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Characterization of the intestinal absorption of inorganic mercury in Caco-2 cells

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ABSTRACT

The main form of mercury exposure in the general population is through food. Intestinal absorption is therefore a key step in the penetration of mercury into the systemic circulation, and should be considered when evaluating exposure risk. Many studies have investigated the transport of mercury species in different cell lines, though the mechanisms underlying their intestinal absorption are not clear. This study evaluates the accumulation and transport of Hg(II), one of the mercury species ingested in food, using Caco-2 cells as intestinal epithelium model with the purpose of clarifying the mechanisms involved in its absorption. Hg(II) shows moderate absorption, and its transport fundamentally takes place via a carrier-mediated transcellular mechanism. The experiments indicate the participation of an energy-dependent transport mechanism. In addition, H^+- and Na^+-dependent transport is also observed. These data, together with those obtained from inhibition studies using specific substrates or inhibitors of different transporter families, suggest the participation of divalent cation and amino acid transporters, and even some organic anion transporters, in Hg(II) intestinal transport. An important cellular accumulation of up to 51% is observed – a situation which in view of the toxic nature of this species could affect intestinal mucosal function. This study contributes new information on the mechanisms of transport of Hg(II) at intestinal level, and which may be responsible for penetration of this mercurial form into the systemic circulation.

1. Introduction

Mercury (Hg) is a metal found in the environment in different chemical forms: elemental [Hg(0)], inorganic [Hg(II)] and organic [methylmercury (CH_3Hg), ethylmercury (CH_3CH_2Hg) and phenylmercury (C_6H_5Hg)]. The toxic effects of mercury are well known, and are dependent upon the chemical form involved. In this context, organic species are regarded as more toxic than the inorganic forms (Berntssen et al., 2004). Methylmercury is classified as a probable carcinogen for humans by the International Agency for Research on Cancer (IARC, 1993). It is also considered to be a neurotoxic agent (Grandjean et al., 2010), and various epidemiological studies have linked its high intake through seafood products to the development of certain disease conditions (Poulin and Gibb, 2008). Regarding Hg(II), the Environmental Protection Agency of United States has classified HgCl as a possible carcinogen (group C; U.S. EPA; 1999); however, the IARC considers this mercurial form to be a non-carcinogenic compound. The main target is the renal system, where damage has been described in the glomeruli and renal tubules (NTP, 1993; JEFCA, 2011).

In humans, the main source of exposure is the diet – mercury being present in food mainly as methylmercury or inorganic mercury (OEHHA, 1999; Clarkson and Magos, 2006; EFSA, 2012). Fish and other seafood products are the foods with the highest levels of mercury (1–11,400 mg/kg), mostly in the form of CH_3Hg (EFSA, 2012). In other food groups, the predominant species is inorganic mercury [Hg(II)] (EFSA, 2012). In these foods, the concentrations are usually low (range 0.1–50 μg/kg), though occasionally very high values have been reported (0.498–10 mg/kg) (EFSA, 2012).

The gastrointestinal epithelium is the first barrier following the ingestion of mercury and could limit penetration of the latter into the bloodstream and therefore its harmful effects upon the different target tissues. *In vivo* studies have described the absorption of the different forms of mercury – inorganic species being less extensively absorbed than organic species (EFSA, 2012). The absorption of Hg(II) in food has been reported to be less than 15% in experimental animals (Piotrowski et al., 1992), whereas human volunteers who ingested an oral tracer dose of Hg(II) nitrate given either in an aqueous solution or bound to protein absorbed an

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average of 5–10% of the administered dose (Rahola et al., 1973). In rats, the gastrointestinal absorption of Hg(II) has been shown to be greater in newborn animals than in adults (38% versus 6.7%) (Kostial et al., 1978, 1983).

Few studies have investigated the mechanisms that transport Hg(II) across the intestinal epithelium. It has been suggested that intestinal absorption in the rat takes place in two stages: initially, Hg(II) accumulates in the intestinal mucosa, and this in turn is followed by internalization and penetration into the systemic circulation (Piotrowski et al., 1992; Foulkes and Bergman, 1993). Regarding the type of transport involved in the intestinal absorption of Hg(II), most studies have been carried out *in situ*, evidencing that transport is modified upon varying the pH (Endo et al., 1988a,b) and concentrations of Cl^- and Na^+ (Endo et al., 1988a,b, 1990), and in response to the presence of chelating agents and cysteine (Endo et al., 1991). *In vitro* studies in intestinal cells are limited to the work of Aduayom et al. (2003), who suggested that Hg(II) is transported by a passive diffusion mechanism. This limited information contrasts with the extensive literature available on the transport of Hg(II) in other cell types, particularly kidney cells (Endo et al., 1995a,b, 1997; Zalups and Ahmad, 2004; Zalups et al., 2004). In these cells, Hg(II) has been found to be transported mainly bound to low molecular weight thiols, with the intervention of amino acid transporters, in view of the existing structural similarities (Ballatori, 2002).

The *in vitro* characterization of intestinal transport can be made using different approaches such as PAMPA assay, intestinal cell lines, everted gut sacs and isolated perfused organs (Le Ferrec et al., 2001; Niazi, 2007). In this respect it has been shown that cell lines derived from human colon adenocarcinoma offer a good correlation to the *in vivo* situation (Niazi, 2007). Specifically, the Caco-2 cell line preserves parts of the intestinal epithelial differentiation program, expressing many of the genes characteristic of the differentiated intestinal epithelium (Hidalgo et al., 1989). Caco-2 cells likewise maintain the capacity to form polarized cell monolayers *in vitro* once confluence has been reached in culture (Pinto et al., 1983). The Caco-2 cell line was initially introduced as an experimental tool for studying intestinal transport mechanisms. Subsequently, the use of this cell model was extended to the monitoring of drug intestinal permeability and for predicting the oral absorption of drug substances (Tavelin, 2003). Subsequent evaluations have shown the expressed transporter profile of Caco-2 cells to be qualitatively similar to that of the human small intestine (Maubon et al., 2007).

The aim of the present study was to characterize the transport mechanisms involved in the intestinal absorption of Hg(II) using the human Caco-2 cell line as a model of the intestinal epithelium.

2. Materials and methods

2.1. Cell cultures

The human colon carcinoma Caco-2 cell line was 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) containing 4.5 g/L glucose and 0.87 g/L glutamine was added, supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) non-essential amino acids, 1 mM 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 (ethylene diamine tetraacetic acid, 0.22 g/L), and reseeded at a density of 5–6.5 $\times 10^4$ cells/cm^2. The assays were performed with cultures between passages 27 and 42. All the reagents used were obtained from PAA Laboratories GmbH (Labclinic, Spain).

The transport assays were carried out in 6-well plates with polyester membrane inserts (24 mm diameter, pore size 0.4 μm, Transwell®, Costar Corporation, Sigma, Spain). In this system the cells are seeded onto the porous membrane of the insert that separates the well into two compartments: apical (upper) and basolateral (lower). Cells were seeded (6.5 $\times 10^4$ cells/cm^2) onto the inserts, with the addition of 1.5 mL of DMEMc to the apical chamber and 2 mL of DMEMc to the basolateral chamber. The cells were incubated at 37 °C, with 5% CO_2 and 95% relative humidity, with a change of medium every 2–3 days until cell differentiation was reached (12–14 days post-seeding). In order to evaluate the evolution of the monolayers during cell differentiation in the two-compartment system, the transepithelial electrical resistance (TEER) was measured using a Millicell®-ERS voltohmmeter (Millipore Corporation, Spain). The cell monolayer was considered completely formed when values of $\geqslant 250\ \Omega\ cm^2$ were recorded.

2.2. Transport assays and calculation of apparent permeability

The transport assays were carried out in Transwell® plates at pH 7.4 and 37 °C. Two transport directions were tested: apical–basolateral (A–B) and basolateral–apical (B–A). The standard solutions of Hg(II) were prepared from commercial $Hg(NO_3)_2$ standard (1000 mg/L, Merck, Spain) in Hanks' balanced salt solution (HBSS) with $NaHCO_3$ (PAA) supplemented with 10 mM HEPES (HBSS–HEPES). For testing in the A–B direction, the cells were exposed to three concentrations of Hg(II): 0.1, 0.5 and 1 mg/L, equivalent to 0.5, 2.5 and 5 μM, respectively. For testing in the B–A direction, the concentrations used were 0.5 and 1 mg/L. Before starting the experiment, the cells were conditioned with HBSS–HEPES for 15 min, followed by addition of the Hg(II) standard solutions to the donor compartment (apical or basal, depending on whether testing was in the A–B or B–A direction, respectively) and HEPES–HBSS to the acceptor compartment.

At pre-established timepoints (5, 15, 30, 60, 90 and 120 min), aliquots were removed from the acceptor compartment (600 μL) and were replaced by the same volume of HBSS–HEPES. The aliquots removed at each timepoint were processed for mercury content analysis following the protocol described by Vázquez et al. (2013). Likewise, mercury in the cell monolayer and in the donor medium collected at the end of the experiment was quantified. The transport and cell accumulation values were corrected for the total number of viable cells.

The apparent permeability coefficients ($P_{app;}$ cm/s) were calculated from equation 1:

$$P_{app} = (dC/dt)(V_r/AC_o) \quad (1)$$

where dC/dt is the flow (mg/mL/s) determined from the linear slope of the equation defining the variation in mercury concentration (corrected for dilution) versus time; V_r is the volume of the receptor compartment (apical 1.5 mL; basal 2 mL); A is the surface of the cell monolayer (4.67 cm^2) and C_o is the initial mercury concentration in the donor compartment (mg/L).

The efflux ratio (E_r) was calculated from equation 2:

$$E_r = P_{app}(\text{basolateral—apical})/P_{app}(\text{apical—basolateral}) \quad (2)$$

2.3. Study of efflux

Hg(II) efflux assays were performed in Transwell® plates at pH 7.4. A solution of 1 mg/L (5 μM) of Hg(II) in HBSS–HEPES (1.5 mL)

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