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Selection of Molecular Biomarkers Relevant to Abnormal Behaviors of Medaka Fish (*Oryzias latipes*) Caused by Diazinon

Sung-Cheol Koh※, Sung-Woo Shin, Hyun-Duk Cho[1], Tae-Soo Chon[1], Jong-Sang Kim[2] and Sung-Kyu Lee[3]

Division of Civil and Environmental Engineering, Korea Maritime University, Busan 606-791, Korea
[1]Department of Biological Sciences, Pusan Naitonal University, Busan 609-735, Korea
[2]Department of Animal Biotechnology, Kyungpook Naitonal University, Daegu 702-701, Korea
[3]Korea Institute of Toxicology, Daejeon 305-600, Korea

다이아지논에 의해 야기된 송사리의 이상행동 연관 분자생물지표의 선발

고성철※, 신성우, 조현덕[1], 전태수[1], 김정상[2], 이성규[3]

한국해양대학교 건설환경공학부, [1]부산대학교 생명과학부,
[2]경북대학교 동물공학과, [3]안전성평가연구소

요 약

본 연구의 목적은 다이아지논(Diazinon; *O*, *O*-diethyl *O*-[6-methyl-2 (1-methylethyl)-4-pyrimidinyl] phosphorothioate)에 노출된 모델 생물체(송사리)의 행동변화와 관련된 분자생물학적 기전 규명을 통하여 비정상적 행동의 모니터링을 위한 생물지표(biomarker)를 개발하는데 있다. 이를 위해 우선 suppression subtractive hybridization (SSH) 및 DNA microarray 기법을 활용하여 다양한 유전자를 스크리닝하였다. 다이아지논에 노출시킨 송사리에서 발현의 차이가 나는 상향 조절된 유전자 97개 (알려지지 않은 유전자 27개 포함)와 하향 조절된 유전자 99개 (알려지지 않은 유전자 60개 포함)를 동정 하였고 이들 중 이상행동과 관련되는 것으로 보이는 유전자 10개 (상향조절 5개, 하향조절 5개)를 선발하였다. 이들 중에서 primer 제작이 잘된 beta-1, Orla C3-1, parvalbumin 및 apolipoprotein E을 선발하여 그 유전자 발현을 real-time PCR 기법을 사용하여 정량적으로 모니터링 하였다. Orla C3-1, parvalbumin 및 apolipoprotein E는 고농도의 다이아지논 처리 (1000 ppb; 24 h)에서 그 발현이 억제됨이 관찰되었다. 다이아지논 처리 시 신경질환 (알츠하이머 병 및 다운 신드롬)에 관련된 apolipoprotein E와 근육세포의 유연화에 작용하는 parvalbumin 등의 발현억제는 송사리의 인지능력 교란 및 근육세포의 경직 등을 각각 유도하여 송사리의 비정상적 행동을 야기하는 것으로 판

※To whom correspondence should be addressed.
Tel: +82-51-410-4418, Fax: +82-51-410-4415
E-mail: skoh@hhu.ac.kr

단되었다. 따라서 이들 생물지표는 신경독성물질에 의한 송사리 및 기타 어류의 이상행동의 변화의 감지에 활용될 수 있을 것으로 사료된다.

Key words : diazinon, biomarkers, *Oryzias latipes*, suppression subtractive hybridization, real-time PCR, behaviors

INTRODUCTION

Organophosphate (OP) pesticides are widely and effectively used all over the world with application in agriculture and horticulture for controlling insects in crops, ornamentals, lawns, fruit and vegetables. Diazinon (*O*, *O*-diethyl *O*-[6-methyl-2-(1-methylethyl)-4-pyrimidinyl] phosphorothioate) is an organophosphorous insecticide widely used for the control of agricultural and household pests, the toxic effects of which are mainly due to the inhibition of cholinesterase (Kim *et al*., 1999). Most of organophosphate pesticides, however, show selective toxicity among fish. Diazinon, a neurotoxic chemical, is relatively highly toxic to fish, and it is well known that it causes vertebral malformation and behavioral change of fish at relatively low concentrations (Dutta *et al*., 1992; Pan and Dutta, 1998). Behavioral change caused by pesticides is most likely related to changed levels in neurotransmitters such as acetylcholine, dopamine, serotonin, and norepinephrine. Few reports have been made regarding a relationship between monoamine neurotransmitter generation and behavior responses in fish. The behavioral changes in fish affected by a few neurotoxic chemicals include increase in surfacing and distance traveled, jumping, erratic movements, convulsions and opercular movements (Murali and Krishna, 1991). Human exposed to narcotics or pesticides can develop Parkinson's disease. The disease is a common neurodegenerative syndrome characterized by loss of dopaminnergic neurons in the substantia nigra, formation of filamentous intraneuronal inclusions (Lewy bodies) and an extrapyramidal movement disorder (Majachrzak *et al*., 1990).

In vertebrates, three catecholamines (CA), dopamine, noradrenaline, and adrenaline, act as major monoamine neurotransmitters in the central nervous system (CNS) and the peripheral nervous system (PNS). They are sequentially synthesized from aromatic amino acids where TH (tyrosine hydroxylase) catalyzes a rate-limiting step (Elaine *et al*., 1987; Coker *et al*., 1998). Acetylcholinesterase (AChE) activity is increased within and around amyloid plaques, which are present in Alzheimer's disease (AD) patient's brain. One of the characteristic changes that occurs in AD is the loss of acetylcholinesterase (AChE) activity, the enzyme responsible for acetylcholine hydrolysis, from both cholinergic and non-cholinergic neurons of the brain (Kim *et al*., 1999; Kim *et al*., 2003). Transcriptome analysis of fish responses to various chemicals have been useful for diagnosing biological effects of chemicals at genomics level (Tyler *et al*., 2008). Little comprehensive transcriptomic studies have, however, been performed to investigate how toxic chemicals can affect the fish behavior and what mode of actions are involved in the behavioral responses. This study has been performed as a portion of the interdisciplinary research project whose focus is on development of an alarm system for monitoring hazardous toxic chemicals in the aquatic environments. Herein, the goal of this study has been to identify and validate molecular biological biomarkers related to the medaka fish behaviors caused by the environmental toxicants using suppression subtractive hybridization (SSH), DNA microarray techniques and real time PCR analysis.

MATERIALS AND METHODS

1. Experimental chemicals and fish

Diazinon (purity: 99%) was obtained from Wako Pure Chemical Ind., Ltd. (Osaka, Japan) and the Japanese medaka was treated under appropriate sublethal concentrations (10, 100 and 1,000 ppb) of diazinon.

Japanese medaka (*Oryzias latipes*) was obtained from Korea Institute of Toxicology (KIT, Daejeon, Korea). The fish was held in a square glass chamber (40 × 22 × 40 cm) containing 30-liter of dechlorinated water (pH 6.5 ~ 7.3) with aeration and was reared with artificial dry diet (Tetramin®) under the light regime of L10 : D14 at a temperature of 25°C. Before observation, tap water in the test aquarium was sufficiently dechlorinated by bubbling air under sunlight for 2 or 3 days.

2. Fish sample preparation and total RNA extraction from fish

For total RNA extraction, the treated fish was first immediately frozen in the liquid nitrogen and preserved in a deep freezer (−70°C) until used. Total RNA was extracted according to a protocol accompanied in RNAwiz (Ambion, Texas) extraction kit. Tissues were homogenized in a Polytron homogenizer after approximately 20 mg of tissue were suspended in 1 mL of phosphate buffer (pH 8.0, 0.1 M). Total RNA was then treated with RQ1 DNase (1 U of DNase/5 g of RNA; Promega) for 60 min at 37°C, extracted twice with phenol/chloroform, and precipitatied with ethanol.

Total RNA isolation using solution D protocol (Sambrook and Russell, 2001) was performed in order to do SSH and cDNA microarray analysis. The fish was treated with diazinon at the concentration of 0 (control), 10, 100, 1,000 ppb under different exposure times: 24 and 48 hrs for 10 ppb; 6 and 36 hrs for 100 ppb; 3 and 24 hrs for 1,000 ppb. Total RNA was isolated from these head tissues using modified acid phenol-guanidinium thiocyanate-chloroform extraction.

3. Suppression subtractive hybridization (SSH) and cDNA microarray analysis

1) SSH and cloning

Driver (control) and tester (treatment) cDNA was synthesized from each 1 μg of medaka fish total RNA. Suppression subtractive hybridization was performed according to protocols of PCR-select™ cDNA Subtraction Kit (Clontech Laboratories Inc., Palo Alto, CA). Total RNA was first reverse transcribed into double strand cDNA. The resulting cDNAs were digested by *Rsa* I and purified. The *Rsa* I digested cDNA was served as driver cDNA. A *Rsa* I digested cDNA of each stage was diluted with sterile water and divided into two portions. One portion was ligated with adaptor 1 while the other portion with adaptor 2R. Such adaptor-ligated cDNAs were served as testers. Two hybridizations were then performed. In first step, an excess of driver cDNA was added to each sample of tester of another stage. During the second hybridization, the two primary hybridization samples of the same stage were mixed together without denaturing, and freshly denatured driver cDNA of another stage was added to further enrich for differentially expressed sequences. After that, two steps of PCR amplifications were performed. First non-specific cDNAs have been removed and differently expressed cDNAs have been enriched. Up- and down-regulated PCR cDNA fragments were directly cloned into pEZ-T vector (Promega, Wisconsin), which would be transfected into the *E. coli* (JM 109) and spread on X-gal agar plate. White colonies were selected and PCR amplifications were performed with primers of nested PCR primer 1 (5′-TCGAGCGGCCGCCCGGGCAGGT-3′) and nested PCR primer 2R (5′-AGCGTGGTCGCG GCCGAGGT-3′).

The following oligonucleotides were used in the SSH experiments:

1) cDNA synthesis primers; SMART ∏ A oligonucleotide, 5′-AAGCAGTGGTATCAACGCAGAGT ACGCGGG-3′; 3′ SMART CDS prime ∏ A, 5′-AA GCAGTGGTATCAACGCAGAGTACT (30)-N-1N-3′ (N=A,C,G or T; N-1=A,G, or C)

2) Adapters; Adaptor 1, 5′-CTAATACGACTCAC TATAGGGCTCGAGCGGCCGCCCGGGCAGGT-3′; 3′-GGCCCGTCCA-5′

Adaptor 2R, 5′-CTAATACGACTCACTATAGG GCGCGATGGTCGCGGCCGAGGT-3′; 3′-GCCGG CTCCA-5′

2) DNA sequencing and BLAST analysis

Plasmid DNAs of hybridization positive colonies

Table 1. Primer pairs used for the real-time RT-PCR to estimate expression of genes responsible for Japanese medaka behaviors

Gene annotation	Primers*	PCR product (bp)
Beta-actin	CCCATCCACCTAGAAGATCA GCTGGGGAGGAGAAAGTCTG	270
P. agilis beta-1 tubulin	TGCGTGAAATCGTTCATGTG TTGGCACCAATCTACAGGGA	209
Orla C3-1	AAAAGAAATGTGCAGCACCG AGCAGGTGACAAAAGCTCCG	210
Parvalbumin	TGCCATGGTCAAGGGTTAAA ACGACATTCGCTTATGCAGG	262
Apolipoprotein E	GTGGATCGCTTTTGGCAGTA CCTCAGTCATGGTTTCGGTG	124

*Upper, forward 5′→3′; lower, reverse 3′→5′

were prepared using Qiagen Miniprep Kit (Qiagen, Germany) according to the manufacturer's protocol. DNA sequencing was performed using an automatic sequencer (ABI Prism 377 DNA Sequencer, Perkin-Elmer). Sequence data were analyzed using the National Center for Biotechnology Information (NCBI, USA) programs (http://www.ncbi.nlm.nih.gov/cgibin/BLAST/).

3) cDNA microarray analysis

DNA microarray technology was employed for a large scale gene expression screening and the procedures were custom-ordered to Pharmacogenechips. Inc. (Chuncheon, Republic of Korea). Inserts in the pEZ-T vector were amplified with PCR using the nested SSH-primers and then the PCR products were purified with the Quick PCR Purification Kit (Qiagen, Germany). One hundred seventy-three unique SSH cDNAs were robotically printed onto glass slides. Labeled cDNA was synthesized by reverse transcription of 10 μg total RNA from a homogenate of pooled brains from three fish in the presence of oligo dT primer and either Cy5- or Cy3-UTP (Amersham, UK). Hybridization temperatures used were 42°C.

4. Real-time RT-PCR

Real-time RT-PCR analysis was carried out to estimate the mRNA expression in the head of medaka fish. A few head portions were pooled and frozen in liquid nitrogen. The cDNA templates for real-time RT-PCR were synthesized from total RNA of the fish using reverse-transcriptase (Superscript II, Gibco-BRL, Gaithersburg, MD). PCR was performed using SYBR Green PCR Core Reagents (Applied Biosystems, Foster City, CA, USA), according to the manufacture's instructions, but with one modification: reaction volume was adjusted to 25 μL. The primer pairs used were shown in Table 1.

RESULTS AND DISCUSSION

1. Observation of medaka fish behaviors caused by the toxic chemicals

Diazinon exposed fish showed neurotoxicity behaviors such as irregular movements, opercular movements, zig-zag motion, repetitive back-and-forth movement compared to controls (Shin *et al*., 2001; Kwak *et al*., 2002; Chon *et al*., 2005). Mandal and Kulshrestha (1980) reported that the pesticide severely reduced the capacity of free swimming and physical stamina of gills, accompanied by structural lesions. Moreover several studies revealed variation in the spinal dopaminergic system that are possibly related to different swimming patterns created by the body trunk. Neurobehavioral changes and signs of the pes-

ticide stress in the form of rapid swimming, enhanced rate of opercular movements may be useful for the biological assessment of pesticide toxicity in aquatic system (Murali and Krishna, 1991). The behavioral changes in fish affected by a few neurotoxic chemicals include increase in surfacing and distance traveled, jumping, erratic movements, convulsions and opercular movements (Lee and Lee, 1998; Erwin *et al.*, 2003). These behaviors were also observed in this study.

The affected behavior appeared mostly 3～8 hours after treating the chemical and the number of frequency rapidly increased in 3～4 hours after the treatment, being maximized in 5～6 hours (Chon *et al.*, 2005). The number of individuals initially responding to the treatment of diazinon peaked in 3～5 hours. Total number of responding behavior decreased from 8 hours on. Although the number of the responding behavior was low after 8 hours, this does not indicate that treatment effect decreased rapidly. Most of the tested individuals, however, showed the response behavior sporadically, although the duration of abnormal behavior was generally short.

2. Screening of new potential behavioral biomarkers by suppression subtractive hybridization (SSH) and DNA microarray techniques

Once the cDNA was synthesized, it was cut with *Rsa* I and ligated to adapters at supplied in the Clontech kit. Forward and reverse were imaged and quantitative subtraction hybridizations were performed between samples from diazinon treated and control groups, and then the subtractive hybridization products were amplified by a suppression PCR (data not shown).

To increase representation and to reduce redundancy of the subtracted cDNA library, cycling conditions of both the first and the second PCR procedures for SSH were optimized. Twenty-seven cycles for the first PCR and seventeen cycles for the second produced a desirable amplification (Fig. 1). The secondary PCR products from the forward subtraction and the reverse

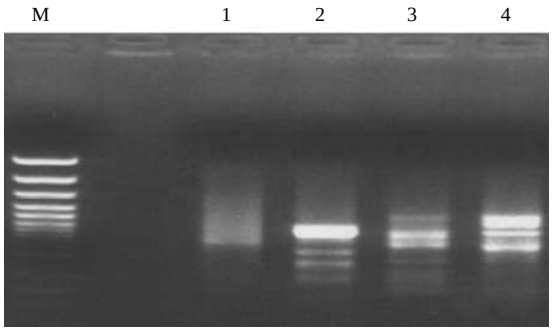

Fig. 1. Secondary PCR products of subtracted cDNA separated on a 1% TAE agarose gel. Lane 1: reverse subtracted cDNA; Lane 2: reverse unsubtracted cDNA; Lane 3: forward subtracted cDNA; Lane 4: forward unsubtracted cDNA (see Materials and Method 4.1 for detail).

subtraction were cloned into the pEZ-T vector, respectively, and transformed into *E-coli*. JM109 through electroporation. The host cells were cultured in Petri dishes in LB medium containing ampicillin 100 μg/mL.

3. Expression analysis of SSH clones by microarray hybridization

Each test sample was derived from separate individuals and was individually hybridized against the same brain control sample. Subsequently, data derived from the control hybridizations were compared with data derived from the treatment hybridizations. A differential gene expression analysis was performed by image scanning and scatter plot analysis with a gene pix program. Table 4 presents some representative up-regulated gene expressions in the head portion of Japanese medaka treated with diazinon (100 ppb) for 24 h. The number of genes up-regulated by diazinon treatment were 97 genes which include 27 of unknown genes. In addition, the number of down-regulated genes were 99 which include 60 unknown genes.

Genes showing 5 or more than 5 as ratio of hybridization signal (treatment/control) were classified as up-regulated genes while the ratio of 0.5 or below as down-regulated genes (Tables 2, 3, and 4).

In treatment of diazinon (100 ppb) for 12 hr (Table

Table 2. Expression of the representative up-regulated genes in the head portion of Japanese medaka treated with diazinon (100 ppb) for 24 hr

Row No.	Gene ID	Gene annotation	Signal ratio* (treatment/control)
Row 16	15	Ribosomal Protein L4	24.3
Row 171	170	Lysophosphatidic acid acyltransferase	19.0
Row 223	222	Unknown	16.7
Row 52	207	*Oryzias latipes* Orla C3-1 mRNA	8.6
Row 450	472	LPAAT	7.6
Row 499	500	〃	7.5
Row 407	248	*P. agilis* beta-1 tubulin (beta-1-tub) gene	6.5
Row 509	13	Unknown	6.5
Row 18	354	〃	6.5
Row 208	26	*Oryzias latipes* choriogenin L gene	5.9
Row 473	435	Unknown	5.7
Row 501	456	Chromosome 17 genomic DNA	5.6
Row 458	156	Signal recogfition particle 54KD (SRP54)	5.4
Row 484	286	Unknown	5.3
Row 38	495	〃	4.9

*Based on the average of two replicates of the microarray experiment

Table 3. Expression of the representative up- and down-regulated genes in the head portion of Japanese medaka when treated with diazinon (100 ppb) for 12 hr

Row No.	Gene ID	Gene annotation	Signal ratio* (treatment/control)
Up-regulated genes			
Row 171	170	Lysophosphatidic acid acyltransferase	19.0
Row 450	449	*Gallus gallus* cardiac-enriched TEA domain transcription factor (DTEF-1B) mRNA	14.7
Row 208	207	*Oryzias latipes* Orla C3-1 mRNA	8.6
Row 473	472	LPAAT	7.6
Row 249	248	*P. agilis* beta-1 tubulin (beta-1-tub) gene	6.5
Row 27	26	*Oryzias latipes* choriogenin L gene	5.9
Row 157	156	Signal recogfition particle 54KD (SRP54)	5.4
Down-regulated genes			
Row 254	116	*Oryzias latipes* Ol-vit1 mRNA for vitellogenin 1	1.7
Row 379	378	G3PDH	0.4
Row 539	538	*Anguilla japonica* GAPDH mRNA for glyceraldehyde-3-phosphatedehydrogenase (170bp)	0.4
Row 245	244	*Cypselurus agoo* mRNA for myosin light chain 2	0.4
Row 50	49	Myosin light chain 2	0.4
Row 130	129	*Oryzias latipes* mRNA for cytoplasmic actin OlCA1	0.4
Row 170	169	*Oryzias latipes* Gb mRNA for adult beta-type globin	0.3

*Based on the average of two replicates of the microarray experiment

3), particularly regulated genes were Orla C3-1, beta-1 tubulin, O1-vit1 and myosin light chain genes. Tubulin, the building block of microtubules, consists of an alpha and beta subunit, each in itself a family of several highly homologous isotypes.

According to Oehlmann *et al*. study (2003), a distinct

Table 4. Expression of the representative up- and down-regulated genes in the head portion of Japanese medaka when treated with diazinon (1,000 ppb) for 24 hr

Row No.	Gene ID	Gene annotation	Signal ratio* (treatment/control)
Up-regulated genes			
Row 206	205	Lysophosphatidic acid acyltransferase	7.4
Row 13	12	*Homo sapience* similar cytochrom oxidise 3	6.2
Row 262	261	*Oryzias latipes* p53 tumor suppressor (TP53) gene	5.7
Row 16	15	Ribosomal Protein L4	5.3
Row 61	60	Lysophosphatidic acid acyltransferase	5.2
Row 27	26	*Oryzias latipes* choriogenin L gene	5.0
Down-regulated genes			
Row 121	120	*Oryzias latipes* DNA for transferrin	4.8
Row 157	156	Signal recognition particle 54KD (SRP54)	4.6
Row 249	248	*P. agilis* beta-1 tubulin (beta-1-tub) gene	3.7
Row 445	444	*Coxiella burnetii* heat shock protein (dnaJ) gene	3.6
Row 99	98	*Cavia porcellus* nucleoside diphosphate kinaseA (NDPK-A) mRNA	0.4
Row 268	267	Apolipoprotein E, partial	0.4
Row 50	49	Myosin light chain 2	0.4
Row 242	241	*Theragra chalcogramma* clone 2 parvalbumin mRNA	0.4
Row 538	537	*Oryzias latipes* DNA for transferrin	0.4
Row 270	269	*Gillichthys mirabilis* nuclease diphosphate kinase B mRNA	0.4
Row 194	193	*Cyprinus carpio* mRNA for parvalbumin	0.3

*Based on the average of two replicates of the microarray experiment

expression pattern of beta-1 tubulin was observed in the zebrafish embryo in restricted regions of the peripheral and central nervous system that comprise early-differentiating neurons. Here, the expression pattern changes during development and in the adult zebrafish expression mostly is confined to a subset of brain zones that include the telencephalic ventricle, and hypothalamic area and in the olfactory epithelium. Beta-1 tubulin level was significantly decreased in the temporal, frontal, parietal cortex and in thalamus of patients with Alzheimer's disease (Schuller *et al*., 2001). Also, in Down syndrome the t-complex polypeptide 1/beta-1 tubulin ratio was significantly increased in frontal and parietal cortex, suggesting a different mechanism for aggregation of microfilament proteins as beta-1.

In case of diazinon treatment (1,000 ppb) for 24 hr, of particular interest were genes for p53 tumor suppressor, choriogenin L, heat shock protein, parvalbumin and transferrin (Table 4). p53 gene is a well-defined tumor suppressor gene that is frequently mutated in human cancers. Recently, two proteins homologous to p53, termed p73 and p63, were identified. Current data demonstrate that both p73 and p63, like p53, can induce cell-cycle arrest and apoptosis, suggesting that they might also be tumor suppressors (Quinones and Rainov, 2001).

Heat shock proteins (HSP), a family of molecular chaperones, are key players in the cellular stress response process and crucial for defending cells from hepatotoxic insults (Salminen *et al*., 1996), including copper toxicity (Ma *et al*., 1998). $CuSO_4$ treatment (0, 25, 50, 100 and 200 μM) resulted in a dose-dependent elevation in heat shock protein 70 (hsp70) expression at 24 and 48 h post-exposure. There was no effect of copper (200 μM $CuSO_4$) on hepatotoxicity at 24 h, whereas longer exposures (48 h) resulted in increased lactate dehydrogenase (LDH) leakage and apoptosis. When living organisms are exposed to thermal and non-thermal stressors, one of the most important functions of HSP is to protect organisms from the toxic effects of heating (Feng *et al*., 2003).

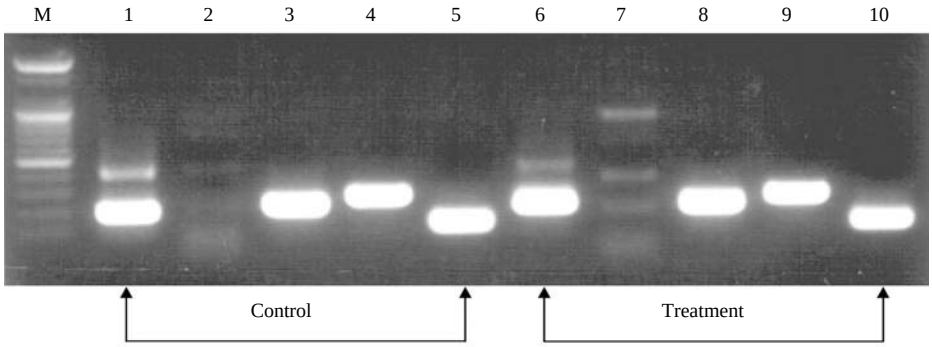

Fig. 2. Efficacy test of designed primers used for real-time RT-PCR for genes encoding the representative biomarker proteins: Lane 1, 6: beta-actin (as a control); Lane 2, 7: beta-1 tubulin; Lane 3, 8: Orla C3-1; Lane 4, 9: parvalbumin; Lane 5, 10: apolipoprotein E.

Transferrin is a beta-globulin that reversibly binds irons and transports it in the bloodstream. The N- and C- terminal lobes of transferrin have similar amino acid sequence, tertiary structure and are believed to have evolved as a result of gene duplication. Stafford and Belosevic (2003) proposed a model of fish macrophage activation that is mediated by a non-cytokine host protein (i.e., transferrin) in combination with highly conserved innate immunity recognition receptors that are almost certain to exist in teleost.

From the results combined so far and comparative analysis from literature study, it is suggested that a few genes are involved in behavior alteration and cytoskeletal protein expression of the cell, and also associated with brain and central nervous system. Therefore, the genes closely regulated with diazinon treatment could be good candidates for behavioral biomarkers to be used in biomonitoring the target chemical and other chemicals of similar toxic effects in the environment.

4. Quantitative monitoring by real time PCR of the putative gene expressions relevant to the abnormal behaviors

The level of potential biomarker gene expression was monitored using real time RT-PCR technique. The primer pairs used for the PCR were shown in Table 1, and efficacy of primers for the representative potential biomarker genes was tested as shown Fig. 2. Gene expression for Orla C3-1, parvalbumin and apolipoprotein E were successfully monitored using the designed primers. These three genes turned out to be suppressed by treatment of diazinon (1,000 ppb) for 24 hr based on Ct values (Table 5). The most significantly suppressed genes were ones encoding parvalbumin and apolipoprotein E. By the way, gene for Orla C3-1 was up-regulated in the treatment condition (diazinon 100 ppb for 24 hr) but this gene was repressed in the more harsh treatment condition (1,000 ppb for 24 hr). This indicates that the gene expression is

Table 5. Real-time RT-PCR analysis of genes for Orla C3-1, parvalbumin and apolipoprotein E in Japanese medaka brain treated with diazinon (1,000 ppb) for 24hr

Genes for	Sample name	Mean Ct value* (threshold cycle)	Standard deviation (SD)
Orla C3-1			
	Control	23.51	0.175
	Treatment	27.86	0.605
Parvalbumin			
	Control	23.21	0.117
	Treatment	36.16	0.737
Apolipoprotein E			
	Control	24.01	4.229
	Treatment	33.82	0.336

*Mean of triplicate experiments

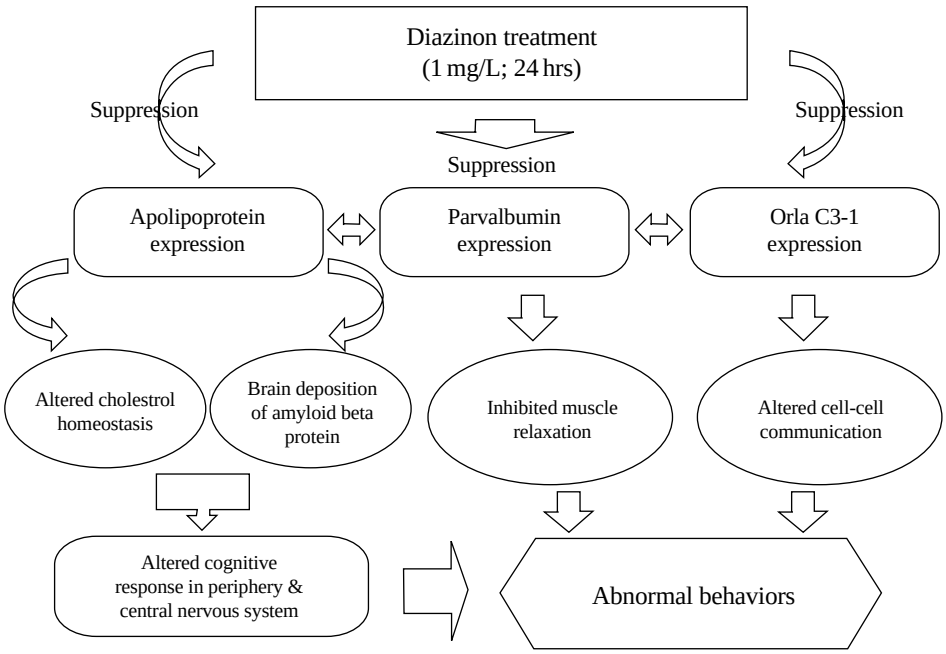

Fig. 3. A proposed mechanistic explanation for the abnormal behaviors of Japanese medaka caused by diazinon treatment.

dependent upon the treatment conditions in which different toxic effects could lead to different behavior patterns. Therefore, the gene expression may be significantly affected by the treatment conditions and hence they will contribute to the abnormal behaviors of the tested fish. In other words, the behaviors will be the outcomes of the interactions among many genes involved including the three genes mentioned above. Therefore a mechanistic understanding of the abnormal behaviors should include the elucidation of the complex gene expressing processes, causing physiological and behavioral changes.

C3 is the most characterized complement component and interacts with many proteins, including some that participate in or control cell adhesion and cell-to-cell communication. Zarkadis *et al.* (2001) have previously identified and characterized three distinct trout C3 proteins (C3-1, C3-3 and C3-4). Complement C3 is the central protein of all three activation pathways, being the major opsonin of the complement system and essential for the generation of the membrane attack complex.

Parvalbumins (PA) are Ca^{2+}-binding proteins with a low molecular mass and an acidic isoelectric point. They are abundant in the white-muscle sarcoplasm of cold-blooded vertebrates, where they can act as soluble muscle-relaxing factors. Adult fish display from two to five parvalbumin isotypes characteristic of the species. Huriaux *et al.* (2002) have shown that parvalbumin isotypes synthesized at different stages of fish growth differ structurally, and that the most marked difference is between larval-juvenile and adult clariid isotypes. Thomas *et al.* (2003) studied effects of cold acclimation on gene transcription in goldfish (*Carassius auratus*) lateral musculature. Array analysis showed that parvalbumin beta was higher in cold acclimated fish muscle when compared to warm acclimated fish muscle and parvalbumin beta message was approximately 6 fold greater in cold acclimated fish muscle.

Apolipoprotein E for Alzheimer's disease (AD) is now well established and widely elucidated. Alzheimer's disease (AD) is a neurodegenerative disease

characterized by alterations of cholesterol homeostasis in both the periphery and the central nervous system. Alterations in cholesterol distribution have been shown to be closely related to the presence of the Apolipoprotein E (ApoE) in AD and control subjects. ApoE polymorphisms related to the pathogenesis of AD have been associated with additional phenotypic or environmental phenomena, such as chronic and acute head injury, response to intracerebral hemorrhage and recovery from cardiopulmonary by pass surgery and stroke (Jordan *et al*., 1997; McCarron *et al*., 1998). ApoE is candidate protein involved in the brain deposition of aggregation of amyloid β protein (Aβ), one of the neuropathological hallmarks in Down's syndrome (Lucarelli *et al*., 2003). ApoE is well known to be related to the pathogenesis of AD, indicating its role in central neurons system. Hence ApoE gene (APOE) could be a good candidate biomarker for behavior responses caused by various environmental factors including toxic chemicals. Taken together, a hypothesis has been proposed regarding the abnormal behaviors of Japanese medaka caused by diazinon treatment (Fig. 3)

CONCLUSION

Identification of differentially expressed genes by SSH and gene chip technique was performed. The number of genes up-regulated by diazinon treatment was 97 which includes 27 of unknown genes and down-regulated genes were 99 which includes 60 of unknown ones. These gene pools were successfully used to provide genes to be used as potential behavioral biomarkers. The expression levels of Orla C3-1, parvalbumin and apolipoprotein E were successfully measured based upon Ct from real-time PCR analysis. Their Ct values increased with diazinon treatment (1 mg/L), indicating their suppression. The suppression of apolipoprotein E (related to AD and down syndrome) and parvalbumin (involved in relaxing muscle) would disrupt cognitive response and rigidity of muscle, respectively, which eventually lead to the abnormal behaviors of medaka. Genes encoding these proteins would be good biomarkers to be used for monitoring behavioral changes caused by toxic chemicals and other relevant impacts. The use of biomarkers and biological effects indices has proven useful in establishing evidence of exposure to pollutant chemicals and damage to the health of sentinel organisms (Depledge *et al*., 1993). In this context, biomarkers including at least three biomarkers identified in this study will be a good start to link molecular endpoints with the ecological (i.e., behavioral) endpoints and consequences. Various molecular biomarkers to be developed in the further study will contribute to establishment of biomonitoring systems to detect the impact of pollutants on organisms in the environment and to use of the biomarkers in monitoring of specific pollutants in water bodies from an engineering perspective.

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