Toxicology 331 (2015) 119–124

Contents lists available at ScienceDirect

Toxicology

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

Participation of divalent cation transporter DMT1 in the uptake of inorganic mercury

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ARTICLE INFO

Article history:
Received 16 February 2015
Received in revised form 6 March 2015
Accepted 10 March 2015
Available online 12 March 2015

Keywords:
Mercury
DMT1
Transport
Intestinal epithelium
Caco-2 cells

ABSTRACT

Mercury (Hg) is found in food in various chemical forms, which differ in terms of accumulation, transport, and toxicity. Although methylmercury (CH_3Hg) is the predominant mercury species in the diet, contributed mostly by seafood products, there is also a contribution of inorganic mercury [Hg(II)] from vegetables, cereals, and seafood products. The main pathway for exposure to mercury is oral, and therefore the gastrointestinal mucosa is the first barrier that the contaminant meets when it enters the systemic circulation. However, the transport mechanisms responsible for the process of mercury absorption are not known.

The aim of this study is to evaluate the possible participation of divalent metal transporter 1 (DMT1) in Hg(II) intestinal uptake. For this purpose, we have used various complementary approaches. We have studied mercury acquisition in a *Saccharomyces cerevisiae* strain expressing murine *DMT1*. Moreover, we have evaluated the effect of a reduction of *DMT1* expression in Caco-2 cells, by means of small interfering RNA and of treatment with hepcidin, on mercury uptake and transport.

The results show that expression of the transporter *DMT1* in yeast produces an increase in Hg(II) accumulation. Furthermore, a decrease in the levels of *DMT1* mRNA in Caco-2 cells in various stages of differentiation leads to a reduction in cellular accumulation and apical-basolateral transport of Hg(II). These data point clearly to the mediation of the divalent cation transporter DMT1 in the entry of Hg(II) into the intestinal epithelium.

1. Introduction

Food is the main source of exposure to mercury (Hg) for most of the population. Methylmercury (CH_3Hg) is the predominant mercury form in the diet and is found mainly in seafood products (Forsyth et al., 2004). Divalent inorganic mercury [Hg(II)] can also be found in substantial concentrations in vegetables, cereals, and seafood products (JECFA, 2011; EFSA, 2012). The toxic effects of mercury differ, depending on the chemical form, the dose, and the pathway of exposure. CH_3Hg mainly affects the nervous system (National Research Council, 2000), whereas, inorganic mercury produces renal pathologies, and also gastrointestinal, immunological, and dermatological disorders (NTP, 1993).

The fact that the main pathway for exposure to mercury is oral means that passage through the gastrointestinal barrier is the step that limits the arrival of this element into the systemic circulation for subsequent distribution to the various target organs. It is known that in general the ratios of intestinal absorption of Hg(II) are lower than those of CH_3Hg (Clarkson and Magos, 2006). Studies on the transport of inorganic mercury have mostly been conducted using kidney, brain, and retinal cells, and have shown that Hg(II) bound to thiol groups can use amino acid transporters (Bridges and Zalups, 2004; Bridges et al., 2004, 2007). With regard to transport in intestinal cells, Vázquez et al. (2015) used techniques of gene silencing with small interfering RNA to show that the transporter $b^{0,+}$ can transport Hg(II) bound to L-cysteine (L-Cys).

The above-mentioned studies concentrated on evaluating transport of Hg(II) bound to thiol groups, mainly L-Cys owing to the high affinity that mercury species have for those groups (Zalups, 2000). However, despite the thermodynamic stability of the (linear I or II) coordinate covalent bonds formed between mercuric ions and various thiol-containing molecules in aqueous solution, the bonding characteristics appear to be more labile within the living organism (Zalups, 2000). Therefore, in the intestinal lumen there could also be Hg(II) forming salts with other elements such as chloride, for which it also has a high affinity. This inorganic mercury form could use the same intestinal transport mechanisms as other divalent cations.

Divalent metal ion transporter 1 (DMT1) is a divalent transition-metal/H^+ cotransporter from the Nramp family located in the

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http://dx.doi.org/10.1016/j.tox.2015.03.005
0300-483X/

apical domain of the epithelial cells of the intestine (Canonne-Hergaux et al., 1999). This protein is able to transport a great variety of essential divalent metal cations, such as Fe, Zn, Mn, Co, Cu, and Ni (Zalups and Koropatnick, 2010). There is also evidence that DMT1 could participate in the absorption of toxic metal cations such as Cd(II) and Pb(II) (Bannon et al., 2002; Okubo et al., 2003; Bressler et al., 2004). However, no studies have been made of its possible participation in the transport of Hg(II).

The aim of this study is to evaluate the possible participation of DMT1 in Hg(II) transport. For this purpose, we have used various approaches: the study of mercury uptake in a *Saccharomyces cerevisiae* strain transformed with *DMT1*, and the evaluation of the effect of a reduction of *DMT1* expression in Caco-2 cells by means of small interfering RNA (siRNA) and of treatment with hepcidin on mercury uptake and transport.

2. Materials and methods

2.1. Yeast strains and growth conditions

The *S. cerevisiae* strains used in this study were BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and *smf1Δsmf2Δ* (XL131 strain; *MATα smf1::URA3 smf2::HIS3 ade2 lys2Δ201 leu2-3 112*), kindly donated by Dr. Valeria Culotta (Department of Environmental Health Sciences, Johns Hopkins University, School of Hygiene and Public Health, Baltimore, MD, USA). Stocks of yeast strains were maintained on standard YPD medium [1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose]. Yeast cells were transformed following the protocol described by Gietz and Woods (2002). Wild-type (BY4741) and *smf1Δsmf2Δ* (XL131) yeast cells transformed with vector alone (p415GPD) or expressing DMT1 (p415GPD-DMT1) were grown overnight in synthetic complete medium without leucine (SC-leu) [0.17% (w/v) yeast nitrogen base without amino acids and ammonium sulfate (Difco), 0.5% (w/v) $(NH_4)_2SO_4$, 2% (w/v) glucose, and the corresponding Kaiser drop-out supplements (ForMediumTM)], and then diluted to an A_{600} of 0.2–0.4.

For yeast mercury uptake experiments, standard solutions were prepared from commercial solution of $Hg(NO_3)_2$ (Merck, Spain). In parallel, yeast were exposed to manganese [$Mn(NO_3)_2$, Merck], used as positive control substrate because DMT1 has a high affinity for this element (Gunshin et al., 1997; Arredondo et al., 2003). Cells were incubated at 30 °C in agitation (190 rpm) in SC-leu containing 30 μM $Mn(NO_3)_2$ or 20 μM $Hg(NO_3)_2$. Yeast cells were collected at 7 and 24 h, washed with 0.9% (w/v) NaCl to eliminate trace element residues, and frozen in liquid nitrogen until analysis.

2.2. Plasmids

Murine *DMT1* coding sequence was amplified by PCR from the pMT2-mDMT1 plasmid (kindly provided by Jaekwon Lee Department of Biochemistry, University of Nebraska-Lincoln, USA) with the Expand High Fidelity PCR System (Roche Applied Science) by using the DMT1-HindIII-F (5′-TGGAAGCTTACCATGGTGTTGGATCC-TAAA-3′) and DMT1-XhoI-R (3′-TTCCTCGAGTTAGTCATCTGGACAC-CACT-5′) primers. The PCR product was digested with HindIII and XhoI restriction enzymes, cloned into p415GPD yeast expression vector (Mumberg et al., 1995), and sequenced.

2.3. Human cell culture

Caco-2 epithelial colorectal adenocarcinoma cells were obtained from the European Collection of Cell Cultures (ECACC, 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) and glutamine (0.87 g/L) at pH 7.2 were added. The DMEM was 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%. When the cell monolayer reached 80% confluence, the cells were detached with a solution of trypsin (0.5 g/L) and EDTA (0.2 g/L) and reseeded at a density of 5–6.5 × 10^4 cells/cm^2. The assays were performed with cultures between passages 30 and 38. All the reagents used were obtained from HyClone Laboratories (Scientific Thermo, Spain).

2.4. DMT1 *mRNA levels in Caco-2 cells exposed to Hg(II)*

Caco-2 cells were grown in 6-well plates at a density of 6.4 × 10^4 cells/cm^2 in DMEMc. After 12–15 days of differentiation, cells were exposed to 1 mg/L Hg(II) in minimal essential medium (MEM) (HyClone) supplemented with 1 mM sodium pyruvate for 24 and 48 h. The Hg(II) solutions were prepared from $Hg(NO_3)_2$. After exposure, cells were recovered for gene expression of *DMT1*. Cells not exposed to Hg(II) were used as a control.

DMT1 mRNA levels were quantified by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RNA was extracted using a NucleoSpin RNA II kit (Macherey-Nagel, Germany). The RNA extracted was quantified spectroscopically in a NanoDrop ND-1000 (NanoDrop Technologies, USA). First-strand complementary DNA (cDNA) was obtained from 200 ng of total RNA using a 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), and 1 μL of each forward and reverse primer (10 μM) (Biolegio, the Netherlands). No-template controls were run to verify the absence of genomic DNA. The oligonucleotides used for *DMT1* were the following: forward 5′-GTGGTCAGCGTGGCTTATCTG-3′; reverse 5′-GATGCTTACCGTATGCCCACAGT-3′. RN18S was used as a reference gene (forward 5′-CCATCCAATCGGTAGTAGCG-3′; reverse 5′-GTAACCCGTTGAACCCCATT-3′). PCR efficiency curves for each gene were calculated using five duplicate 2-fold dilutions of cDNA.

The PCR 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. Data was analyzed with Relative Expression Software Tool (REST 2009, QIAGEN), using standard mode. All experiments were performed in quadruplicate.

2.5. siRNA transfections in Caco-2 cells: effect on DMT1 expression and mercury accumulation

On average, 5 × 10^4 cells/cm^2 were seeded on a 24-well plate in DMEMc without antibiotics and antifungics, and incubated overnight under their normal growth conditions. siRNAs obtained from Qiagen (Hs_SLC11A2_7, Hs_SLC11A2_14) were diluted to a concentration of 60 nM in DMEM without supplementation. Lipofectamine transfection reagent (Life Technologies, 3 μL in 50 μL DMEM without supplementation) was added to the diluted siRNAs in a 1:1 proportion and the mixture was incubated for 5 min to allow formation of transfection complexes. Then the complexes were added drop-wise to the cells (100 μL) and incubated under their normal growth conditions. Transfection efficiencies were estimated by the evaluation of cell viability (trypan blue staining) after transfection of AllStars Hs Cell Death Control siRNA (Qiagen) at different time points (6, 24, and 48 h). After siRNA transient transfection (24 h), cells were recovered for gene expression assays as described previously.