Ecotoxicology and Environmental Safety 87 (2013) 80–88

Contents lists available at SciVerse ScienceDirect

Ecotoxicology and Environmental Safety

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

Selenium (sodium selenite) causes cytotoxicity and apoptotic mediated cell death in PLHC-1 fish cell line through DNA and mitochondrial membrane potential damage

Vellaisamy Selvaraj, Justin Tomblin, Mindy Yeager Armistead, Elizabeth Murray *

Department of Integrated Science and Technology, Marshall University, Huntington, WV 25755, USA

ARTICLE INFO

Article history:
Received 25 July 2012
Received in revised form
26 September 2012
Accepted 28 September 2012
Available online 13 November 2012

Keywords:
Selenite toxicity
PLHC-1
Apoptosis
Reactive oxygen species
Mitochondrial membrane potential

ABSTRACT

Elevated concentration of selenium poses a toxic threat to organisms inhabiting aquatic ecosystems influenced by excessive inputs from anthropogenic sources. Selenium is also an essential micronutrient in living things, particularly in fish, and provides antioxidant properties to tissues. Whole fish and hepatocytes in primary culture show selenite toxicity above threshold levels. The present study was designed to investigate the process by which selenite exposure causes cellular toxicity and apoptotic and necrotic cell death in fish hepatoma cell line PLHC-1. PLHC-1 cells were exposed to various selenite concentrations (1, 10, 50 and100 μM) for 10, 20 and 40 h intervals. The 24 h inhibitory concentration 50 (IC_{50}) of selenite in PLHC-1 cell line was found to be 237 μM. Flow cytometery data showed that selenite exposed cells promote apoptotic and necrotic mediated cell death when selenite concentrations were $\geq$ 10 μM compared to control. Selenite exposure was associated with a significant increase of caspase-3 activities suggesting the induction of apoptosis. Selenite exposure at high levels ($\geq$ 10 μM) and longer exposure times ($\geq$ 20 h) induces mitochondrial membrane potential damage ($\Delta\Psi_m$), DNA damage and elevated production of ROS which could be associated with cell death.

1. Introduction

Selenium (Se) is a naturally occurring metalloid that is an essential element required for the health of humans and other animals, including fish. Selenium has a narrow range between levels required for organismal health and toxic levels (NRC, 1980). The toxicity of selenium in aquatic environments has drawn much attention due several high profile examples (Chapman et al., 2009). Selenium exposure in freshwater fish has been linked to gill damage, increase in lymphocytes and anemia, reproductive failure, teratogenic deformities and pathological alterations in liver, kidney, heart and ovary (Lemly, 2002). Selenium is found in a variety of forms (inorganic, organic and elemental) which vary greatly in toxicity (Lemly, 2002). In recent years, coal combustion wastes have drawn attention as sources of selenium in aquatic systems (Reash, 2012). Wastewater from these processes may contain elevated selenium. Selenium can also be mobilized through anthropogenic activities such as mining of coal, phosphate and metals (Hamilton, 2004; Palmer et al., 2010). These can expose selenium rich strata to greatly enhanced leaching and subsequent transport (Lindberg et al., 2011). In aquatic ecosystems, inorganic selenium is rapidly and efficiently assimilated by primary producers (bacteria, fungi and plant) and transformed into organic selenium species. These organic selenium species are transferred throughout the food web via the diet to primary and secondary consumers invertebrate and vertebrate (Chapman et al., 2009). Oviparous (egg-laying) vertebrates, such as fish and birds, are the most sensitive organisms to elevated selenium concentration ranging from 10 to 50 μg/l in the aquatic ecosystem (Hamilton, 2004).

Selenium may replace sulfur in selenoamino acids (selenomethionine, selenocysteine, selenocystine) and is incorporated into vital selenoproteins such as glutathione peroxidase which are essential for normal growth and development (Maier and Knight, 1994). Selenium is characterized as an antioxidant, since selenium is part of the active site of glutathione peroxidases, which catalyze the conversion of hydrogen peroxide to water (Victoria and Stefanos, 2010). However there is also research evidence that both organic and inorganic selenium exposure increase oxidative stress (Seko and Imura, 1997; Shen et al., 1999; Wycherly et al., 2004; Misra and Niyogi, 2009). Spallholz and Hoffman (2002) suggested that reactive

Abbreviations: MTT, (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; IC_{50}, inhibitory concentration; JC-1 dye, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethyl-benzamidazolocarbocyanin iodide; DEVD-AFC, 7-amido-4-methylcoumarine derivatives; GFP, green fluorescent filter; DMSO, dimethyl sulfoxide; μM, micromolar; nM, nanomolar; ANOVA, analysis of variance; LC_{50}, lethal dose concentration

* Corresponding author. Fax: +11 304 696 6533.
E-mail address: murraye@marshall.edu (E. Murray).

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http://dx.doi.org/10.1016/j.ecoenv.2012.09.028

oxygen species (ROS) play a significant role in inducing selenium toxicity in birds exposed to high levels of selenium. The accumulating free radicals can oxidize the cellular membrane and macromolecules, resulting in the loss of structural integrity of the cell, and ultimately lead to the cell death (Kelly et al., 1998). The selenium induced cell death can occur due to the induction of either apoptosis or necrosis (Shen et al., 1999; Weiller et al., 2004). However, many of these cellular effects depend on selenium concentration and chemical form, and results of in vitro studies could help to delimitate those conditions in which toxicity becomes evident.

In vivo bioassays have long been used as a test system for studying the toxicity of selenium compounds. The acute lethality test has been used to characterize the toxicity of selenium in several fish species (Adams, 1976) as LC_{50}, the dose of selenium fatal to 50 percent of the individuals exposed. In the aquatic environment, fish have been proven to be valuable test species to study the effects of selenium uptake and bioaccumulation on metabolic activities and other functions. It is challenging to use whole fish in metabolic studies. Such experiments can be inconvenient, time consuming, expensive, difficult to reproduce, and require sacrificing organisms (Wang et al., 2004). Additionally, measurable endpoints in whole organism evaluations, such as condition indices, may be less sensitive than cellular changes. Cell-based assays are a sensitive and convenient method of identification cellular repose to toxins. The use of in vitro assays in ecotoxicological studies provides the opportunity for extrapolation from in vitro to in vivo systems, while generating information on biological responses at a relatively high level of biological organization (Castano et al., 2003). In vitro studies can assess early cellular responses to selenium and may predict organism level effects. In vitro assays have been developed to serve as an alternative, or a supplementary bioassay, for toxicity ranking of chemicals (Fent, 2001).

Fish liver is a major target organ of selenium toxicity (Sato et al., 1980), since selenium can accumulate in the liver to a moderately high level and mediate its effect in a dose dependent manner. Misra and Niyogi (2009) demonstrated that primary cultures of trout hepatocytes experienced oxidative stress and increased cell death at high levels of selenite (100–200 μM). The purpose of the present study was to characterize selenite induced cellular toxicity, as evidenced by DNA damage as DNA damage is one of the hallmarks of apoptotic or necrotic mediated cell death, mitochondrial membrane potential changes and apoptosis, and to relate in vitro effects to those demonstrated by in vivo systems. We used *Poeciliopsis lucida* hepatoma cell line (PLHC-1) as a test system to evaluate selenite induced cellular toxicity resulting from selenite exposure by measuring DNA damage and apoptotic and necrotic cell death using a variety of assays. This fish cell line was chosen for four reasons: (i) This cell line retains liver cell properties, providing sensitivity to a range of chemicals (Pichardo et al., 2005), (ii) it is more sensitive to chemicals than other commonly used fish cell lines such as RTG-2 and RTL-21 (Caminada et al., 2006), (iii) unlike RTG-2 and RTL-21, which require refrigerated temperatures for propagation, this cell line thrives readily at room temperature (Caminada et al., 2006) and (iv) the PLHC-1 cell line has proven a versatile test system for evaluating cytotoxic effects following exposure to various reference agents. Several techniques were used to evaluate the cellular toxicity of selenite including (i) MTT and Neutral Red uptake assays to determine the cell proliferation rate, (ii) cytosolic lactate dehydrogenase activity to monitor the cell membrane integrity, (iii) comet assay to evaluate the DNA damage, (iv) caspase-3 activity, TUNEL assay and flow cytometry to assess and confirm the apoptosis mediated cell death, (v) DCFH-DA and JC-1 staining to determine the production of reactive oxygen species and changes in mitochondrial membrane potential ($\Delta\Psi_m$) damage.

2. Materials and methods

2.1. Materials

Sodium selenite (Na_2SeO_3) was purchased from Sigma Chemicals; JC-1 Mitochondrial Membrane Potential Detection Kit from Cell Technology (Mountain View, CA, USA); OxiSelect™ ROS Assay Kit from Cell Bio Labs, Inc. (San Diego, CA, USA); Caspase-3 Fluorometric Assay Kit, LDH leakage assay kit and TUNEL assay kits from Bio Vision Research Products (Mountain View, CA, USA); Comet assay™ kit from Trevigen, Inc (Gaithersburg, MD, USA); Media and other supplements from ATCC (Manassas, VA, USA); Serum from Atlanta Biological (Atlanta, GA, USA); Bio-Rad Protein Assay Reagent (Hercules, CA, USA). All other tissue culture products were purchased from BD (Franklin Lakes, NJ, USA).

2.1.1. Cell line

PLHC-1 cells (ATCC CRL-2406) were grown in 25 cm^2 cell culture flasks at 30 °C with 5 percent CO_2 in minimum essential medium containing 2 mM/L-glutamine, 1 percent Pen/Strep (10,000 units penicillin and 10 mg streptomycin/ml) and supplemented with 5 percent of fetal bovine serum.

2.2. Determination of cytotoxicity dose 50 (IC_{50} of selenite)

At first, we determined 50 percent inhibitory concentration of selenite (IC_{50}) by MTT assay (Mosmann, 1983). Briefly, concentrated stock solution of selenite (10 M) was prepared each time in sterile PBS. Thereafter stock solution was serially diluted in PBS to desired concentrations and added to growth media. Cells were first seeded in 24-well plates and propagated to 80 percent confluence. Once reaching the desired density, the culture medium was replaced with the fresh medium containing different concentration of selenite (0–1000 μM), and the cells were incubated for another 24 h. Cell survival (percent) was calculated three times in independent experiments using six wells per concentration. The 24 h IC_{50} of selenite was determined by curve fitting (nonlinear regression) using Graph Pad Prism software and found to be 237 μM.

2.3. Propagation of PLHC-1 cells under experimental conditions

To investigate the time dependent effects of selenite on functional assays, the PLHC-1 cells (1×10^5/ml) were grown in 24, 12 and 6 wells tissue culture plates for 2 or 3 days until 70–80 percent confluent. Once the desired cell density was reached, the culture medium was replaced with the fresh medium containing different doses of selenite (0–100 μM) for intervals of 10, 20 and 40 h. For functional assays evaluation, the selenite concentration was selected on the basis of IC_{50} value. Negative control cells were treated with media and 100 μl PBS.

2.3.1. Determination of dose- and time-dependent cytotoxicity of selenite

2.3.1.1. Cell proliferation study. Cells were seeded in 12-well plates, grown to 80 percent confluence and treated with various concentration of selenite at various time points as mentioned in the experimental conditions. The cell proliferation was assessed based on MTT assay (Mosmann, 1983). Percentage of cell survival was determined three times in independent experiments using six wells per concentration.

2.3.1.2. Neutral red uptake assay. The neutral red uptake (NR) assay is based on the accumulation of neutral red (2-methyl-3-amino-7-dimethyaminophenanzine0 dye in lysosomes of viable cells (Borenfreund and Puerner, 1985). Percentage of cell surviving was determined three times in independent experiments using six wells per concentration.

2.3.1.3. Lactate dehydrogenase (LDH) assay. Cytotoxicity induced by selenite was assessed by lactate dehydrogenase (LDH) leakage into the culture medium following the method of Fotakis and Timbrell (2006) with modifications below. Following exposure to selenite concentrations, culture medium was aspirated and centrifuged at 2000 rpm for 10 min to obtain a cell free supernatant. LDH activity in medium was estimated by conversion of lactate to pyruvate using a commercially available kit from Bio-Vision (Mountain View, CA, USA). The percentage of injured cells in the total cell population was determined in three independent experiments using six wells per concentration.

2.4. Evaluation of mitochondrial membrane potential damage ($\Delta\psi_m$)

The extent of mitochondrial membrane potential damage ($\Delta\Psi_m$) was determined by using JC-1 dye, both quantitatively and qualitatively, (Cell Technology, Mountain View, CA, USA) according to the manufacturer's specification. Briefly, media was aspirated from the cells from each plate (selenite treated and control