

Original Paper

Low Doses of Cadmium Chloride and Methallothionein-1-Bound Cadmium Display Different Accumulation Kinetics and Induce Different Genes in Cells of the Human Nephron

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Key Words

Cadmium · Cd7MT-1 · HO-1 · Human tubular cells · MT-1

Abstract

Background/Aims: The present study was conducted to investigate the renal tubular handling of inorganic cadmium (Cd^{2+}) by exposing primary human tubular cell cultures to physiologically relevant doses of cadmium chloride ($CdCl_2$). Furthermore, the cellular accumulation of Cd^{2+} was compared to that of metallothionein-1-bound Cd (Cd7MT-1). Finally, this study aimed to investigate the effect of the accumulation of Cd (both Cd^{2+} and Cd7MT-1) in renal cells on the expression of genes relevant to nephrotoxic processes. **Methods:** Cd concentration was measured using atomic absorption spectrometry. mRNA expression was evaluated by quantitative real-time RT-PCR. **Results:** Cd^{2+} accumulated into human tubular cells in a concentration- and time-dependent way. Furthermore, cellular accumulation of Cd^{2+} was different from the cellular accumulation of Cd7MT-1, indicative for different uptake routes. Finally, mRNA expression of the genes encoding the anti-oxidative proteins metallothionein-1 (MT-1) and heme-oxygenase-1 (HO-1) as well as the pro-apoptotic Bcl-2-associated X protein (Bax) were upregulated by $CdCl_2$ and not by Cd7MT1. **Conclusion:** In the presence of physiologically relevant Cd concentrations, tubular accumulation of the element in its inorganic form is different from that of Cd7MT-1. Furthermore, the tubular accumulation of inorganic Cd induces mRNA expression of genes of which the protein products may play a role in Cd-associated renal toxicity.

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Introduction

Cadmium (Cd) is one of the most toxic metals present in our environment. Major sources of Cd exposure include contaminated food and water, industrial fumes and dusts [1], but cigarette smoke is by far the largest source of Cd exposure in the general human population [2]. Each cigarette may contain 1–2 μg of Cd, and 40–60% of the Cd in inhaled smoke generally passes through the pulmonary epithelium into systemic circulation [3, 4].

In humans and other mammals, increased exposure to Cd adversely affects a number of organs and tissues, including the kidneys, liver, lung, pancreas, testis, placenta, and bone. Out of these, the effect on the kidneys is the earliest and most important and consists primarily of tubular damage and concomitant low-molecular-weight proteinuria [5–9]. In fact, based on epidemiological and toxicological studies in humans and investigations in animals, the International Agency for Research on Cancer (IARC) has classified Cd as a Category I carcinogen (IARC, 1993).

Environmental/occupational exposure to the non-essential metal Cd, together with various other elements such as lead, mercury and arsenic, is a known hazard to human health. The first reliable information on Cd levels in humans originates from studies in the seventies together with the availability of instrumentation and techniques allowing accurate measurement of the low concentrations in which the element circulates in the blood compartment or accumulates in tissues. Already in 1979, Lauwerys et al. [10] reported that blood Cd levels up to 0.1 μM (11.2 $\mu\text{g}/\text{l}$) in workers chronically exposed to Cd correlated with an increased risk for renal dysfunction, predominantly of tubular origin. Normal whole-blood Cd levels in the general population have been reported to be $<0.02 \mu\text{M}$ (2.25 $\mu\text{g}/\text{l}$). In 1990, Minoia et al. [11] reported normal-blood Cd levels ranging from 0.005 to 0.05 μM (0.56 up to 5.6 $\mu\text{g}/\text{l}$) in the healthy European population. Significantly elevated values were found in occupationally exposed workers and in people living in the proximity of Cd-processing industries, particularly in smokers [12, 13]. Most of the *in vitro* and experimental studies investigating the toxic effects of Cd were performed in the presence of Cd concentrations that were orders of magnitude higher than those encountered *in vivo*.

Cd taken up via inhalation and/or ingestion undergoes hepatic conversion into the Cd-metlothionein (Cd7MT-1) complex. Cd7MT-1 (MW: 3,500–14,000 Da) is filtered by the glomerulus and taken up by the proximal tubular cells via receptor-mediated endocytosis (RME). However, arguments exist that also inorganic (free ionic) Cd (Cd^{2+}) plays a substantial role in renal toxicity. In this context, the finding that Cd bound to small peptides may be released at the brush border after which it is taken up in its free ionic form by renal cells is of particular interest [14]. Furthermore, besides affecting the paracellular transport of essential divalent cations [14, 15], tubular damage caused by Cd diminishes the active transcellular transport of Na^+ and K^+ [16]. Cd exposure has also been associated with increased urinary calcium (Ca^{2+}) excretion and renal stone formation, which can be the consequence of a Cd-induced bone pathology either manifested by an increased calcium efflux out of the bone, a decreased incorporation of the element into the bone [17], and/or blockage of the epithelial calcium channels [18]. In view of these findings, it seems reasonably that inorganic Cd, along with the methylated form, is taken up by renal tubular cells.

Little is known concerning the renal (tubular) handling of Cd in its inorganic form (Cd^{2+}), and although it probably accounts for only a minor fraction of the renal Cd uptake *in vivo*, studying tubular handling of inorganic Cd is of particular interest in view of its potential toxicity.

The present study was conducted to investigate the renal tubular handling of Cd^{2+} by exposing primary human tubular cell cultures to physiologically relevant doses of Cd^{2+} (administered as CdCl_2). Furthermore, the cellular accumulation of Cd^{2+} was compared to that

of Cd7MT-1 and, finally, this study aimed to investigate the effect of the accumulation of Cd (both Cd²⁺ and MT complexes) in renal cells on the expression of several genes relevant to nephrotoxic processes.

Materials and Methods

Isolation, Purification and Cultivation of Primary Human Kidney Tubular Cells

Human kidney tubular epithelial cells were isolated as described previously [19]. Briefly, normal human kidney tissue that became available through nephrectomy performed upon oncological indication was collected and processed in a sterile manner. The cortex and outer stripe of outer medulla were dissected, cut into pieces of about 1 mm³ and digested in collagenase D solution (Roche, Ottweiler, Germany). The suspension was shaken vigorously for 2 h at 37°C and sieved through a 120-µm sieve. The resulting cell suspension was loaded on top of a discontinuous Percoll (Amersham Pharmacia Biotech, Uppsala, Sweden) gradient with densities of 1.04 and 1.07 g/ml. After centrifugation tubular cells were carefully aspirated from the intersection, washed, and brought into culture. Cells were seeded in 24-well plates (25,000 cells/well) in α-MEM medium (Life Technologies, Rockville, Md., USA) modified according to Gibson d'Ambrosio, supplemented with 10% fetal calf serum and grown until confluence (±6 days). Twenty-four hours before studying the Cd²⁺ uptake, the serum-containing medium was replaced by serum-free α-MEM Gibson d'Ambrosio.

Measurement of the Cellular Cd Concentration

Cell cultures were exposed to CdCl₂ dissolved in α-MEM medium (37°C) at different concentrations (0–1,000 nM) for 5 h. Furthermore, cell cultures were exposed to CdCl₂ or Cd7MT-1 (Bestenbalt, Tallinn, Estonia) for different time periods (3–72 h) at 500 nM (for both compounds, concentrations correspond to the metal concentration).

After incubation, cells were extensively rinsed with PBS (3 times) and lysed during 20 min with 0.1% Triton X-100 in PBS. The lysate was divided in aliquots and stored at –20°C (some aliquots were also stored at –80°C for gene expression studies).

At the time of analysis, 0.5 ml of the lysates were digested in ultrapure HNO₃ (Merck Suprapur 4371) at 70°C for 24 h. Digests were subsequently mixed with a matrix modifier consisting of diammonium hydrogen phosphate (0.5 g/100 ml), magnesium nitrate [0.24 g/100 ml and Triton X-100 (0.1% m/v)]. The Cd concentration was measured against a matrix-modified aqueous calibration curve with standards prepared from a stock solution of 1,000 µg/ml Cd (Merck Titrisol 9960) and using a Zeeman 3030 atomic absorption spectrometer equipped with an HGA graphite furnace, an AS-60 autosampler, and an Anadex DP-9500B silent scribe printer, all from Perkin-Elmer (Norwalk, Conn., USA).

In order to evaluate the cellular response to the anti-oxidant compound ebselen, the latter compound was added to serum-free α-MEM at a 10 µM concentration for 1 h. Cells were then washed 3 times with serum-free medium and were exposed to different CdCl₂ concentrations (in the absence of ebselen) for 5 h.

To check the effect of zinc (Zn) on cellular Cd²⁺ uptake, 5 µM ZnCl₂ was administered to confluent monolayers 1 h before addition of 500 nM CdCl₂. Cd²⁺ uptake was then assessed (still in the presence of Zn) after 1, 5, and 20 h.

To evaluate the effect of pH on Cd²⁺ uptake, parallel experiments were performed in glucose-containing HEPES or 2-(N-morpholino)ethanesulfonic acid (MES) solutions buffered at pH 7.4 and 5.5, respectively, for 1, 5, 15, and 20 h.

In order to allow the cellular Cd concentration to be expressed per milligram protein, the protein concentration of the cell lysates was determined using a BCA kit (Perbio, Belgium) according to the manufacturer's instructions.

RNA Isolation and Quantitative Real-Time RT-PCR Analysis

Total RNA was extracted from cell lysates using the RNeasy Micro Kit (Qiagen, Hilden, Germany). cDNA was synthesized using the High-Capacity cDNA Archive Kit (ABI, Nieuwerkerk a/d IJssel, The Netherlands), and mRNA expression was evaluated by quantitative real-time PCR on the ABI Prism 7000 Sequence Detection System (ABI) using the fluorescent Taqman methodology. Ready-to-use primer and probe sets were pre-designed by Applied Biosystems for the following genes: divalent metal transporter 1 (DMT-1; Hs00167206_m1), metallothionein-1 (MT-1; Hs00831826_s1), heme-oxygenase-1 (HO-1; Hs00157965_m1), B-cell CLL/lymphoma 2 (Bcl-2; Hs00153350_m1), and Bcl-2-associated X protein (Bax; Hs99999001_m1). Instructions of the supplier were followed for PCR conditions, which were uniform for all assays under study. The relative mRNA expression of the different genes was analyzed in triplicate, normalized against glyceraldehyde 3-phosphate dehydrogenase (GAPDH; Hs99999905_m1) as a control housekeeping gene and expressed in relation to a calibrator sample using the comparative Ct method. Control (untreated) cells served as calibrator sample which was given a gene of interest/GAPDH mRNA expression ratio value of 1.

Statistics

Data are expressed as the means \pm SEM. To analyze saturation curves, non-linear regression was fitted to a Michaelis-Menten equation using OriginLab version 8. Differences in Cd uptake and mRNA levels were tested for significance using the non-parametric Kruskal-Wallis test for multiple group comparison, followed by the Mann-Whitney U test for comparisons between 2 groups using SPSS Statistics 17.0 software.

Results

Intracellular Cd Accumulation in Primary Human Tubular Kidney Cells

We first examined whether physiologically relevant concentrations of Cd²⁺ (0, 20, 100, 200, and 500 nM of CdCl₂) are taken up by renal tubular cells during an incubation time of 5 h. The dose-response curve depicted in figure 1a shows that the accumulation of Cd in the tubular cells is saturable, with a maximal cellular uptake of 641 ± 76 pmol Cd/mg protein.

Figure 1b shows the kinetics of intracellular Cd accumulation by confluent cells incubated with serum-free culture medium containing 500 nM CdCl₂ in function of time (filled circles curve). Cellular Cd rapidly increased with time and more or less reached a plateau after 24 h with a maximal value of 772 ± 354 pmol/mg protein after 72 h of exposure. We performed concomitant measurements of the Cd concentration in the extracellular medium. During 72 h of exposure and in line with the cellular uptake, the Cd concentration in the extracellular medium gradually decreased to 21.5 ± 2 pmol/mg protein. The time-dependent intracellular accumulation of Cd²⁺ was compared to that of Cd7MT-1 (500 nM; open circles curve). The final amount of Cd present in the tubular cells after 72 h of incubation, as well as the accumulation kinetics with either CdCl₂ or Cd7MT-1 were significantly different. Whilst the Cd concentration in cells treated with inorganic CdCl₂ reached a plateau after 24 h of incubation ($p > 0.05$ between values noted after 24, 48, or 72 h of exposure), addition of Cd to the culture medium in the Cd7MT-1 complex resulted in a linear and steady increase in the Cd levels from 5 h ($p < 0.05$ vs. 24, 48, and 72 h) until 72 h of exposure, reaching a maximal cellular concentration of 407 ± 28 pmol/mg protein.

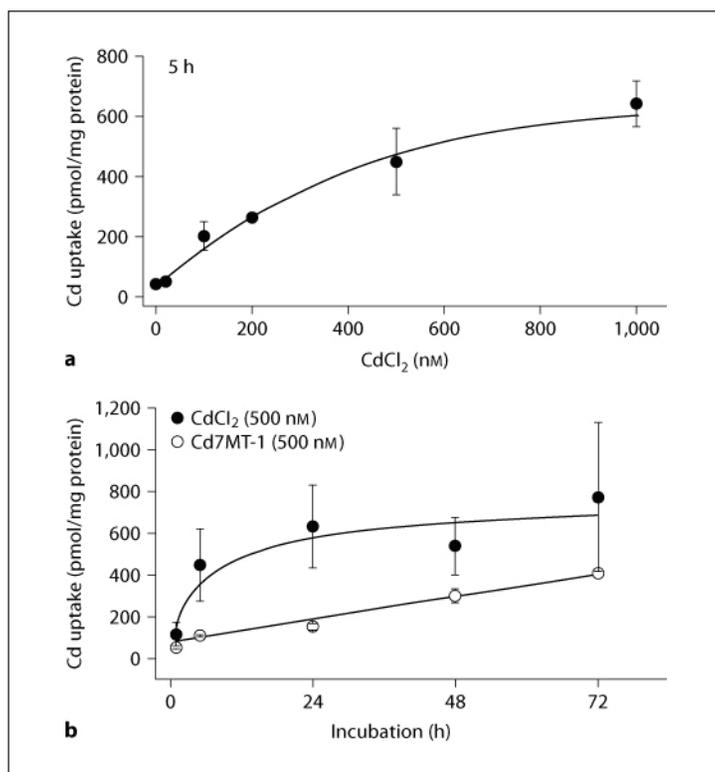


Fig. 1. Intracellular accumulation of Cd in human kidney tubular cell cultures is dependent on concentration (a) and incubation time (b). **a** Cells were grown until confluence and incubated at 37°C in α -MEM medium with different CdCl₂ concentrations (0, 20, 100, 200, and 500 nM) for 5 h. Cd accumulation in the tubular cells was saturable, with a maximal cellular uptake of 641 ± 76 pmol Cd/mg protein. **b** Confluent cells were incubated with 500 nM CdCl₂ (●) or 500 nM Cd7MT-1 (○) for periods of 3, 5, 24, 48, and 72 h. Intracellular accumulation kinetics were clearly different for both. Values are means \pm SEM of triplicate determinations, performed on cells originating from 3 different nephrectomies. In each of these 3 experiments, triplicate determinations (3 wells) were performed for each condition.

Effect of pH and Zn on Intracellular Cd Accumulation

To evaluate the effect of pH on the intracellular Cd accumulation during exposure to CdCl₂, cells were incubated for different periods with 20 nM CdCl₂ in glucose-containing HEPES- (pH 7.4) and MES- (pH 5.5) buffered solutions. The intracellular Cd accumulation was significantly higher ($p < 0.05$) in cells that were incubated in the acidic buffer (fig. 2a).

To determine whether Zn would affect Cd accumulation, cultures were incubated with 5 μ M ZnCl₂ (1 h before and during the whole CdCl₂ incubation period). The intracellular Cd concentration decreased from 632 ± 199 to 149 ± 100 pmol/mg protein in the presence of Zn²⁺ (fig. 2b).

Possible Involvement of DMT Proteins in Cellular Cd Uptake

The observation that the accumulation of Cd²⁺ in the tubular cells is pH dependent and dramatically affected by the presence of other divalent cations (such as Zn) points to a possible important contribution of DMT proteins to the uptake of Cd into the cells. As DMT-1 is expressed in the kidney [20], it was investigated by quantitative real-time RT-PCR analysis whether exposure of the tubular cells to CdCl₂ goes along with an upregulation of the DMT-1 gene expression.

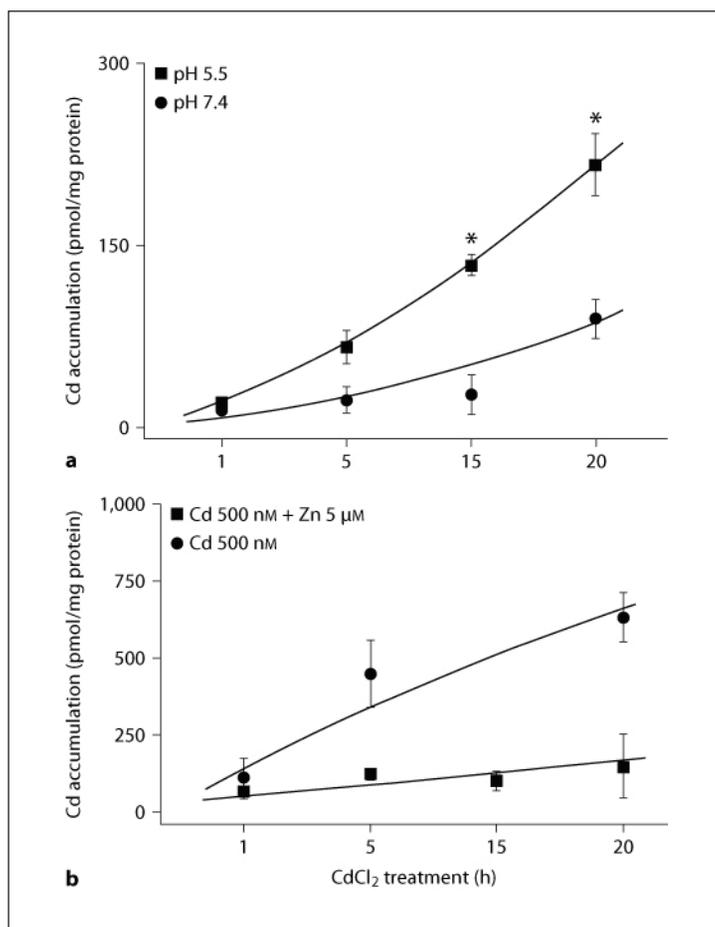


Fig. 2. Intracellular accumulation of Cd, added to the cell culture medium as CdCl₂, is pH dependent (a) and inhibited by Zn (b). **a** Confluent cells were incubated in glucose-containing buffered solution of pH 5.5 or 7.4; 20 nM CdCl₂ was added for indicated time periods. Mean values of Cd²⁺ accumulation at pH 5.5 and 7.4 were significantly different from each other for incubation times 15 and 20 h. **b** Confluent cells were incubated with 500 nM CdCl₂ either in the presence or in the absence of 5 μM ZnCl₂. The intracellular Cd²⁺ accumulation was significantly inhibited by the presence of Zn. Each data point represents means ± SEM of 3 wells derived from tissue of the same nephrectomy. * p < 0.05.

After 5 h of CdCl₂ treatment, mRNA expression level of the gene encoding DMT-1 was not different from that in cultured human renal cells at control conditions (absence of Cd; fig. 3, open column). However, long-term (24–72 h) exposure to 500 nM CdCl₂ resulted in a significant (p < 0.05 vs. control) increase in the DMT-1/GAPDH ratio.

CdCl₂-Induced Effects on the Expression of Genes Relevant to Nephrotoxic Processes

In this part of the study, we investigated whether inorganic CdCl₂ had an effect on the gene expression of key proteins in pathways relevant to nephrotoxic processes: MT-1 and HO-1 as protectors against oxidative stress; and Bax and Bcl-2 as pro- and anti-apoptotic genes, respectively. In addition, we examined in which way pre-incubation of the cells with an anti-oxidative agent (ebselen) influenced the expression of these genes.

Treatment of the cells with CdCl₂ at physiologically relevant concentrations (20, 100, 200, 500, and 1,000 nM) during 5 h resulted in a concentration-dependent increase of MT-1

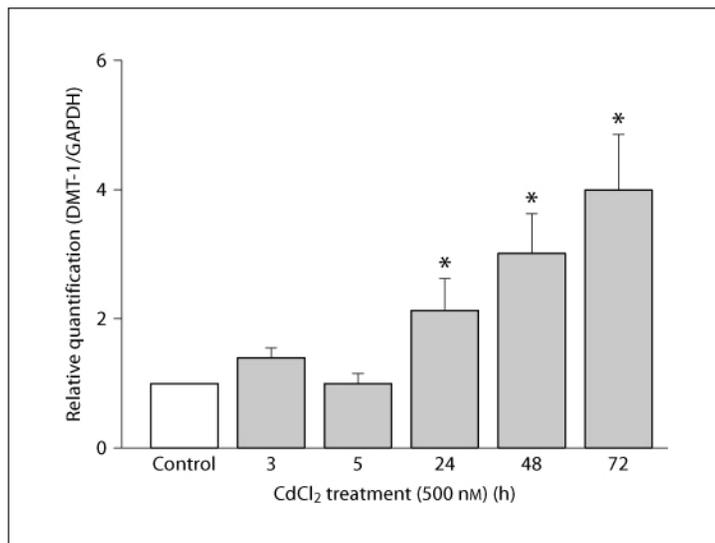


Fig. 3. Expression of DMT-1 (DMT-1/GAPDH) mRNA in human tubular cells affected by CdCl₂ exposure. Confluent primary cultured cells were treated with 500 nM CdCl₂ during 0 (control), 3, 5, 24, 48, and 72 h. DMT-1 and GAPDH mRNA levels were quantified by quantitative real-time RT-PCR; values obtained from untreated (control) cells were set to 1. Relative DMT-1 expression (DMT-1/GAPDH) is significantly upregulated starting at 24 h CdCl₂ incubation. Data represent means ± SEM of triplicate experiments on cells originating from the same nephrectomy. * p < 0.05 as compared to control.

mRNA levels, ranging from a 3-fold increase at 100 nM CdCl₂ up to a 140-fold increase at a concentration of 1,000 nM CdCl₂ (fig. 4a, open columns). When the cells were treated with 10 μM ebselen for 1 h, prior to CdCl₂ administration, a significantly (p < 0.05) lower Cd²⁺-induced production of MT-1 mRNA was observed. Although production of MT-1 was not completely abolished in any condition, at the highest CdCl₂ concentration (1,000 nM), mRNA levels of MT-1 were up to 3 times lower in ebselen-treated cells as compared to the non-treated cultures (fig. 4a, closed columns). With regard to the temporal effect of CdCl₂ exposure on the MT-1 gene expression, a peak level was observed after 5 h of exposure of the cells to culture medium containing 500 nM CdCl₂. With longer incubation periods, MT-1 mRNA expression again decreased reaching baseline levels after 48 h of treatment (fig. 4b).

Similar to MT-1, induction of HO-1 is an important cellular response and a well-recognized biomarker for oxidative stress. Treatment of the cells with CdCl₂ for 5 h significantly and dose-dependently (CdCl₂ >200 nM) upregulated (4-fold) the expression of HO-1 mRNA in human tubular cells as compared to controls (fig. 5a). This upregulation of HO-1 was completely prevented by pre-incubation of the cells with 10 μM ebselen for 1 h. With regard to the temporal effect, relative expression of HO-1 mRNA after exposure to CdCl₂ for more than 5 h was not significantly different anymore from cells not incubated with CdCl₂ (fig. 5b).

In comparison to controls, the Bax mRNA level was significantly higher when cells were exposed to 500 nM CdCl₂ for longer time periods (24–72 h). In contrast, the expression of Bcl-2 mRNA significantly decreased during this period. As a result, the Bax/Bcl-2 mRNA ratio significantly shifted in favor of Bax, yielding values of 1.4, 2.3, and 4.5 versus controls (set at 1.0) after 24, 48, and 78 h, respectively (fig. 6). No significant effect was observed after a short (<24 h) exposure to CdCl₂.

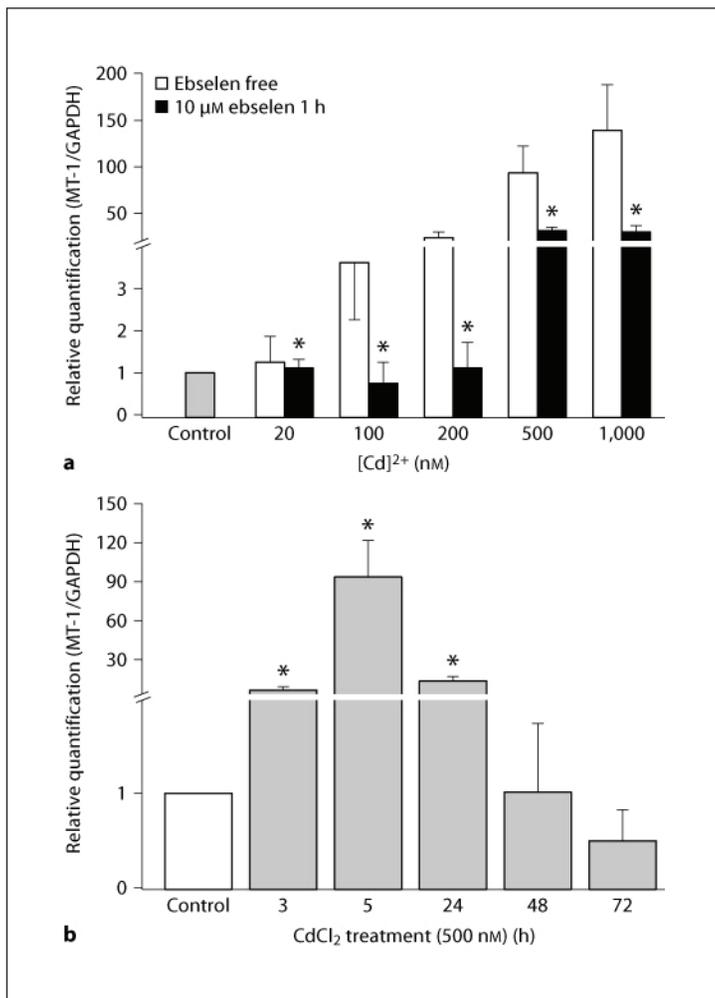


Fig. 4. Expression of MT-1 mRNA in human tubular cells. **a** Relative expression of MT-1 (MT-1/GAPDH) mRNA in tubular cells treated for 5 h with different CdCl₂ concentrations (20–1,000 nM), either or not pre-incubated with 10 μM ebselen. MT-1 mRNA was upregulated by CdCl₂. Upregulation was significantly prevented by ebselen pre-treatment. **b** Time course (3–72 h) of relative MT-1 (MT-1/GAPDH) mRNA expression in tubular cells after exposure to CdCl₂ (500 nM). MT-1 mRNA expression was significantly upregulated versus control during the short incubation times only. Columns represent means ± SEM of triplicate experiments on cells originating from the same nephrectomy as in figure 3. * p < 0.05.

Cd7MT-1-Induced Effects on the Expression of Genes Relevant to Nephrotoxic Processes

MT-1 mRNA expression of cells incubated with 500 nM Cd7MT-1 remained below baseline levels (data not shown). Moreover, in contrast to CdCl₂, the presence of Cd7MT-1 resulted in a reduction in HO-1 mRNA levels at 24, 48, and 72 h (fig. 7a). Additionally, whereas CdCl₂ treatment significantly shifted the Bax/Bcl-2 mRNA ratio in favor of Bax, Cd7MT-1 treatment significantly shifted the Bax/Bcl-2 mRNA ratio in favor of the anti-apoptotic Bcl-2, yielding values of 1.4, 2.3, and 4.5 versus controls (set at 1.0) after 24, 48, and 78 h, respectively (fig. 7b).

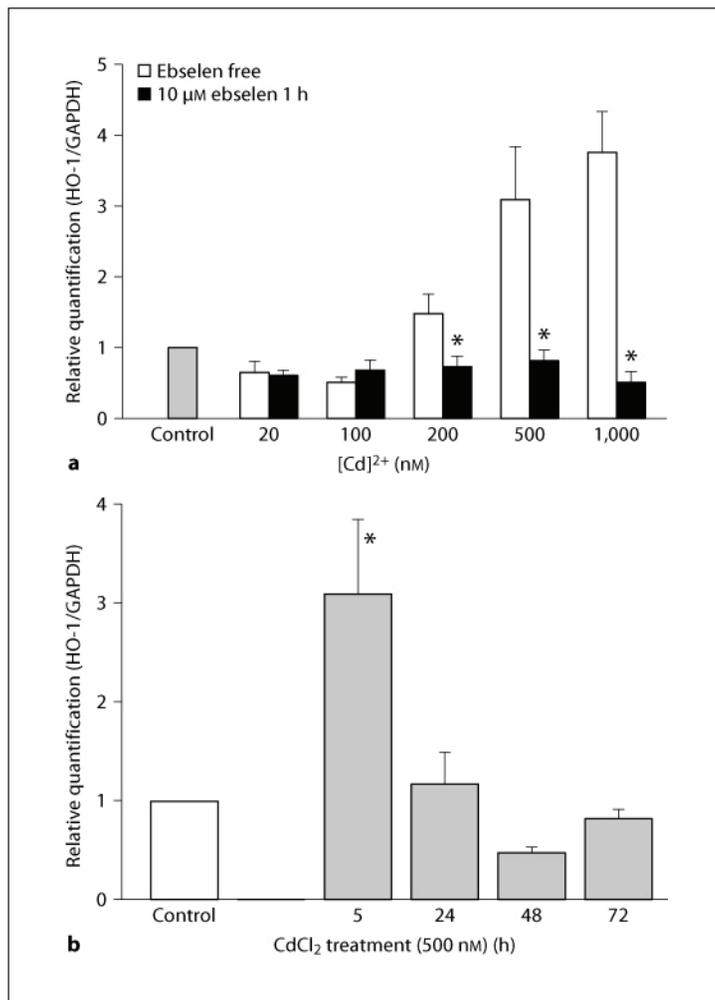


Fig. 5. Expression of HO-1 mRNA in human tubular cells. **a** Relative expression of HO-1 (HO-1/GAPDH) mRNA in tubular cells treated for 5 h with different CdCl₂ concentrations (2–1,000 nM), either or not pre-incubated with 10 μM ebselen. HO-1 mRNA was upregulated by CdCl₂. Upregulation was significantly prevented by ebselen pre-treatment. **b** Time course (3–72 h) of relative HO-1 (HO-1/GAPDH) mRNA expression in tubular cells after exposure to CdCl₂ (500 nM). HO-1 mRNA expression was significantly upregulated versus control after 5 h of incubation, while baseline expression was seen for the longer incubation periods. Columns represent means ± SEM of triplicate experiments on cells originating from the same nephrectomy as in figures 3 and 4. * p < 0.05.

Discussion

In the present study, the renal tubular handling of Cd²⁺ was investigated by exposing primary human tubular cell cultures to physiologically relevant concentrations (20–1,000 nM) of Cd²⁺ (administered as CdCl₂). Cd²⁺ accumulated into those cells in a concentration- and time-dependent manner. Furthermore, (time-dependent) cellular accumulation of Cd²⁺ was different from the cellular accumulation of Cd bound to metallothionein. Indeed, in contrast to the quick plateauing of Cd administered as CdCl₂, Cd accumulation when administered as Cd7MT-1 steadily increased over time during 72 h of treatment, suggesting different routes of uptake for both compounds.

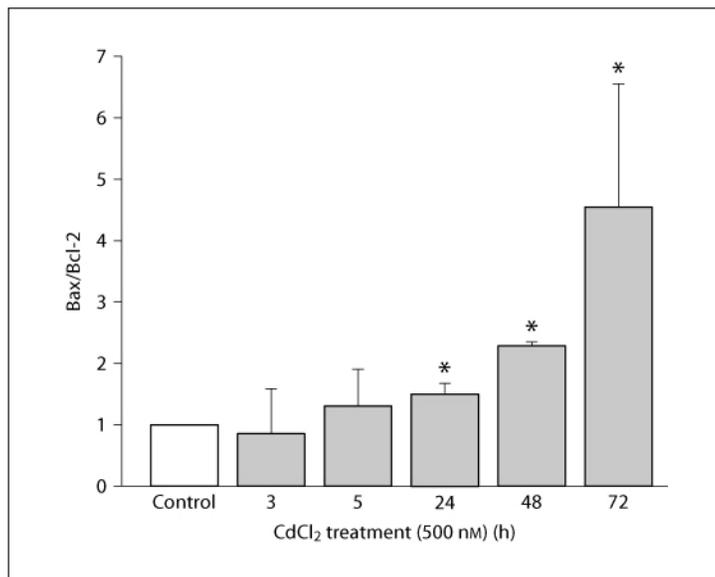


Fig. 6. Bax/Bcl-2 ratio in human tubular cells chronically treated with CdCl₂. Confluent tubular cell cultures were exposed to 500 nM CdCl₂ for different time periods (3–72 h). CdCl₂ treatment during 24 h or longer significantly increased the Bax/Bcl-2 ratio versus untreated control. Ratios of Bax/Bcl-2 mRNA levels were calculated from relative expression ratios (to GAPDH) of both genes. The results are means ± SEM of triplicate experiments on cells originating from the same nephrectomy. * p < 0.05.

Consensus exists in the literature regarding the uptake of Cd²⁺ by the renal cell, and one may assume that Cd²⁺ is internalized at the apical membrane of renal cells, especially proximal tubular cells, via RME [21]. RME of proteins has previously been studied in the primary human tubular cell culture model proposed here [19]. We found that this process, in line with the in vivo situation, occurs in cells originating from the proximal part of the tubule, while it was absent in cells originating from the more distal parts of the nephron. This result together with the notion that uptake of Cd²⁺ is not saturable at the concentration under study allows us to presume that the uptake of Cd²⁺ in our cell cultures also took place through RME by the proximal tubular cells.

In contrast to Cd²⁺, the uptake pathway of Cd²⁺ is much less known. Cd is a non-essential metal and therefore most likely uses cellular entry pathways of other (essential) metals such as iron (Fe) and Zn. The finding that in our cell culture model, Cd accumulation in the cells was significantly higher at pH 5.5 as compared to pH 7.4, points to the involvement of DMT-1 in the cellular uptake of Cd [22, 23]. This is further supported by our data showing that long-term exposure to CdCl₂ (fig. 3) goes along with a significant and time-dependent increase of the DMT-1 mRNA expression. An in-detail study of DMT-1 expression in the kidney indicated that, next to its lysosomal presence in the proximal tubule, DMT-1 is also present at the apical membrane of cells in the thick ascending limb and more distal parts of the nephron [20]. Preliminary investigations of our group on Cd²⁺ uptake in pure cultures of distal tubular cells revealed that Cd²⁺ indeed is taken up by distal tubular cells as well. This is in line with a study of Olivi et al. [24] reporting DMT-1-mediated uptake of Cd (administered as CdCl₂) into MDCK cells, a cell line originating from the distal tubular part of the nephron.

Although DMT-1 primarily is an Fe-transporting molecule, a recent study identified DMT-1 as a Zn-transporting molecule as well [25]. Therefore, the dramatic inhibition of cel-

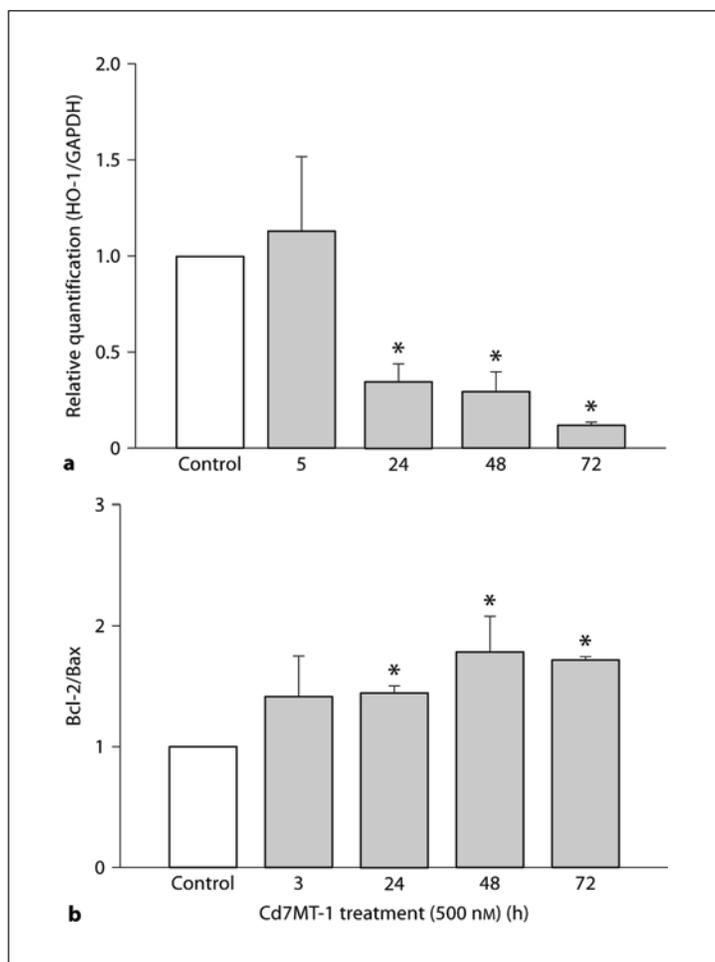


Fig. 7. The relative expression of the genes associated with the effects of Cd7MT-1. **a** Time course (3–72 h) of relative HO-1 (HO-1/GAPDH) mRNA expression in tubular cells after exposure to Cd7MT-1 (500 nM). HO-1 mRNA expression was significantly downregulated versus control for 24, 48, and 72 h incubation periods. **b** Cd7MT-1 treatment during 24 h or longer significantly increased the Bcl-2/Bax ratio versus untreated control. Ratios of Bcl-2/Bax mRNA levels were calculated from relative expression ratios (to GAPDH) of both genes. The results are means \pm SEM of triplicate experiments on cells originating from one nephrectomy (same as in figure 6). * $p < 0.05$.

lular Cd accumulation in the presence of Zn may also be indicative for a role of DMT-1 in cellular Cd transport. However, it also points to the potential contribution of other (Zn) transporters such as those of the ZIP family [26]. The further unraveling of the pathways involved in cellular Cd accumulation in different parts of the nephron would be an interesting challenge for future investigations.

In conclusion, the results described above make clear that the handling of the same concentrations of Cd in either its inorganic (CdCl_2) or organic form (Cd7MT-1) by human tubular kidney cells is very different. These different kinetics point to different uptake pathways, which end up in different intracellular concentrations and most probably in a different intracellular localization as well. This incited us to check whether differences in cellular handling of Cd also result in different effects on the expression of genes of which the protein products are known to play a role in cellular toxicity.

Unlike Fe and copper (Cu), Cd is not a Fenton metal and thus does not generate free radicals by itself. Hence, nowadays there is increasing evidence that ROS are generated in the presence of Cd [27] and that several proteins such as metallothioneins and heme-oxygenases may act as early protective tools against oxidative stress. Our results are in line with this as they show that acute exposure (5 h) to high doses of CdCl₂ stimulates production of MT-1 mRNA up to 140-fold as compared to controls (no exposure to CdCl₂). These results are in agreement with literature data showing increased transcription of MT-1 genes in response to acute exposure to particular metals. Moreover, in previous studies, it has been found that overexpression of MT-1 mRNA levels correlates well with increased production of the MT-1 protein, suggesting that transcription rather than translation determines the Cd²⁺-induced upregulation of MT-1 [28].

Together with MT-1, the induction of HO-1 has been established as a defense mechanism against oxidative stress. HO-1 expression increases following exposure to a wide variety of cellular stressors. HO-1 mRNA expression showed a similar temporal pattern as that seen for MT-1 mRNA, as the mRNA levels of HO-1 rapidly increased after an acute 5-hour exposure to CdCl₂, after which they again decreased to baseline values after longer exposure periods (24–72 h).

In contrast to CdCl₂, culturing of our cells in Cd7MT-1-containing cell culture medium did not result in upregulation of HO-1 or MT-1 mRNA expression. As a next step, the expression of MT-1 and HO-1 mRNA in response to Cd²⁺ exposure was measured after pre-incubating the cells with ebselen. Ebselen [2-phenyl-1,2-benzisoselenazol-3(2H)-one] is a selenium-containing compound which has been shown to exhibit anti-oxidant, anti-nociceptive, neuroprotective and anti-inflammatory properties in various experimental models [29–31]. Thus, the rationale for pre-treating cells with ebselen was that if cellular upregulation of MT-1 and HO-1 mRNA protects cells against Cd²⁺-induced oxidative stress, a reduced upregulation of both genes must reasonably be expected in the presence of ebselen. Results indicated that pre-incubation with ebselen indeed prevented the upregulation of HO-1 and MT-1 mRNA during Cd²⁺ exposure.

During the last decade, studies at the cellular level indicated that Cd may induce various biochemical changes associated with apoptosis [32]. Different families of proteins have been identified to play an important role in the apoptotic process. Of these, Bcl-2, an intracellular membrane-associated protein family, functions to inhibit apoptosis, whereas the Bax and BH3 families promote apoptosis [33, 34]. Hence, a shift in the Bax/Bcl-2 balance may modulate the cellular function either to pro- or to anti-apoptotic processes.

The present study demonstrates that the Bax/Bcl-2 gene expression ratio is shifted towards the apoptotic pathway when cells are treated with CdCl₂ (fig. 6). Interestingly, the temporal increase in the Bax/Bcl-2 gene expression ratio at 24–72 h of incubation went along with the normalization (end of upregulation) of both the MT-1 and HO-1 mRNA expression. In this context, the observation that, in the presence of Cd7MT-1, the cellular response in contrast to CdCl₂ is in favor of Bcl-2 mRNA expression is interesting and in agreement with previous studies obtained on renal cells of porcine origin, in which overexpression of Bcl-2 mRNA completely inhibited Cd-induced apoptosis [35].

In summary, in the presence of physiologically relevant Cd concentrations, tubular accumulation of the element in its inorganic form is different from that of Cd7MT-1. Furthermore, the tubular accumulation of inorganic Cd induces genes of which the protein products may play a role in Cd-associated renal toxicity, while this is not the case for Cd7MT-1. Indeed, whereas exposure to CdCl₂ induces an increase in MT-1 and HO-1 mRNA, expression of the genes remained below baseline levels in the presence of Cd7MT-1. Moreover, in contrast to Cd7MT-1, cellular exposure to CdCl₂ does result in an upregulation of pro-apoptotic genes. Finally, this study demonstrates that, in combination with a set of appropriate analytical

techniques, our human primary cell culture system offers an excellent model to study the characteristics and toxicological consequences of cellular uptake of heavy metals at the level of the kidney.

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Disclosure Statement

The authors confirm that there are no conflicts of interest.

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