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Superoxide anion generation and oxidative stress in methylmercury-induced endothelial toxicity *in vitro*

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ABSTRACT

Emerging evidence has pointed to mercury exposure as a risk factor for hypertension, atherosclerosis, myocardial infarction and coronary heart disease. However, the underlying mechanisms are not well understood. This study investigated potential toxic effects of low concentrations of methylmercury (MeHg) in cultured bovine aortic endothelial cells (BAECs) and the possible involvement of reactive species, particularly superoxide anion, in mediating such toxicity. MeHg treatment increased the oxidation of 2′,7′-dichlorodihydrofluorescein diacetate (a general probe for reactive species) and dihydroethidium, a specific probe for superoxide anion. MeHg-induced 2′,7′-dichlorodihydrofluorescein diacetate and dihydroethidium oxidations were significantly decreased by apocynin, an inhibitor of the enzyme NADPH oxidase, which represents a main source of superoxide anion in endothelial cells. MeHg treatment significantly disrupted mitochondrial membrane potential and this event was also reversed by apocynin. MeHg treatment also decreased glutathione levels and this event preceded glutathione peroxidase inhibition, which was observed only at 24 h after treatment. These results indicate that MeHg induces oxidative stress in cultured BAECs and that this event is related to the production of superoxide anion. Moreover, the observed protective effects of apocynin in BAECs suggest the potential involvement of NADPH-oxidase in MeHg-induced endothelial dysfunction, which represents a pivotal event in most cardiovascular diseases.

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1. Introduction

Mercury (Hg) is a hazardous metal present in the environment due to either natural or anthropogenic sources. Hg is present in nature in different chemical forms, such as inorganic (*i.e.*, Hg(0), Hg(I), Hg(II)) and organic (*i.e.*, methylmercury and ethylmercury) mercurials (Clarkson, 2002). Methylmercury (MeHg) is a widespread and highly toxic Hg compound that results from the methylation of inorganic Hg in a reaction catalyzed mainly by aquatic microorganisms. In the aquatic environment, MeHg presents an enormous biomagnification potential, being accumulated by more than seven orders of magnitude from sub ng/L concentrations in water to over 1 mg/kg in piscivorous fish (Hintelmann, 2010). Dietary intake of MeHg, through ingestion of contaminated fish and seafood, has been recognized as a significant public health concern (Clarkson et al., 2003; Roman et al., 2011).

In 2000, the National Research Council's Committee (NRC, 2000) issued a report on the toxicological effects of MeHg, in which it revised the various adverse health effects associated with the exposure to this compound. MeHg-induced neurotoxicity, especially in fetuses and children, is the most prominent and well documented health effect. However, there is growing evidence pointing to the potential toxic effects of MeHg toward the cardiovascular system. In fact, MeHg has been associated with hypertension, carotid atherosclerosis, myocardial infarction and coronary heart disease (Salonen et al., 1995, 2000; Virtanen et al., 2005; Houston, 2007; Bautista et al., 2009; Choi et al., 2009). In a case-control study conducted by Guallar et al. (2002), the toenail Hg levels were directly associated with the risk of myocardial

Abbreviations: BAECs, bovine aortic endothelial cells; DCFH-DA, 2,7-dichlorofluorescein acetate; DHE, dihydroethidium; DMEM, Dulbecco's Modified Eagle medium; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; FCCP, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; HBSS, Hank's Balanced Salt Solution; Hg, mercury; IMT, intima media thickness; IP, Propidium Iodide; JC-1, tetraethylbenzimidazolylcarbocyanine iodide; LDH, lactate dehydrogenase Lactate dehydrogenase; MeHg, methylmercury; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NADPH, β-Nicotinamide adenine dinucleotide phosphate reduced; $O_2^{\cdot-}$, Superoxide anion; RS, reactive species; t-buOOH, tert-Butyl hydroperoxide.

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infarction. In Faroese whaling men, toenail and hair mercury levels were significantly associated with increased carotid intima media thickness (IMT) and hypertension (Choi et al., 2009). Similarly, a study from Salonen et al. (2000) found a significant association between mean IMT and hair Hg content in adult men. More recently, it was reported that Hg levels in the blood may be associated with an increased risk of hypertension and myocardial infarction or angina in the Korean population (Kim et al., 2014). Although the mechanisms mediating MeHg-induced cardiovascular toxicity are not fully understood, experimental evidence has shown that MeHg causes hypercholesterolemia (Moreira et al., 2012) and induces direct hazardous effects toward endothelial cells (Hirooka and Kaji, 2012).

Based on experimental *in vitro* and *in vivo* protocols, we have observed that oxidative stress and metabolic deficits represent important events mediating MeHg-induced neurotoxicity (for a review, see Farina et al., 2011). In addition, we have observed that MeHg decreases the activity of peroxidases (Farina et al., 2009; Franco et al., 2009) and the levels of reduced glutathione (GSH) (Franco et al., 2006; Stringari et al., 2008), which in turn leads to increased levels of hydrogen peroxide (Franco et al., 2007) and occurrence of lipid peroxidation and oxidative stress-related cytotoxicity (Farina et al., 2009). Based on the crucial role of oxidative stress in mediating MeHg-induced neurotoxicity, we hypothesized that oxidative stress could also mediate MeHg-induced endothelial toxicity. Specifically, we investigated the potential formation of superoxide anion in MeHg-treated BAECs. Our findings provided first evidence that exposure to low MeHg concentrations increased superoxide anion generation and decreased the mitochondrial membrane potential in cultured BAECs. Moreover, these events were blunted by cotreatment with apocynin, a NADPH inhibitor.

2. Materials and methods

2.1. Chemicals and reagents

2,7-dichlorofluorescein acetate (DCFH-DA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), β-Nicotinamide adenine dinucleotide phosphate sodium salt reduced form, apocynin, ascorbic acid, carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), dimethyl sulfoxide (DMSO), dihydroethidium (DHE), glutathione reductase from baker's yeast, hydrogen peroxide, probucol, propidium iodide, reduced glutathione and tetraethylbenzimidazolylcarbocyanine iodide (JC-1) were purchased from Sigma (St. Louis, MO, USA). Dulbecco's Modified Eagle medium (DMEM), fetal bovine serum (FBS), L-glutamine, HEPES and penicillin and streptomycin were obtained from GIBCO (Carlsbad, CA, USA).

2.2. Cell culture

Bovine aortic endothelial cells (BAECs) were obtained from Genlantis (Torreyana, San Diego, CA, USA). BAECs provide a cellular model to study arterial cardiovascular events, such as endothelial homeostasis and dysfunction, and to investigate the mechanisms underlying cardiotoxicity of compounds (Panus et al., 1993). All endothelial cells were characterized positive for uptake of acetylated LDL. Cells were cultivated in DMEM supplemented with 2 mM glutamine, 10 mM HEPES, 100 units/ml penicillin, 100 μg/ml streptomycin and 10% fetal bovine serum (FBS) at 37 °C, in a humidified atmosphere of 5% CO_2. Cells were subcultured at confluences and used between the fourth and fourteenth passage.

2.3. Cell viability assays

Cell viability was measured by two different assays, that evaluate different events of the cellular homeostasis. Cells were plated onto a 96-well plate at equal density (1.0×10^4 cells/well) in DMEM medium. After 24 h, cells were treated with increasing concentrations of MeHg (0.1 to 10 μM) for 24 h, at 37 °C. The reduction of 3-(4, 5-dimethylthiazol-2-yl)-2, 5- diphenyl-tetrazolium bromide (MTT) assay, which assesses the activity of labile mitochondrial dehydrogenases, was conducted as described by Mosmann (1983). Lactate dehydrogenase (LDH) release, that evaluates plasma membrane integrity, was measured according to previously described protocols (Farina et al., 2009). In addition to LDH assay, the Propidium Iodide (PI) staining of nuclei was used as an indicator of a loss in cell viability. Briefly, cells was incubated with PI probe (0,147 mg/ml) for 25 min at 37 °C. The PI staining intensity was measured in a fluorometric microplate reader with excitation at 535 nm and an emission at 617 nm. Results of MTT assays were expressed as percentage of control values (nontreated cells) and results of LDH and PI assays were expressed as percent of LDH released and PI staining, respectively, where the 100% value represents control cells treated with 2% Triton X-100 for 15 min.

2.4. Measurement of general reactive species (RS) production

Intracellular RS production was detected using the nonfluorescent cell permeating compound, 2′, 7′ -dichlorodihydrofluorescein diacetate (DCFH-DA) (Ali et al., 1992). Once inside the cells, DCFH-DA is hydrolyzed by cellular esterases to form DCFH, which is trapped intracellularly due to its membrane impermeability. DCFH reacts with intracellular RS (oxygen and nitrogen species) to form the fluorescent product 2′,7′-dichlorofluorescein (DCF). Cells were plated onto 12-well plates at equal density (1.0×10^5 cells/well) and treated with MeHg (1 μM) or vehicle for 1, 3 or 6 h. In another set of experiments, cells were pretreated with apocynin (300 μM), ascorbic acid (200 μM), probucol (10 μM) or vehicle for 1 h and thereafter challenged with MeHg (1 μM) for 6 h at 37 °C. Hydrogen peroxide (1 mM) was used as a positive control. After the indicated treatments, cells were loaded with DCFH-DA (3 μM) for 30 min at 37 °C in Hanks' balanced salt solution (HBSS). Cells were washed tree times with HBSS, harvested using trypsin and collected for immediate determination of RS generation by flow cytometry (FACS Canto II, BD Bioscience, USA) (Hort et al., 2014). Results were expressed as the percentage of control (non-stimulated cells; 100%) fluorescence intensity.

2.5. Measurement of superoxide anion production

Superoxide anion production in MeHg-treated cells was determined by using dihydroethidium (DHE). This assay is based on the reduction of ethidium by superoxide anion to a fluorescent compound ethidium (Bindokas et al., 1996). Cells were plated into 24-well plates at equal density (5.0×10^4 cells/well) and treated with MeHg (1 μM) or vehicle for 1, 3 or 6 h. In some experiments, cells were pre-treated with apocynin (300 μM) for 1 h and then stimulated with MeHg (1 μM) for 6 h. After the treatment, the media was removed and cells were incubated with DHE (10 μM) for 30 min at 37 °C. Then, cells were whased with HBSS and celullar fluorescense was recorded with excitation at 535 nm and emission at 610 nm in a fluorimetric microplate reader (Tecan, Mannendorf, Switzerland). DHE stained cells were examined under a fluorescence microscope connected to a digital camera and image acquisition software capture (Zeiss®, Germany).

2.6. Measurement of mitochondrial membrane potential

The lipophilic cationic probe fluorochrome JC-1 was used to determine the mitochondrial membrane potential ($\Delta\Psi m$) (Reers et al., 1991). In the presence of physiological mitochondrial membrane potentials, JC-1 forms aggregates that fluoresce with an emission peak at 588 nm. Loss of membrane potential favors the monomeric form of JC-1, that has an emission peak at 530 nm. At first, cells were seeded onto 24-well plates at equal density (5.0×10^4 cells/well) and treated with MeHg (1 μM) for 12 h. In another set of experiments, cells were pre-treated with apocynin (300 μM) for 1 h followed by MeHg challenge

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