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Impact of deltamethrin exposure on mRNA expression levels of metallothionein A, B and cytochrome P450 1A in rainbow trout muscles

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ABSTRACT

Metallothioneins (MT) are widely utilized to identify specific responses to heavy metal pollution. In addition, there is evidence demonstrating that in vertebrates MT synthesis is stimulated by different endogenous and exogenous agents in particular compounds leading to production of ROS. Also, cytochrome P450 1A can enhance the generation of ROS. On this basis, MT and CYP 1A induction can be considered as biomarkers of oxidative stress. In the current study, we examined the influences of pesticide administration on the expression of *MT-A*, *MT-B* and *CYP 1A*. For this purpose, we produced muscle metallothionein-A, metallothionein-B and cytochrome P450 1A cDNAs and used quantitative RT-PCR to assay mRNAs in rainbow trout exposed to acute and long-term deltamethrin administration. We observed that deltamethrin exposure significantly ($p<0.05$) increased the expression levels of *Cyp1A*, *MT-A* and *MT-B* in a time dependent manner. Results of our study contributes to the identification of inducers of such biomarkers in addition to well known agents.

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1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) and halogenated aromatic hydrocarbons (HAHs) are widely distributed and persistent environmental toxins found in cigarette smoke, smoked food, air pollution, and industrial by products (Costa, 1998). These compounds can transcriptionally induce phase I and phase II metabolic enzymes through activation of the aryl hydrocarbon receptor (Hankinson, 1995; Poellinger, 1995). One predictable response to PAH and HAH exposure is the induction of cytochrome P450 1A isoform (CYP 1A).

Metallothioneins (MTs) are small proteins (~7 kDa) that bind and are induced by free cytosolic metal ions, especially Cd, Cu, Zn and Hg (Kägi and Schäffer, 1988). These proteins are involved in Zn-homeostasis as well as sequestering and detoxification of non-essential metals. Because of a high content of cysteine, MT can also function as an antioxidant. These properties have implicated MT to be an important factor in metal resistance in both aquatic invertebrates and vertebrates (Xie and Klerks, 2004). Brown trout populations inhabiting different metal environments, have MT levels that correlate well with the metal concentrations in the habitats (Linde et al., 1999; Olsvik et al., 2000).

In aquatic animals, and especially with the invertebrates, the development of procedures for the study of specific MT forms and MT gene expression has been relatively recent. There is still a need to better understand the basic features associated with MT expression and their relationship to cellular mechanisms of metal sequestration and toxicity (Roesijadi, 1994).

Deltamethrin is a non-composite synthetic pyrethroid; a broad spectrum, non-cumulative insecticide; a fast-acting neurotoxic agent with good contact and stomach action and no fumigant action (Fig. 1). Its biological activity is very stable in the environment since it has good residual activity on many insects and most surfaces. Deltamethrin is non-systemic in plants. It is of moderately high toxicity in mammals, and is readily metabolized with rapid loss of toxicity. It has a repellent effect for some insects. It is used in the following concentrations: emulsifiable concentrates (25 g a.i./l); ULV (Ultra Low Volume) concentrates (4, 5 and 10 g/l); wettable powder (25 and 50 g/kg); dusts (0.5 and 1.0 g/kg); granules (0.5 and 1.0 g/kg); and flowable (25 g a.i./l). Deltamethrin metabolism and excretion have been extensively studied in rats, mice, and cows. The pattern does not vary significantly among these species. Deltamethrin's effect on the parasite is mainly through contact and ingestion. As deltamethrin is extremely lipophilic it easily penetrates the cuticles of insects and acarines. Deltamethrin is metabolized by liver microsomal esterases and oxidases. Hydrolysis initially cleaves the parent compound into two fragments — cyclopropanecarboxylic acid and 3-phenoxybenzyl

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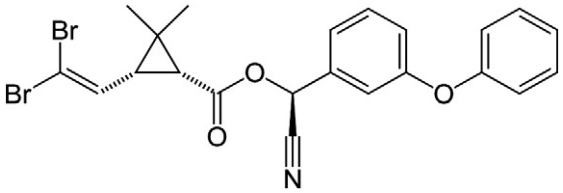


Fig. 1. Molecular structure of deltamethrin.

alcohol. The latter is then oxidized to 3-phenoxybenzoic acid which is the major excretion compound of this moiety. Hydroxylation of a significant portion of this moiety, primarily at the carbon 4′ site but also to some extent at sites 5 and 2, can occur either before or after hydrolysis (Krasnykh et al., 1982).

Pesticides can reach in fish from water, sediments, food, and suspended particulate material and this may be hazardous for living systems, including specific enzymes, proteins and genes (Erdogan et al., 2007). Identification of the interactions between environmental toxicants and biomarkers is of great importance and merits more investigation. Thus, we focused this research on the influences of deltamethrin on expression profiles of Metallothionein-A (*MT*-A), Metallothionein-B (*MT-B*) and cytochrome P450 1A (*Cyp 1A*) genes in rainbow trout (*Oncorhynchus mykiss*) muscle tissue.

2. Materials and methods

2.1. Fish husbandry and maintenance

One year old rainbow trout samples (mixed sex) were obtained from the Fisheries Department of the Agricultural Faculty at Atatürk University in Erzurum and weighed of 150 ± 10 g in autumn, 2009. They were fed twice a day with a commercial pelleted trout feed at a daily ration of 1% of their wet body mass during the study. Trout feed was purchased from Pınar Yem Company, İzmir-Turkey. Feed was given by hand. Fish treatments were conducted according to Applied Research Ethics National Association (ARENA) (2002).

Prior to experiments, fish in each group were kept in 1 × 1.2 m (wide-deep) fiber-glass tanks for adaptation during 1 month. Aeration was provided along the experiments. The average water temperature was 9 °C during the tests. The water quality parameters were analyzed in Laboratory of Erzurum Hygiene Institute. Fiber-glass tanks contained fifteen fish in 600 l water. The average dissolved oxygen level was adjusted to 8.8 ppm and maintained by means of ventilation system. One tank was used as control, and did not contain pesticide while others were added 0.25 μg/l, 1 μg/l and 2.5 μg/l deltamethrin, respectively. The muscle samples were collected after the pesticide treatment from each group fish at 6 h, 12 h, 24 h, 48 h, 72 hand 30 days. All fish samples were frozen with liquid nitrogen and stored at −86 °C until RNA isolation.

2.2. RNA isolation and cDNA synthesis

Total RNA was isolated from 500 mg of frozen white muscle tissues, between the dorsal fin and lateral line, of both deltamethrin treated and control fish using TRIzol reagent (Invitrogen). RNA was treated with Dnase (DNA-free™) in order to avoid genomic contamination. RNA concentrations and quality were verified by means of spectrophotometer (Nanodrop) and RNA gel electrophoresis, respectively. Following isolation, cDNA synthesis was performed using the ThermoScript™ RT-PCR System for First-Strand cDNA Synthesis Kit (Invitrogen) according to the manufacturer's protocol. All cDNA was stored at −20 °C until use.

2.3. TaqMan probe and primer design

Primers and TaqMan probes were designed in Primer3 software (v. 0.4.0) (http://frodo.wi.mit.edu/) using rainbow trout *Cyp 1A* (GenBank accession number AF015660), *MT-A* (GenBank accession number M18103) and *MT-B* (GenBank accession number M18104) sequences and BLASTed to ensure correct mRNA sequences. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as the housekeeping gene (GenBank accession number NM_001124246). In order to perform multiplex Real Time PCR, TaqMan probe of the housekeeping gene was conjugated with Cy5/Blackhole Quencher 2, the fluorophore and quencher molecules, whereas the probes for target genes were conjugated with FAM/TAMRA. The primer and probe sequences are provided in Table 1.

2.4. Real-time PCR

Quantification of gene expression by real-time PCR analysis was performed using a thermal cycler Stratagene MxPro3000. The real-time PCR was carried out in a reaction volume of 50 μl containing template DNA, 900 nM of both target and reference forward and reverse primers, 250 nM both target and reference TaqMan probes, and 25 μl FastStart TaqMan Probe Master (Applied Biosystems) which consists of AmpliTaq Gold DNA Polymerase, AmpErase uracil N-glycosylase (UNG), dNTP with dUTP, and optimized buffer component. Amplification and detection of the samples and the standards were performed using the following thermal cycling conditions: 50 °C for 2 min for activation of optical AmpErase UNG enzyme, 95 °C for 10 min as hot start to activate AmpliTaq Gold DNA polymerase followed by 45 cycles of denaturation at 95 °C for 15 s, and annealing and extension at 60 °C for 1 min. Real-Time PCR data were analyzed using the efficiency $(e)^{(-\Delta\Delta Ct)}$ method (Pfaffl, 2001), which is used to determine mean fold changes in gene expression against control group and housekeeping gene GAPDH. Analytical sensitivity was confirmed by running standard curves.

Table 1
The primer and probe sequences.

Primers and prob names	Sequence (5′→3′)	Amplification length (bp)	GenBank accession number
CYP1A forward	AGTGATGAGTTTGGGCAGGT	179	AF015660
CYP1A reverse	TCACGGATGTTGTCCTTGTC		
CYP1A probe	^{FAM}-TCGTTACCTGCCCAACCGCAC-^{TAMRA}		
MT-A forward	ATCCTGCAAGTGCTCCAAC	214	M18103
MT-A reverse	ACAAAAGCTATGCTCAAGATGG		
MT-A probe	^{FAM}-TGCGTGTGCAAGGGCAAGACT		
MT-B forward	ATCCTGCAAGTGCTCAAACT	168	M18104
MT-B reverse	ATGACTGCATTGTCACGGTA		
MT-B probe	^{FAM}-AAAGTTGCTGCCCCTGCTGTCC-^{TAMRA}		
GAPDH forward	ATCAAAGGGGCTGTCAAGAA	106	NM_001124246
GAPDH reverse	AGGAGTGGGTGTCTCCAATG		
GAPDH probe	^{Cy5}-CGCCGAAGGACCCATGAAGG-^{BQ2}		

Amplification efficiency (e) was calculated based on the slopes of the curves (slope) using the formula $e = 10^{(-1/slope)}$ (Pfaffl, 2001), and the slope value via Stratagene MxPro3000 software.

2.5. Statistical analysis

All experiments were triplicated. Presented data show differences between control and treated groups. Following Arc-sine, Two-Way ANOVA and Duncan's multiple range test via SPSS 17.0 was used to determine the toxic effects of deltamethrin and $p<0.05$ was considered statistically significant.

3. Results

We analyzed the acute and chronic effects of increasing deltamethrin concentrations on the expression levels of *MT-A*, *MT-B* and *Cyp 1A* mRNAs. For this purpose, different concentrations (0.25 μg/l, 1 μg/l and 2.5 μg/l) of deltamethrin were administrated to fish, and the mRNA expression levels were calculated at 6 h, 12 h, 24 h, 48 h and 72 h to determine the acute effects. In the long-term period, the mRNA levels were calculated at 30th day to define the chronic effects. In deltamethrin treated rainbow trouts, sampling was managed until 30 days for 0.25 μg/l dosage. However, it was able to be managed up to 48 h for 1 μg/l dosage and up to 24 h for 2.5 μg/l dosage. The values of amplification efficiency were found as 1.902, 1.945 and 1.925, for MT-A, MT-B and CYP 450 1A respectively.

In general, deltamethrin caused significant ($p<0.05$) increase in *Cyp 1A* mRNA levels in muscles for all concentrations tested depending on exposure time (Fig. 2). When acute and chronic effects compared, the increase remained at 30 days and was significant ($p<0.05$). If 6 h, 12 h and 24 h of each dose compared among each other, *Cyp 1A* mRNA levels significantly increased in parallel with exposure time (Fig. 2). Only a decrease was observed at 48 h of 0.25 mg/L administration whereas a very strong elevation was seen at 72 h.

Similarly, *MT-A* and *MT-B* expression levels elevated significantly ($p<0.05$) in a time and dose dependent manner in general (Figs. 3, 4). The difference in *MT-A* mRNA levels is not significant between 6 h and 12 h of 1 μg/l and 2.5 μg/l administrations. Also a decrease was observed between 6 h and 12 h of 0.25 μg/l administration. We did not observe the similar results for MT-B levels between 6 h and 12 such that significant elevations were detected in all concentrations. The most similar behavior between *MT-A* and *MT-B* levels is that both levels significantly decreased after 72 h. (Figs. 3, 4).

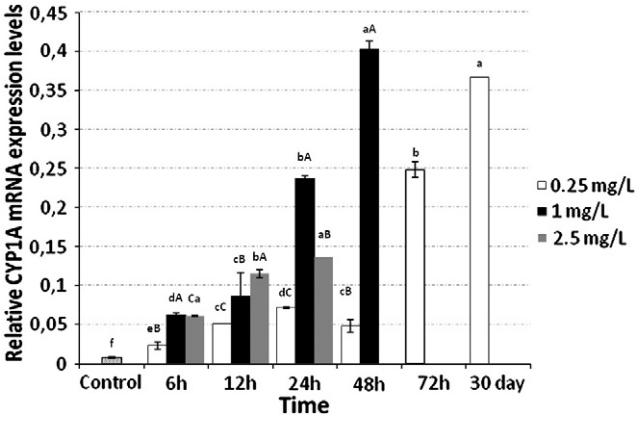


Fig. 2. Relative mRNA expression levels of CYP 1A in the muscles of rainbow trout exposed to different deltamethrin dosages. GAPDH was used as the reference gene. a, b, c, d = statistical differences between different time points of each dosage. A, B, C = statistical differences between different dosages of each time point.

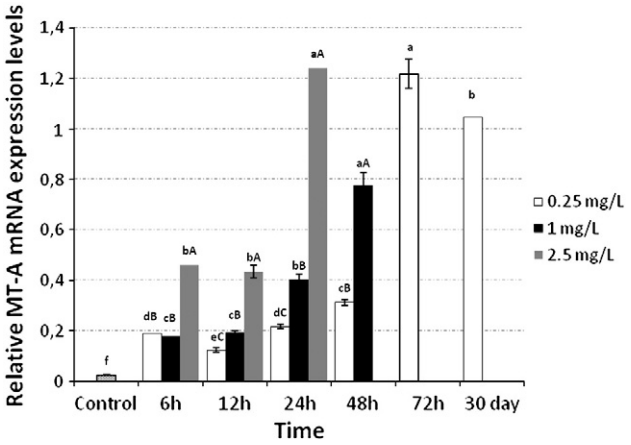


Fig. 3. Relative mRNA expression levels of MT-A in the muscles of rainbow trout exposed to different deltamethrin dosages. GAPDH was used as the reference gene. a, b, c, d = statistical differences between different time points of each dosage. A, B, C = statistical differences between different dosages of each time point.

Overall results indicate that although there is no direct correlation between dosages and elevation, deltamethrin exposure significantly increased the expression levels of *Cyp1A*, *MT-A* and *MT-B* time dependently.

4. Discussion

Extensive research in the recent years has established potential links between oxidative damage and neurological injury and diseases (Simonian and Coyle, 1996). High oxygen consumption, high content of polyunsaturated lipids, and relatively low catalase activity, make the brain particularly vulnerable to oxidative damage (Halliwell, 1989). Only a few studies, however, have addressed the interaction between CYP induction and oxidative damage in the brain. Induction of CYP 2E1, the isoform induced by ethanol, increases the generation of oxygen radicals in rat brain and cultured astrocytes (Montoliu et al., 1994, 1995). Induction of the CYP 1A isoforms by PAHs and HAHs also increases generation of ROS, such as superoxide anion, hydroxyl radical, and hydrogen peroxide (Mishin et al., 1975). Park et al. demonstrated that induction of CYP 1A by 2,3,7,8-tetrachloro dibenzo-p-dioxin (TCDD), a HAH CYP 1A inducer, can generate sufficient levels of ROS to cause oxidative DNA damage in hepatoma cells (Park et al., 1996). Potent hepatic CYP 1A inducers such as 3-methylcholanthrene (3-MC)

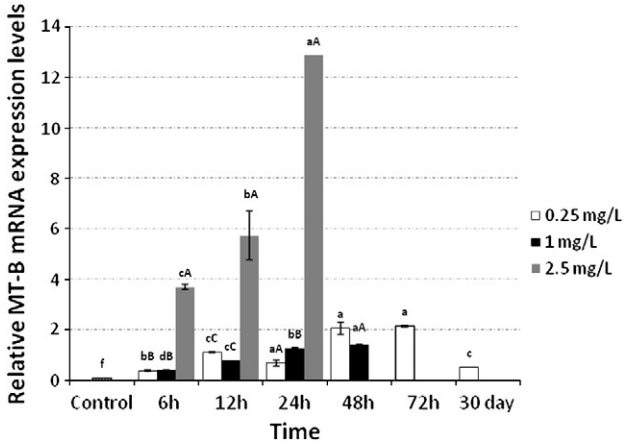


Fig. 4. Relative mRNA expression levels of MT-B in the muscles of rainbow trout exposed to different deltamethrin dosages. GAPDH was used as the reference gene. a, b, c, d = statistical differences between different time points of each dosage. A, B, C = statistical differences between different dosages of each time point.

also induce these isoforms in rat brain (Dhawan et al., 1990), although exposure to PAHs and HAHs does not lead to documented neurotoxicity (Unkila et al., 1995).

Although MTs have been widely utilized to identify specific responses to heavy metal pollution, there is now a body of evidence demonstrating that in vertebrates (mammals and fish) MT synthesis is stimulated by different endogenous and exogenous agents (Kägi and Schäffer, 1988), e.g. glucocorticoid hormones, various kinds of stress (cold, heat, extreme exercise), cytokines and in particular compounds leading to production of ROS (Dalton et al., 1994). Therefore, in mammals and fish, not only inorganic pollutants such as heavy metals but also organic contaminants may activate MT neosynthesis (Bauman et al., 1991; Pedrajas et al., 1995; Sato et al., 1989; Wormser and Calp, 1988).

The molecular mechanisms by which different agents can induce MT synthesis in vertebrate cells have been recently investigated by studies concerning the structure of the MT gene promoter. Metallothionein gene expression is induced by a high variety of stimuli, as metal exposure, oxidative stress, glucocorticoids, hydric stress, etc. The level of the response to these inducers depends on the MT gene. MT genes present in their promotors specific sequences for the regulation of the expression, elements as metal response elements (MRE), glucocorticoid response elements (GRE), GC-rich boxes, basal level elements (BLE), and thyroid response elements (TRE).

Authors have also focused their interest on the response of MT to inductors other than heavy metals. Olsson et al. (1995) demonstrated that in RTH-149 cells hydrogen peroxide could activate MT gene transcription probably through an antioxidant response element (ARE) consensus sequence. Similar in vitro experiments concerning organic xenobiotics are lacking, but it can be inferred from in vivo experiments on fish that polar xenobiotics such as paraquat, but not apolar xenobiotics such as BaP or PCB-156 (Hylland et al., 1996), can stimulate MT neosynthesis. Moreover, it has been shown that the pretreatment of fish with BaP or PCB-156 prior to heavy metals can inhibit MT induction (Sandvik et al., 1997), showing that the use of MT as a biomarker of exposure to heavy metal can be complicated by interactions with other chemicals.

As known, in mussel cells there is a low level of mixed function oxygenase activity (about 1% compared with mammalian MFO activity) and whether tissue accumulation of organic xenobiotic compounds enhance the cytochrome p450 level is not well-identified. Similarly, even though the mussel MT gene promoter might be ROS-activated, whether organic pollutants stimulate MT synthesis must be considered.

Xenobiotics are derived from pharmaceutical, nutraceutical and environmental exposure, and many of the mechanisms involved are highly complex in nature, not easily predictable from existing in vitro tests and do not always predict well from in vivo animal models. Rainbow trout is one of the most important cultivated species in the world. Molecular determination of how specific genes such as *Cyp1A*, *MT-A* and *MT-B* are affected in positive or negative ways is thus very important for molecular biological purposes.

As known, MTs may reduce the dangerous effects of free radicals and ROS by releasing zinc to the neuronal membrane. Thus, the significant increase in mRNA levels of MTs in response to Deltamethrin and DDVP administration may be considered as a defense mechanism in addition to the antioxidants against oxidative stress.

The metabolism of xenobiotics usually involves two distinct stages, commonly referred to phases I and II. Phase I metabolism involves an initial oxidation, reduction or dealkylation of the xenobiotic by cytochrome P-450 monooxigenases (Guengerich, 1991). This step is often needed to provide a molecule with hydroxyl- or amino-groups, which is essential for phase II reactions. Phase II metabolism generally adds hydrophilic moieties, thereby making a toxin more water soluble and less biologically active (Erat et al., 2003).

Taking into account all these questions, we aimed to analyze the acute and chronic effects of deltamethrin on the mRNA expression levels of CYP 1A, *MT-A* and *MT-B*. We observed that deltamethrin exposure significantly ($p<0.05$) increased the expression levels of *Cyp1A*, MT-A and MT-B in time dependent manner. In our previous studies, we demonstrated that deltamethrin inhibits the activities of various proteins including antioxidant enzymes and induces the expression of Heat Shock Protein 70 in addition to reducing the expression levels of insuline-like growth factors and growth hormone in rainbow trout (Aksakal et al., 2010; Ceyhun et al., 2010; Ekinci and Beydemir, 2010) and we claimed that alterations of these genes might be resulted through the formation of oxidant agents in response to deltametrin exposure. The current study supports that theory such that induction of the *CYP 1A* increases generation of ROS, such as superoxide anion, hydroxyl radical, and hydrogen peroxide, and MT synthesis is stimulated by different endogenous and exogenous agents in particular compounds leading to production of ROS.

5. Conclusions

Here we demonstrate that acute and long-term deltamethrin exposure induces the expressions of *CYP 1A, MT-A* and *MT-B* in rainbow trout muscles. Our findings thus indicate that deltamethrin may cause considerable oxidative stress leading to production of ROS and that it interferes with the sensitivity of these biomarkers to PAHs and metal ions. Results of this study also decipher inducers of these genes in addition to well known agents being one of the few investigations in this regard.

Conflict of interest

The authors report no conflict of interest.

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