Cadmium affects osmotic phase and regulatory volume decrease in cultured human embryonic kidney cells

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Abstract

The present investigation aims to verify whether cadmium (Cd^{2+}), a metal possibly accumulated in body tissues from air and food, affects cell volume regulation capability in cultured human embryonic kidney (HEK 293 Phoenix) cells. The osmotic phase (OP), which is the expected cell swelling due to aquaporins involvement after hyposmotic challenge, and regulatory volume decrease (RVD), bringing cell volume back to control values through Ca^{2+}-dependent ion efflux (K^+ and Cl^-), have been monitored in HEK 293 cells treated with Cd^{2+} (1-10-100 µM) for different time intervals (30 min, 3 h, overnight) and then submitted to 15 % hyposmotic shock. The results show that both 1 and 10 µM Cd^{2+} significantly reduced OP, whereas 100 µM impaired Cd^{2+} RVD mechanisms. The use of glutathione (GSH, 200 µM) confirmed that Cd^{2+} elicited its effect *via* oxidative damage, being RVD inhibition after Cd^{2+} treatment prevented by this antioxidant compound. Our findings show that: i) HEK 293 cells are a suitable model to assay the effect of xenobiotics on cell homeostasis; ii) Cd^{2+}, depending on its concentration, affects cell homeostasis at different levels, *i.e.* water and ion permeability, responsible for, respectively, OP and RVD mechanism, adding thus more information to the knowledge of Cd^{2+} toxicology.

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Introduction

Heavy metals and their toxic effects on cells and tissues have been widely investigated, as they can be accumulated through respiration, adsorption and ingestion.[1,2] In this regard, cadmium (Cd^{2+}) accumulates in the atmosphere being produced, from one hand, by natural processes such as erosion and abrasion of rocks and soils, forest fires and volcanic eruptions[3] and, from another hand, by anthropogenic factors due to industrial development[4] concerning production of pigments, electronic compounds and rechargeable nickel-cadmium batteries. Hence, Cd^{2+} accumulation in humans may result from water, food and air contaminations. In this regard, cigarette smoking should be also considered as a source of intoxication, due to high Cd^{2+} concentrations in cigarettes.[5]

Cd^{2+} in humans may provoke acute or chronic intoxication effects, as already observed in liver, lungs, thyroid, bones, testis and immunity system. Its detrimental effects, reviewed elsewhere,[6-8] could be associated to its low renal excretion rate, thus contributing to its accumulation in the organism. The effect of Cd^{2+} on cells has been already described,[9-12] though this issue has not been completely clarified.

One of the most critical feature for cell survival is the ability of a cell to regulate its volume under osmotic perturbations.[13,14] In a hyposmotic environment, after the expected initial swelling, termed osmotic phase (OP), cells undergo a regulatory shrinkage through the loss of intracellular solutes, mainly K^+ and Cl^-, along with osmotically obliged water, leading then to regulatory volume decrease (RVD).[13,15] RVD mechanisms are highly conserved and common to many cells from evolutionary distant species.[14,16-18] Similarly, the uptake of osmolytes, followed by gain of osmotically obliged water occurs under hypertonic shrinkage, leading to regulatory volume increase (RVI), a process exhibited by many cell types and mainly due to Na^+ influx.[13,14,19]

Studies on the volume sensor transduction pathway for volume regulation[20,21] provide evidence for intracellular Ca^{2+} involvement, protein phosphorylation, arachidonic acid and phosphoinositide turnover, as well as mechanical sensors, such as stretch-activated channels and

cytoskeleton. These pathways may work together producing an integrated and effective volume regulatory response,[22] which allows cell adaptation to osmolarity changes of the external medium. Both RVD and RVI processes account for cell viability and homeostasis[23] and, as already shown in other cell types,[24] they can be affected by exogenous compounds like pollutants.

On this basis and in an attempt to consolidate RVD response as a tool to monitor cell function under perturbations of the external medium, the aim of the present investigation is to verify whether OP and/or RVD are affected by Cd^{2+} in human embryonic kidney (HEK 293 Phoenix) cells, chosen as a model of renal cells continuously exposed to hyposmotic and hyperosmotic conditions during the process of urine production, and, therefore, capable of adaptive mechanisms to regulate cell volume.[14]

Materials and Methods

Human embryonic kidney cells culture

Human renal HEK 293 Phoenix cells[25] were kindly provided by Paracelsus Medizinische Privatuniversität (Salzburg, Austria) and frozen in liquid nitrogen before culturing. Cells were seeded in 100 mm diameter Petri dishes containing 10 mL of Minimum Essential Eagle Medium (MEM; Sigma Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Cambrex Bio Science, East Rutherford, NJ, USA), 2 mM l-glutamine, 100 µg/mL streptomycin, 100 U/mL penicillin and 1 mM pyruvic acid (sodium salt). Subcultures were routinely established every second to third day by seeding cells into 100 mm diameter Petri dishes following trypsin/ethylenediaminetetraacetic acid (EDTA) treatment. Cultured cells were placed in an incubator chamber (humidified 95% air, 5% CO_2, 37°C). Confluent cells were detached with 1 mL trypsin-EDTA solution, re-suspended in 10 mL of fresh medium and then seeded in 60 mm diameter Petri dishes, as described below.

In order to perform RVD test, HEK 293 cells were seeded on 18x18 mm coverslip glasses, previously placed into 60 mm diameter Petri dishes, and then grown for 24 h (humidified 95% air and 5% CO_2 at 37°C) before the experiment.

At the end of incubation, cells were used for either control or experimental tests after Cd^{2+} treatment. With regard to control experiments, a single coverslip glass with adherent cells, taken from the 60 mm diameter Petri dish, was mounted on a slide and used for RVD test. For experimental tests, after 24 h incubation in 60 mm diameter Petri dish, Cd^{2+} at different concentrations (1-10-100 M) was added to the medium and cells incubated for, alternatively, 30 min, 3 h or overnight. Coverslips with adherent cells were then mounted on a slide and used for RVD tests.

Chemicals

The hyposmotic solution was composed by NaCl 125 mM, $CaCl_2$ 2.5 mM, $MgCl_2$ 2.5, HEPES 10 mM, at pH 7.4 and osmotic pressure 275 $mOsm/Kg_{H2O}$. Isosmotic physiological solution was obtained by adding mannitol to the hyposmotic medium (pH 7.4, osmotic pressure, 325 $mOsm/Kg_{H2O}$). pH was measured with an Orion pH-meter. Osmolality of solutions was measured with a Fiske OS osmometer. Stock solutions for either $CdSO_4$ or glutathione (GSH) were dissolved in distilled water. The compound was then added to the experimental solution to yield the final concentrations (1-10-100 µM for $CdSO_4$, 200 µM for GSH), used on HEK 293 cells. All chemicals were purchased from Sigma Aldrich.

Regulatory volume decrease tests

To perform RVD tests, a make-shift perfusion chamber was prepared by placing at first two strips of double sided tape (Scotch 3M; 3M, St. Paul, MN, USA) on the long edges of a glass slide, then a 18x18 mm coverslip with adherent cells and, finally, a 24x32 mm coverslip. This allowed either isosmotic or hyposmotic solution to be added at one side and absorbed at the other side of the coverslip 24x32 mm with strips of filter paper, so that they could be rapidly exchanged.

Control tests

With regard to control RVD tests, the experimental design consisted of three periods: period 1: pH 7.4, isosmotic physiological solution for 5 min; period 2: pH 7.4, hyposmotic physiological solution (15% hyposmotic shock) for 30 min; period 3: pH 7.4 isosmotic physiological solution for 5 min. Cell volume measurements were taken from cells strongly adherent to the slide. About 40 images/cell were taken, minute-by-minute during the whole experiment, with a phase contrast microscope (Leica DMLS; Leica Microsystems GmbH, Wetzlar, Germany) connected to a video camera (charge-coupled device digital camera) and a computer equipped with suitable software (Movie Maker; Microsoft, Redmond, WA, USA). Since each chosen cell was considered as a sphere, the diameter (needed to calculate cell volume) of each recorded image was measured (Image J; http://imagej.net/) as a function of time. The results were then expressed as the relative volume – V/V_0 – where V and V_0 represent, respectively, the volume of a cell at a given time and the average of volume of the same cell in isosmotic physiological solution.

Regulatory volume decrease test on cadmium-treated cells

RVD tests on Cd^{2+}-treated cells were performed upon cell viability assessment by Trypan blue dye exclusion test (Sigma Aldrich). Concentrations and time of exposure to this metal have been chosen according to what reported by Bertin and Averbeck.[6] As no cell damage was seen, treated cells, after incubation with Cd^{2+} (1 or 10 or 100 µM) for, respectively, 30 min - 3 h - overnight, were used for RVD test according to the following protocol: pH 7.4, isosmotic physiological solution for 5 min (period 1); pH 7.4, hyposmotic physiological solution (15% hyposmotic shock) for 30 min (period 2); pH 7.4 isosmotic physiological solution for 5 min (period 3). Image analysis was performed as described for control tests.

Regulatory volume decrease test on glutathione-cadmium-treated cells

To possibly counteract the effect of Cd^{2+} on RVD, cells were pretreated with the antioxidant GSH (reduced glutathione, 200 µM). With this aim, HEK 293 cells, after overnight incubation, were treated with 200 µM GSH for 20 min before adding either 1 or 10 or 100 µM Cd^{2+}. After incubation for 30 min - 3 h - overnight, RVD test and image analysis were performed according to the protocol reported above for control tests.

Statistical analysis

Statistical analysis was performed using GraphPad Prism (version 5.03; GraphPad Software Inc., San Diego, CA, USA). The results are expressed as means±standard error of means. Comparisons among groups were performed with Student's *t* test and, for multiple comparisons, with two-way analysis of variance (ANOVA), followed by Bonferroni's *post-hoc* test. Statistically significant differences were assumed at $P<0.05$ (*$P<0.05$ low significance, **$P<0.01$ medium significance, ***$P<0.001$ high significance). *N* represents the number of independent experiments. Statistical analysis was reported in Table 1 and, accordingly, on graph panels and legends.

Results

Control tests

Untreated cells exposed to the hyposmotic shock (Figure 1) exhibited the expected swelling, reaching a V/V_0 peak value of 1.294±0.025, within 4 min of the hyposmotic challenge. This V/V_0 value was significantly higher than that observed in the isosmotic period (1st period, ***P<0.001), consistent with the OP. After the peak value, being unchanged the external medium, cell volume decreased towards the control values, reaching, at the end of the hyposmotic period a V/V_0 value of 1.030±0.016, significantly lower than the V/V_0 peak value (2nd period, §§§P<0.001) and comparable to the V/V_0 observed in the isosmotic period, thus showing 90% RVD capability. Once replaced the hyposmotic medium with an isosmotic one, to restore the control conditions (3rd period), cell volume was brought back to the initial values. No post-RVD RVI was detected within the observation time chosen for the test.

Cadmium treatment

Cell integrity assessment by Trypan blue dye exclusion test was performed on Cd^{2+}-treated cells before RVD experiments, to exclude any possible cell damage. Neither cells exposed for 30 min, nor for 3 h or overnight to Cd^{2+} at all concentrations (1-10-100 M) exhibited damage.

Treatment with 1 µM cadmium

HEK 293 cells treated with 1 M Cd^{2+} for 30 min (Figure 2a), 3 h (Figure 2b) or overnight (Figure 2c) exhibited the OP after the hyposmotic challenge, reaching a V/V_0 peak value of, respectively, 1.158±0.015 (2nd period, Figure 2a), 1.192±0.016 (2nd period, Figure 2b), 1.180±0.021 (2nd period, Figure 2c). This is significantly higher than the V/V_0 measured in isosmotic conditions (***P<0.001), while it is significantly lower than the V/V_0 peak value observed in untreated cells (°°°P<0.001, Figure 2a; °°P<0.01, Figure 2b and c). The V/V_0 peak value observed in both treated and control cells, at any time of incubation, was observed within 5 min of hypotonic shock application (2nd period). At the end of the hyposmotic period (2nd period) in each experimental condition (30 min, 3 h, overnight), cell volume decreased to a V/V_0 value of, respectively, 1.030±0.009 (30 min treatment, 2nd period, Figure 2a), 1.025±0.0025 (3 h treatment, 2nd period, Figure 2b), 1±0.002 (overnight treatment, 2nd period, Figure 2c), which is signifi-

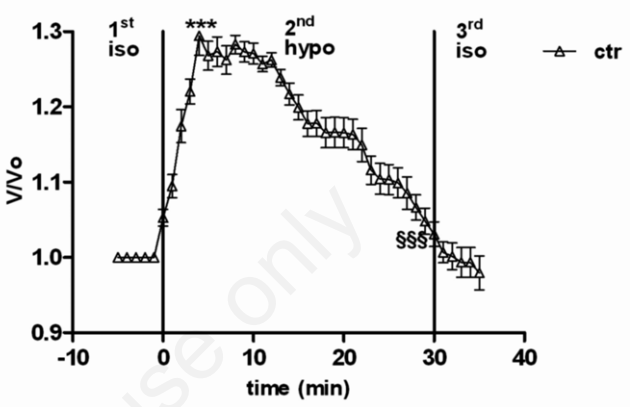

Figure 1. Relative volume (V/V_0) of cells measured after exposure to isosmotic (1st and 3rd period) and hyposmotic (2nd period) solution (15% shock). The peak value in hyposmotic solution was significantly higher than in isosmotic solution (*P<0.001), while it was significantly lower at the end of the 2nd period (§§§P<0.001).**

Table 1. Relative volume in human embryonic kidney cells measured in control conditions or treated with either cadmium at different concentrations (1-10-100 µM), with or without 200 µM glutathione, and at different incubation times (30 min, 3 h, overnight).

Experimental condition	V/V_0 1st period (isotonic)	V/V_0 2nd period (hyposmotic) - peak value	V/V_0 2nd period (hyposmotic) - last value	*N*
Control	1.000±0.015	1.294±0.025***	1.030±0.016§§§	15
1 µM Cd^{2+}				
30 min	1.000±0.015	1.158±0.025***,°°°	1.030±0.009§§§	13
3 h	1.000±0.002	1.192±0.016***,°°	1.025±0.025§§§	14
Overnight	1.000±0.001	1.180±0.021***,°°	1.000±0.002§§§	16
10 µM Cd^{2+}				
30 min	1.000±0.001	1.119±0.0025**,°°°	1.030±0.009§§§	13
3 h	1.000±0.005	1.138±0.013***,°°°	1.043±0.007§§§	14
Overnight	1.000±0.001	1.138±0.012***,°°°	1.010±0.0014§§§	14
100 µM Cd^{2+}				
30 min	1.000±0.003	1.210±0.0025***,°	1.033±0.009§§§,ns	18
3 h	1.000±0.003	1.213±0.013***,°	1.131±0.007§§,##	17
Overnight	1.000±0.002	1.246±0.012***,ns	1.178±0.014§§,###	16
200 µM GSH + 100 µM Cd^{2+}				
30 min	1.000±0.002	1.229±0.002***,ns,^^^	1.063±0.005§§§,+++	17
3 h	1.000±0.002	1.262±0.002***,ns,^^^	1.071±0.020§§§,+++	19
Overnight	1.000±0.002	1.253±0.005***,ns,^^^	1.112±0.007§§§,+++	15

V/V_0, relative volume; V, volume of a cell at a given time; V_0, the volume of a cell at a given time and the average of volume of the same cell in isosmotic physiological solution; *N*, number of independent experiments; GSH, glutathione; ns, not significant. Data are presented as means±standard error of mean from separate *N* experiments, where: ***P<0.001 significantly different compared to isotonic; §§§P<0.001 or §§P<0.01 significantly different compared to peak value; °°°P<0.001 and °°P<0.01 significantly different compared to control; ^^^P<0.001 significantly different compared to 100 µM Cd^{2+}-treated cells; 200 µM GSH + 100 µM Cd^{2+} 30 min ns compared to peak value after 100 µM Cd^{2+}; +++P<0.001 significantly different compared to Cd^{2+}-treated cells; ###P<0.001 significantly different compared to last hyposmotic value in untreated cells, as determined by two way ANOVA followed by Bonferroni's *post-hoc* test or paired Student's *t* test.

cantly lower than V/V_0 peak value observed in each experimental condition ($^{§§§}P<0.001$), thus showing, respectively, 80.3, 87, 100% RVD capability. Once restored the isosmotic medium (3rd period), cell volume was totally recovered (Figure 2a-c).

Treatment with 10 µM cadmium

After exposure of HEK 293 cells to 10 µM Cd^{2+} for 30 min, 3 h and overnight, OP was observed after hyposmotic challenge, reaching a V/V_0 value of, respectively, 1.119±0.0025 (2nd period, Figure 3a), 1.138±0.013 (2nd period, Figure 3b), 1.138±0.012 (2nd period, Figure 3c), *i.e.* significantly higher than values observed in the isosmotic period ($^{**}P<0.01$, Figure 3a; $^{***}P<0.001$, Figure 3b and c), and significantly lower than the V/V_0 peak value observed in untreated cells ($^{°°°}P<0.001$). At the end of the hyposmotic period (2nd period), cell volume decreased to a V/V_0 value of, respectively, 1.030±0.009 (Figure 3a), 1.043±0.007 (Figure 3b), 1.010±0.0014 (Figure 3c), which is significantly lower than the peak value ($^{§§§}P<0.001$), showing, respectively, 74, 69 and 93% RVD capability. The V/V_0 peak value observed in Cd^{2+}-treated cells (at any incubation time) was reached within 10 min of hypotonic shock, significantly later than in untreated cells. Once restored the isosmotic medium (3rd period), cell volume was brought back to control values (Figure 3a-c).

Treatment with 100 µM cadmium

HEK 293 cells exposed to 100 µM Cd^{2+} for 30 min, 3h or overnight exhibited OP after the hyposmotic challenge, reaching a V/V_0 value of, respectively, 1.210±0.0025 (2nd period, Figure 4a), 1.213±0.013 (2nd period, Figure 4b), 1.246±0.012 (2nd period, Figure 4c), significantly higher than values observed in isotonic period ($^{***}P<0.001$) and significantly lower than the V/V_0 peak value observed in untreated cells

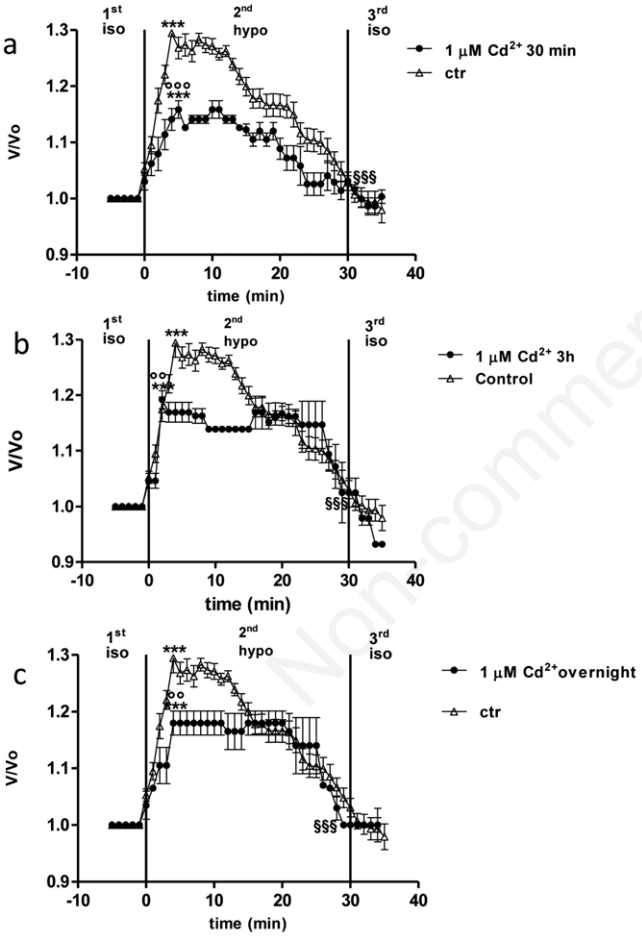

Figure 2. Relative volume (V/V_0) of cells exposed to 15% hyposmotic shock after treatment with 1 µM Cd^{2+} for 30 min (a), 3 h (b), or overnight (c). Comparison with control cells (ctr) is also provided. The peak value in hyposmotic medium (2nd period) was significantly higher than in isosmotic solution ($^{***}P<0.001$), and significantly lower than in control cells ($^{°°°}P<0.001$). At the end of the 2nd period, V/V_0 in treated cells was significantly lower than the peak value ($^{§§§}P<0.001$) at any incubation time.

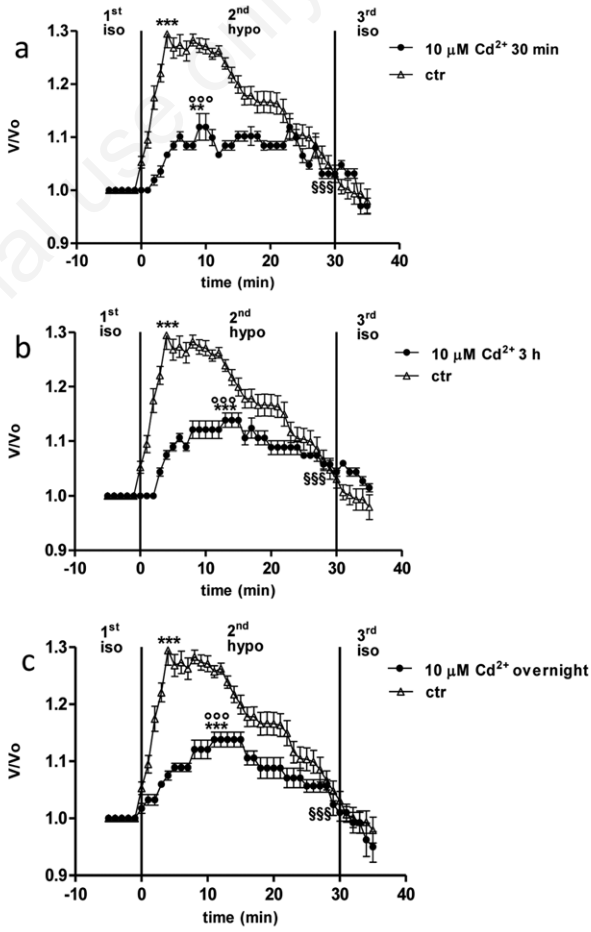

Figure 3. Relative volume (V/V_0) of cells exposed to 15% hyposmotic shock after treatment with 10 µM Cd^{2+} for 30 min (a), 3 h (b), or overnight (c). Comparison with control cells (ctr) is also provided. The peak value in hyposmotic medium (2nd period) was significantly higher than in isosmotic solution ($^{**}P<0.01$ at 30 min, $^{***}P<0.001$ at both 3 h and overnight), and significantly lower than in control cells ($^{°°°}P<0.001$). At the end of the same period, V/V_0 was significantly lower than the peak value ($^{§§§}P<0.001$) at any incubation time.

(°P<0.05, Figure 4a and b). V/V_0 peak value observed in overnight 100 M Cd^{2+}-treated cells was not significant with respect to control cells. At the end of the hyposmotic period (2nd period), cell volume decreased to a V/V_0 value of, respectively 1.033±0.009 (Figure 3a), 1.131±0.007 (Figure 3b), 1.178±0.014 (Figure 3c) significantly lower than the V/V_0 peak value (Figure 3a, §§§P<0.001; Figure 3b and c, §§P<0.01) and significantly higher than that one measured in untreated cells only after 3 h and overnight treatment (##P<0.01 and ###P<0.001, respectively), showing, respectively, 74, 69 and 93% RVD capability. The V/V_0 peak value in Cd^{2+}-treated cells was reached within 5 min of hypotonic shock at 30 min or 3 h, comparable to control conditions, while, on the other hand, it was reached within 10 min of hypotonic shock after overnight treatment, significantly later than in untreated cells. Once restored the isosmotic medium (3rd period), cell volume was not brought back to control values (Figure 4a-c) within the time of observation chosen for the present protocol.

Treatment with 200 µM glutathione

In a separate set of experiments, 200 µM GSH as an antioxidant compound was used to possibly counteract the inhibitory action of Cd^{2+} (10-100 µM) on OP and/or RVD. Since cell response of both 10 and 100 Cd^{2+}-treated cells to GSH application was comparable, data from 100 µM Cd^{2+} plus 200 µM GSH experiments have been depicted in Figure 5. GSH alone (200 µM) did not damage HEK 293 cells and both OP and

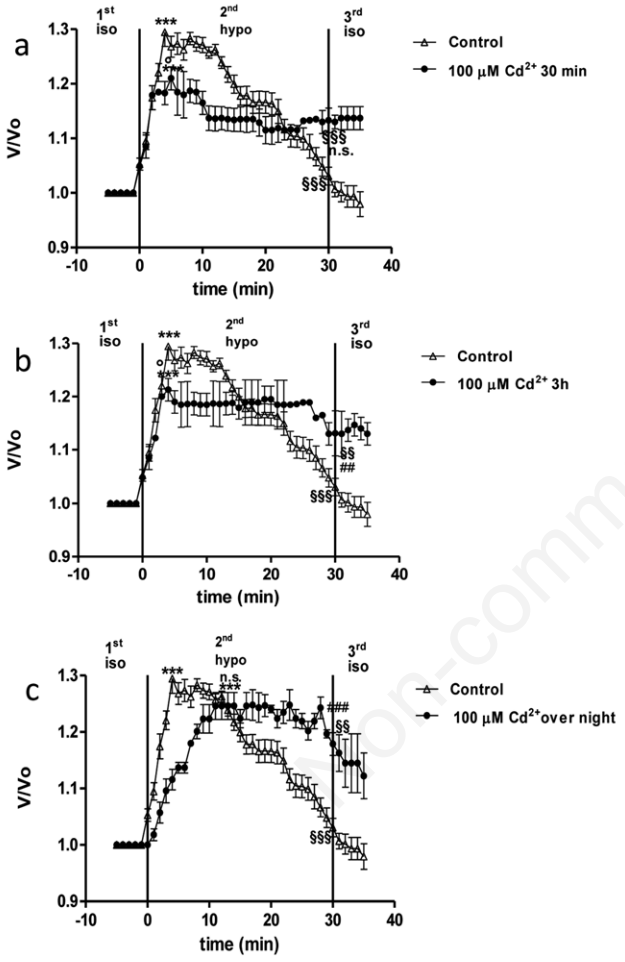

Figure 4. Relative volume (V/V_0) of cells exposed to 15% hyposmotic shock after treatment with 100 µM Cd^{2+} for 30 min (a), 3 h (b), or overnight (c). Comparison with control cells (ctr) is provided. The peak value in hyposmotic medium (2nd period) was significantly higher than in isosmotic solution (*P<0.001). V/V_0 at the end of the 2nd period in treated cells was significantly lower than the peak value (a, §§§P<0.001; b,c, §§P<0.01), while it was significantly higher than the last value in untreated cells (###P<0.001). In cells treated for both 30 min and 3 h, the peak value was significantly lower than in control (a,c, °P<0.05), while, after overnight treatment, it was not significantly different with respect to control (b, ns).**

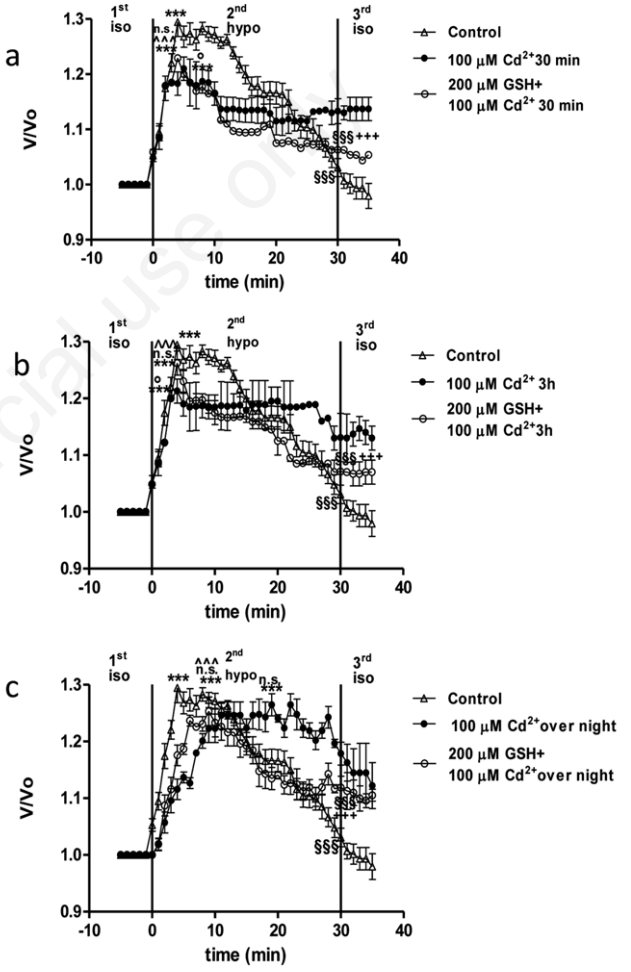

Figure 5. Relative volume (V/V_0) of cells exposed to 15% hyposmotic shock after treatment with 200 µM glutathione (GSH) plus 100 µM Cd^{2+} for 30 min (a), 3 h (b), or overnight (c). Comparison with both control cells (ctr) and Cd^{2+}-treated cells is also provided. Peak value of GSH-Cd^{2+}-treated cells at any time of incubation was significantly different with respect to Cd^{2+}-treated cells (^^^P<0.001), while not significantly different with respect to untreated cells. At the end of the hyposmotic challenge in GSH-Cd^{2+}-treated cells, V/V_0 was significantly lower than peak value (§§§P<0.001) and significantly different with respect to what observed in both Cd^{2+}-treated and control cells (+++P<0.001).

RVD exhibited after hyposmotic shock application were comparable to those observed in untreated cells, at any time of incubation (30 min, 3 h or overnight; data not shown). HEK 293 cells exposed to 200 µM GSH plus 100 µM Cd^{2+} for 30 min, 3 h or overnight, after hyposmotic shock application swelled reaching a V/V_0 peak value of, respectively, 1.229±0.002 (Figure 5a), 1.262±0.002 (Figure 5b), 1.253±0.005 (Figure 5c), significantly higher than V/V_0 observed in isosmotic conditions (***P<0.001). The V/V_0 peak value of GSH-Cd^{2+} treated cells, at any time of incubation was significantly higher than that one observed in Cd^{2+}-treated cells (^^^P<0.001) while not significant with respect to V/V_0 peak value of control cells. At the end of the hyposmotic challenge in GSH-Cd^{2+}-treated cells, cell volume decreased to a V/V_0 value of, respectively, 1.063±0.005 (Figure 5a) 1.071±0.020 (Figure 5b), 1.112±0.007 (Figure 5c), significantly lower than peak value (§§§P<0.001) and significantly different with respect to what observed in both Cd^{2+}-treated cells and control condition (+++P<0.001), showing, respectively 72.5, 73.3 and 50% RVD capability. Once restored isosmotic conditions (3rd period), V/V_0 was only partially recovered in both GSH-10 µM Cd^{2+}- and GSH-100 µM Cd^{2+}-treated cells.

Discussion

It has been shown that long-term environmental exposure of cells to Cd^{2+} results in high blood levels of this metal, which is associated with organ-specific toxic effects and distinct pathologies in a variety of tissues and organs, including kidney.[26] In this regard, the proximal tubule as well as renal glomeruli, exposed to circulating metals during plasma filtration, have been recognized as a major target in Cd^{2+}-induced effects.[27-29]

On this basis, cultured human embryonic kidney (HEK 293 Phoenix) cells have been chosen as a model to define whether heavy metals like Cd^{2+}, deriving from environmental pollution,[4] may affect cell volume regulation under hyposmotic shock, a homeostatic parameter recognized to be essential for survival of many cells.[13,30] Being renal cells continuously exposed to physiological changes in the osmolarity of the external medium,[31] cell lines derived from kidney are a suitable model for monitoring homeostatic cell functions, such as the regulation of cellular volume, in the presence of xenobiotics.[32,33]

Our results show that HEK 293 cells exhibit the expected OP within 4 min of 15% hyposmotic challenge, leading to water influx, as shown in other cell types.[18] After OP, RVD phase occurs and is completed within 30 min of hyposmotic shock application, with cell volume recovering, due to both K^+ and Cl^- outflow, resulting in water efflux.[18,30]

Here we demonstrate that time exposure comprised between 30 min and overnight did not induce cell death under either 1 or 10 or 100 µM Cd^{2+}. Furthermore, both 1 and 10 µM Cd^{2+}-treated HEK 293 cells did not exhibit a peak value comparable to that observed in untreated cells. This result provides evidence for an inhibition of OP inhibition presumptively due to aquaporins,[18,34,35] rather than an inhibition of RVD mechanisms, suggesting thus a possible action of the metal on water flux. On the other hand, OP was preserved in 100 µM Cd^{2+}-treated HEK 293 cells, while RVD inhibited, suggesting a possible action of the metal on ion transport, namely both K^+ and Cl^- flux, playing a major role in RVD response.[13]

The evidence that sensitivity to Cd^{2+} varies from one cell type to another and that Cd^{2+} toxic effect highly depends on its concentration, duration of exposure and tissues/cells target, has been already demonstrated.[6] This point implies that multiple effects on cells after Cd^{2+} exposure can be considered. In this regard, Cd^{2+}, at concentrations above 1 µM,[36] has been described to affect cell cycle progression, proliferation, differentiation, DNA replication and repair as well as apoptotic pathways.[37] However, at lower concentrations, it seems to enhance DNA synthesis and cell proliferation.[38]

As concerns the inhibition of OP and/or RVD phase after Cd^{2+} exposure, some authors have already correlated the effect of heavy metals to cell membrane transport, hence supporting our hypothesis that both ion and water movement may be modified after exposure to Cd^{2+}. Metals have been shown to inhibit epithelial Na^+ channels (ENaC),[39] mainly through Cys and His, possibly after internalization of this metal. In this respect, it has been reported that Cd^{2+} may compete with Ca^{2+} uptake through Ca^{2+} channels[40] and not through Cd^{2+}-specific uptake pathways, being Cd^{2+} uptake reduced by Ca^{2+} channel blockers in several cell types.[9] Moreover, Cd^{2+} has been demonstrated to enter mitochondria via Ca^{2+} transport pathways,[41] so that the site of action of Cd^{2+} seems to be inside the mitochondria, the major source of reactive oxygen species (ROS) production.[42] As a matter of fact, Cd^{2+} toxicity may be associated to ROS induction,[43] even though the mechanism of their formation, not occurring via Cd^{2+} triggered fenton-like reaction, is yet unknown.[44]

It has been already shown that Cd^{2+} doses lower than 50 µM lead to increased H_2O_2 levels as early as 5 min after exposure with a maximum at 15 min, affecting membranes permeability. On this basis, we may suggest that Cd^{2+}-induced inhibition of RVD mechanisms may rely on an oxidative damage targeting HEK 293 cell membrane. Investigations on Cd^{2+}-induced ROS also reveal an increase in both superoxide anion (O_2^-) and H_2O_2 levels in HeLa and bovine endothelial cells, under 1-20 µM Cd^{2+} concentrations.[43] Shih and co-authors[45] confirmed these results and assessed that in normal human lung fibroblasts (MRC5) H_2O_2 is 2.9-fold elevated after 3 h of Cd^{2+} treatment.

Previous data obtained on HEK 293 cells treated with H_2O_2 in isosmotic conditions[46] clearly show a cell volume decrease, putatively due to ion loss and obliged water efflux, considered as an early stage of the apoptotic process.[47] Since in the present investigation an impaired swelling capacity under hyposmotic challenge was seen under both 1 and 10 µM Cd^{2+} treatment, an oxidative effect of the metal comparable to what observed by H_2O_2-treated HEK 293 cells may be even suggested.

In addition to its role as a generator of ROS, Cd^{2+} may also affect antioxidative enzymes that play an important role in ROS elimination. In this respect, in normal rat liver cell line, high concentrations of Cd^{2+} (100-300 µM), after 4 or 8 h exposure, have been demonstrated to reduce the activity of antioxidant enzymes such as catalase and glutathione reductase as well as reduced and oxidized glutathione. The reduction in glutathione levels after Cd^{2+} administration has been also shown in mice.[48] Other studies demonstrate a relationship between Cd^{2+} exposure and lipid peroxidation, as reported in skeletal muscle cells C2C12, where oxidation of lipids occurs at concentrations of Cd^{2+}>7.5 µM.[49] Lipid peroxidation may cause cross-linking and polymerization of membrane components,[50] affecting thus lipid composition of cell membranes and, in turn, cell functions.

As reviewed by Bertin and Averbeck[6], metallothioneins and glutathione can mainly detoxify Cd^{2+} in cells. Reduced-glutathione levels, observed after intoxication, may be brought about by the affinity of Cd^{2+} to thiol groups and may be responsible for the decrease in cellular antioxidant activities. Both events, *i.e.* the reduction of both metallothionein expression and glutathione levels, enhance cellular injury due to Cd^{2+} exposure. On this basis, GSH has been here used as an antioxidant compound to treat cells before Cd^{2+} application, similarly to what already performed in H_2O_2-treated HEK 293 cells.[46] In this latter case 200 µM GSH was effective in impairing cell shrinkage due to H_2O_2. Since in the present experiments 200 µM GSH impairs the inhibitory effects of Cd^{2+} (at both 10 and 100 µM, at any time of Cd^{2+} treatment), we may support the hypothesis that Cd^{2+} may affect cell function acting via oxidizing events such as glutathione levels reduction.

Conclusions

The present investigation would add information about the effect that heavy metals, such as Cd^{2+}, may exert on a cell target, demonstrating that: i) renal cells, usually exposed to metabolites and toxicants, are confirmed as a good model for investigating the impact of heavy metals at cellular level; ii) RVD capability has been revealed as a suitable tool to verify the effect of both short and long term exposure to the metal; iii) the effect of Cd^{2+} on HEK 293 cells depends on both time of exposure to the metal and on metal concentration; iv) both OP, depending on aquaporins involvement, and RVD, depending on K^+ and Cl^- fluxes, are affected by Cd^{2+}; v) though no mechanism of action is here proposed to explain Cd^{2+} effect, the use of GSH as antioxidant compound significantly reduced RVD alterations due to Cd^{2+} exposure.

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