Toxicology and Applied Pharmacology 314 (2017) 55–62

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

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

Multidrug and toxin extrusion proteins mediate cellular transport of cadmium

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ARTICLE INFO

Article history:
Received 22 September 2016
Revised 15 November 2016
Accepted 17 November 2016
Available online 18 November 2016

Keywords:
Multidrug and toxin extrusion protein
Cadmium
Transporter
Toxicity
Detoxification
Kidney

ABSTRACT

Cadmium (Cd) is an environmentally prevalent toxicant posing increasing risk to human health worldwide. As compared to the extensive research in Cd tissue accumulation, little was known about the elimination of Cd, particularly its toxic form, Cd ion (Cd^{2+}). In this study, we aimed to examine whether Cd^{2+} is a substrate of multidrug and toxin extrusion proteins (MATEs) that are important in renal xenobiotic elimination. HEK-293 cells overexpressing the human MATE1 (HEK-hMATE1), human MATE2-K (HEK-hMATE2-K) and mouse Mate1 (HEK-mMate1) were used to study the cellular transport and toxicity of Cd^{2+}. The cells overexpressing MATEs showed a 2–4 fold increase of Cd^{2+} uptake that could be blocked by the MATE inhibitor cimetidine. A saturable transport profile was observed with the Michaelis-Menten constant (K_m) of 130 ± 15.8 μM for HEK-hMATE1; 139 ± 21.3 μM for HEK-hMATE2-K; and 88.7 ± 13.5 μM for HEK-mMate1, respectively. Cd^{2+} could inhibit the uptake of metformin, a substrate of MATE transporters, with the half maximal inhibitory concentration (IC_{50}) of 97.5 ± 6.0 μM, 20.2 ± 2.6 μM, and 49.9 ± 6.9 μM in HEK-hMATE1, HEK-hMATE2-K, and HEK-mMate1 cells, respectively. In addition, hMATE1 could transport preloaded Cd^{2+} out of the HEK-hMATE1 cells, thus resulting in a significant decrease of Cd^{2+}-induced cytotoxicity. The present study has provided the first evidence supporting that MATEs transport Cd^{2+} and may function as cellular elimination machinery in Cd intoxication.

1. Introduction

Cadmium (Cd) is a toxic heavy metal which can accumulate in multiple organs, including kidney, liver, testis, lung, and pancreas. In addition to daily life exposure such as foods and cigarette smoking, Cd has been extensively disseminated in the environment as a pollutant due to a strong demand worldwide, particularly in the battery industry. Cd has no known physiological benefit. It may induce mitochondrial damage and culminate in cell death either by apoptosis or necrosis (Thevenod and Lee, 2013b; Thevenod and Lee, 2013a). Emerging evidence has linked chronic low level of Cd exposure to the causes of cancer, cardiovascular diseases, and diabetes (Prozialeck et al., 2008; Edwards and Prozialeck, 2009; Tellez-Plaza et al., 2012; Son et al., 2014).

Kidney has been determined as a major target organ in Cd intoxication (Klaassen et al., 1999; Thevenod and Lee, 2013b; Yang and Shu, 2015). Cd is found both as the free form (Cd^{2+}) and bound to carriers such as albumin, metallothionines (MT), glutathione (GSH), and cysteine (Cys) after absorption from intestine and lung (Klaassen et al., 1999; Zalups, 2000; Wang et al., 2010). All of the Cd-MT, Cd-GSH, Cd-Cys and Cd^{2+} could be filtered through the glomerulus, and reabsorbed by tubular epithelial cells. The bound form Cd-MT could be transported into the proximal tubular cells *via* endocytosis, while the complexes Cd-GSH and Cd-Cys reabsorbed by certain amino acid transporters in the apical membrane of the proximal tubular cells. As for the free Cd^{2+}, several apical membrane transporters, which are responsible for reabsorption of essential metals such as zinc, iron, manganese, and calcium, have been identified as Cd^{2+} transporters (Bannon et al., 2003; Fujishiro et al., 2012; Kovacs et al., 2013; Marchetti, 2013). Recent studies have provided evidence supporting organic cation transporters (OCTs) as basolateral transporters for Cd^{2+} uptake in the proximal tubular cells (Soodvilai et al., 2011; Thevenod et al., 2013). Interestingly, additional transporters such as organic anion transporters (OATs) have also been suggested to play a role in the basolateral uptake of certain bound forms of Cd (Zalups et al., 2004).

As compared to the comprehensive understanding in Cd accumulation, its elimination is poorly recognized. Since an extremely long half-life of Cd has been observed both in rodents and humans (10–30 years) (Thomas et al., 1980; Jarup and Akesson, 2009), an elimination mechanism, while presumably being not as efficient as those for uptake and accumulation, could be critical to Cd detoxification. As a cellular protective mechanism, endogenous thiol-containing groups such as GSH, Cys and MT sequester most Cd present in the cell. Cd^{2+} could be released from those degradable bound complexes and there is

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http://dx.doi.org/10.1016/j.taap.2016.11.007
0041-008X/

equilibrium between the bound and the free forms of Cd in the cell. The released Cd^{2+} might be either chelated by thiol-containing groups or transported out of the cell. Hence we postulated that the efflux transporters resided in the apical side of the renal tubular epithelial cells for Cd^{2+}, if any, might serve as a detoxification mechanism. Inspiringly, Endo and colleagues have found that efflux of Cd^{2+} occurred *via* a proton antiport exchanger (Cd^{2+}/H^{+} antiport) (Endo et al., 1998a; Endo et al., 1998b). Moreover, the identification of OCTs as basolateral Cd^{2+} transporters also provides a clue that the organic cation transporters in the apical membrane may be involved in transport of Cd^{2+} (Soodvilai et al., 2011; Thevenod et al., 2013).

Multidrug and toxin extrusion proteins (MATEs) are located in the apical membrane in hepatocytes and renal proximal tubules (Otsuka et al., 2005; Masuda et al., 2006). Functioning as organic cation transporters, MATEs are responsible for elimination of various, structure unrelated endogenous and exogenous compounds in the kidney. The present study was to determine whether Cd^{2+} was a substrate of MATEs. In particular, since the free Cd^{2+} is mainly responsible for Cd-induced cytotoxicity, we sought to test whether MATEs could ameliorate such cytotoxicity by transporting Cd^{2+} out of cells.

2. Materials and methods

2.1. Materials

[^{14}C]-metformin (1.0 mCi, 90 mCi/mmol) was purchased from Moravek Biochemicals. Cd chloride was purchase from Sigma-Aldrich (St. Louis, MO). Cd Standard (1000 μg/mL in 2% HNO_3) was purchased from SPEXCertiPrep Inc. (Metuchen, NJ). Indium Standard (100 μg/mL in 2% HNO_3) was purchased from ULTRA Scientific Inc. (N. Kingstown, RI). Dulbecco's Modified Eagle's medium (DMEM), PBS buffer, Opti-MEM reduced serum medium, Lipofectamine 2000, hygromycin, and fetal bovine serum (FBS) were purchased from Invitrogen. All other reagents were commercial available. All the reagents used for Inductively Coupled Plasma mass Spectrometry (ICP-MS) were of trace ICP-MS grade.

2.2. Cell lines

The generation of HEK-293 cell lines stably expressing human MATE1 (HEK-hMATE1) and human MATE2-K (HEK-hMATE2-K) using the Flp-In system (Invitrogen) has been described previously (Li et al., 2013). In brief, the cDNAs of hMATE1 and hMATE2-K were constructed into pcDNA5 empty vector, and the stable cell lines were established by selection against hygromycin (75 μg/mL). Transient transfection was used to overexpress mouse Mate1 in HEK-293 cells (HEK-mMate1) according to manufacturer's instruction (Lipofectamine 2000, Invitrogen). The overexpression of the MATE transporters in HEK-293 cells was confirmed by real-time PCR and functional tests.

2.3. Cell culture

HEK-293 cells were cultured in DMEM supplemented with 10% FBS, 100 μg/mL streptomycin, and 100 U/mL penicillin, and maintained in 75 cm^2 plastic flask under 37 °C in a humidified atmosphere with 5% CO_2.

2.4. Cd^{2+} cellular uptake and quantification

The cellular uptake experiments were performed in the 24-well plates coated with poly-D-lysine. The protocol has been described elsewhere with minor modification (Li et al., 2013; Muller et al., 2013). In order to characterize MATE function in the heterogeneously expression system (HEK-293 cells), uptake studies were performed under an artificial intracellular acidic environment established by pre-incubation with NH_4Cl. 3.5×10^5 cells were seeded in each well and incubated for 18–24 h to reach confluence. Once ready, the cells were washed by pre-warmed K^{+} based buffer (KBB, 140 mM KCl, 0.4 mM KH_2PO_4, 0.8 mM $MgSO_4$, 1.0 mM $CaCl_2$, 25 mM glucose, and 10 mM HEPES, pH 7.4), then incubated in KBB buffer containing 30 mM NH_4Cl for 15 min at 37 °C, thereafter incubated for 5 min in NH_4Cl-free KBB buffer which were replaced with the KBB buffer containing different concentrations of Cd^{2+} with or without MATE inhibitors for different periods of time. The uptake was stopped by adding 750 μL ice-cold KBB buffer, and the wells washed 3 times by KBB buffer of room temperature. After thorough aspiration of KBB buffer, 200 μL of nitric oxide (67–70%, Sigma-Aldrich, St. Louis, MO) was added to each well and the plate then shaken for 15 min. Thereafter, 100 μL of cell lysate was transferred to a 2 mL tube and incubated under 56 °C for at least 4 h. 20 μL internal standard (Indium, 100 μg/mL) and 1880 μL 2% nitric oxide was then added into each tube to make a final volume of 2 mL which was ready for quantification by ICP-MS (Agilent 7700). Cellular protein levels in parallel wells were determined by a bicinchoninic acid (BCA) protein assay kit (Bio-Rad Co. Hercules, CA). The protein levels were used to normalize the Cd^{2+} concentration values determined from ICP-MS analysis.

2.5. Metformin cellular uptake

The protocol for performing metformin uptake study is similar to that for Cd^{2+} as described above. The substrate metformin (50 μM: 10 μM [^{14}C]-metformin plus 40 μM non-radioactive metformin) was incubated with or without MATE inhibitors to probe cellular MATE activities. The reaction was stopped by adding ice-cold KBB and the cells were washed 3 times with KBB. The cells were shaken for another 15 min after adding 300 μL of cell lysis buffer (PBS with 1% triton X-100). 250 μL of cell lysate was then added to the scintillation tube which was pre-loaded with 3 mL Biodegradable Counting Cocktail buffer (Fisher Scientific Inc., Pittsburgh, PA). The radioactivity was counted by a liquid scintillation analyzer (PerkinElmer, Tri-Carb 2910 TR) normalized by the protein level in the lysate.

2.6. Determination of cell viability

The HEK-293 cells overexpressing a MATE transporter and mock cells were plated at a density of 3.5×10^5/well in 24-well plates in a serum-containing medium for 18–24 h. After grown to confluent, Cd^{2+} treatment was started with varied length of incubation periods. MATE transporters are bi-directional *per se*. Depending on the direction of their driving force of proton gradient, MATEs can either take up the substrates into the cell as uptake transporters or transport them out of the cell as efflux transporters. We analyzed Cd^{2+} cytotoxicity with two treatment protocols. First, the cells were intra-acidified by pre-incubation with NH_4Cl-containing KBB buffer as described above. The acidified cells which are supposed to take up the substrates of the expressing MATE transporters were then treated by different concentration of Cd^{2+} for 30 or 60 min with or without MATE inhibitors. After incubation, the uptake medium was aspirated, and the cells were washed once by PBS and then incubated in serum-containing medium. After incubation for another 18 h, the cell viability was determined by using the cell counting kit-8 (CCKi-8, Enzo Life Science Inc.) according to the manufacturer's instruction. In brief, 200 μL of the cell counting medium (10% of CCKi-8 in the serum-containing medium) was added into each well. After incubation under 37 °C for 30 to 60 min, the medium was transferred to a 96-well and absorbance was measured at 450 nm. The second treatment protocol was followed to test if MATE transporters could efflux Cd^{2+} out of the cell and reduce Cd^{2+}-induced cytotoxicity. The cells were firstly intra-acidified and treated with Cd^{2+} as described above. After this Cd preloading period, the cells were washed by KBB buffer for 3 times, then changed to Krebs-Ringer HEPES (KRH) buffer (125 mM NaCl, 4.8 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM HEPES, 5.6 mM glucose,