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Intestinal transport of methylmercury and inorganic mercury in various models of Caco-2 and HT29-MTX cells

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ABSTRACT

Food is the main pathway of exposure to mercury for most of the population. In food, mercury is generally present as inorganic mercury [Hg(II)] or methylmercury [MeHg]. Both chemical forms have some degree of toxicity, especially MeHg, which is considered a powerful neurotoxicant during development and is classified as a possible human carcinogen. Since the main exposure pathway is oral, gastrointestinal absorption is a decisive step in the process by which mercury reaches the systemic circulation. However, there are few studies that characterize this absorption process.

The present work evaluates transport and cellular retention of Hg(II) and MeHg, using various models of the intestinal epithelium (Caco-2 monocultures and Caco-2/HT29-MTX co-cultures in various proportions). Additionally, a study was made of the influence of the mucus secreted by HT29-MTX cells and of substances normally present in the gastrointestinal tract (L-cysteine, bile salts and food components) on mercury transport and accumulation.

The results show that incorporation of HT29-MTX reduces the permeability coefficient of Hg(II) and MeHg. This decrease coincides with an increase in cellular accumulation, since mercury is retained in the layer of mucus secreted by HT29-MTX cells [Hg(II): 40%; MeHg: 70%]. The presence of L-cysteine, bile salts and food matrix components increases the percentage of both species that is not absorbed. It is noteworthy that in all the conditions assayed the intracellular accumulation of mercury was very high (37–77%). This study shows the importance of the cell model and assay conditions for an *in vitro* evaluation of intestinal transport of mercury species.

1. Introduction

Mercury is a global pollutant which is present in the environment in various chemical forms: metal mercury, inorganic mercury and organic mercury. For most of the population the diet is the main pathway of exposure to this contaminant. Fish and other seafood products mainly contribute organic mercury in the form of methylmercury (MeHg), while it is accepted that inorganic mercury is the predominant form in foods other than fish and shellfish (EFSA, 2012).

The critical target for the toxicity of inorganic mercury is the kidney, and it also produces toxic effects on the liver, nervous immune and reproductive system (EFSA, 2012). MeHg causes adult and developmental neurotoxicity (EFSA, 2012) and is also classified by the International Agency for Research on Cancer as possibly carcinogenic for humans (Group 2B; IARC, 1993). Considerable *in vitro* research has been done to elucidate cell transport mechanisms for mercury species in target organs. Kidney cells (Zalups and Ahmad, 2004, 2005), retinal pigment epithelial cells (Bridges et al., 2007) and neural cells (Heggland et al., 2009) have been used for this purpose. However, although the intestinal wall is the first barrier to prevent mercury species from reaching the systemic circulation, transport of these species across the epithelium of the intestinal mucosa has not been characterized.

For *in vitro* intestinal transport studies it is customary to use the Caco-2 cell line. In culture, these cells differentiate spontaneously, forming monolayers with tight junctions and polarized apical/mucosal and basolateral/serosal membranes that are structurally and functionally similar to enterocytes. However, they have some limitations that could be important for a study of intestinal transport depending on the characteristics of the compound. They express many of the enzymes and transporters that are present in the small intestine (Pinto et al., 1983), although in most cases the expression levels differ with regard to human intestine. It is well known that permeabilities of compounds that are transported *via* carrier-mediated absorption are lower in the Caco-2 cell system as compared to the human small intestine (Hilgendorf et al., 2000). Moreover, because they come from the colon, the size of the pore radius of the cell junctions (5 Å) is less than that of pores

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in the human intestinal epithelium (8–13 Å) (Tavelin, 2003). This limitation might lead to an underestimate of the permeability of compounds that are transported predominantly by the paracellular pathway (Wikman-Larhed and Artursson, 1995). Another important limitation is the absence of the layer of mucus secreted by goblet cells (Mahler et al., 2009), which *in vivo* covers the human intestinal epithelium and which has been seen to affect transport of some compounds (Wikman et al., 1993; Mahler et al., 2009). One possible way of overcoming some of these limitations is to add HT29-MTX cells to the Caco-2 monoculture. The HT29-MTX cell line, which is also of colonic origin, differentiates in postconfluence into mucosecretory cells that form a monolayer with an intercellular pore size closer to that of the human small intestine (Walter et al., 1996; Hilgendorf et al., 2000; Mahler et al., 2009). The use of co-cultures of Caco-2 and HT29-MTX allows the model to incorporate the two major cell types found in the small intestinal epithelium (enterocytes and goblet cells) and therefore provides a model that represents the human intestinal epithelium more accurately. This model has been used to evaluate intestinal absorption of drugs (Hilgendorf et al., 2000) and some trace elements (Mahler et al., 2009; Calatayud et al., 2012a; Rocha et al., 2012).

The aim of the present study is to evaluate transport and cellular accumulation of Hg(II) and MeHg, using various models of the intestinal epithelium (Caco-2 monocultures and Caco-2/HT29-MTX co-cultures in various proportions). A further aim is to evaluate the influence of compounds normally present in chyme, either endogenous or derived from the diet (bile salts, forms of cysteine, soluble food matrix components), on transport of mercury species.

2. Materials and methods

2.1. Mercury species

The solutions of Hg(II) and MeHg were prepared by diluting commercial standards of $Hg(NO_3)_2$ (1000 mg/L, Merck, Spain) and CH_3HgCl (1000 mg/L, Alfa Aesar, Spain), respectively.

2.2. Cell cultures

The human colon carcinoma Caco-2 cell line was obtained from the European Collection of Cell Cultures (ECACC, number 86010202, Salisbury, UK), and used between passages 11 and 36. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L glucose and 0.87 g/L glutamine, and 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 HT29-MTX cell line was kindly provided by Dr. Técla Lesuffleur (Institut National de la Santé et de la Recherche Médicale, INSERM UMR S 938, Paris, France) and used between passages 15 and 24. Cell maintenance was done in DMEM 4.5 g/L glucose and 0.87 g/L glutamine, supplemented with 10% (v/v) fetal bovine serum, 1 mM sodium pyruvate, 10 mM HEPES, 100 U/mL of penicillin, 0.1 mg/mL of streptomycin and 0.0025 mg/L of amphotericin B (HT-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 $5–6 \times 10^4$ cells/cm^2. All the reagents used were obtained from PAA Laboratories GmbH (Labclinics, Spain).

The transport and accumulation assays were carried out in 6-well plates with polyester membrane inserts (24 mm diameter, pore size 0.4 μm, Transwell®, Costar Corporation, Sigma). In this system the cells are seeded on the porous membrane of the insert that separates the well into two compartments: apical (upper) and basolateral (lower). The cells were seeded (5.5×10^4 cells/cm^2) on the apical side to produce monocultures of Caco-2 and HT29-MTX and co-cultures of Caco-2/HT29-MTX in different proportions (70/30 and 50/50), depending on the assay. Then, 1.5 mL of DMEMc (for Caco-2) or HT-DMEMc (for co-cultures and HT29-MTX monocultures) was added to the apical chamber, and 2 mL of the same medium was added to the basolateral chamber. The cells were maintained in a humidified atmosphere of 5% CO_2 and 95% air at 37 °C, with a change of medium every 2–3 days until differentiation (11–12 days post-seeding). During cell differentiation in the bicameral systems the transepithelial electrical resistance (TEER) was measured with a Millicell®-ERS voltohmmeter (Millipore Corporation, Spain) to evaluate the progress of the monolayers.

2.3. Transport and accumulation assays

Caco-2 monocultures and Caco-2/HT29-MTX co-cultures (70/30 and 50/50) differentiated for 11–12 days in the Transwell® systems were used. The medium employed for the assays was Hanks' Balanced Salt Solution (HBSS) (PAA) supplemented with 10 mM HEPES (pH 7.2). Before beginning the experiment, the cells were conditioned with the medium for 15 min.

Transport of standards of Hg(II) and MeHg at a concentration of 0.8 mg/L (4 μM) was studied in the apical–basolateral direction (A–B). For the assays, 1.5 mL of the standard prepared in HBSS-10 mM HEPES together with 100 μM of the paracellular transport marker Lucifer Yellow, LY (Sigma, Spain), was added to the apical compartment, and 2 mL of HBSS-10 mM HEPES to the basolateral compartment. At the stipulated assay times (5, 15, 30, 45, 60, 90 and 120 min), aliquots (600 μL) were taken from the acceptor compartment and replaced with an equal volume of HBSS-10 mM HEPES without addition of mercury species. The total mercury concentration was determined in the aliquots taken at each time, and in the cell monolayer and the apical medium collected at the end of the experiment.

The apparent permeability coefficients (P_{app}) were calculated using Eq. (1).

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

where dC/dt is the flow (μg/s) determined by the linear slope of the equation that governs the variation in the mercury species concentrations, corrected for dilution, *versus* time. V_r is the volume of the receptor compartment (2 mL). A is the surface occupied by the cell monolayer (4.67 cm^2). C_0 is the initial mercury concentration in the apical compartment (0.8 mg/L).

For the accumulation assays, 1.5 mL of standard solutions of MeHg and Hg(II) (0.8 mg/L, 4 μM) prepared in HBSS-10 mM HEPES (pH 7.2) together with LY (100 μM) was added to the apical compartment. After 120 min of exposure, the cell monolayers were washed with phosphate buffered saline (PBS, PAA) and recovered by trypsinization with a solution of trypsin (0.5 mg/L) and EDTA (0.22 mg/L). Finally, the total mercury concentration in the apical and basolateral media and in the cell monolayers was determined.

2.4. Retention of mercury species in mucus layer secreted by HT29-MTX cell line

To confirm the possible influence of the mucus layer on mercury species transport, the mercury concentration in the mucus of differentiated HT29-MTX cells (12 days post-seeding) exposed for 120 min to 0.7 mg/L (3.5 μM) of MeHg or Hg(II) was determined. After exposure, the medium was recovered and the mucus layer was separated from the cell monolayer, using the method described by Mahler et al. (2009) with some modifications. Briefly, the monolayer was washed with 1.5 mL of 10 mM N-acetylcysteine (NAC, Sigma) in HBSS at 37 °C with agitation (135 rpm) for 30 min. Subsequently, medium (HBSS + NAC) was recovered to quantify the mercury concentration in the mucus.

2.5. Influence of L-cysteine (L-Cys) on transport and accumulation of mercury species

Transport of Hg(II) and MeHg (0.4 mg/L, 2 μM) was assayed in the presence of 5 μM of L-Cys (Merck) in differentiated Caco-2 monocultures and Caco-2/HT29-MTX (70/30) co-cultures. During the test period (120 min), aliquots were collected from the basolateral compartment after 5, 15, 30, 60, 90 and 120 min. The total mercury concentration was determined in the aliquots taken at each time, and in the cell monolayer and the apical medium collected at the end of the experiment.

2.6. Influence of taurocholic acid on transport and accumulation of mercury species

This study was conducted on the Caco-2/HT29-MTX (70/30) co-culture and on the Caco-2 monocultures. To evaluate the effect of taurocholic acid, the cells were exposed to a standard solution of 0.7 mg/L (3.5 μM) of Hg(II) or MeHg and 20 mM of taurocholic acid (Sigma) prepared in HBSS-10 mM HEPES. During the test period (90 min), aliquots were collected from the basolateral compartment after 5, 15, 30, 60, and 90 min for determination of the total mercury concentration. The mercury concentrations in the cell monolayer and in the apical medium collected at the end of the assay were also determined.

2.7. Influence of the food matrix on transport and accumulation of mercury species

Three samples of swordfish (*Xiphias gladius*) were digested using an *in vitro* simulated gastrointestinal digestion (Laparra et al., 2003). Briefly, after the gastric step (0.02 g pepsin/g sample, pH 2, 37 °C, 2 h and 120 strokes/min) and the intestinal step (0.0005 g of pancreatin/g sample and 0.003 g of bile extract/g sample, pH 6, 37 °C, 2 h and 120 strokes/min), samples were centrifuged at 26,891 × *g* for 30 min at 4 °C to separate the soluble fraction (bioaccessible). Mercury was analyzed in the bioaccessible fractions. Digestion blanks were obtained in the same conditions. The enzymes employed for the gastrointestinal digestion were purchased from Sigma.