Aquatic Toxicology 101 (2011) 31–37

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aquatox

Differential expression of ribosomal protein gene, gonadotrophin releasing hormone gene and Balbiani ring protein gene in silver nanoparticles exposed *Chironomus riparius*

Prakash M. Gopalakrishnan Nair, Sun Young Park, Si-Won Lee, Jinhee Choi*

School of Environmental Engineering and Graduate School of Urban Sciences, University of Seoul, 90 Jeonnong-dong, Dongdaemun-gu, Seoul 130-743, Republic of Korea

ARTICLE INFO

Article history:
Received 29 April 2010
Received in revised form 18 August 2010
Accepted 26 August 2010

Keywords:
Chironomus riparius
Silver nanoparticles
Differential gene expression
Ecotoxicity
Genotoxicity

ABSTRACT

The eco- and genotoxicity of silver nanoparticles (AgNPs) was investigated in the fourth instar larvae of the aquatic midge, *Chironomus riparius*. AgNPs did not have acute toxicity in *C. riparius*, but did exhibited chronic toxicity on development (pupation and emergence failure) and reproduction. Genotoxicity also occurred in AgNPs exposed *C. riparius*. Differential Display PCR (DD-PCR), based on the Annealing Control Primer (ACP) technique, was conducted to investigate the underlying toxic mechanism, which identified altered gene expression in *C. riparius* after treatment with AgNPs. The possible toxicity mechanism of AgNPs in *C. riparius* involves the down regulation of the ribosomal protein gene (CrL15) affecting the ribosomal assembly and consequently, protein synthesis. Up regulation of the gonadotrophin releasing hormone gene (CrGnRH1) might lead to the activation of gonadotrophin releasing hormone mediated signal transduction pathways and reproductive failure. Up regulation of the Balbiani ring protein gene (CrBR2.2) may be an indication of the organism's protection mechanism against the AgNPs. The overall results suggest that the toxicity of AgNPs towards aquatic organisms should be thoroughly investigated to allow for their safe use, as they seem to exhibit important toxicity towards *C. riparius*.

1. Introduction

There has recently been great scientific concern about the possible adverse effects associated with manufactured nanomaterials because of the rapid development of commercialized nanoproducts (Perelshtein et al., 2008; Arora et al., 2008; Li et al., 2008; Chen and Schluesener, 2008). Of these nanomaterials, due to their effective antimicrobial property, silver nanoparticles (AgNPs) are extensively used in many commercial products, surgical instruments, water purification products and paints, as well as in various house hold items, and could end up in the environment during disposal (Maynard et al., 2006). The release of AgNPs into aquatic environments occurs through various means, and is becoming of great concern due to their high surface area and mobility (Benn and Westerhoff, 2008).

Despite the recent increase in research on the ecotoxicity of nanoparticles (NPs), limited investigations have been conducted on the toxicity of AgNPs on aquatic organisms (Griffitt et al., 2009; Asharani et al., 2008). Moreover, most of the ecotoxicity studies on aquatic organism have been conducted using organism level endpoints; such as mortality and growth, (Kahl et al., 1997; Watts and Pascoe, 1998; Crane et al., 2002), with only a few having been performed that include genotoxic endpoints. However, the presence of genotoxic and potentially carcinogenic compounds in aquatic environments is of major concern with respect to the health of aquatic media biota; therefore, the genotoxicity of NPs need to be identified before their widespread release to the aquatic environment.

In the present study, the eco- and genotoxicities of AgNPs were investigated on the aquatic larvae of nonbiting midges, (Chironomidae, Diptera) *Chironomus riparius*, the most abundant group of insects found in freshwater ecosystems, which hold an important position in the aquatic food chain (Cranston, 1995). Taking into account the ecological importance of *Chironomus* larvae in freshwater, studying the effects of AgNPs on *C. riparius* could contribute to a better understanding of the aquatic toxicity of AgNPs. Pupation and adult emergence were used as development descriptors, with the number of egg masses per treatment and number of eggs per egg mass as parameters for reproduction. Genotoxicity was investigated by measuring tail moments using the Comet assay.

Gene expression analysis has also been increasingly used in aquatic ecotoxicology, as it offers high sensitivity and mechanistic values in the diagnosis of environmental contamination (Ankley et al., 2006) and several studies have focused on the responses to chemical stressors at the molecular level in aquatic invertebrates

* Corresponding author. Tel.: +82 2 2210 5622; fax: +82 2 2244 2245.
E-mail address: jinhchoi@uos.ac.kr (J. Choi).

0166-445X/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.aquatox.2010.08.013

(Lee et al., 2006; Ha and Choi, 2009; Park et al., 2010). However, integrating gene expression profiling into an ecotoxicological study using organisms without sequenced genomes is a difficult and challenging task (Snell et al., 2003). Since genomic information on *C. riparius* is limited, the genes that are differentially expressed due to AgNPs toxicity need to be studied and sequenced to predict their possible impacts. Therefore, to gain an insight into the mechanisms of the eco- and genotoxicities of AgNPs in *C. riparius*, a differential display technique (Liang and Pardee, 1992) based on annealing control primers (ACPs) was used to identify Differentially Expressed Genes (DEGs) whose expressions were affected by AgNPs exposure. The toxicity mechanisms of AgNPs toward *C. riparius* are discussed on the basis of the experimental evidence obtained.

2. Materials and methods

2.1. Organism culture and exposure to AgNPs

Using an original strain provided by the Korea Institute of Toxicology (Daejeon, Korea), *C. riparius* larvae were obtained from adults reared in our laboratory, as described previously (Lee et al., 2008). Briefly, *C. riparius* larvae were reared in a 2 l glass chamber, containing dechlorinated tap water (800 ml) and acid washed sand (60 g), fed with fish flake food (Tetramin, Tetrawerke, Melle, Germany), with aeration under a 16–8 h light–dark photoperiod at $20 \pm 2\,^{\circ}C$.

AgNPs (size < 100 nm, Sigma–Aldrich Chemical, St. Louis, MO, USA) were homogenously dispersed in deionized water by sonication for 13 h (Branson-5210 sonicator, Branson Inc., Danbury, CT) and stirring for 7 d, and then filtered through a cellulose membrane (pore size 100 nm, Advantec, Toyo Toshi Kaisha, Japan) to remove nanoparticle aggregations. To determine the size and shape of the AgNPs, 20 μl of the particle suspension were dried onto a 400 mesh carbon-coated copper grid and imaged with a Transmission Electron Microscope (TEM), LIBRA 120 (Carl Zeiss, Oberkochen, Baden-Wrttemberg, Germany) at 80–120 kV. The size distribution of the AgNPs was evaluated using a Photal dynamic light scattering (DLS) spectrometer, DLS-7000 (Otsuka Electronics Co., Inc., Osaka, Japan).

2.2. Exposure analysis of silver in water and sediment

Exposure tanks containing dechlorinated water (800 ml) and sand (60 g) were spiked with 1 mg/l AgNPs and fifty-fourth instar larvae were added to each chamber. Water samples (10 ml) were taken from the middle of the water column after 15 min and different days (1, 2, 5, 20 and 25) of exposure and filtered through 0.2 μm filters, for analysis of dissolved silver in water. To check the total silver content in sediment, 1.5 g sand was collected after 15 min and 1, 2, 5, 20 and 25 d and digested with aqua-regia extraction procedure (ISO, 1995). All samples were frozen immediately after collection until analysis. The metal content in water and sediment was determined using Inductively Coupled Plasma-Mass Spectrometer (ICP-MS, Varian 820-MS, Palo Alto, CA, USA).

2.3. Acute and chronic ecotoxicity

For the acute toxicity test, groups of 10 larvae were exposed to four concentrations (0.5, 1, 2 and 4 mg/l) of AgNPs, with another group made the control. After 24 h of exposure, the number of individuals died was determined.

For the chronic ecotoxicity study, fifty-fourth instar larvae were introduced at the beginning of the experiment, and exposed to different concentrations (0, 0.2, 0.5 and 1 mg/l) of AgNPs until the end of the experiments (25 d). The emerging adults were retained with steel wire mesh until the emergence in the control and experimental aquaria were complete. The numbers of pupae and emerged adults from each vessel were counted and their sexes were determined. The two sexes were easily distinguished by the form and length of their antennae and abdominal terminalia. Additionally, the dead pupae were counted and the time to their complete emergence also investigated. For the reproduction parameters, the numbers of egg masses oviposited by the emerged adults and the numbers of eggs per egg mass in the control and AgNPs-treated vessels were counted. Every 2 d, 50 mg of Tetramin fish food flakes was supplied to each aquarium. The test solutions were not renewed. All the data were recorded at daily intervals.

2.4. Comet assay

An alkaline Comet assay was performed, as described previously (Park and Choi, 2007). Briefly, a total of 10 fourth instar larvae of *C. riparius* were collected 24 h after treatment with AgNPs (0, 0.2, 0.5 and 1 mg/l) from both experimental and control tanks, and pooled for a Comet assay. Treated organisms were placed in 1 ml of phosphate-buffered saline (PBS), containing 20 mM ethylene diamine tetra acetic acid (EDTA) and 10% dimethyl sulfoxide (DMSO), and disintegrated mechanically by mincing. The cell suspension was precipitated by vortexing, and then immediately mixed with 100 μl of 1% low-melting-point (LMP) agarose for use in the Comet assay. To prepare slides, 100 μl of 1% LMP agarose was spread onto a normal agarose pre-coated microscope slide and incubated at 4 °C for 5 min to allow for solidification. The cells were lysed in high salt and detergent (10 mM Tris, 100 mM EDTA, 2.5 M NaCl, 10% DMSO, 10% Triton X-100, pH 10), and subsequently exposed to alkaline conditions (300 mM NaOH, 1 mM EDTA, pH > 13) for 20 min at 4 °C to allow for DNA unwinding and expression of alkali-liable sites. For electrophoresis, an electric current of 300 mA (25 V) was applied for 20 min. After the electrophoresis, the slides were neutralized and dehydrated in 70% ethanol. The slides were stored in a dry place until the image analysis. Before analysis, the slides were stained with 50 μl ethidium bromide (5 μg/ml), then analyzed at 400× magnification using a fluorescence microscope, equipped with an excitation filter of BP 546/12 nm and barrier filter of 590 nm. Three slides were prepared per treatment and 50 cells per slide were examined. DNA damage was expressed as the tail moment (tail length × tail % DNA/100) using an image analysis computerized method (Komet 5.5, Kinetic Imaging Limited, Nottingham, UK).

2.5. Differentially expressed gene analysis

Based on the eco- and genotoxicity tests, fifty-fourth instar larvae were treated with 1 mg/l of AgNPs for 24 h to study the AgNPs induced DEGs. Total RNAs from treated and control larvae were isolated using Trizol (GibcoBRL), according to the manufacturer's protocol, and purified using an RNeasy mini kit (Qiagen, Valencia, CA, USA) suspended in 30 μl of RNase free water, was then quantified using a spectrophotometer (Thermospectronic, Rochester, NY, USA). Samples were stored at −80 °C until analyzed. Three replicates were maintained for each treatment.

GeneFishing™ DEG kits were used for screening DEGs, using the ACP based method, with 80 different arbitrary ACPs, as per the manufacturer's instructions (Seegene™, Seoul, South Korea). The DNA fragments produced by PCR were separated by electrophoresis on a 2% agarose gel and extracted from the agarose gel using the Geneclean® II kit (Q-BIOgene, Cambridge, UK). The DNA sequencing was performed by Macrogen (Seoul, South Korea). Sequence data were identified by comparing with the GenBank database through the BlastX program of the NCBI