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Combined effect of temperature and zinc on *Caenorhabditis elegans* wild type and *daf-21* mutant strains

Yunbiao Wang [a,*,1], Anastasia N. Ezemaduka [b,1]

[a] Key Laboratory of Wetland Ecology and Environment, Northeast Institute of Geography and Agroecology, Chinese Academy of Sciences, Changchun 130102, China

[b] National Laboratory of Protein Engineering and Plant Genetic Engineering, School of Life Sciences, Peking University, Beijing 100871, China

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ABSTRACT

Heavy metal pollution in aquatic ecosystems is a far reaching environmental problem. The possible influences of heavy metal exposure and the potential harm to organisms when combined with other environmental stressors such as temperature have been largely unexplored. An aquatic toxicity test of *Caenorhabditis elegans* was performed to estimate the 24 h median lethal concentration (LC_{50}) of different zinc concentrations at different temperatures (15 °C, 20 °C, 25 °C, and 30 °C). We also examined the time course thermotolerance on wild type (N2) and *daf-21* null (JT6130) adults exposed to 6.1 mM zinc at 37 °C. Hsp90 protein expression level in response to the combined effect of temperature and zinc toxicity was also investigated by both Western blots and ELISA. Our results show that *C. elegans* wild type nematodes exhibit severe lethal toxicity after a 24 h exposure to zinc at higher temperatures. In addition, the expression level of Hsp90 was highly inhibited in adult worms subjected to zinc stress. This toxicity assay at different temperatures provides insight into organism response to combined effects of temperature and zinc toxicity.

1. Introduction

Zinc is one of the most important environmental and biotoxic metals (Yang et al., 2013). It is an essential trace metal for vertebrates, which becomes very toxic at high concentrations and causes adverse effects (Kaplanski et al., 2000). Temperature has been recognized as an important factor influencing biological systems at various levels of organization. Many organisms have evolved different behavioral, biochemical and molecular mechanisms to assist in their continuous adjustment to naturally occurring temperature variation (Wang et al., 2007). Although studies have been published which have attempted to investigate the possible interaction of temperature and heavy metal toxicity using adult invertebrates, we still lack studies elucidating the combined potential harm of temperature and metals (Wang et al., 2013).

Heat shock or stress proteins (Hsps) are highly conserved intracellular molecular chaperones, expressed in response to various stressors, including heavy metal exposure (Mayer, 2010). Hsps are classified into small molecular weight (14–43 kDa) and high molecular weight (40–110 kDa) (Ruell et al., 2009). They play key roles in in vivo proteostasis by assisting in the correct folding of proteins in the highly crowded cellular environment (Devaney et al., 2005; Gillan et al., 2009; Him et al., 2009). However, expression levels of specifically Hsp90 do not appear to be significantly affected by temperature in *Caenorhabditis elegans* (Devaney et al., 2005). A series of studies has demonstrated that *hsp90* is essential in parasitic and free-living nematodes (Him et al., 2009; Gillan et al., 2009). Notably, the gene encoding Hsp90 was identified as *daf-21*, one of many dauer formation (*daf*) genes in *C. elegans*. Dauer larvae in this organism are formed in response to unfavorable conditions such as high temperature, starvation, and overcrowding (Wang et al., 2009). The free-living soil nematode *C. elegans* is frequently used for toxicological studies in various exposures, and it is considered a useful animal model for the study of eco-toxicological relevance of chemical-induced responses (Leung et al., 2008).

Although metal toxicity studies involving *C. elegans* focus mainly on organism-level endpoints, such as mortality, behavior, growth, or reproduction (Leung et al., 2008; Lagido et al., 2009), studies elucidating the effects of temperature and metal stressors are still lacking. In the current study, in order to investigate the combined effects of zinc and temperature on the nematode *C. elegans*, we performed a toxicity test estimating the 24 h median lethal concentration (LC_{50}) and lethality endpoint at different

Abbreviations: Hsp90, 90-kDa heat shock protein; LC_{50}, lethal concentrations to 50%; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis.

* Corresponding author. Tel.: +86 431 85542266; fax: +86 431 85542298.
E-mail address: wangyb@neigae.ac.cn (Y. Wang).

[1] These authors contributed equally to this work.

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temperatures. Zinc(II) chloride was chosen as the test chemical due to its relevant abundance in the environment. We also investigated *C. elegans* Hsp90 protein expression levels in response to zinc exposures at various temperatures by Western blots and ELISA. Our findings suggest that Hsp90 could be indicative of zinc toxicity, and that the nematode *C. elegans* could serve as a biosensor model for Hsp90 stress related induced expression.

2. Materials and methods

2.1. C. elegans growth and maintenance

All experiments were performed using *C. elegans* strains N2 (wild type) and JT6130 (*daf-21* null) obtained from the *Caenorhabditis* Genetics Center (Minneapolis, MN, USA). Nematodes were grown on NGM (nematode growth medium) agar plates seeded with *Escherichia coli* strain OP50 as previously described (Brenner, 1974), and kept at 20 °C unless specified.

2.2. Zinc lethality assay

Synchronized eggs isolated from gravid hermaphrodites using a mild bleaching mixture (0.5N NaOH, 5% NaClO) were grown to young adult stage. Zinc chloride (pure analytical-grade, Sigma-Aldrich, St. Louis, MO, USA) was prepared in K-medium (0.032 M KCl and 0.051 M NaCl; Williams and Dusenbery, 1990). Each test consisted of five metal concentrations. Approximately 9–11 (average 10) day 3 adult N2 wild type and *daf-21* (JT6130) nematodes were transferred into 24-well microtitre plates containing 0.5 mL of the test solution. Worms were incubated at 15 °C, 20 °C, 25 °C, and 30 °C for 24 h. For each treatment, three replicates for each zinc concentration and control were conducted. Worms were scored dead by visual inspection and prodding with a platinum wire under a dissecting microscope (Wang et al., 2013). For time course survival, 50–60 day 3 adult N2 wild type and *daf-21* mutants (JT6130) were exposed to 6.1 mM zinc at 37 °C. Worms were counted for survival every 1 h. The time course survival percentage was scored using three independent replicates.

2.3. Hsp90 expression detected by Western blotting

Hsp90 induction was assessed in day 3 wild type adults after exposure to zinc chloride. Approximately 110 nematodes were incubated at 15, 20, 25 and 30 degrees centigrade for 24 h following exposure to different concentrations of zinc (0.061 mM, 0.61 mM, and 6.1 mM). The highest zinc concentration used was the LC_{50} (24 h) value determined in the lethal toxicity tests.

Worms were resuspended in ice cold extraction buffer (50 mM sodium phosphate [pH 7.2], 0.1 mM dithiothreitol, 1% Triton, and 0.1 mM phenylmethylsulfonyl fluoride) and homogenized on ice using chilled mortar and pestle. The homogenate was then centrifuged at $10{,}000 \times g$ for 30 min. Protein concentration was measured by the BCA assay (Pierce) according to the manufacturer's instructions.

Western blot analysis was performed according to established protocols (Wang et al., 2007, 2013). In brief, samples were first diluted 1:1 with loading buffer [112 mM Tris, 72% SDS (w/v), 5% mercaptoethanol (w/v), 5% glycerol (w/v), 0.005% bromophenol blue (w/v)], and boiled for 8 min. Protein extracts (100 μg) were separated by 10% SDS-PAGE (polyacrylamide gel electrophoresis), transferred to polyvinylidene difluoride (PVDF) membranes in a Semi Dry Transfer Unit (Bio-Rad) at 100 V for 90 min. Membranes were blocked overnight at 4 °C in blocking buffer (5% non-fat dry milk in TBS-T (Tris 20 mM pH 7.5, NaCl 150 mM, 0.05% (v/v) Tween-20)). After washing three times with TBST for 5 min each, blots were incubated for 1 h at room temperature with rabbit anti-Hsp90 polyclonal antibody (1:5000 dilution in blocking buffer). After another washing, membranes were incubated for 1 h at room temperature with secondary antibody horse anti-mouse IgG conjugated with alkaline phosphatase (1:5000 dilution in blocking buffer). Protein bands were detected with 5-bromo-4-chloro-3 indolyl phosphate toluidinium (BCIP) and nitroblue tetrazolium chloride (NBT). The values of Hsp90 band intensities were determined with the KODAK Image Station 4000R Digital Imaging System. The relative densities of bands were calculated as a percentage of the control at 20 °C.

2.4. Hsp90 enzyme-linked immunosorbent assay (ELISA)

Hsp90 protein expression in wild type *C. elegans* was analyzed using the classical sandwich enzyme-linked immunosorbent assay (ELISA) performed in 96-well microplates. The 96-well microtiter plates were coated with 100 μL of worm protein samples ($10\ \mu g\ mL^{-1}$), then covered with foil and incubated at 4 °C overnight. After washing three times with 200 μL TBST containing 0.05% (v/v) Tween-20 each, plates were blocked with 100 mL blocking buffer (5% bovine serum albumin in PBS) and incubated in 37 °C water bath for 1 h. After all unbound substances were removed by washing, 100 μL of anti-Hsp90 polyclonal antibody 1:5000 was added to the wells followed by incubation for 1 h in 37 °C water bath. After another wash, goat anti-rabbit IgG conjugated with horseradish peroxidase (IgG-HRP) 1:15,000 was added to the wells and incubated in 37 °C water bath for 1 h. After the final wash, TMB microwell peroxidase substrate was added to the wells and incubated for 15 min in the dark. The reaction was stopped with 50 μL of $2\ mol\ L^{-1}$ of phosphoric acid (H_2SO_4). The color intensity was measured at 450 nm with a microtiter plate reader (Bio-Rad Model 550™). Each sample was independently replicated at least three times to obtain an average. The OD value of the differences between the sample wells and control wells (no sample added) represented the level of Hsp90 protein expression in the sample. Control (no zinc) at 20 °C was taken as a reference to calculate the relative value of the amount of Hsp90 expression.

2.5. Statistics

The median lethal concentrations value (LC_{50}) from each replicate was generated using log-transformed data and is reported as the mean value of three replicates. Standard deviations and coefficients of variation were also calculated for individual LC_{50} values. All data tested were found to conform to assumptions of equal variance and normality, and expressed as mean ± SEM. Student's *t*-test was applied to study the relationship between exposures at different temperatures. A *P*-value less than 0.05 was considered to be significant. All statistical analyses were conducted using SPSS 12 (SPSS, Chicago, IL, USA).

3. Results

3.1. Combined effect of temperature and Zn^{2+} on 24 h LC_{50} values

To determine the effect of temperature and zinc stress on *C. elegans* wild type and *daf-21* mutants, we subjected both strains to zinc stress at different assayed temperatures. An 24 h LC_{50} on wild type adults exposed to 6.12 mM zinc (95% confidence interval: 3.98–11.60 mM) at 20 °C was obtained using Probit analysis (Fig. 1). A decrease in the LC_{50} value, indicating higher toxicity, was observed in wild-type adults at low zinc concentrations at 25 and 30 °C. Significant differences were detected in the LC_{50} values of