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Differential toxicity and gene expression in Caco-2 cells exposed to arsenic species

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HIGHLIGHTS

- As(III) and MMA(III) induce cell death by necrosis and apoptosis respectively.
- For DMA(III) the percentages of apoptosis and necrosis are similar.
- Trivalent forms of arsenic induces oxidative stress.
- Trivalent arsenic species alter the activity of glutathione peroxidase and catalase.
- Trivalent arsenic species induces expression of stress proteins and metallothioneins.

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ABSTRACT

Inorganic arsenic [As(V) + As(III)] and its metabolites, especially the trivalent forms [monomethylarsonous acid, MMA(III), and dimethylarsinous acid, DMA(III)], are considered the forms of arsenic with the highest degree of toxicity, linked to certain types of cancer and other pathologies. The gastrointestinal mucosa is exposed to these forms of arsenic, but it is not known what toxic effect these species may have on it. The aim of the present work was to evaluate the toxicity and some mechanisms of action of inorganic arsenic and its metabolites [monomethylarsonic acid, MMA(V), dimethylarsinic acid, DMA(V), MMA(III) and DMA(III)] in intestinal epithelial cells, using the Caco-2 human cell line as a model.

The results show that the pentavalent forms do not produce toxic effects on the intestinal monolayer, but the trivalent species have a different degree of toxicity. As(III) induces death mainly by necrosis, whereas only apoptotic cells are detected after exposure to MMA(III), and for DMA(III) the percentages of apoptosis and necrosis are similar. The three forms produce reactive oxygen species, accompanied by a reduction in intracellular GSH and lipid peroxidation, the latter being especially notable in the dimethylated form. They also alter the enzyme activity of glutathione peroxidase and catalase and induce expression of stress proteins and metallothioneins. The results indicate that the trivalent forms of arsenic can affect cell viability of intestinal cells by mechanisms related to the induction of oxidative stress. Further studies are needed to evaluate how the effects observed in this study affect the structure and functionality of the intestinal epithelium.

1. Introduction

The inorganic arsenic present in water and some foods is the form of arsenic with the highest degree of toxicity among those detected in these sources of exposure. The current EPA *Guidelines for Carcinogen Risk Assessment* categorize inorganic arsenic as "carcinogenic to humans" because of convincing epidemiological evidence of a causal relationship between oral exposure of humans to inorganic arsenic and cancer (USEPA, 2005). Chronic exposure to inorganic arsenic is associated with a greater prevalence of cancer of the lung, urinary tract, liver and skin (Chen et al., 1992; Smith et al., 1992). There is also an increase in other pathologies, such as type 2 diabetes, cardiovascular and cerebrovascular problems, chronic obstructive respiratory diseases and non-carcinogenic skin disorders (hypo- and hyperpigmentation, palmar keratosis) (EFSA, 2009). In children, a population group highly susceptible to chronic exposure to arsenic, among the most commonly reported effects are those related to reduction in cognitive capabilities (Wasserman et al., 2004).

It is now known that some of the intermediate species in inorganic arsenic metabolism are even more toxic than the inorganic arsenic from which they are derived. Some studies demonstrate the toxicity of the trivalent methylated metabolites monomethylarsonous acid (MMA(III)), dimethylarsinous acid (DMA(III)) and dimethylarsinous glutathione DMA(III)-GSH (Styblo et al., 2000; Sakurai et al., 2006). Kojima et al. (2009) have shown that cells with

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a higher rate of inorganic arsenic metabolism are more susceptible to oxidative damage to DNA and cell transformations towards tumorigenic phenotypes. Therefore the effects observed in chronic exposure to inorganic arsenic may be due to inorganic arsenic or its metabolites or possibly the joint action of all of them. Calatayud et al. (2012a) have recently shown the ability of intestinal epithelial cells to metabolize inorganic arsenic, highlighting the importance of considering the methylated arsenic species when evaluating the toxicity of arsenic at enteric level.

The cellular and molecular mechanisms involved in the toxicity of arsenic have been extensively studied, but they are subjects of ongoing debate. Evaluation of these mechanisms is partly hampered by the diversity of signal transduction pathways that are affected during exposure to forms of arsenic and by the fact that the effects observed vary in relation to time, dose, arsenic species and individual susceptibility. Arsenic has been implicated in promoting alterations in multiple cellular pathways, including suppression of cell cycle checkpoint proteins, altered expression of growth factors, resistance to apoptosis, inhibition of DNA repair, decreased immunosurveillance, alteration of DNA methylation and increased oxidative stress (Lantz and Hays, 2006). One of the mechanisms of action that has been studied most is oxidative stress. Arsenic may induce oxidative stress by cycling between oxidation states of metals such as iron, by interacting with intracellular levels of antioxidants or by increasing inflammation, leading to the chronic presence of cells that produce radicals and/or growth factors (Flora, 2011).

The intestinal epithelium is the first physiological barrier that arsenic meets after ingestion. The toxic effect that arsenic can have on the intestinal mucosa has scarcely been studied. Clinical signs of gastrointestinal irritation are observed in cases of short-term high-dose exposure to inorganic arsenic and dimethylarsinic acid (Uede and Furukawa, 2003; Lee et al., 1995). Acute inflammation and haemorrhage of the small intestine have been reported in rhesus monkeys chronically exposed to high doses of arsenic trioxide (Heywood and Sortwell, 1979). Long-term exposure of rodents to MMA led to a thickened wall, oedema and haemorrhagic, necrotic, ulcerated or perforated mucosa in the large intestine and a significant increase in the incidence of squamous metaplasia of the epithelial columnar absorptive cells in the colon and rectum (Arnold et al., 2003). In other types of cells or tissues the modes and mechanisms of action of inorganic arsenic and its metabolites have been extensively evaluated, but there are few studies on intestinal cells (Davis et al., 2000; Nakagawa et al., 2002; Laparra et al., 2006, 2008). According to the guidelines for arsenic risk assessment published by the US Environmental Protection Agency (USEPA, 2005), the mode of action and human relevance cannot necessarily be generalized to other toxic endpoints or tissues or cell types without additional analyses. The aim of the present work was to evaluate the toxicity and some mechanisms of action of inorganic arsenic and its metabolites in intestinal epithelial cells. The Caco-2 cell line, originally isolated from a colon adenocarcinoma, was used was used as a model of intestinal epithelium.

2. Materials and methods

2.1. Arsenical species

The As(V) standard solution (1000 mg/L) was acquired from Merck (Merck, Germany). The As(III) standard (1000 mg/L) was prepared by dissolving 1.320 g of As_2O_3 (Riedel de Haën, Germany) in 25 mL of KOH at 20% (m/v), neutralizing with 20% H_2SO_4 (v/v) and making up to a final volume of 1 L with 1% H_2SO_4 (v/v). The standard solutions of MMA(V) and DMA(V) were prepared by dissolving the appropriate amount of the following salts in water: $CH_3AsO(ONa)_2{\cdot}6H_2O$ for MMA(V) (Carlo Erba, Italy), and $(CH_3)_2AsNaO_2{\cdot}3H_2O$ for DMA(V) (Fluka Chemika Biochemika, Spain). The standard solutions of MMA(III) and DMA(III) were prepared from CH_3AsI_2 and $(CH_3)_2AsI$ (Argus Chemicals, Italy), respectively. Throughout this article the concentrations of the various arsenical species are expressed as arsenic rather than as species, to facilitate comparison between them.

2.2. Cell culture

The Caco-2 cells were obtained from the European Collection of Cell Cultures (ECACC; number 86010202, Salisbury, UK). The cells were maintained in 75 cm^2 flasks to which 10 mL of Dulbecco's Modified Eagle Medium (DMEM) with glucose (4.5 g/L) was added at pH 7.4. The DMEM was supplemented with 10% (v/v) of fetal bovine serum (FBS), 1% (v/v) of non-essential amino acids, 1 mM of sodium pyruvate, 10 mM HEPES (N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid), 100 U/mL of penicillin, 0.1 mg/mL of streptomycin and 0.0025 mg/L of amphotericin B (DMEMc). The cells were incubated at 37 °C, in an atmosphere with 95% relative humidity and a CO_2 flow of 5%. The medium was changed every 2–3 days. When the cell monolayer reached 80% confluence, the cells were detached with a solution of trypsin (0.5 g/L) and EDTA (ethylenediaminetetraacetic acid, 0.2 g/L). The assays were performed with cultures between passages 5 and 25. All the assays described below were performed by seeding 5×10^4 cells/cm^2 in multiwell culture plates in Minimum Essential Medium Eagle (MEM). All the reagents used were obtained from PAA Laboratories GmbH (Germany).

2.3. Mitochondrial metabolic activity assay

For the assays of mitochondrial activity, sodium resazurin was used (7-hydroxy-3H-phenoxazin-3-one-10-oxide sodium salt, Sigma, Sigma–Aldrich Química, S.A., Spain). The Caco-2 cells were seeded in 96-well plates and after differentiation (11–12 days) they were exposed to the following treatments: 0.1, 0.5, 1, 10 and 100 μM of As(V), MMA(V) or DMA(V); 1, 10, 25, 50 and 75 μM of As(III); 0.05, 0.1, 0.5, 1 and 5 μM of MMA(III) or DMA(III). The exposure times were 24, 48 and 72 h for As(V), As(III), MMA(V) and DMA(V) and 2 and 24 h for MMA(III) and DMA(III). After exposure, the medium was removed and the culture was washed three times with phosphate buffered saline (PBS) (PAA). Then 500 μL of resazurin solution (10 μg/mL in MEM) was added and the result was incubated at 37 °C for 4 h in an atmosphere with 95% relative humidity and a CO_2 flow of 5%. Resazurin reduction was measured colorimetrically (570 and 600 nm) using a PowerWave HT Microplate Scanning Spectrophotometer (Bio-Tek instruments). The results were expressed as percentages with respect to the absorbance of cells not treated with arsenic.

2.4. Analysis of reactive species (ROS) generation

2′,7′-Dichlorofluorescein diacetate (DCFDA) (Sigma) was used for ROS measurement. The Caco-2 cells were seeded in 96-well plates and after differentiation (11–12 days) they were exposed for 24 h to the following treatments: 0.1, 0.5, 1, 10 and 100 μM of As(V), MMA(V) or DMA(V); 5, 10, 25 and 50 μM of As(III); 0.01, 0.1, 0.5 and 1 μM of MMA(III); 0.1, 1, 5 and 10 μM of DMA(III). Cells treated with 2 mM H_2O_2 (Prolabo, VWR, Spain) were used as a positive control.

After the treatments, the medium was eliminated and the cells were washed three times with PBS and then incubated with 100 μL of 100 μM of DHCF-DA in PBS at 37 °C for 30 min. Then the DHCF-DA solution was eliminated and the cells were lysed with Triton X-100 (Merck, 0.1% (m/v) in PBS). The cell lysate was transferred to a 96-well plate and fluorescence was determined in a microplate reader (PolarSTAR OPTIMA, BMG-Labtech, Germany) (excitation λ = 488 nm; emission λ = 530 nm). The fluorescence values obtained were normalized per mg of protein, quantified by the Bradford method (Bio-Rad Protein Assay, Bio-Rad, USA) and expressed as percentages of the untreated control.

2.5. Measurement of lipid peroxidation

The colorimetric method used to determine the barbituric acid reactive species (TBARS) in Caco-2 cells was based on the protocol described by Aviello et al. (2011). Briefly, the Caco-2 cells were seeded in 6-well plates and after differentiation (14–15 days) they were treated for 24 h with As(III) (10, 25 and 50 μM), MMA(III) (0.1, 0.5 and 1 μM) or DMA(III) (0.1, 1 and 5 μM). Cells treated with 1 mM of H_2O_2 were used as a positive control. After this exposure time, the medium was eliminated and the cells were washed three times with PBS, recovered in Triton X-100 (0.1% (m/v) in PBS) and centrifuged at 11,000 rpm for 10 min at 4 °C. The supernatant was removed and 0.5 mL of trichloroacetic acid (10% (m/v), Merck) was added to the cell residue. After centrifugation at 11,000 rpm for 10 min, 0.5 mL of 0.67% (m/v) thiobarbituric acid (Fluka) was added to the supernatant and the mixture was heated at 80 °C for 30 min. After cooling, MDA equivalents formation was recorded at the wavelength of 532 nm, using a microplate reader (PolarSTAR OPTIMA). An MDA standard curve (0, 0.625, 1.25, 2.5, 5, 10, 25 and 50 μM) was used to quantify the levels of MDA equivalents formed during the experiments, and the results were expressed as a percentage of MDA equivalents/mg protein with respect to a control not treated with arsenic.

2.6. Reduced and oxidized glutathione levels (GSH/GSSG)

The Caco-2 cells were seeded in 96-well plates and maintained for 11–12 days until differentiation. Then they were treated for 24 h under various conditions: 0.1,