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In vitro study of intestinal transport of arsenite, monomethylarsonous acid, and dimethylarsinous acid by Caco-2 cell line

M. Calatayud, V. Devesa, R. Montoro, D. Vélez*

Instituto de Agroquímica y Tecnología de Alimentos (IATA-CSIC), Apdo. 73, 46100 Burjassot (Valencia), Spain

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ABSTRACT

Arsenic is a pollutant widely distributed in the environment. There are numerous studies on the toxicity of trivalent arsenic forms As(III), MMA(III), and DMA(III), but few data are available on the processes of digestion and absorption of these arsenic species and the mechanisms involved are unknown. The present study evaluated the processes involved in intestinal absorption of trivalent arsenic species, using the Caco-2 cell model as system. The apparent permeability values obtained for As(III) in apical-basolateral direction $(4.6 \pm 0.3) \times 10^{-6}$ cm/s, showing moderate intestinal absorption. Transport of MMA(III) [$P_{app} = (7.0 \pm 0.9) \times 10^{-6}$ cm/s] and DMA(III) [$P_{app} = (10.6 \pm 1.4) \times 10^{-6}$ cm/s] is greater than that of As(III). The cellular retention of As(III) (0.9–2.4%) was less than that observed for MMA(III) (30%) and DMA(III) (35%).

A substantial paracellular component was observed in transport of As(III) and MMA(III), whereas DMA(III) does not use this pathway for its absorption. For all the trivalent species, transport depends on temperature, with an active transcellular component for MMA(III) and DMA(III). Variations in pH do not affect transport of these species. The presence of GSH and green tea extract significantly alters transport of As(III) across Caco-2 cells.

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1. Introduction

Arsenic (As) is a contaminant widely distributed in the environment. The main pathway of exposure for the populations affected is consumption of water and food. The As species present in these matrices can differ considerably in terms of toxicity, from inorganic As, which is considered carcinogenic (IARC, 2004), to arsenobetaine, considered practically innocuous.

Toxicological studies of As species have concentrated mainly on inorganic As and the monomethylated and dimethylated As species. The oxidation state of the As atom in these species determines their toxicity, with As(III), MMA(III) [monomethylarsonous acid], and DMA (III) [dimethylarsinous acid] having much greater toxicity than that of their pentavalent equivalents (Petrick et al., 2000; Styblo et al., 2000; Hirano et al., 2003).

Studies on experimental animals associate exposure to As(III) with a greater incidence of certain kinds of tumors through exposure *in utero* (Tokar et al., 2010). It has been related with the greater prevalence of type 2 diabetes observed in places where exposure to As is chronic (Navas-Acien and Guallar, 2008). There are also reports of its effects on cognitive capabilities (Xi et al., 2009), functions of the central nervous system (Krüger et al., 2006), and a whole series of processes that affect the immune system (Kozul et al., 2009). The trivalent methylated forms MMA(III) and DMA(III) have been studied in less detail. MMA(III) inhibits the entrance of insulin-dependent glucose in adipocyte cultures with an IC_{50} less than that observed for As(III) (Paul et al., 2007), as well as inducing over-production of many pro-inflammatory cytokines (Escudero-Lourdes et al., 2010). This growing knowledge about the effect of the trivalent species after they reach systemic circulation contrasts with the few studies that exist about the mechanisms involved in their absorption.

Insufficient information is available about the presence of trivalent As species in foodstuffs. Most methods currently used for quantification of As species employ extraction procedures that modify redox states (Heitkemper et al., 2001). The As(III) and As(V) concentrations reported in many studies that characterize foodstuffs must therefore be taken cautiously. With regard to the methylated species, most studies do not indicate the oxidation state of the arsenic atom. The only report available is of the presence of MMA(III) in carrots grown in an area polluted with arsenic (Yathavakilla et al., 2008).

Although the presence of trivalent As species in foodstuffs may be questioned, it has been shown that these species can be generated during intestinal processes (gastrointestinal digestion, metabolism by the intestinal epithelium, the effect of colonic flora).

* Corresponding author. Tel.: +34 963 900 022; fax: +34 963 636 301.
E-mail address: deni@iata.csic.es (D. Vélez).

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Studies conducted in our laboratory show reduction of As(V) to As(III) during the processes of digestion and intestinal metabolism (unpublished data). There are also reports of the reduction of As(V) to As(III) by cecal microflora, and even its conversion to species with a greater degree of methylation (Van de Wiele et al., 2010).

In view of the above, one cannot rule out the possibility that trivalent As species may reach the intestinal epithelium, which is the first barrier that exogenous toxic agents must cross in order to be absorbed. The absorption of these species has not been characterized in depth. *In vitro* models can be used to obtain information prior to *in vivo* study in laboratory animals. The Caco-2 human cell line, initially isolated from a colon adenocarcinoma, is one of the cell lines most frequently used for these studies. It differentiates spontaneously, producing a monolayer with the physiological and morphological characteristics of mature enterocytes, with many of the enzymes and transporters that exist in the small intestine (Hidalgo et al., 1989), which indicates its suitability for studying the mechanisms of intestinal transport. Laparra et al. (2006) used this cell line to study the apparent permeability coefficient and cytotoxic effects of As(III), but there have been no investigations for MMA(III) and DMA(III).

Given the toxicity of the trivalent forms and the sparse information about their passage across the intestinal epithelium, the aim of the present work was to study the intestinal transport of As(III), MMA(III), and DMA(III), using the Caco-2 cell line as a model system. The results obtained from altering the exposure conditions (concentration, pH, opening of cell junctions, temperature) and the study of the influence of certain dietary constituents and additives increase our knowledge about intestinal absorption of As species.

2. Materials and methods

2.1. Arsenical species

The standard of As(III) (1000 mg/l) was prepared by dissolving 1.320 g of As_2O_3 (Riedel de Haën, Germany) in 25 ml of 20% (m/v) KOH, neutralizing with 20% (v/v) H_2SO_4, and diluting to 1 l with 1% (v/v) H_2SO_4. The standard solution of monomethylarsonous acid, MMA(III), was prepared from CH_3AsI_2 (Argus Chemicals, Vernio, Italy). The standard solution of dimethylarsinous acid, DMA(III), was prepared from $(CH_3)_2AsI$ (Argus Chemicals).

2.2. Cell culture

The Caco-2 cells were acquired from the European Collection of Cell Cultures (ECACC; number 86010202, Salisbury, UK). The cells were kept in 75 cm^2 flasks to which 10 ml of Dulbecco's Modified Eagle Medium (DMEM) with glucose (4.5 g/l) and pyruvate (Gibco, BRL Life Technologies, Paisley, Scotland) was added at pH 7.4. The DMEM was supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco), 1% (v/v) non-essential amino acids (NEAA) (Gibco), 1% (v/v) HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) 1 M, 1% (v/v) of antibiotics (penicillin/streptomycin) (Sigma), and 1% (v/v) fungizone (Sigma). The cells were incubated at 37 °C, in an atmosphere with a relative humidity of 95% and a CO_2 flow of 5%. Medium was replaced every 2–3 days. When the cell monolayer reached 80% confluence, the cells were detached with a solution of trypsin (2.5 g/l) and EDTA (0.2 g/l) (Sigma). All the cultures were used between passes 5 and 25.

Cell differentiation and transport tests were performed in two-chamber wells with Transwell® polyester membranes (24 mm diameter, pore size 0.4 μm; Costar Corp., NY, USA). In this system the Caco-2 cells are kept on a porous support that separates the well into two compartments: apical and basal. The cells were seeded at a density of 7.5×10^4 cells/cm^2, adding 1.5 ml of culture medium to the apical chamber and 2 ml to the basal chamber. The cells were incubated at 37 °C, 5% CO_2, and 95% relative humidity, changing the medium every 2–3 days until cell differentiation was attained (14–15 days post seeding).

2.3. Cell monolayer integrity

During the period of growth and differentiation, cell monolayer integrity was monitored every 2–3 days, measuring the transepithelial electrical resistance (TEER) with a Millicell®-ERS (Millipore Corporation, Madrid, Spain). The cell monolayer was considered completely formed when stable TEER values were obtained ($\geq$250 Ω cm^2). TEER was also measured at various times during the uptake and transport tests, including the start and finish times of the experiment.

Monolayer integrity was also evaluated by calculating the apparent permeability (P_{app}) of the paracellular transport marker Lucifer Yellow, LY (Sigma). This compound was added at a concentration of 100 μM to the control wells and to the wells treated with As. The fluorescence of the LY transported to the acceptor side was measured at excitation/emission wavelengths of 485/520 nm by a fluorescence microplate reader (PolarSTAR OPTIMA, BMG-Labtech, Germany). To evaluate possible interactions of LY with uptake and transport of As species, we performed parallel experiments with and without the paracellular marker which showed the absence of interference between LY and the As compounds studied.

2.4. Cell viability

At the end of each experiment, the trypan blue exclusion technique was used to quantify the number of viable cells (trypan blue solution, 0.4%, Sigma).

2.5. Uptake, transport, and permeability tests

The tests were performed in Hanks buffered solution salts medium (HBSS) with $NaCO_3$ (Sigma, Spain) supplemented with 10 mM HEPES (pH 7.2). Before the experiment started the cells were kept in contact with the medium for 15 min.

The standard solutions of As(III), MMA(III), and DMA(III) were prepared in HBSS and added individually to the apical compartment to study transport in the apical-basolateral direction (A-B). As(III) was also studied in the basolateral-apical direction (B-A) by adding the standard solution to the basal compartment. The concentrations assayed were 1 μM, 7 μM, 13 μM, and 67 μM for As(III) and 1 μM for MMA(III) and DMA(III). At the stipulated test times (5 min, 15 min, 30 min, 45 min, 60 min, 90 min, 120 min, 180 min, and 240 min), aliquots were taken from the acceptor compartment (400 μL) and replaced with an equal volume of HBSS without the addition of the As species. As(III) was also evaluated at higher times: 6 h, 8 h, 12 h and 24 h.

The apparent permeability coefficients (P_{app}) up to 120 min of test time were calculated in both directions (A-B, B-A), using Eq. (1).

$$P_{app} = \left(\frac{dC}{dt}\right)\left(\frac{V_r}{AC_0}\right) \tag{1}$$

where:

dC/dt is the flow (μM/s) determined by the linear slope of the equation that governs the variation in the concentrations of As species, corrected by dilution, against time.
V_r is the volume of the acceptor compartment (apical 1.5 ml; basal 2 ml).
A is the surface occupied by the cell monolayer.
C_0 is the initial concentration in the donor compartment.

The efflux ratio (E_r) for As(III) was calculated using Eq. (2).

$$E_x = \frac{P_{app}(\text{basal} - \text{apical})}{P_{app}(\text{apical} - \text{basal})} \tag{2}$$

2.6. Effect of temperature on arsenic transport

Permeability was evaluated at 4 °C and 15 °C in the A-B direction for 67 μM As(III), 1 μM MMA(III), and 1 μM DMA(III) prepared in HBSS. During the test period (60 min), aliquots were collected after 5 min, 15 min, 30 min, 45 min, and 60 min. The P_{app} values were calculated from Eq. (1).

Applying the Arrhenius equation to the experimental data, the activation energy of permeation, E_P, was calculated as follows (Eq. (3)):

$$P_{app} = P_0 e^{-E_P/RT} \tag{3}$$

where P_{app} represents the permeability coefficient, P_0 is a pre-exponential factor, R is the gas constant, and T is the absolute temperature in Kelvin degrees. By plotting the logarithm of P_{app} versus $1/T$, E_P was determined from the slope of the linear fitting.

2.7. Effect of pH on permeability

The effect of pH on transport of 67 μM As(III) was evaluated in the A-B and B-A directions, whereas only the A-B direction was studied for 1 μM MMA(III) and 1 μM DMA(III). The study was carried out in HBSS medium supplemented with 10 mM o-2-(N-morpholino) ethanesulfonic acid (MES) (pH 5.5) in the donor compartment (apical or basal, depending on the transport direction being studied), and HBSS-HEPES (pH 7.2) in the acceptor compartment. During the test period (120 min), aliquots were collected after 5 min, 15 min, 30 min, 45 min, 60 min, 90 min, and 120 min. The P_{app} and E_r values were calculated from Eqs. (1) and (2), respectively.

2.8. Study of paracellular transport

Paracellular transport was evaluated in the A-B direction through the modulation of intercellular junctions using 5 mM ethylenediaminetetraacetic acid (EDTA) in phosphate buffered saline (PBS) without Ca^{2+} and Mg^{2+}. The cell monolayers were preincubated with the PBS–EDTA solution for 5 min. Subsequent, 1.5 ml of As species [67 μM As(III), 1 μM MMA(III), or 1 μM DMA(III), prepared in HBSS medium without Ca^{2+}] were added. Incubation was carried out for a maximum of 120 min, collecting aliquots after 5 min, 15 min, 30 min, 45 min, 60 min, 90 min, and 120 min. The P_{app}

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