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Fe- and Zn-induced inhibition of Cd uptake in human lung cell lines: Speciation studies with H441 and A549 cells

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ABSTRACT

Cadmium transport was studied in human lung cell lines A549 and H441 as a function of inorganic metal speciation. A 2-fold higher equilibrium accumulation was obtained in the A549 cells, but a specific system of transport of high affinity and low capacity was characterized in both these cells. Exposure conditions optimizing $[Cd^{2+}]$ increased Cd uptake, but $CdCl_n^{2-n}$ species are also taken up. Studies on Cd-Ca reciprocal inhibition do not support a role for Ca pathways in Cd uptake. Nramp2 and Zip8 mRNAs were detected in both cell lines. Fe inhibited Cd uptake in the nitrate medium with an apparent at pH 5.5. NRAMP2 may contribute to Cd uptake but a major role is precluded since the overall process of accumulation was inhibited by acidic conditions. Zn and Mn inhibited Cd uptake with the following observations: (1) Similar apparent K_i values for Zn-induced inhibition in the chloride and nitrate media. (2) Whatever the medium, a higher Mn-induced inhibition at pH 5.5 compared to pH 7.4, with much lower K_i values under acidic conditions. (3) Elimination of the stimulatory effect of HCO_3^- by Zn but not by Mn. Zn may inhibit a ZIP8-mediated Cd uptake, whereas the Mn-sensitive component of uptake would be related to other transport processes.

1. Introduction

Cadmium (Cd) is a widespread metal that has various adverse effects on health. For the general nonsmoking population, the main exposure to Cd is oral intake through contaminated food products. However, uptake by inhalation can exceed that from ingestion in specific workplaces as well as in industrial and polluted residential areas where atmospheric Cd levels have been shown to be rather high (Järup and Akesson, 2009). Cd lung toxicity includes chronic edema and bronchitis, emphysema as well as cancer (Ando et al., 1996; Bell et al., 2000; Fiévez et al., 2009; Wild et al., 2009). Pulmonary absorption far exceeds oral bioavailability of Cd as it ranges from 10% to 50% compared to less than 5% (Järup, 2002). The size of the inhaled particles is critical for Cd accumulation along the respiratory tract, and significant particle deposition in the terminal bronchioli and alveoli occurs for particle diameters smaller than 2–3 μm, whereas larger particles deposit in the nasopharyngeal regions (Heyder, 1982). Metal speciation also affects Cd availability: chloride, sulfide, and oxide are relevant metal compounds for human exposure but the availability of oxide and sulfide is rather low because of their poor solubility (Glaser et al., 1986). How Cd enters the lung cells remains to be clarified, however.

It has been hypothesized that Cd enters the cells using transport systems devoted to essential elements. For decades, huge efforts have been put into the identification of transport processes responsible for Cd accumulation in target cells. Earlier *in vivo* studies showed that Ca, Zn, or Fe dietary status may modify the absorption of Cd (Flanagan et al., 1978; Waalkes, 1986; Hoadley and Johnson, 1987). Cd is known as an L-type channel blocker, and a number of studies have suggested the involvement of voltage-gated channels in Cd uptake (Flanagan and Friedman, 1991; Limaye and Shaikh, 1999; Baker et al., 2003; Fotakis and Timbrell, 2006). The contribution of voltage-independent Ca transporters, namely CaT1 (TRPV6) in Cd uptake should also be considered (Min et al., 2008), and the role that Ca transport systems play in the accumulation of Cd in lung cells remains unknown. The Fe^{2+}/H^+ cotransporter NRAMP2 has also been shown to contribute to Cd^{2+} accumulation (Elisma and Jumarie, 2001; Bannon et al., 2003; Okubo et al., 2003), and iron responsive element (IRE)-dependent upregulation of NRAMP2 by low iron body status is believed to contribute to the well-known higher susceptibility to Cd toxicity of individuals suffering from anemia. NRAMP2 is expressed early in the bronchiolar epithelium during embryonic development (Chong et al., 2005). It has been suggested that lung NRAMP2 plays a role in metal detoxification and would protect the epithelial surface from iron-induced oxidative stress (Ghio et al., 2005). More recently, the Slc39a8 gene has been demonstrated to be the genetic locus *Cdm* associated with sensitivity to Cd-induced injury in testis (Dalton et al., 2005; Wang et al., 2007). The Zn transporter ZIP8 would be responsible for

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increased Cd uptake in the testis vascular endothelial cells. Human lung epithelial cells express ZIP8 which has been suggested as playing a role in lung protection in response to inflammation (Besecker et al., 2008). Cd transport via ZIP8 has been shown in some expression systems (He et al., 2006; Liu et al., 2008) but very few studies have investigated the role that ZIP8 could play in Cd uptake in lung cells (Aiba et al., 2008).

Hence, it appears that there is no unique mechanism of transport that may be involved in Cd entry into cells, and that many systems may contribute to the overall Cd accumulation in the same cell. This reality would be rather more complex if we consider that Cd^{2+} is unlikely to be the sole metal species that could be transported: our previous studies performed under well-controlled inorganic speciation conditions have shown that $CdCl_n^{2-n}$ chlorocomplexes could also be taken up (Jumarie et al., 2001). It is conceivable that the contribution of each of the transport systems would vary depending on the exposure conditions (transport properties and metal speciation) and cell phenotype (level of expression). The human lung carcinoma cell line A549, which expresses numerous features of alveolar type II cells, including surfactant synthesis, expression of CYP450 isozymes, cation transporters, and amiloride-sensitive Na^+ channels (Hukkanen et al., 2000; Boesewetter et al., 2006; Dagenais et al., 2006), is one of the most-used in vitro lung cell models. In recent decades, increasing uses of human lung carcinoma cells H441, with characteristics of bronchiolar (Clara) cells, also appear in the scientific literature for studying a number of lung functions (Kulaksiz et al., 2002; Nie et al., 2009; Wong et al., 2010). The aim of this study was to characterize Cd uptake in these two widely used cell models and to compare them in estimating the contribution of Ca, Fe, and Zn pathways in Cd accumulation. Data will provide information on uptake mechanisms responsible for Cd accumulation in different lung cell types in addition to providing further characterization on the cell models per se.

2. Material and methods

2.1. Cell culture

Cell lines A549 and H441, obtained from the American Type Culture Collection, were maintained in Ham's F12 and RPMI 1640 media, respectively (25 mM glucose, 2 mM glutamine, 1 mM sodium pyruvate, 50 units/ml penicillin, 50 μg/ml streptomycin) (Gibco BRL, Grand Island, NY, USA), supplemented with 10% and 7.5% heat-inactivated fetal bovine serum (FBS) (PAA Inc., Montréal, QC, Canada). Cells were routinely grown in 75-cm^2 culture flasks at 37 °C in a 5% CO_2-95% humidified air atmosphere and were passaged weekly by trypsinization (0.05% trypsin-0.053 mM EDTA). For all experiments, cells were seeded at 12.5×10^3 cells/cm^2 in 35-mm diameter petri dishes (Corning Inc., Corning, NY, USA). The A549 and H441 cells were grown for 14 and 9 days, respectively, and the culture media were changed every 2 days. The A549 cells were used between passages 80 and 92, and the H441 cells between passages 60 and 72.

2.2. Transport measurements

Cellular Cd uptake experiments were performed at 37 °C on a tray in a heating bath in inorganic defined transport media referred to as chloride (Cl^-) or nitrate (NO_3^-) media and containing, respectively: 137 mM $NaCl/NaNO_3$, 5.9 mM KCl/KNO_3, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2/Ca(NO_3)_2$, 4 mM D-glucose, 10 mM HEPES, adjusted to pH 5.5, 6.5, or 7.4 with NaOH. Chloride and nitrate media were used to study Cd uptake as a function of inorganic metal speciation (Cd^{2+} vs. $CdCl_n^{2-n}$). In contrast to Cl^-, NO_3^- does not bind as much to Cd^{2+}, thus 14% and up to 80% of the total dissolved metal is present as Cd^{2+} in the chloride and nitrate media, respectively, Elisma and Jumarie, 2001). The cells were washed four times with 2 ml of tracer-free Cl^- transport medium (pH 7.4) in order to remove the serum-containing culture medium. Accumulation was started by incubating the cells with 2 ml transport medium containing 0.3 μM ^{109}Cd (specific activity of 0.5 μCi/nmol, Ecker & Ziegler, Valencia, CA, USA) or 0.5 mM ^{45}Ca (specific activity set at 3 μCi/μmol, Perkin Elmer Life Sciences, Woodbridge, ON, Canada). Some experiments were carried out in the presence of unlabeled Cd, Fe, Zn, or Mn added as $CdCl_2/(NO_3)_2$, $FeCl_2/(NO_3)_2$, $ZnCl_2/(NO_3)_2$, or $MnCl_2/(NO_3)_2$ to the Cl^-, or NO_3^- transport media, respectively. In all cases, values of accumulation are reported relative to tracer ^{109}Cd exclusively: unlabeled Cd was used as a pure competitive inhibitor to discriminate nonspecific and specific uptake processes. Tracer uptake was stopped by removing the transport medium, and the monolayers were rapidly rinsed four times with 2 ml of ice-cold $^{109}Cd(^{45}Ca)$-free transport medium containing 2 mM EDTA used to extract the labile metal fraction from the outer surface of the cell membrane. Cells were then solubilized in 1 N NaOH (0.5 ml), and aliquots of 0.3 ml were used for radioactivity determination using a Cobra II gamma counter (Canberra Packard, Canada), while 50 μl of the cell suspension was used for protein assay according to Bradford (1979).

2.3. RT-PCR analyses

The levels of Nramp2 and Zip8 mRNA were estimated by semi-quantitative RT-PCR. Total RNA of 14- and 9-day-old A549 and H441 cell cultures was extracted using Trizo® Reagent (Invitrogen Life Technologies, Burlington, ON, Canada). Reverse transcriptase was performed with 2 μg mRNA using 1 μM random hexamere pd(N)6 (Amersham Biosciences, UK) and the Omniscript® RT Kit (Qiagen, Mississauga, ON, Canada), and PCR was conducted with 1 ml cDNA using the Tap PCR Core Kit (Qiagen). The sense and antisense primer sequences for Nramp2 (SLC11A2) were 5′-TATCATCGGCTCAGACATGC and 5′-GTCATGGTGGAGCTCTGTCC, respectively (GenBank AB004857, bases 556–1254), 5′-TGTGAGGATCGGCCCAAGCAC and 5′-GTGGAGGGCATCGCAGAGCG for Zip8 (SLC39A8) (GenBank MN_022154, bases 866–1471), and 5′-AGAAAATCTGGCACCACACC and 5′-CCATCTCTTGCTCGAAGTCC for β-actin (GenBank M001101, bases 332–766). Forty cycles consisting of: denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min, and extension at 72 °C for 1 min were carried out for cDNA amplification. The resulting PCR products were resolved on 2% (w/v) agarose gels containing 0.05 μg/ml ethidium bromide and visualized under UV trans-illumination using an LKB 2011 Macrovue Fluorescence system controlled by Alphamager™ 2200 software (Alpha Innotech Corporation, San Leandro, CA, USA). Nramp2 and Zip8 mRNAs were both normalized to that of β-actin.

2.4. Speciation calculation

The chemical forms of the metals at equilibrium in each of the transport media used were calculated using MINEQL$^+$ 3.01 software (Environmental Research Software, Hallowell, ME) and the NIST 5.0 stability constant data base (US Department of Commerce, Gaithersburg, MD). The formation constants ($\log K$) for Cd, Fe, and Zn complexation in the media used (corrected to zero ionic strength) are listed in Table 1. Note that $\log K$ values for $CdCl_n^{2-n}$ have been experimentally tested under our experimental conditions (ionic strength of 0.2 M) using an ion-exchange technique (Fortin and Campbell, 1998).