress in fish, and the primary stress responses induce secondary responses that affect energy requirements, such as increases in plasma glucose levels (Carmichael et al., 1984), changes in the homeostasis of electrolytes in blood and tissues (Carmichael et al., 1984), and increases in AST and ALT (Pan et al., 2003; Choi et al., 2007b).

Plasma cortisol levels were highest after 6 h exposure to both 10 and 30 μg/L BaP. This implies that BaP was absorbed by the tissue and identified as a stressor, activating the hypothalamus–pituitary–interrenal axis (Redding and Schreck, 1983). Considering that cortisol is involved in metabolism (Mommsen et al., 1999), increasing cortisol levels may reflect an increase in energy metabolism in response to BaP, which in turn may lead to an increase in glucose levels (Carmichael et al., 1984). In time, cortisol levels recovered to normal. Therefore, it can be concluded that stress is reduced as BaP is metabolized and becomes nontoxic.

Like cortisol, plasma glucose levels were highest after 6 h exposure to both 10 and 30 μg/L BaP. This suggests that stress-induced increases in cortisol promote gluconeogenesis in the liver. Hyperglycemia via this mechanism supplements the increased energy requirements due to stress (Vijayan et al., 1997).

In summary, exposing olive flounder to BaP led to oxidative stress and the generation of ROS, which were subsequently scavenged by the antioxidants SOD and MT. Changes in plasma glucose and cortisol levels, in particular their return to baseline

levels in time following BaP exposure, suggest that the effects of ROS were counteracted by MT and SOD. Our results can be used in the future as the baseline data for measuring the degree of oxidative stress induced by xenobiotics such as BaP.

References

Ahmad, I., Hamid, T., Fatima, M., Chand, H.S., Jain, S.K., Ather, M., Raisuddin, S., 2000. Induction of hepatic antioxidants in freshwater catfish (*Channa punctatus* Bloch) is a biomarker of paper mill effluent exposure. Biochem. Biophys. Acta 1523, 37–48.

Andrews, G.K., 2000. Regulation of metallothionein gene expression by oxidative stress and metal ions. Biochem. Pharmacol. 59, 95–104.

Basha Siraj, P., Rani Usha, A., 2003. Cadmium-induced antioxidant defense mechanism in freshwater teleost *Oreochromis mossambicus* (Tilapia). Ecotoxical. Environ. Saf. 56, 218–221.

Baudrimont, M., Andress, S., Durrei, G., Boudou, A., 2003. The key role of metallothioneins in the bivalve *Corbicula fluminea* during the depuration phase, after in situ exposure to Cd and Zn. Aquat. Toxicol. 63, 89–102.

Boleas, S., Fernandez, C., Beyer, J., Tarazona, J.V., Goksøyr, A., 1998. Accumulation and effects of benzo[a]pyrene on cytochrome P450 1A in waterborne exposed and intraperitoneal injected juvenile turbot (*Scophthalmus maximus*). Mar. Environ. Res. 46, 17–20.

Borhoumi, R., Mouneimne, Y., Awooda, I., Safe, S.H., Donnelly, K.C., Burghardt, R.C., 2002. Characterisation of calcium oscillations in normal and benzo[a]pyrene-treated clone 9 cells. Toxicol. Sci. 68, 444–450.

Canova, S., Degan, P., Peters, L.D., Livingstone, D.R., Voltan, R., Venier, P., 1998. Tissue dose, DNA adducts, oxidative DNA damage and CYP1A-immunopositive protein in mussels exposed to waterborne benzo[a]pyrene. Mutat. Res. 399, 17–30.

Carlson, E.A., Li, Y., Zelikoff, J.T., 2002. The Japanese medaka (*Oryzias latipes*) model: applicability for investigating the immunosuppressive effects of the aquatic pollutant benzo[a]pyrene (BaP). Mar. Environ. Res. 54, 565–568.

Carmichael, G.J., Tomasso, J.R., Simco, B.A., Davis, K.B., 1984. Characterization and alleviation of stress associated with hauling largemouth bass. Trans. Am. Fish. Soc. 113, 778–785.

Cavaletto, M., Ghezzi, A., Burlando, B., Evangelisti, V., Ceratto, N., Viarengo, A., 2002. Effect of hydrogen peroxide on antioxidant enzymes and metallothionein level in the digestive gland of *Mytilus galloprovincialis*. Comp. Biochem. Physiol. C 131, 447–455.

Cho, Y.S., Choi, B.N., Kim, K.H., Kim, S.K., Kim, D.S., Bang, I.C., Nam, Y.K., 2006. Differential expression of Cu/Zn superoxide dismutase mRNA during exposures to heavy metals in rockbream (*Oplegnatus fasciatus*). Aquaculture 253, 667–679.

Choi, C.Y., An, K.W., Nelson, R.E., Habibi, H.R., 2007a. Cadmium affects the expression of metallothionein (MT) and glutathione peroxidase (GPX) mRNA in goldfish, *Carassius auratus*. Comp. Biochem. Physiol. C 145, 595–600.

Choi, C.Y., Min, B.H., Jo, P.G., Chang, Y.J., 2007b. Molecular cloning of PEPCK and stress response of black porgy (*Acanthopagrus schlegeli*) to increased temperature in freshwater and seawater. Gen. Comp. Endocrinol. 152, 47–53.

Hansen, B.H., RØmma, S., Garmo, Ø.A., Olsvik, P.A., Anderson, R.A., 2006. Antioxidative stress proteins and their gene expression in brown trout (*Salmo trutta*) from three rivers with different heavy metal levels. Comp. Biochem. Physiol. C 143, 263–274.

Jifa, W., Zhiming, Y., Xiuxian, S., You, W., 2006. Response of integrated biomarkers of fish (*Lateolabrax japonicus*) exposed to benzo[a]pyrene and sodium dodecylbenzene sulfonate. Ecotoxicol. Environ. Saf. 65, 230–236.

Kägi, J.H.R., 1993. Overview of metallothionein. Metallobiochemistry Part B: metallothionein and related molecules. Methods Enzymol. 205, 613–626.

Kim, M.O., Phyllis, E.B., 1998. Oxidative stress in critical care: is antioxidant supplementation beneficial? J. Am. Diet. Assoc. 98, 1001–1008.

Kiruthiga, P.V., Beema Shafreen, R., Karutha Pandian, S., Arun, S., Govindu, S., Pandima Devi, K., 2007. Protective effect of silymarin on erythrocyte haemolysate against benzo[a]pyrene and exogenous reactive oxygen species (H_2O_2) induced oxidative stress. Chemosphere 68, 1511–1518.

Klaassen, C.D., Liu, J., 1998. Induction of metallothionein as an adaptive mechanism affecting the magnitude and progression of toxicological injury. Environ. Health Perspect. 106, 297–300.

Kojima, Y., Berger, C., Vallee, B.L., Kägi, J.H., 1976. Amino-acid sequence of equine renal metallothionein-1B. Proc. Natl. Acad. Sci. U. S. A. 73, 3413–3417.

Lee, S.S., Yang, S.F., Ho, Y.C., Tsai, C.H., Chang, Y.C., in press. The upregulation of metallothionein-1 expression in areca quid chewing-associated oral squamous cell carcinomas. Oral Oncol. doi:10.1016/j.oraloncology.2007.01.019.

Lehtinen, K.J., 1990. Mixed function oxygenase enzyme responses and physiological disorders in fish exposed to kraft mill effluents: a hypothetical model. Ambio 19, 259–265.

Livingstone, D.R., 1993. Biotechnology and pollution monitoring: use of molecular biomarkers in the aquatic environment. J. Chem. Tech. Biotechnol. 57, 195–211.

Marklund, S., Marklund, C.T., 1974. Involvement of the superoxide anion radical in the autooxidation of pyrogallol and a convenient assay for superoxide dismutase. Eur. J. Biochem. 47, 469–474.

McFarland, V.A., Inouye, L.S., Lutz, C.H., Jarvis, A.S., Clarke, J.U., McCant, D.D., 1999. Biomarkers of oxidative stress and genotoxicity in livers of field-collected brown bullhead, Ameiurus nebulosus. Arch. Environ. Contam. Toxicol. 37, 236–241.

Mcgroddy, S.E., Farrington, J.W., 1995. Sediment porewater partitioning of polycyclic aromatic hydrocarbon in three cores from Boston harbor, Massachusetts. Environ. Sci. Technol. 29, 1542–1550.

Mommsen, T.P., Vijayan, M.M., Moon, T.W., 1999. Cortisol in teleosts: dynamics, mechanisms of action, and metabolic regulation. Rev. Fish Biol. Fish. 9, 211–268.

Olafson, R.W., McCubbin, W.D., Kay, C.M., 1988. Primary- and secondary-structural analysis of a unique prokaryotic metallothionein from a *Synechococcus* sp. cyanobacterium. Biochem. J. 251, 691–699.

Palace, V.P., Dick, T.A., Brown, S.B., Baron, C.L., Klaverkamp, J.F., 1996. Oxidative stress in Lake Sturgeon (*Acipenser fulvescens*) orally exposed to 2,3,7,8-tetrachlorodibenzofuran. Aquat. Toxicol. 35, 79–92.

Pan, C.H., Chien, Y.H., Hunter, B., 2003. The resistance to ammonia stress of *Penaeus monodon* Fabricius juvenile fed diets supplemented with astaxanthin. J. Exp. Mar. Biol. Ecol. 297, 107–118.

Redding, J.M., Schreck, C.B., 1983. Influence of ambiental salinity on osmoregulation and cortisol concentration in yearling coho salmon during stress. Trans. Am. Fish Soc. 112, 800–807.

Romèo, M., Cosson, R.P., Gnassia-Barelli, M., Risso, C., Stien, X., Lafaurie, M., 1997. Metallothionein determination in the liver of the sea bass *Dicentrarchus labrax* treated with copper and B(a)P. Mar. Environ. Res. 44, 275–284.

Sato, M., Bremner, I., 1993. Oxygen free radicals and metallothionein. Free Radic. Biol. Med. 14, 325–337.

Vijayan, M.M., Pereira, C.E., Grau, G., Iwama, G.K., 1997. Metabolic responses associated with confinement stress in tilapia: the role of cortisol. Comp. Biochem. Physiol. C 116, 89–95.

Wells, P.G., Kim, P.M., Loposa, R.R., Nicol, C.J., 1997. Oxidative damage in chemical teratogenesis. Mutat. Res. 396, 65–78.

Winzer, K., Van Noorden, C.J.F., Koföhler, A., 2002. Sex-specific biotransformation and detoxification after xenobiotic exposure of primary cultured hepatocytes of European flounder (*Platichthys flesus* L.). Aquat. Toxicol. 59, 17–33.

Zelko, I.N., Mariani, T.J., Folz, R.J., 2002. Superoxide dismutase multigene family: a comparison of the CuZn-SOD (SOD1), Mn-SOD (SOD2), and EC-SOD (SOD3) gene structures, evolution, and expression. Free Radic. Biol. Med. 33, 337–349.

Zhang, J.F., Shen, H., Wang, X.R., Wu, J.C., Xue, Y.Q., 2004. Effects of chronic exposure of 2,4-dichlorophenol on the antioxidant system in liver of freshwater fish *Carassius auratus*. Chemosphere 55, 167–174.