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Manganese is highly effective in protecting cells from cadmium intoxication

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Abstract

Cadmium poisoning results in cell death. Although several intracellular pathways have been identified in this response, transport systems responsible for cadmium entry into cells remain poorly understood and controversial. Here, we analyzed the effects of several divalent cations on cadmium toxicity in different cell types. We found that zinc, previously reported as a protective agent against cadmium poisoning, is actually much less efficient than manganese. We show that manganese dramatically reduces cadmium intake, and that this is associated with the inhibition of our recently reported sustained activation of ERK, characteristic of cadmium intoxication. Finally, we show that this inhibition of cadmium entry and ERK-sustained activation perfectly correlates with a high cellular resistance to cadmium exposure. Our results, together with previously published data, support the idea that the yet to be characterized manganese transporter system(s) may be responsible for cadmium entry into cells.

Keywords: Cadmium; ERK; Transport; Manganese; Zinc; Protection

Cadmium is an environmental pollutant whose toxicity is amplified in organisms by its long biological half life of 15–30 years [1]. Since cadmium is a nonessential metal not naturally present in organisms, it must enter cells via transport mechanisms normally used for other purposes. More than 25 years ago it was suggested that cadmium could enter cells via divalent ion transporters, such as zinc transporters [2]. Supporting this notion are early studies showing that zinc can indeed protect against cadmium toxicity [3]. However, the rationale and mechanism of zinc protection remain somewhat controversial. More recent data suggest that DMT1 (divalent metal-ion transporter-1) could also be a transport system used by cadmium [4]. However, no definitive information is available as to what transport systems are indeed used by cadmium for entry into cells. What is clear is that cadmium does enter cells and causes cell death. We recently showed that cadmium toxicity is closely associated with an unconventional activation of ERK, which can last as long as 6 days [5], and that this sustained ERK response appears to be associated with the onset of cell death mechanisms involving caspase activation. In this present study we wanted to investigate the effects of several divalent ions both on cadmium entry and on cell survival to cadmium exposure. We were interested in following cadmium exposed cells for over 24 h after other divalent cation treatments, in order not to miss physiological responses that could be overlooked in classical biochemical studies which analyze cadmium entry seconds or minutes after treatment with other divalent ions. Our approach was very interesting since it revealed that over time the classical zinc protection is actually much less effective than manganese, as evidenced by the complete lack of toxicity of cadmium on different cell culture types

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co-treated with manganese. Our results show that the protective effect of manganese is likely to be the result of the inhibition of cadmium entry into cells, and that this inhibition by manganese is more pronounced than that obtained with zinc. Furthermore, we show that manganese protective effect is nicely associated with the inhibition of the sustained ERK activation that we recently reported [5]. Finally, our results, together with data reported by others, suggest that manganese transporter(s) may be responsible for cadmium entry into cells. Cloning and characterization of such transporters will be required to further investigate this possibility.

Materials and methods

Cell culture. All cells were incubated in DMEM complemented with 10% fetal calf serum except for primary rat osteoblasts. Primary rat osteoblasts were obtained from calvaria of 3- to 4-day-old new born rats after three serial digestions with collagenase II and trypsin under rotation at 37 °C for 20–40 min as previously described [6]. Cells were maintained in 45% DMEM and 45% MEM α supplemented with 10% serum, 1 mM L-glutamine, and 1 mM sodium pyruvate. All cultures were maintained at 37 °C in water saturated atmosphere at 5% CO_2. DCT and PCT (distal and proximal convoluted tubule cells, respectively) are spontaneously immortalized lines obtained from primary murine cells transfected with a pSV2neo vector and selected for G418 resistance.

Cell treatments. $CdCl_2$, $CaCl_2$, $CuSO_4$, $MgCl_2$, $MnCl_2$, and $ZnCl_2$ were purchased from Sigma and solubilized in deionized water. Cells were concomitantly treated with the indicated solutions at the indicated concentrations, and kept in culture until lysed or photographed. For comparison of protective effects of manganese versus zinc from cadmium toxicity, (Fig. 2), the different cell types were inoculated in 24-well plates with a range of manganese and zinc concentrations (2, 4, 8, 16, 32, and 64 μM) in the presence or absence of 2 μM cadmium. Cells were monitored on a daily basis for growth, and concentrations of manganese for which cell growth was indistinguishable from that of control cells were identified for each cell type. This method, which is completely noninvasive, turned out to be the most reliable and reproducible one.

Cadmium entry determination. When mentioned, pH was adjusted with 20 mM Hepes at the indicated pH. Cells were cultivated in the presence of: Fig. 1A, HEK293 cells with 0.5 μM ^{109}Cd at 443 μCi/μmole for 24 h at 37 °C, 5% CO_2, with a 10-fold molar excess of indicated competitors; Fig. 1B, 1 μM ^{109}Cd at 114 μCi/μmole for 24 h at 37 °C, 5% CO_2, with a 10-fold molar excess of manganese when indicated; and Fig. 1C, primary osteoblasts with 1 μM ^{109}Cd at 290 μCi/μmole for 24 h at 37 °C, 5% CO_2, with a 5- or 10-fold molar excess of manganese and/or zinc when indicated. Cells were washed 3× with (Hepes 20 mM, pH 7, NaCl 150 mM, and EGTA 5 mM) and lysed in the same buffer supplemented with 0.1 N NaOH. Aliquots were mixed in scintillation liquid and counted. Results were plotted relative to control devoid of competitor, arbitrarily set as 100%. Fig. 4: HEK293 cells and primary osteoblasts were grown for 5 h in the presence of 0.2 μM ^{109}Cd at 207 μCi/μmole at 37 °C, 5% CO_2, at indicated pH, which remained constant throughout the experiment. Cells were washed 3× with (Hepes 20 mM, pH 7, NaCl 150 mM, and EGTA 5 mM) and lysed in the same buffer supplemented with 0.1 N NaOH. Aliquots were mixed in scintillation liquid and counted. This experiment was performed in the absence of serum. Similar results were also obtained in 10% serum (data not shown).

Western blot analyses. Nonadherent cells found in the culture supernatants were collected by centrifugation and added to adherent cells that were scraped off the dishes. Lysis was performed on ice in (50 mM Hepes, 150 mM NaCl, 100 mM NaF, 10 mM EDTA, 10 mM $Na_4P_2O_7$, 2 mM orthovanadate, 25 mM glycophosphate, and 1% Triton X-100). Protein concentrations were determined by Bio-Rad assays. Equivalent amounts of proteins were analyzed by SDS–PAGE and then transferred onto PVDF membranes. Membranes were blocked in 5% nonfat dried milk in TBS buffer, and antibodies were incubated at the following concentrations: ERK rabbit polyclonal antibodies (Sigma M5670): 1/10,000, phospho ERK (Sigma M8159) mouse monoclonal antibodies 1/5000. Secondary antibodies (DAKO) were used at 1/10,000 final dilution. Signals were revealed by chemiluminescence.

Statistical analysis. Results were analyzed for statistical significance using one-way ANOVA parametric test and Tukey pairwise comparisons. *p* are indicated on figures.

Results

Cadmium toxicity is a time- and dose-dependent process. Cadmium entry is required for its toxic effect, as demonstrated by invalidation studies of the metallothionein genes, which code for proteins binding free cadmium present within cells. Indeed, mice devoid of metallothioneins are more affected by cadmium than wild type animals [7]. We investigated the effects of several divalent metal cations upon cadmium entry into cells. To this end, we cultivated HEK293 cells in the presence of 0.5 μM $CdCl_2$ spiked with

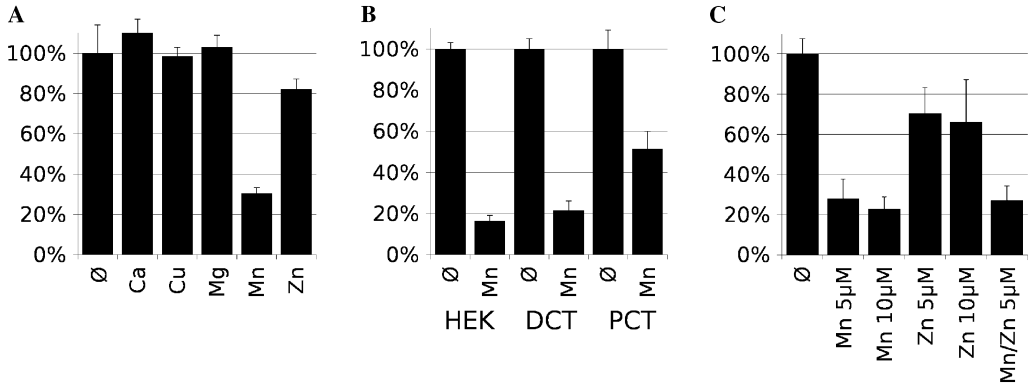

Fig. 1. Effects of divalent cations on cadmium incorporation. (A) HEK293 cells were treated with 0.5 μM cadmium and a 10-fold molar excess of indicated salts for 24 h. Incorporation of radioactive cadmium was determined and that of control cells incubated with cadmium alone (Ø) was arbitrarily set to 100%. (B) Manganese effect on cadmium incorporation was analyzed as in (A) in parallel experiments in HEK293, DCT, and PCT cells exposed to 1 μM cadmium. (C) Comparison of manganese and zinc effects on cadmium incorporation. Primary osteoblasts treated with 0.5 μM cadmium for 24 h were co-treated with the indicated concentrations of zinc and/or manganese and cadmium incorporation was determined as in (A).

traces of radioactive ^{109}Cd. Tenfold molar excesses of other divalent cations were added to the culture media in parallel experiments (Fig. 1A). Rather than comparing transport modifications occurring within the first minutes following cation addition, as is routinely done in biochemical studies of transporters, we analyzed their effects after a 24 h delay to allow cells to reflect the physiological consequences of these putative modulations of cadmium entry. Consequently, cadmium incorporation for each condition was determined by radioactivity counting after 24 h in culture. We found that cadmium accumulation was linear ($R^2 > 0.99$) in at least three different cell types tested (HEK293, HeLa, and REF) up to a maximum level, corresponding to a calculated internal cadmium concentration of about 20 μM. After that level was reached, a decrease in accumulated cadmium was observed, reflecting a loss of cellular integrity (data not shown). $CaCl_2$, $CuSO_4$, and $MgCl_2$ had no significant effects on cadmium incorporation into cells (Fig. 1A). In agreement with previously published results, we observed a competitive effect of $ZnCl_2$ on cadmium incorporation, even though this competition was rather modest in our hands ($p = 0.032$). However, we found that $MnCl_2$ resulted in an over 80% decrease of cadmium incorporation in HEK293 cells ($p = 2 \times 10^{-6}$). To determine whether this effect is restricted to HEK293 cells or is a more general phenomenon, we repeated this experiment with other cell types including two cell lines derived from renal tubules (PCT and DCT for proximal and distal convoluted tubule, respectively), that play a central role in the detoxification processes of the organism. We found that at 2 μM $CdCl_2$, a 10-fold molar excess of $MnCl_2$ resulted in approximately 80% decrease in cadmium incorporation for both HEK293 cells and DCT cells, while the effect was limited to 50% in PCT cells, still being highly significant ($p = 1 \times 10^{-5}$, Fig. 1B; data not shown). Similar results were observed with other cell types, including primary osteoblasts (Fig. 1C). Since zinc has been reported as a competitor for cadmium entry, we analyzed whether manganese and zinc could cooperate to protect against cadmium toxicity. Primary osteoblasts were incubated in the presence of cadmium, and molar excesses of either $MnCl_2$, $ZnCl_2$, or a combination of both. As indicated in Fig. 1C, a 10-fold molar excess of manganese reduced cadmium uptake by more than 70% ($p = 2 \times 10^{-6}$). A 10-fold excess of zinc resulted in a more moderate 30% reduction of cadmium uptake ($p = 0.025$). A higher molar excess (20-fold) did not result in any substantial increase in the competition observed. When both Mn and Zn were present in the culture medium, the reduction in cadmium uptake was not statistically different from that observed with Mn alone ($p = 0.99$), suggesting that there is no additive effect between Zn and Mn for the inhibition of cadmium uptake.

To correlate these results with cell survival, we investigated growth and phenotypic changes of the different cell types to cadmium in the presence of $MnCl_2$ or $ZnCl_2$ for 120 h. First, we determined the toxic concentrations of Mn and Zn for PCT and DCT cells, HEK293 cells, and primary osteoblasts derived from calvaria. We consistently observed that both $ZnCl_2$ and $MnCl_2$ became deleterious to cells at 100 μM after 50 h of culture. However, while at 150 μM $ZnCl_2$ all the cells were dead, some cells were still viable up to 500 μM $MnCl_2$, suggesting that $MnCl_2$ is less toxic to cells in vitro than $ZnCl_2$ (data not shown). Second, we plated these four cell types either in fresh

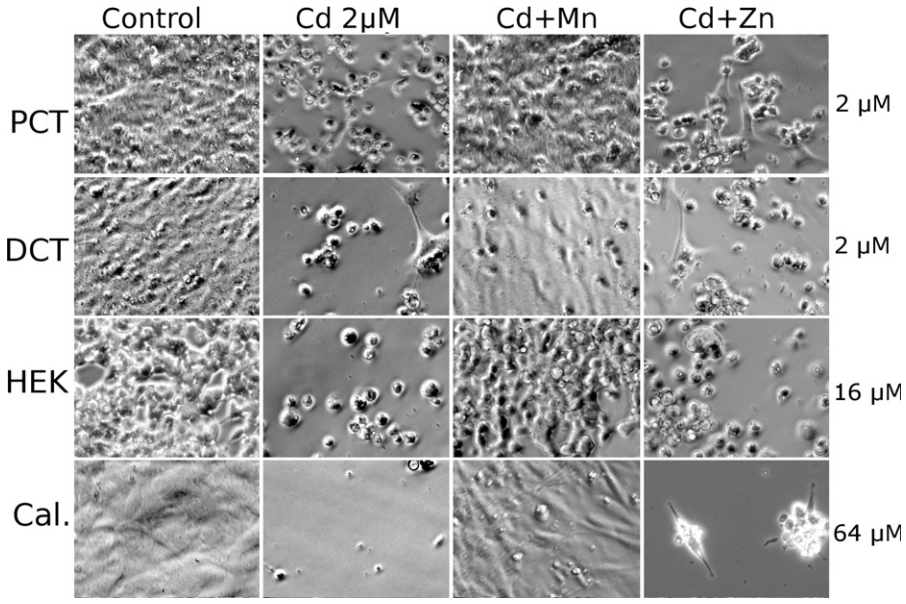

Fig. 2. Comparison of the protective effects of manganese and zinc in different cell types. Cells were left untreated (control), or were cultivated for 120 h in the presence of 2 μM cadmium (Cd, 2 μM), with manganese (Cd+Mn) or zinc (Cd+Zn). Pictures illustrate the lowest concentrations of manganese (indicated on the right side of the figure) that permitted a proliferation indistinguishable from that of control cells. The effects of the same concentrations of zinc on the different cell types are also presented in the (CD+Zn) column.

culture medium, or in the presence of 2 μM $CdCl_2$. In addition to $CdCl_2$, we added increasing concentrations of either $MnCl_2$ or $ZnCl_2$. Fig. 2 illustrates the behavior of the different cell types after 120 h in culture. For each cell type, we selected the minimal $MnCl_2$ concentration for which cells were apparently completely protected from cadmium toxicity (indicated on right hand side of Fig. 2). This concentration varied widely from one cell type to another, since equimolar concentrations of $CdCl_2$ and $MnCl_2$ were sufficient to protect the renal DCT and PCT cell lines. A 8-fold molar excess was necessary to protect HEK293 cells, while a 32-fold molar excess was needed to protect primary osteoblasts. Nevertheless, this experiment clearly demonstrates that Mn protects cells more efficiently than Zn at identical concentrations (compare Cd+Mn column with Cd+Zn column). In addition, we found that the protective concentrations of Zn is consistently about four times higher than that of Mn, independent of the cell type considered (data not shown).

Since we previously showed that sustained ERK activation is tightly associated with cadmium intoxication, we analyzed the status of ERK in cells co-treated with cadmium and manganese for 24 h. As depicted in Fig. 3, $MnCl_2$ treatment resulted in the complete disappearance of sustained ERK activation. This strongly suggests that Mn protective effect is an upstream event as compared to ERK sustained activation and is most likely the exclusive result of manganese competition for cadmium entry. It should be noted that we observed a perfect inverse correlation between the level of protection from cadmium toxicity by different manganese concentrations and the level of ERK activation.

DMT1 (divalent metal-ion transporter-1), a H^+/metal-ion cotransporter, has been shown to transport cadmium into cells [4]. DMT1 transport activity was shown to be maximal at pH 6.75, while up to five times lower at pH 7.5 [8]. We thus tested the effects of extracellular pH on cadmium entry into different cell types (Fig. 4). The highest cadmium influx that we observed takes place at pH 8.0, whereas that influx is dramatically and significantly decreased at pH 6.8 (Fig. 4). This suggests that, at least under our experimental conditions, DMT1 does not act as the major means of entry for extracellular cadmium.

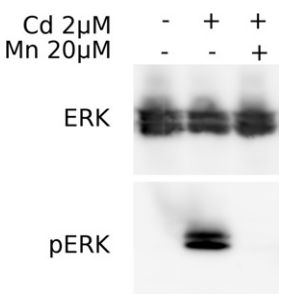

Fig. 3. Cadmium-induced sustained ERK activation is completely inhibited by manganese treatment. Western analysis of total ERK (ERK, upper panel) and phosphorylated ERK (pERK, lower panel) in HEK293 cells, following control treatment (−/−) or treatments for 24 h with 2 μM cadmium (+/−), and 2 μM cadmium and 20 μM manganese (+/+).

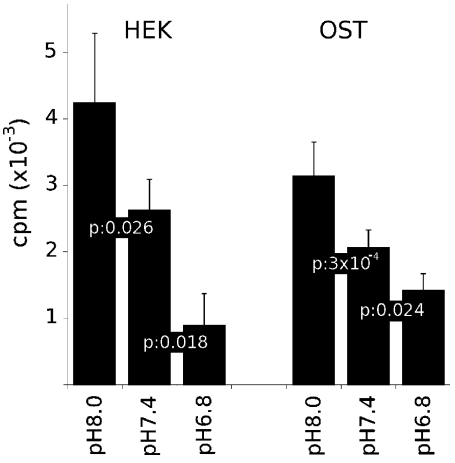

Fig. 4. Effect of extracellular pH on cadmium incorporation. Extracellular pH was adjusted with 20 mM Hepes at either pH 8.0, 7.4, or 6.8. Cadmium incorporation was determined as in Fig. 1A. The differences observed between the different pH conditions were highly significant ($0.0003 < p < 0.024$).

Discussion

Several reports have shown that zinc can compete for cadmium entry, both in vitro [2] and in vivo [9,10]. As a result, zinc is considered as a way to protect against cadmium intoxication [11]. In this study, we tested and compared several divalent ions for their effects on cadmium entry in different cell types. Unexpectedly, manganese turned out to be much more effective than zinc in protecting cells from cadmium toxicity in all cell types tested. We previously showed that sustained ERK activation is linked to cadmium intoxication [5]. Our results demonstrate that the protective effect of manganese also results in the inhibition of long-term ERK activation. Protection against cadmium by manganese is thus an event occurring upstream of ERK and is likely restricted to the inhibition of cadmium entry. This indicates that ERK sustained activation is not the result of the triggering of some extracellular sensors or receptors, but a response to intracellular cadmium.

Even though we found that the protective effect of manganese is due to a strong reduction of cadmium entry into cells, we could not detect any displacement of cadmium already present within cells (data not shown). A report from 1985 [12] indicated that manganese acts as a protective agent against cadmium in rats. Our observations suggest that this protective effect on the whole organism is most likely due to the protection that we observed at the cellular level, that is itself due to a competition for cadmium entry into cells.

DMT1 (divalent metal-ion transporter-1) has been shown to be a possible entry way for cadmium into cells [4]. Most published results describe DMT1 as a H^+/metal-ion

cotransporter, with an optimal activity at an extracellular pH of 6.75 [8]. We found however that cadmium entry was inversely correlated with extracellular H^+ concentration in different cell types (Fig. 4), suggesting that at least under our experimental conditions, DMT1 does not act as the major means for extracellular cadmium entry. This apparent discrepancy is not the result of cadmium insolubility at a lower pH, since the lower the pH, the higher the cadmium solubility [13]. Since manganese very efficiently protects against cadmium toxicity, it is possible that transporter(s) for manganese could be involved in cadmium entry. This hypothesis is reinforced by a study performed on metallothionein null cell lines, that were selected for their resistance to cadmium. Interestingly, the authors found that a high-affinity manganese transport system was suppressed in their cadmium resistant lines [14]. Unfortunately, specific manganese transporters, if any, have not yet been identified [15].

It is interesting to note that manganese protection appears to be most effective in cells derived from kidney proximal tubules (PCT and DCT), which are precisely the cells absorbing most of the cadmium present in the lumen in the case of cadmium toxicity [16,17]. This protection may reflect the highly efficient uptake systems present on renal tubules, inherent to their reabsorption functions. In these tubule cells, manganese is also more efficient than zinc in protecting against cadmium toxicity. We systematically found that the molar ratio between the protective dose of manganese and the protective dose of zinc is more or less constant (around four times more zinc is required than manganese for a similar level of protection), independent of the cell type tested. This, taken together with the observation that adding manganese and zinc together does not have an additive effect on decreasing cadmium toxicity, suggests that zinc, manganese, and cadmium target the same transport system(s), present on all cell types tested. Manganese would interfere with this(ese) transport system(s) more efficiently than zinc, suggesting again that manganese transporters could be the major entry way of cadmium into cells. Finally, not only is manganese more effective than zinc in protecting cells against cadmium toxicity, it is also less toxic than zinc, since cells survive at higher manganese concentrations than zinc concentrations. In the animal, the LD_{50} for $MnCl_2$ is comparable to that of $ZnCl_2$: 331 mg/kg [18] versus 350 mg/kg [19], respectively. It is worth mentioning that while inhalation of high levels of manganese induces neurological disorders [20], manganese does not appear to be toxic in food and drinkable water up to relatively high concentrations, nor has it been shown to be carcinogenic or genotoxic [21]. Consumption of water containing at least 0.3 mg/l and up to 2 mg/l of $MnCl_2$ over several years does not lead to any neurological symptoms [22], and heavy tea drinkers have daily manganese intakes exceeding 20 mg, without any consequences [21]. Thus, despite its clear toxicity when inhaled, manganese is no more toxic than zinc for the organism. Our observation raises the question as to whether manganese should not be considered as a more effective competitor for cadmium toxicity than zinc.

Acknowledgments

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