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Mercury-induced dysfunctions in multiple organelles leading to cell death

C.S. Vergilio [a], C.E.V. Carvalho [b], E.J.T. Melo [a,*]

[a] Laboratório de Biologia Celular e Tecidual, Centro de Biociências e Biotecnologia, Universidade Estadual do Norte Fluminense, 28013-602 Campos dos Goytacazes, RJ, Brazil
[b] Laboratório de Ciências Ambientais, Centro de Biociências e Biotecnologia, Universidade Estadual do Norte Fluminense, 28013-602 Campos dos Goytacazes, RJ, Brazil

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ABSTRACT

Mercury (Hg) is a highly toxic metal that can exert multiple adverse effects, ultimately leading to cell death. Before causing death, the Hg enters the cells and affects diverse intracellular targets. The present study aimed to investigate the structure and function of several organelles or cellular structures, including mitochondria, acidic compartments and vesicles, endoplasmic reticulum elements and microfilaments, following Hg exposure of a human hepatic cell line (HuH-7 cells) to examine the sequence and coordination of the events associated with Hg-induced cell death. Hg exposure led to a progressive decrease in cell viability and induced alterations in cell morphology including cytoplasmic shrinkage and nuclear fragmentation. Hg treatment (10 μM for 12 h) affected multiple intracellular targets simultaneously. These included loss of mitochondrial functionality, pronounced cytoplasmic acidification and dysfunctions in the cytoskeleton and endoplasmic reticulum. This overall Hg-induced toxicity in the human hepatocyte cell line (HuH-7 cells) led to cell death through both apoptosis and autophagy.

1. Introduction

Mercury (Hg) is a highly toxic metal that can induce severe adverse effects (Liao et al., 2006). Among the organs, the liver is the main site of metal toxicity due to its central role in physiological metabolism and its involvement in various detoxification reactions (Hinton et al., 2008). Hepatocytes are the main cellular type present in the liver and exert important functions in the liver injury response (Malhi et al., 2010).

Hg effects result from its high affinity for thiol groups, which are present in important biomolecules as constituents of proteins, transcription factors, and nucleic acids (Fabbri et al., 2012; Syversen and Kaur, 2012). This strong reactivity with distinct molecules is reflected by the capacity of Hg to disturb a variety of cellular and biochemical processes (Vallee and Ulmer, 1972). However, due to the complexity and diversity of the events associated with the cell–Hg interactions, the wide-ranging effects induced in the cellular organelles and their involvement in cell death remain incompletely understood.

Hg treatment induced apoptosis (Araragi et al., 2003; Carranza-Rosales et al., 2005; Duncan-Achanzar et al., 1996; Park and Park, 2007; Shenker et al., 2000) and necrosis (Chen et al., 2010; Duncan-Achanzar et al., 1996; Kuo and Lin-Shiau, 2004) in different cell types. Recently, some reports have demonstrated the occurrence of autophagy following Hg exposure (Chang et al., 2013; Chatterjee et al., 2013b, 2012; Zhang et al., 2014). Several studies implicated the mitochondria as an organelle target for Hg-induced cell death. Importantly, mitochondrial dysfunction is the main source of reactive oxygen species (ROS), which can trigger various adverse effects in cellular processes (Bertin and Averbeck, 2006; Gobe and Crane, 2010; Wang et al., 2004). However, the roles of other cellular organelles in the Hg toxicity process have been neglected.

The present study aimed to investigate the structure and function of several organelles and cellular structures, including mitochondria, acidic organelles and vesicles, endoplasmic reticulum elements and microfilaments, following Hg exposure in a human

Abbreviations: AO, acridine orange; DAPI, 4′,6-diamidino-2-phenylindole; $DiOC_6$, 3,3′-dihexyloxacarbocyanine iodide; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; MDC, monodansylcadaverine; LTR, LysoTracker Red; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NR, neutral red; PMA, Phorbol 12-myristate 13-acetate; ROS, reactive oxygen species; RP, rhodamine phalloidin; YP, YO-PRO-1.

* Corresponding author at: Universidade Estadual do Norte Fluminense, Centro de Biociências e Biotecnologia, Laboratório de Biologia Celular e Tecidual, Av. Alberto Lamego, 2000, Parque Califórnia, CEP 28013-602 Campos dos Goytacazes, RJ, Brazil. Tel.: +55 22 2739 7175.

E-mail addresses: cristianevergilio@uenf.br, cristianesvergilio@gmail.com (C.S. Vergilio), carvalho.cev@gmail.com (C.E.V. Carvalho), ejtm1202@gmail.com (E.J.T. Melo).

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hepatic cell line (HuH-7 cells). The evaluation of the metal-induced damage in these intracellular compartments is important in understanding the sequence and cooperative events associated with Hg-induced cell death.

2. Materials and methods

2.1. Materials

Medium (DMEM-1152), mercury chloride ($HgCl_2$), MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), rhodamine 123 (R123), acridine orange (AO), monodansylcadaverine (MDC), $DiOC_6$ (3,3′-dihexyloxacarbocyanine iodide), anti-LC3 and anti-Rabbit IgG-FITC antibodies, Phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was from Gibco (Grand Island, NY, USA). LysoTracker Red (LTR), rhodamine phalloidin (RP) and YO-PRO-1 were obtained from Molecular Probes (Eugene, OR, USA).

2.2. Cell culture

HuH-7 cells (a human hepatic carcinoma cell line) were grown in Dulbecco's modified Eagle's medium (DMEM-1152) supplemented with 10% fetal bovine serum (FBS) in a humidified atmosphere containing 5% CO_2 at 37 °C. The cells were plated at a density of 10^5 cells/mL and allowed to attach for 24 h before the Hg treatments.

2.3. Hg treatments

For the Hg toxicity assays, a stock solution (0.1 M $HgCl_2$) was prepared using ultra-pure quality water, and dilutions with culture medium were made to 1 μM, 5 μM, 10 μM, 15 μM and 20 μM final concentrations. To observe the progression of the Hg-induced damage, the cell cultures were incubated with these concentrations for 2, 6, 12 and 24 h.

2.4. Cell viability assessment through MTT assay

Following each Hg treatment, the cells were incubated with the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution (6 mg/mL) in medium for 4 h at 37 °C (Mosmann, 1983). After the removal of the MTT-containing medium, 200 μL of dimethylsulfoxide (DMSO) was added for 5 min, and the absorbance at 540 nm was measured in a microplate reader (Thermoplate© TP reader). The results are expressed as the means ± standard deviation of three independent experiments.

2.5. Morphological analysis of Hg effects

The control and Hg-exposed cells were fixed in Bouin's solution and stained with Giemsa (10%) for observation by light microscopy. All preparations were examined using a Zeiss Axioplan light microscope equipped with 20× and 40× objectives. Digital images were obtained using Olympus Cell Imaging software (Münster, NW, DE).

2.6. Hg detection in cell culture medium

Following each Hg treatment, the culture medium was collected and frozen at −20 °C until Hg analysis. The procedure for total Hg extraction was performed according to methodology described by Bastos et al. (1998). Analytical control blanks were prepared for each group of 10 samples. The determination was performed using ICP-AES (Varian, Liberty II model) with a cold vapor accessory (VGA-77).

2.7. Apoptosis detection

YO-PRO-1 dye (YP-1) was used for detection of apoptosis. This dye is taken up only by the apoptotic cells and is excluded by the viable cells (Idziorek et al., 1995; Plantin-Carrenard et al., 2003). YO-PRO-1 (1 μM) was added to the HuH-7 cell cultures for 30 min for subsequent observation in a Zeiss Axioplan fluorescence microscope using a fluorescein excitation filter (485 nm). The incidence of apoptosis was analyzed by determining the percentage of YO-PRO-1 positive in approximately 300 cells for each condition. The results are expressed as the means ± standard deviation of three independent experiments.

2.8. Observation of DNA fragmentation

DAPI (4′,6-diamidino-2-phenylindole) staining was used to evaluate the occurrence of DNA fragmentation following Hg treatment. Formaldehyde-fixed control and Hg-treated cells (10 μM for 12 h) were stained with the DAPI solution (300 nM) for 3 min for subsequent observation in a Zeiss Axioplan fluorescence microscope using a UV excitation filter (350 nm).

2.9. Scanning and transmission electron microscopy (SEM and TEM)

Control and Hg-treated cells (10 μM for 12 h) were fixed in 2.5% (v/v) glutaraldehyde and 4% (v/v) formaldehyde in 0.1 M cacodylate buffer (pH 7.2). For SEM preparations, the samples were washed, dehydrated with a graded series of ethanol, critical-point dried in CO_2, positioned on a specimen holder and sputtered with gold. All micrographs were recorded using a Zeiss Evo 40 microscope employing secondary electrons. For TEM, the fixed samples were post-fixed with (1:1) 1% osmium tetroxide and 0.8% potassium ferricyanide, dehydrated with acetone and embedded in Epon. Ultra-thin slices (70 nm) were obtained with a Leica Reichert Ultracut S ultramicrotome, contrasted with uranyl acetate (5%) and lead citrate and observed using a Zeiss 900 transmission electron microscope.

2.10. Autophagy detection

The selective marker monodansylcadaverine (MDC) and the anti-LC3 antibody were used for autophagic vacuole detection. For MDC staining, the cells were incubated with 0.05 mM MDC in PBS for 10 min for subsequent observation in a Zeiss Axioplan fluorescence microscope using a UV excitation filter (350 nm) (Biederbick et al., 1995). The incidence of autophagy was analyzed determining the percentage of MDC-positive autophagic vacuoles in approximately 300 cells for each condition. The results are expressed as the means ± standard deviation of three independent experiments.

For LC3 localization, formaldehyde-fixed cells were permeabilized with 0.1% Triton-X. The cells were first incubated with a 1:20 dilution of anti-LC3 primary antibody for 60 min followed by the incubation with a 1:10 dilution of anti-Rabbit IgG-FITC secondary antibody for 70 min and subsequently observed in a Zeiss Axioplan fluorescence microscope using a fluorescein excitation filter (485 nm).

2.11. Mitochondria, acid compartments and lysosome staining

For the assessment of the mitochondrial function, the control and Hg-exposed cells were incubated with rhodamine 123 (10 μg/mL) for 30 min in 5% CO_2 at 37 °C (Johnson et al., 1980).