Toxicology Letters 224 (2014) 40–46

Contents lists available at ScienceDirect

Toxicology Letters

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

Trivalent arsenic species induce changes in expression and levels of proinflammatory cytokines in intestinal epithelial cells

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HIGHLIGHTS

- Cytokines expression and release in Caco-2 cells exposed to arsenic were studied.
- Cells were exposed to trivalent arsenic species during 2, 4, 6 and 24 h.
- Effect of As coexposure with lipopolysaccharide (LPS) was also investigated.
- Trivalent species induce expression and release of the proinflammatory cytokines.
- As/LPS exposure modifies the cell response compared with exposure to As alone.

ARTICLE INFO

Article history:
Received 26 July 2013
Received in revised form
25 September 2013
Accepted 27 September 2013
Available online 17 October 2013

Keywords:
Arsenic
Intestinal epithelium
Caco-2
Inflammation
Cytokines

ABSTRACT

Chronic arsenic (As) toxicity in humans has been documented in many countries where exposure mostly occurs through drinking water. The As immunotoxic effects have been demonstrated in animal models as well as in humans. The studies of the immunotoxicity of As have centered on organs related to immune response or target organs, with few data being available at intestinal level. The present study has evaluated the changes in the expression and release of cytokines in Caco-2 cells, widely used as an intestinal epithelial model. Differentiated cells were exposed to 1 μM of As(III), 0.1 μM of monomethylarsonous acid [MMA(III)] and 1 μM of dimethylarsinous acid [DMA(III)] during 2, 4, 6 and 24 h. Additionally, the effect of As coexposure with lipopolysaccharide (LPS, 10 ng/mL) has been evaluated. The results show trivalent species to induce increases in the expression and release of the proinflammatory cytokines tumor necrosis factor alpha (TNFα), IL6, IL8 – the magnitude and time of response being different for each As species. The response of greatest magnitude corresponds to DMA(III), followed by As(III), while MMA(III) generates a limited response. Furthermore, the presence of LPS in the co-exposed cells could affect the expression and secretion of cytokines compared with individual exposure to arsenicals, especially for As(III)/LPS and DMA(III)/LPS.

1. Introduction

The main sources of exposure to arsenic (As) are drinking water and certain foods in which the metalloid can be found in different chemical forms. In drinking water, it is mainly found as inorganic As [As(III) (arsenite) and As(V) (arsenate)], while in food the composition is more diverse. In these matrices, inorganic As has been detected as well as monomethylarsonic acid [MMA(V)], dimethylarsinic acid [DMA(V)] and a series of organic arsenical forms of greater structural complexity. Once ingested, the As species can undergo chemical modification. In this context, the intestinal microbial flora is able to transform As(V) to monomethylarsonous acid [MMA(III)] and to thiolated metabolites (Van de Wiele et al., 2010). Likewise, the intestinal epithelial cells produce methylated As metabolites, fundamentally in the trivalent state [MMA(III) and dimethylarsinous acid, DMA(III)], following exposure to inorganic As species (Calatayud et al., 2012).

The toxicity of As depends on its chemical form. Inorganic As has been classified by the International Agency for Research on Cancer as a human carcinogen (type IA) (IARC, 2004). *In vitro* toxicity studies have shown the trivalent methylated forms to be even more toxic than inorganic As (Styblo et al., 2000; Huang et al., 2007; Kumagai and Sumi, 2007). In populations chronically exposed to As, there have been reports of increased prevalence of certain cancers (lung, liver, skin and bladder) as well as of certain non-neoplastic diseases such as cardiovascular and cerebrovascular disorders, type II diabetes, respiratory diseases, and reduction of cognitive capabilities in the child population (Haque et al., 2003; Wasserman et al., 2004; Wang et al., 2007; Navas-Acien et al., 2008; Parvez et al., 2011). The development of some of these diseases, such as

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http://dx.doi.org/10.1016/j.toxlet.2013.09.016

diabetes mellitus, is associated with disruptions in immune function (Banerjee and Saxena, 2012).

In recent years, many *in vivo* studies have contributed information on the effects of As upon the immune system. The study published by Ahmed et al. (2011) in As endemic areas reflects that As exposure in early pregnancy is associated with reduced T cell counts and an increased production of proinflammatory cytokines at placental level. Hernández-Castro et al. (2009) found that individuals exposed to As *via* drinking water show a significant negative correlation between urinary As levels and the proportion of natural T regulatory lymphocytes in peripheral blood. Kozul et al. (2009), in a study conducted in mice, observed changes in the regulation of inflammatory pathways after chronic $NaAsO_2$ exposure through drinking water. Most studies that have evaluated As immunotoxicity have been carried out in human peripheral blood mononuclear cells (PBMC) (Hernández-Castro et al., 2009; Bourdonnay et al., 2009a,b) or target cells of this metalloid (Vega et al., 2001). However, little information is available on the immunotoxic effects of arsenical species upon gastrointestinal epithelial cells. *In vivo*, symptoms of gastroenteritis have been recorded in populations exposed to inorganic As in drinking water (Borgoño et al., 1977; Majumdar et al., 2009), and *Rhesus* monkeys chronically exposed to As(III) have shown signs of acute inflammation and bleeding of the small intestine (Heywood and Sortwell, 1979).

The intestinal mucosa actively participates in the inflammatory response to different stimuli, as well as in the establishment of interactions with immune cells (Jung et al., 1995). The epithelial cells that constitute the intestinal mucosa can express molecules involved in antigen presentation, complement components, receptors for cytokines, eicosanoids and an array of proinflammatory cytokines and chemokines, and may therefore initiate and modulate the mucosal immune response (Jung et al., 1995; Yang et al., 1995). It has been shown that exposure to food contaminants gives rise to increased proinflammatory cytokine secretion by intestinal epithelial cells (Hyun et al., 2007; Lynes et al., 2010), though to date only one study has evaluated intestinal cell response to As exposure (Hershko et al., 2002).

The aim of the present study was to evaluate the effect of the trivalent As species [As(III), MMA(III) and DMA(III)] upon the expression and release of proinflammatory cytokines (IL6, IL8, TNFα) in intestinal epithelial cells, as well as the combined effect of these arsenical forms with *Salmonella enterica* lipopolysaccharide, a known proinflammatory antigen. To this effect, use was made of the Caco-2 human cell line derived from a colon adenocarcinoma, and which has been previously used by other authors in intestinal inflammation studies (Jung et al., 1995; Amin et al., 2008; Brozek et al., 2008; Van de Walle et al., 2010).

2. Materials and methods

2.1. Chemicals

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(III) and DMA(III) were prepared from CH_3AsI_2 and $(CH_3)_2AsI$ (Argus Chemicals, Italy), respectively. Throughout this article, the concentrations of As species are expressed as As rather than as species, to facilitate comparison between them. Lipopolysaccharide (LPS) from *S. enterica* serotype *typhimurium* (Sigma, Spain) was prepared in phosphate buffer saline (PBS, PAA, Labclinics, Spain) at 1 mg/mL and stored at −20 °C until use.

2.2. Caco-2 cell line culture conditions

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). All the reagents used were obtained from PAA.

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, PAA) and EDTA (ethylenediaminetetraacetic acid, 0.2 g/L, PAA). The assays were performed with cultures between passages 20 and 30.

2.3. Caco-2 cells treatments

The experiments were carried out in 12-well plates. The cells were seeded at a density of 2.5×10^4 cells/cm^2 in DMEMc and were maintained for 12–14 days until differentiation, with replacement of the medium every 2–3 days. After this time, the different treatments were carried out: (a) LPS 10 ng/mL; (b) As species [1 μM of As(III), 0.1 μM of MMA(III), 1 μM of DMA(III)]; and (c) As/LPS combinations at the previously described concentrations, both added simultaneously to the cell cultures. The treatments were prepared in Minimal Essential Medium with Earle's salts (MEM) (PAA) supplemented with HEPES (1 mM) and sodium pyruvate (1 mM).

After different exposure times (2, 4, 6 and 24 h), the medium was removed and kept at −80 °C until analysis of cytokine contents. Cell monolayers were washed three times with PBS, scraped in the same buffer and centrifuged. Pellets were washed once with PBS and stored at −80 °C until RNA extraction.

2.4. Differential expression of cytokines by reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

RNA extraction was performed with the NucleoSpin RNA II kit (Macherey-Nagel, Germany). The RNA extracted was spectroscopically quantified in a Nanodrop ND-1000 (NanoDrop Technologies, USA), adjusting the samples with RNase-free water in order to work with the same concentrations. First-strand complementary DNA (cDNA) was obtained from 200 ng of total RNA using the Reverse Transcriptase Core Kit (Eurogentec Headquarters, Belgium).qPCR was performed using the LightCycler® 480 Real-Time PCR Instrument (Roche Diagnostics, USA). Reactions were carried out in a 10 μL final volume containing 5 μL LightCycler® 480 SYBR Green I Master Mix (2×, Roche), 2.5 μL cDNA (20 ng/μL), 1 μL of each forward and reverse primer (10 μM, Biolegio, The Netherlands) and nuclease-free water. No-template controls were run to verify the absence of genomic DNA. The oligonucleotide sequences used are shown in Table 1. 18S ribosomal RNA was employed as reference gene. PCR efficiency curves for each gene were calculated using 5 duplicate 2-fold dilutions of cDNA.

The qPCR conditions were 95 °C for 5 min, followed by 40 cycles: 10 s denaturation at 95 °C, 10 s annealing at 55 °C, and 20 s elongation at 72 °C. The melting curve of each sample was analyzed after each PCR run to confirm PCR product specificity. Data were analyzed with the Relative Expression Software Tool (REST 2009, QIAGEN). All experiments were performed in quadruplicate in two independent experiments.

2.5. Determination of cytokine levels

IL6, IL8 and TNFα levels were assayed in the Caco-2 cell culture media using Gen Probe Human ELISA Kits (Diaclone, Bionova) for each cytokine, following the instructions of the manufacturer. All the results were standardized in terms of total protein contents determined by the Bio-Rad Protein Assay (Bio-Rad, USA).

2.6. Cell viability

Caco-2 cell viability after the different treatments was evaluated using the trypan blue staining technique. Cells were stained with trypan blue 4% (v/v) (Sigma) and counted under a light microscope.

2.7. Statistical analysis

All assays were performed at least in triplicate in independent cultures. The results were statistically analyzed by one-factor analysis of variance (ANOVA) with Tukey's HSD *post hoc* multiple comparison or using the Student *t*-test (SPSS, version 15.0). Differences were considered significant for $p < 0.05$.

3. Results

The As(III) concentrations used in this study (1 μM; 75 μg/L) are consistent with the inorganic As levels found in drinking water in many countries, or in foods such as rice or seaweed (EFSA, 2009). On the other hand, the methylated species concentrations used [MMA(III): 0.1 μM, 7.5 μg/L; DMA(III) 1 μM, 75 μg/L] may be common within intestinal epithelial cells as a consequence of the metabolism of inorganic As (Calatayud et al., 2012) and human gut microbiota transformations (Van de Wiele et al., 2010; Alava