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Toxicological responses of environmental mixtures: Environmental metal mixtures display synergistic induction of metal-responsive and oxidative stress genes in placental cells

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ABSTRACT

Exposure to elevated levels of the toxic metals inorganic arsenic (iAs) and cadmium (Cd) represents a major global health problem. These metals often occur as mixtures in the environment, creating the potential for interactive or synergistic biological effects different from those observed in single exposure conditions. In the present study, environmental mixtures collected from two waste sites in China and comparable mixtures prepared in the laboratory were tested for toxicogenomic response in placental JEG-3 cells. These cells serve as a model for evaluating cellular responses to exposures during pregnancy. One of the mixtures was predominated by iAs and one by Cd. Six gene biomarkers were measured in order to evaluate the effects from the metal mixtures using dose and time-course experiments including: heme oxygenase 1 (*HO-1*) and metallothionein isoforms (*MT1A*, *MT1F* and *MT1G*) previously shown to be preferentially induced by exposure to either iAs or Cd, and metal transporter genes aquaporin-9 (*AQP9*) and ATPase, Cu^{2+} transporting, beta polypeptide (*ATP7B*). There was a significant increase in the mRNA expression levels of *ATP7B*, *HO-1*, *MT1A*, *MT1F*, and *MT1G* in mixture-treated cells compared to the iAs or Cd only-treated cells. Notably, the genomic responses were observed at concentrations significantly lower than levels found at the environmental collection sites. These data demonstrate that metal mixtures increase the expression of gene biomarkers in placental JEG-3 cells in a synergistic manner. Taken together, the data suggest that toxic metals that co-occur may induce detrimental health effects that are currently underestimated when analyzed as single metals.

1. Introduction

Exposure to elevated levels of the toxic metals inorganic arsenic (iAs) and cadmium (Cd) is a global health problem affecting millions of people (Ratnaike, 2003; Satarug et al., 2003; Waalkes, 2003; Jomova et al., 2011). Both iAs and Cd occur naturally in the environment, and their concentrations may be elevated to potentially toxic levels at certain waste sites (Fay and Mumtaz, 1996). There is evidence that metals predominantly occur as mixtures, also potentially in association with other non-metal toxic substances (Balistrieri and Mebane, 2014). Exposure to these metals is of concern as elevated levels have been associated with neurological, reproductive, cardiovascular and carcinogenic effects (Liaw, 2009; Moore et al., 2002; Rahman et al., 2007; Smith et al., 2006; Tsai et al., 2003; von Ehrenstein et al., 2006; Wasserman et al., 2007; Yuan et al., 2007).

In addition to the health effects associated with exposures during adulthood, exposures to susceptible populations such as pregnant women and the developing fetus are of concern. This is because iAs can readily cross the placenta (Concha et al., 1998) and Cd, while doing so less readily, also crosses the placenta (Iyengar and Rapp, 2001; Zhang et al., 2004). Epidemiologic studies support relationships between chronic iAs exposure and increased risk of spontaneous abortion, stillbirth, preterm birth, and neonatal death in pregnant women (Ahmad et al., 2001; von Ehrenstein et al., 2006; Rahman et al., 2007). Similar to iAs, exposure to elevated levels of Cd is also associated with reproductive and developmental effects. This stems from the limited capacity of the body to respond to Cd exposure, as the metal does not undergo metabolic degradation to less toxic species and is poorly excreted (Waalkes, 2003; Arita and Costa, 2009). In pregnant women, Cd accumulation in the placenta causes inhibition of trophoblastic invasion, decreased steroidogenesis, and adjusted handling of nutritive essential metals that are deleterious to fetal and maternal health (*reviewed by* Estaban-Vasallo et al., 2012). Consequently, Cd exposure has been

Abbreviations: iAs, inorganic arsenic; Cd, cadmium; mRNA, messenger RNA; *HO-1*, heme oxygenase 1; *MT*, metallothionein; *AQP9*, aquaporin-9; *ATP7B*, Cu2+ transporting ATP-ase; DGT, diffusive gradient in thin film; O_2 stress, oxidative stress.

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associated with birth outcome effects such as lower birth weight and decreased birth height (Chisolm and Handorf, 1996; Zhang et al., 2004).

Changes in mRNA expression levels can be used as biomarkers that indicate disturbances in cellular metabolic pathways leading to cell death or disease and as such, are valuable predictors of exposure and/or xenobiotic toxicity. Since iAs and Cd activate the induction of specific metal-responsive and oxidative stress-inducible genes such as those that encode for metallothioneins and heme oxygenase (Choi and Alam, 1996; Menzel et al., 1998), it is possible to use these genes as biomarkers/indicators of exposure to iAs and/or Cd. Heme oxygenase 1 (*HO1*) has been shown to be induced by iAs and its importance in cellular stress response has been established (Choi and Alam, 1996; Menzel et al., 1998). *HO1* has also been implicated as a gene biomarker of iAs exposure (Menzel et al., 1998). Cd is also capable of inducing the *HO1* gene (Menzel et al., 1998), but is a preferential potent inducer of metallothionein expression. Metallothioneins (*MTs*) are the primary gene biomarkers of Cd exposure because of their high capacity to bind Cd through the thiol group of their cysteine residues, thereby resulting in increased expression levels relative to Cd. (Chisolm and Handorf, 1996; Wang and Fowler, 2008). In addition to *HO1* and *MTs* detailed above, other genes of interest related to metals response are the metal transporter genes aquaporin-9 (*AQP9*) and Cu2 + transporting ATPase *ATP7B*). *AQP9* is a transmembrane, solute transporting protein that facilitates the passage of glycerol and other non-charged solutes (Tsukaguchi et al., 1999). It has been shown to control the transmembrane transport of iAs, thereby playing a critical role in sensitivity of cells towards iAs cytotoxicity (Leung et al., 2007). *ATP7B* belongs to the P-type adenosine triphosphatase family that includes a number of membrane proteins specialized in the transport of cations across cell membranes (Lutsenko and Kaplan, 1995). *ATP7B* plays a key role in the normal cellular distribution of copper in liver, kidney, placenta (Petrukhin et al., 1994) and brain (Lutsenko et al., 2003). Cd binding can also occur at the thiol group of cysteine residues of *ATP7B*.

Previous studies have shown that concurrent exposure to mixtures of iAs and Cd may result in additive or synergistic effects that are not seen in single component exposures (Liu et al., 2000; Madden, 2002; Wang and Fowler, 2008). In the present study the metal responsive stress biomarkers *HO-1* and *MT* and metal transporter genes *AQP9* and *ATP7B* were measured in placental JEG-3 cells exposed to iAs or Cd alone, and environmental and laboratory mixtures of both metals. The environmental mixtures were obtained as water samples collected from two highly contaminated sites in China in order to evaluate interactive effects from concomitant exposures. The first site represents one of the largest known Cd spills in history, on the Longjiang River in Guangxi Province, China that serves as a drinking water source for 3.7 million residents (Zhang et al., 2013). The second site was at an iAs herbicide operation in the Pearl River watershed in Guangdong Province, China. The iAs collected was present as arsenite. To assess uniformity to the environmental mixtures, comparable laboratory samples were prepared using the same concentrations of iAs and Cd present in the environmental samples. Given the interest in understanding potential environmental factors that could impact reproductive health, the JEG-3 human choriocarcinoma cell line was used in the present study as a model for investigating the effects of metals exposure in the placenta (Guiñazú et al., 2012; Huang and Leung, 2009; Letcher and Holsteijn, 1999; Ronco et al., 2010).

2. Materials and methods

2.1. Water sample collection and analysis

Water samples were collected from two contaminated sites in China. Water samples were obtained using a diffusive gradient in thin film (DGT) passive sampler (DGT Research Ltd., Lancaster, UK) with Chelex gel for Cd and ferrihydrite gel for iAs. These samplers provide a surrogate measurement for the bioavailable or labile fraction of metals in water as described elsewhere (Huynh et al., 2012). The DGT devices were deployed in triplicate for a period of two days in the surface water near the sites of the putative sources of the metals and downstream from the sources providing a gradient of exposure. For each of the contaminated sites, the DGT samplers were deployed and collected at six points along a transect downstream from the putative source, representing a total of 12 collections. Upon retrieval, the DGTs were eluted with 1.5 mL of 3 M Ultrapure HNO_3 for 24 h, prior to analysis by inductively coupled mass spectroscopy (ICP-MS, X Series 2 Thermo Fischer Scientific, Waltham, MA, USA). The resin gels were removed from the DGT samplers and metals eluted in 1 M HNO_3 (pH 5.0) for 24 h. Procedural blanks were less than 1% of the lowest measured concentration, matrix spike samples resulted in mean recoveries of 98% (As) and 101% (Cd), and laboratory duplicate analyses were always less than 5% relative percent difference.

Six total treatments were used for the in vitro assays. Two represented dilutions of the environmentally collected samples with the highest levels of iAs or Cd selected from 12 total river samples. Two were laboratory-generated treatments with the same concentrations of iAs and Cd as the environmental samples. The final two treatments represented single metals. The first treatment, referred to as the iAs-environmental mixture (iAs-EM), was carried out at a final concentration of 0.08 μM iAs and 0.0013 μM Cd. This resulted from a 60-fold dilution of the original environmental sample with the highest iAs concentration from the Pearl River watershed (4.8 μM iAs). The original sample also had measurable levels of Cd (0.08 μM). The second treatment, referred to as the iAs laboratory-generated mixture (iAs-LM), was modeled after the environmental mixture with a final concentration of 0.08 μM iAs and 0.0013 μM Cd. The third treatment, referred to as the Cd environmental mixture (Cd-EM), had final concentrations of 0.1 μM Cd and 0.002 μM iAs. This resulted from a 60-fold dilution of the original environmental sample with the highest concentration of Cd from the Longjiang River (6.07 μM Cd). The original sample also had measurable levels of iAs (0.12 μM). The fourth treatment, referred to as the Cd-laboratory-generated mixture (Cd-LM), was modeled after the environmental mixture with a final concentration of 0.1 μM Cd and 0.002 μM iAs. These were compared to two single metals treatments of iAs (iAs) 0.08 μM or Cd (Cd) 0.1 μM, both prepared in the laboratory. All the mixtures were stored under the same conditions as controls in the laboratory with the iAs samples made fresh prior to treatment.

2.2. Cell culture and iAs and Cd treatments

The JEG-3 choriocarcinoma cell line was purchased from the American Type Culture Collection (Manassas, VA). JEG-3 cells were grown in Dulbecco's modified Eagle's Minimum Essential Medium, supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin and 1 mM sodium pyruvate at 37 °C in 5% CO_2. Cells were plated at 5×10^6 cells per 25 cm^3 flask and incubated under standard conditions until achieving 80–90% confluence. To study the effects of iAs and Cd mixtures in vitro, JEG-3 cells were cultured in a 6-well culture plate for 24 h at 0.5×10^6 cells per well. Cells were serum starved in 2 mL of serum-free DMEM supplemented with 1% penicillin/streptomycin, and 1 mM sodium pyruvate for 4 h. Cells were exposed to identical concentrations of iAs and Cd present in the environmental mixtures by combining 0.08 μM sodium arsenite (Na_2AsO_3) and 0.0013 μM cadmium chloride ($CdCl_2$) for the iAs-LM and 0.1 μM cadmium chloride ($CdCl_2$) and 0.002 μM sodium arsenite (Na_2AsO_3) for the Cd-LM. To assess the effect of the mixtures, cells were either treated with the iAs-EM, iAs-LM, Cd-EM or the Cd-LM. For the metal only treatments, the concentrations were selected to be identical to the final concentrations of iAs and Cd in the mixtures. Therefore, cells were treated with 0.08 μM sodium arsenite (Na_2AsO_3) or 0.1 μM cadmium chloride ($CdCl_2$). Control cells were treated with dilute nitric acid at (pH 5.0). All the cells in