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Modulation in the mRNA expression of ecdysone receptor gene in aquatic midge, *Chironomus riparius* upon exposure to nonylphenol and silver nanoparticles

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ABSTRACT

Chironomus riparius, a non-biting midge (Chironomidae, Diptera), is extensively used as a model organism in aquatic ecotoxicological studies, although little is known about its genome sequences. Ecdysteroids are steroid hormones that play an important role in development, growth, moulting of larva, and reproduction in *Chironomus* spp. The effect of ecdysteroids is mediated by their binding to the ecdysteroid receptor (*EcR*). To study the effect of environmental stressors, nonylphenol and silver nanoparticles (AgNPs), on the modulation of *EcR* mRNA, in this study, full length cDNA of *C. riparius* ecdysone receptor (*CrEcR*) was identified from the Expressed Sequence Tags (ESTs) database and expression of the corresponding mRNA was analyzed following exposure to nonylphenol and AgNPs. The *CrEcR* cDNA was 2548 base pairs (bp) in length, with a 5′ untranslated region (UTR) of 242 bp and a 3′ UTR of 684 bp. The open reading frame contains 1617 nucleotides, encoding 539 amino acids with a predicted molecular weight of 61 kDa and pI of 5.89, and revealed the presence of several domains associated with DNA binding, dimerization, ligand binding and transcriptional activation characteristic of steroid receptor family members. It was found that the expression level of *CrEcR* was significantly up-regulated on exposure to nonylphenol and significant up or down regulation was observed on exposure to AgNPs. These finding shows that nonylphenol as well as AgNPs could modulate the ecdysone nuclear receptor and may have significant implications in different developmental stages in *C. riparius*.
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1. Introduction

The endocrine system within invertebrates is well characterized; it coordinates and regulates growth, development, reproduction and other physiological processes and in insects it is controlled by the steroid hormone ecdysone (Thummel, 1995). The active metabolite 20-hydroxyecdysone binds to the ecdysone heterodimeric complex, comprising various nuclear receptors, the ecdysone receptor (*EcR*) and the ultraspiracle protein. The hormone's action is mediated by this complex at the transcriptional level via binding to ecdysteroid response elements (Yao et al., 1993).

Nonylphenol is widely used in many industrial applications, as well as household cleaning products (Ying et al., 2002) and its oestrogenic effects as an endocrine disruptor have been extensively studied (Nimrod and Benson, 1996; Bandiera, 2006; Lye et al., 2008). According to some studies, nonylphenol concentrations in rivers receiving effluents from sewage water could reach the level of 180 μg/L (Blackburn and

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Waldock, 1995; Tyler et al., 1998). In surface sediments of rivers 700–900 μg/kg concentrations of nonylphenol, nonylphenol monoethoxylate, and nonylphenol diethoxylate have been reported (Marcomini and Giger, 1987). The acute and chronic toxicities of nonylphenol were previously investigated in *C. ripaius* (Lee and Choi, 2006), as well as in other aquatic organisms (Nimrod and Benson, 1996; Lussier et al., 2000). In recent years, with the fast growth of nanotechnology, the application of nanomaterials in various products has increased and their release to aquatic environments could cause many adverse effects at various levels in aquatic ecosystems (Handy et al., 2008). Silver nanoparticles (AgNPs) are widely used in many commercial products, and the AgNPs released from these products could ultimately end up in aquatic environment (Benn and Westerhoff, 2008), where they could cause adverse effects to many aquatic organisms (Asharani et al., 2008; Griffitt et al., 2009; Nair et al., 2011).

Chironomus riparius (Chironomidae, Diptera), is widely used in aquatic exotoxicological studies for assessing acute and sub-lethal toxicities of contaminated sediments and for water monitoring due to their widespread occurrence, short life-cycle, ease with which they are reared in the laboratory, physiological tolerance to various environmental conditions and the fact that they undergo a metamorphosis under hormone control (Bettinetti et al., 2002; Crane et al., 2002; Taenzler et al., 2007). The endocrine system is known to regulate different developmental stages such as embryogenesis, growth, metamorphosis, and reproduction in *C. riparius* (Watts et al., 2001). In *Chironomus* low receptor levels were found when the moulting hormone tiers were low, and high receptor levels corresponded to stages with high moulting hormone levels. This is similar to the sequence of hormone and receptor level changes reported in *D. melanogaster* and in vertebrates (Syms et al., 1985; Deak and Laufer, 1995; Deak et al., 1998).

It has been reported that the expression profiles of the ecdysone receptor (*EcR*) genes can be modulated by environmental chemicals thereby disrupting the hormone regulating mechanisms of insects (Zou and Fingerman, 1997; Mu et al., 2005; Rodriguez et al., 2007; Planelló et al., 2008). Nonylphenol exposure in *C. riparius* induced a reduction in the rates of total adult emergence and emergence failures (Lee and Choi, 2006). Recently we observed that exposure to AgNPs could cause significant impairment on development, emergence, male to female ratio and egg production in *C. riparius* (Nair et al., 2011). Therefore in this study, the cDNA of *CrEcR* was selected from the previously developed *C. riparius* ESTs database and characterized. Expression patterns in *C. riparius* fourth instar larvae were analyzed after exposure to different concentrations nonylphenol and AgNPs for different time intervals in order to determine the effects of nonylphenol and AgNPs in the modulation of *CrEcR* in *C. riparius*.

2. Materials and methods

2.1. Animals

C. riparius larvae were obtained from the Toxicological Research Center of the Korea Institute of Chemical Technology (Daejon, South Korea). The larvae were reared on an artificial diet of fish flake food (Tetramin, Tetrawerke, Melle, Germany) in glass chambers containing dechlorinated tap water and acid washed sand, with aeration under a 16–8 h light–dark photoperiod at room temperature (20 ± 1 °C).

2.2. Identification and sequence analysis of CrEcR

Gene sequences were retrieved from the ESTs database and were manually annotated to predict transcription initiation and termination sites using BlastX comparisons of putative amino acid translations deduced using translation tool (http://www.expasy.ch/tools/) and aligned using ClustalW (Thompson et al., 1994). The ligand binding domains were aligned and a phylogenetic tree was constructed by the neighbour-joining method using MEGA4.1 (Tamura et al., 2007).

2.3. Chemical exposure, RNA isolation and cDNA synthesis

Groups of 15 fourth instar larvae were exposed to nonylphenol (Sigma–Aldrich Chemical, St. Louis, MO, USA) (10 and 50 μg/L) and AgNPs (Sigma, USA) (0, 0.2, 0.5 and 1 mg/L) for 0, 12, 24, 48 and 72 h. The exposure concentrations of nonylphenol and AgNPs were selected based on our previous studies (Lee and Choi, 2006; Nair et al., 2011). The control group for nonylphenol was exposed to the solvent acetone (Sigma, USA). Aqueous suspensions of AgNPs were prepared and characterized as described previously (Nair et al., 2011). Three independent treatments and three replications were maintained for each treatment. After the exposure the larvae were collected, immediately frozen in liquid nitrogen and stored at −80 °C. Total RNA from samples was isolated using TRIZOL™ Reagent (Invitrogen Life Technology, USA). One microgram of total RNA was used for making cDNA by reverse transcription, using an oligo dT_{18} primer and RT-Premix (Bioneer, South Korea), in a total reaction volume of 20 μl, following the manufacturer's instructions.

2.4. Expression analysis

The primers were checked for their substrate specificity using RT-PCR using the thermal cycle program consisting of an initial denaturation at 94 °C for 2 min followed by 25 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, and a final extension at 72 °C for 5 min using 1 μl cDNA with an RT-PCR premix (Bioneer, South Korea) as per the manufacturer's instructions. Primer3 (http://frodo.wi.mit.edu/primer3/) programme was used to design all the PCR primers (*CrEcR* – Forward – 5′-CATCCAATGCAACAGCTTTTACCAGA-3′ and *CrEcR* – Reverse – 5′-TGGCTGTTCGTAACCGTCTTGATA-3′ and *CrActin* – Forward – 5′-GATGAAGATCCTCACCGAACG-3′; *CrActin* – Reverse – 5′-TTCGAGTGAGGTTGATGCAG-3′). To study the gene expression of AgNPs exposed larvae, 1 μl cDNA was used for quantitative real time PCR using a 0.2 μM of the sense and antisense oligonucleotide primers of each gene, 10 μl of 2× IQ SYBR Green Super Mix (Bio-Rad, USA) and 1 μL template cDNA in

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