Ecotoxicology and Environmental Safety 79 (2012) 90–100

Contents lists available at SciVerse ScienceDirect

Ecotoxicology and Environmental Safety

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

Exposure to mercury causes formation of male-specific structural deficits by inducing oxidative damage in nematodes

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ARTICLE INFO

Article history:
Received 3 December 2010
Received in revised form
9 December 2011
Accepted 10 December 2011
Available online 28 December 2011

Keywords:
Male reproductive toxicity
Oxidative stress
Insulin signaling
CLK-1
Mercury exposure
Caenorhabditis elegans

ABSTRACT

Metal exposure causes reproductive damage in hermaphrodite nematodes, but effects of metals on male development are unclear. We here investigated the effects of mercury chloride exposure on development of males. Hg exposure severely increased the percentage of abnormal males, disrupted the development of male-specific structures, and caused high reactive oxygen species (ROS) production in male tails. Pre-treatment with antioxidant (vitamin E) protected the nematodes against toxicity from Hg exposure on development of male-specific structures. The ROS production in tails was closely correlated with formation of abnormal male-specific structures in males induced by Hg exposure. Moreover, mutations of *clk-1*, encoding ortholog of COQ7/CAT5, and *daf-2*, encoding an insulin/IGF receptor, functioned in two different pathways to suppress the formation of deficits in development of male-specific structures. Thus, three different lines of evidence support our conclusion that $HgCl_2$ causes male structure-specific teratogenesis via production of oxidative stress.

1. Introduction

Annually tons of mercury chloride ($HgCl_2$) are released into the atmosphere by industrial processing and municipal waste incineration (Boujbiha et al., 2009). Contamination and toxicity from mercury (Hg) have posed a serious hazard to human health (Tan et al., 2009). It has been proven that mercury compounds have been associated with male reproductive toxicity *in vitro* and *in vivo*. Exposure to Hg resulted in reduced sperm motility and curvilinear velocity, and acrosome breakage in sperm head membranes with formation of various sized microvesicles (Alabi et al., 1985; Castellini et al., 2009). Exposure to mercury compounds can cause adverse effects on testicular spermatogenic and steroidogenic functions in experimental animals and men (Mohamed et al., 1987; Khan et al., 2004; Rao and Gangadharan, 2008). The abnormal reproductive performance was observed in inorganic Hg exposed mice (Khan et al., 2004). Decrements in sperm count, motility, and morphology were observed in methyl mercury exposed monkeys (Mohamed et al., 1987). So far, although oxidative stress, inflammation, induced-apoptosis, ionic and molecular mimicry could be the basis for metal toxic activity on male reproductive function, underlying mechanisms for the adverse effects of Hg exposure are still largely uncertain (Guzzi and La Porta, 2008; Castellini et al., 2009).

Nematodes are abundant in soil ecosystems, and play important roles in cycling, degradation, and decomposition of key nutrients in the environment. The potential of *Caenorhabditis elegans*, a nonparasitic bacterial feeder that lives in soil interstitia, as a model organism was recognized by Brenner (1974) early in the process of its initial characterization. The success of *C. elegans* as a model has also attracted increased attention in the field of biomedical and environmental toxicology (Leung et al., 2008). *C. elegans* is a valuable model for toxicology study since many of its basic physiological processes and stress responses that can be observed in higher organisms (e.g., humans) are conserved in *C. elegans* (Leung et al., 2008). The unique features of *C. elegans* make it being able to complement mammalian models in toxicological research. So far, a series of studies have been performed on the toxicity from metal exposure in *C. elegans* (Leung et al., 2008). Among the examined toxicities in nematodes, reproductive toxicity is one of the ecologically relevant and sensitive ones for acute metal toxicity testing. A 72 h median effective concentration (EC50) derived for reproduction was explored to evaluate the reproductive toxicity from metal exposure in hermaphrodite nematodes (Donkin and Williams, 1995; Dhawan et al., 1999; Anderson et al., 2001; Boyd and Williams, 2003). Endpoints of brood size and generation time were further employed to assess

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doi:10.1016/j.ecoenv.2011.12.007

the reproductive toxicity from metal exposure in hermaphrodite nematodes (Swain et al., 2004; Roh et al., 2007; Wang and Wang, 2008a). The reproduction of hermaphrodite nematodes is sensitive to adverse effects of several toxic metals at micromolar concentrations and specific developmental stages (Guo et al., 2009). Moreover, our previous studies have suggested that the deficits in brood size and generation time induced by metal exposure can be largely transferred from exposed hermaphrodite nematodes to their progeny (Wang and Yang, 2007; Wang et al., 2007a, b; Wang and Wang, 2008b). Furthermore, it has been proven that stress response and oxidative stress are important factors in inducing the formation of severe toxicity from metal exposure in nematodes (Wang and Wang, 2008a; Lin et al., 2006; Ye et al., 2008; Wang et al., 2010; Wu et al., 2011). Nevertheless, it is still unclear whether the metal exposure will result in adverse effects on the male reproductive development and functions. Consequently, mechanisms explaining the possible induced severe reproductive toxicity on male nematodes from metal exposure are also still unknown.

Male formation is an important reproductive event for nematodes. The features that differentiate the male from the hermaphrodites arise during the postembryonic development, and the postembryonic cell lineages and development for adult male have already been described (Sulston et al., 1980). The formation of major male mating structures, consisting of the blunt tail with fan and rays, the hook, the spicules and proctodeum, and the thin body is just before the last molt (Nguyen et al., 1999). In *C. elegans*, males are very infrequent under normal physiological conditions, since the male formation results from an event that may be caused by chromosomal non-disjunction (Goldstein, 1986). Genes required for male development have been identified in genetic screens for defective males or among hermaphrodite developmental genes because of their pleiotropic effects on the male (Hodgkin, 1983; Shen and Hodgkin, 1988; Jiang et al., 2001; Lints and Emmons, 2002). Moreover, environmental cues, such as heat-shock, also play important roles in regulating sexual development (Chow and Chan, 1999). In the present study, we investigated the adverse effects of $HgCl_2$ exposure on male-specific structures and their functions in nematodes. Moreover, we examined the possible important roles of oxidative stress and insulin signaling in the formation of abnormal male-specific structures and functions in nematodes induced by Hg exposure.

2. Materials and methods

2.1. Reagents

Metal concentrations used in this study were referred to our previous descriptions (Wang and Xing, 2010; Wang et al., 2010). Ten measured concentrations of Hg solutions were used in the current work, and they were 0.1 μg/L, 1 μg/L, 2.5 μg/L, 5 μg/L, 10 μg/L, 0.5 mg/L, 9.8 mg/L, 19.3 mg/L, 29.5 mg/L, and 39.7 mg/L. The metal salt was $HgCl_2$ (purity, 99.9 percent). Metal concentrations of exposed solutions were analyzed by atomic absorption spectrophotometry (Model 4100ZL; PerkinElmer, Boston, MA, USA) equipped with a mercury hollow cathode lamp (Table 1). The reagent of 5′,6′-chloromethyl-2′,7′dichlorodihydro-fluorescein diacetate (CM-H2DCFDA) was purchased from Molecular Probes (Eugene, OR, USA). All the other chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA). The vitamin E was dissolved in ethanol, and then diluted into the working concentration of 200 μg/mL.

2.2. Strain preparation

Nematodes used in the present study were wild-type Bristol (N2), and mutants of CB4876 [*clk-1*(*e*2519)], CB1370 [*daf-2*(*e*1370)], and MQ513 [*daf-2*(*e*1370)*clk-1*(*e*2519)] with an increased resistance to oxidative stress, originally obtained from the *Caenorhabditis* Genetics Center (funded by the NIH National Center for Research Resource, USA). They were maintained on nematode growth medium (NGM) plates seeded with *E. coli* OP50 at 20 °C as described (Brenner, 1974). Gravid nematodes were washed off the plates, and age synchronous populations of early L2-larval and late L3-larval animals were obtained by the collection as described (Donkin and Williams, 1995). The collected nematodes were washed with modified K medium (50 mM NaCl, 30 mM KCl, 10 mM NaOAc, pH 5.5) (Williams and Dusenbery, 1990). Metal exposures at concentrations from 0.5 mg/L to 39.7 mg/L were performed on late L3-larval hermaphrodite nematodes in 12-well sterile tissue culture plates, and the exposures were 24 h long. Metal exposures at concentrations from 0.1 μg/L to 10 μg/L were performed on L1-larval hermaphrodite nematodes in 12-well sterile tissue culture plates, and the nematodes were used for the examination of endpoints when they developed into young adults. All exposures were carried out in 20 °C incubator in the presence of food.

Table 1
Measured concentrations of examined Hg solutions.

Measured concentration	Standard deviation (SD)
0.1 μg/L	0.3
1 μg/L	0.1
2.5 μg/L	0.2
5 μg/L	0.4
10 μg/L	0.1
0.5 mg/L	0.1
9.8 mg/L	0.2
19.3 mg/L	0.4
29.5 mg/L	0.3
39.7 mg/L	0.4

2.3. Male formation assay

When Hg exposed late L3-larval nematodes developed into adults, nematodes were transferred into NGM plates. The male nematodes were identified as previously described, and male nematodes have specific features of radically distinct male gonad full of sperm joined to a posterior cloaca rather than a mid-body vulva, a smaller, thinner body, and a differentiated tail with a cellular fan, rays, and a hook (Hodgkin, 1983; Shen and Hodgkin, 1988). To evaluate the ratio of male formation, the number of male nematodes in a population with an approximately 5000 nematodes was counted. Experiments were repeated three times.

2.4. Abnormal male-specific structures

Abnormal males were mainly identified to have abnormal male-specific structures, including cellular fan and rays, in the differentiated tail of metal exposed nematodes. To evaluate the percentage of abnormal males, approximately 50 males were picked into a NGM plate, and the number of abnormal male nematodes was counted. To ensure the consistent positions for taking pictures, the nematodes were sticked onto the dried agar pad. To ensure the consistent developmental stage, the male nematodes with the similar body size were selected. Experiments were repeated three times.

2.5. ROS (reactive oxygen species) determination

To quantify whether Hg treatment increases ROS levels in *C. elegans*, nematodes were transferred to 1 mL of M9 buffer containing 1 μmol/L CM-H2DCFDA and pre-incubated for 3 h at 20 °C. Nematodes were mounted on 2 percent agar pads and examined with a laser scanning confocal microscope at 488 nm of excitation wavelength and 510 nm of emission filter. The fluorescent figures of fans in tails were focused and collected. The relative fluorescence intensities of fans in tails were semi-quantified using the Adobe Photoshop software. The examined nematodes were male nematodes with abnormalities except for the control male nematodes. Nevertheless, the males without abnormalities induced by Hg exposure also had fluorescent signals, which were not so strong as detected in males with abnormalities induced by Hg exposure. The relative fluorescence intensities of fans in Hg exposed nematodes were normalized to the control nematodes. The control was CM-H2DCFDA treated male nematodes without Hg exposure.

2.6. Paraquat and vitamin E treatment

For paraquat pre-treatment, the method was performed largely as described (Oeda et al., 2001). Before Hg exposure, synchronous early L2-larval nematodes were treated with 2 mM paraquat for 6 h in the 12-well sterile tissue culture plates, and then further picked onto the normal NGM plates. For the vitamin E pre-treatment, the method was basically performed as previously described (Ye et al., 2008). Before Hg exposure, synchronous early L2-larval nematodes were treated